p*Silencer*™ 5.1 Retro Kit

(Part Number AM5782, AM5784)

Protocol

P/N 5782M Revision B **Revision Date: December 5, 2008**

Literature Citation: When describing a procedure for publication using this product, please refer to it as the p*Silencer*™ 5.1 Retro Kit.

Warranty and Liability: Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

Limited Label License This product is covered by United States Patent Application No. 10/195,034, and PCT patent application No. US02/22010, owned by the University of Massachusetts. The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to resell, repackage, or further sublicense) under these patent rights to perform the siRNA vector synthesis methods claimed in those patent applications for research and development purposes solely in conjunction with this product. No other license is granted to the buyer whether expressly, by implication, by estoppel or otherwise. In particular, the purchase of this product does not include nor carry any right or license to use, develop, or otherwise exploit this product commercially, and no rights are conveyed to the buyer to use the product or components of the product for any other purposes, including without limitation, provision of services to a third party, generation of commercial databases, or clinical diagnostics. This product is sold pursuant to an exclusive license from the University of Massachusetts, and the University of Massachusetts reserves all other rights under these patent rights. For information on purchasing a license to the patent rights for uses other than in conjunction with this product or to use this product for purposes other than research, please contact the University of Massachusetts, Office of Commercial Ventures and Intellectual Property, 365 Plantation Street, Suite 103, Worcester, Massachusetts 01655.

This product is the subject of the following patents: U.S. Patent No. 6,573,099 and foreign counterparts co-owned by Benitec Australia Ltd. Not-for-profit and academic entities who purchase this product receive the non-transferable right to use the product and its components in non-Commercial Purpose internal research. All commercial and for-profit purchasers using this product for a Commercial Purpose are required to execute a commercial research license with Benitec by contacting Benitec at www.licensing@benitec.com Entities wishing to use ddRNAi as a therapeutic agent or as a method to treat/prevent human disease also should contact Benitec at www.licensing@benitec.com. Commercial Purpose means any activity by a party for tangible consideration and may include, but is not limited to, (i) use of the product in manufacturing; (ii) use of the product to provide services, information or data for a compensatory fee; (iii) use of the product for therapeutic, diagnostic or prophylactic purposes; (iv) use of the product in drug discovery or target validation and/or (v) resale of the product or its components whether or not the product is resold for use in research. If purchaser is not willing to accept the limitations of this label license, seller is willing to accept the return of the product with a full refund. For terms and additional information relating to a commercial research license, or for licensing for any human therapeutic purpose, contact Benitec by email at www.licensing@benitec.com

I. Introduction

A. siRNA and RNA Interference

Small Interfering RNAs (siRNAs) are short, double-stranded RNA molecules that can target mRNAs with complementary sequence for degradation via a cellular process termed RNA interference (RNAi) (Elbashir et al. 2001a). RNAi is an extremely powerful tool for analysis of gene function in mammalian cells. siRNA is typically prepared in vitro and transfected into cells, or it is introduced into mammalian cells by transfecting plasmids that express functional siRNA or hairpin siRNA (also called short hairpin RNA or shRNA) in mammalian cells (Brummelkamp et al. 2002, Paddison et al. 2002, Paul et al. 2002, Lee et al. 2002, Sui et al. 2002). Using siRNA expression vectors has the advantage that expression of target genes can be reduced for weeks or even months (Brummelkamp et al. 2002, Ford et al. 2004), eclipsing the 6–10 days typically observed with in vitro prepared siRNA used for transient transfection (Byrom 2002). The presence of an antibiotic resistance gene in an siRNA expression vector enables selection for cells that stably express the hairpin siRNA and observation of long-term effects of target gene suppression.

B. p*Silencer* 5.1 Retro Retroviral Expression Vectors

IMPORTANT

The recombinant retroviruses produced with this kit are classified as a Biosafety Level 2 hazard. We strongly recommend that you implement the recommendations for safe handling of Biosafety Level 2 hazards described in the following CDC/NIH publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition May 1999. (See section I.G on page 11 for information on obtaining this publication.)

One of the biggest challenges in the use of RNAi to study gene function in mammalian systems is delivering the siRNA or siRNA expression vector into cells. The p*Silencer*™ 5.1 Retro siRNA expression vectors employ retroviral mediated gene transfer—a well characterized and effective tool for the delivery of DNA sequences both in vivo and in vitro. Derived from the murine stem cell virus (MSCV), the p*Silencer* 5.1 Retro vectors can be transfected into the retroviral packaging cell system of your choice to make infectious, replication-incompetent retrovirus for efficient delivery of hairpin siRNA templates into most mammalian cell types, even difficult-to-transfect cell types. Alternatively, you can transfect cells with the plasmid construct for hairpin siRNA expression without preparing infectious recombinant viral particles. Once inside a mammalian cell, one of two powerful RNA polymerase III promoters drives high level expression of cloned hairpin siRNA in a wide variety of cell types.

- p*Silencer* 5.1-U6 Retro uses the U6 promoter; see the vector map in Figure 1 on page 2.
- p*Silencer* 5.1-H1 Retro uses the H1 promoter; see the vector map in Figure 2 on page 2.

p*Silencer* 5.1 Retro vectors harness retroviruses' ability to stably integrate into the host genome of nearly all mitotically active cells and include a puromycin resistance gene for selection of cells that express the recombinant DNA. The p*Silencer* 5.1 Retro vectors enable researchers to study the long-term effects of reducing the expression of specific genes in cell culture models. This technology expands the capabilities of RNA interference research to address long-term alteration of gene expression.

Figure 1. p*Silencer* 5.1-H1 Retro Vector Map

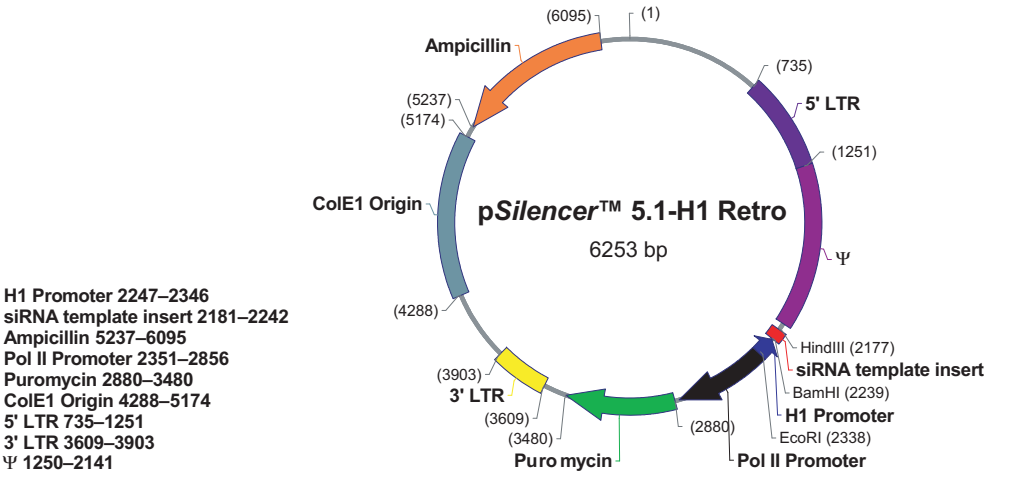
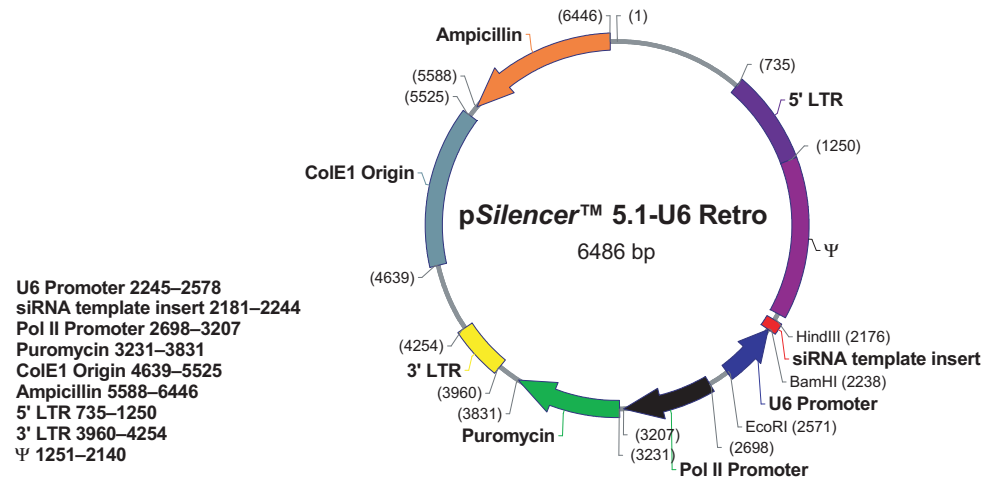


Figure 2. p*Silencer* 5.1-U6 Retro Vector Map



Puromycin resistance gene for selection of stable transfectants

The p*Silencer* 5.1 Retro siRNA expression vectors contain a puromycin resistance gene to enable antibiotic selection in mammalian cells. Puromycin selection can be used to enrich for cells that were successfully infected with recombinant virus by killing off cells that lack the resistance gene. For long-term gene knockdown studies, the puromycin resistance gene makes it possible to select cell populations, or clonal cell lines, that stably express the hairpin siRNA. It can also be used to select for packaging cells that stably express the introduced DNA. Short term antibiotic selection is very useful for experimental systems where low levels of infection would otherwise preclude detection of a reduction in target gene expression.

Puromycin is an aminonucleotide antibiotic produced by *Streptomyces alboniger* that specifically inhibits peptidyl transfer by acting as an analog of the 3' terminal end of aminoacyl-tRNA (De la Luna and Ortin 1992). The puromycin acetyltransferase gene confers puromycin resistance by catalyzing N-acetylation of the tyrosine group of puromycin, rendering a product that cannot accept peptidyl moieties. Puromycin in culture medium at 50–4000 ng/mL is commonly used to select cells containing the resistance gene.

Differences in puromycin selection between p*Silencer* 5.1-U6 Retro and p*Silencer* 5.1-H1 Retro

Puromycin selection is effective regardless of which p*Silencer* 5.1 Retro vector is used, but we have observed that cells infected with p*Silencer* 5.1-H1 Retro typically grow more quickly under puromycin selection pressure than do cells infected with p*Silencer* 5.1-U6 Retro. It is not clear what causes this difference in cells' response to puromycin selection, but it may be due to differential interference between the U6 or H1 RNA polymerase III (pol III) promoters and the pol III promoter driving the expression of the puromycin resistance gene.

RNA polymerase III promoter for high levels of siRNA expression

The p*Silencer* 5.1 Retro vectors employ either the U6 or H1 RNA pol III promoter which generate large amounts of transcript using relatively simple promoter and terminator sequences. These promoters are well characterized (Myslinski 2001, Kunkel 1989), and they provide high levels of constitutive expression across a variety of cell types. The terminator consists of a short stretch of uridines (usually 3–4 nt); this is compatible with the original siRNA design that terminates with a two uridine 3' overhang (Elbashir et al. 2001b).

We have identified differences between the H1 and U6 promoters in target knockdown in some cases. With some inserts, cloning the hairpin siRNA template into the p*Silencer* 5.1-H1 Retro vector vs. the p*Silencer* 5.1-U6 Retro vector resulted in significantly different levels of target gene silencing. Some inserts were found to elicit higher levels of gene knockdown when expressed from the H1 promoter, while in other cases,

the U6 promoter was found to be far superior (see Figure 5 on page 7. It may be beneficial to test hairpin siRNA template inserts in both p*Silencer* 5.1 Retro vectors to find the optimal vector for your system.

p*Silencer* 5.1 Retro plasmids are supplied ligation-ready

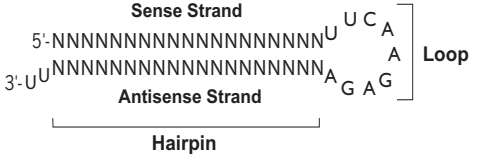
The p*Silencer* 5.1 Retro siRNA Expression vectors are linearized with both BamH 1 and Hind III to facilitate directional cloning. They are also purified to remove the digested insert so that it cannot re-ligate with the vector. This greatly increases the percentage of clones bearing the siRNA template insert after ligation, reducing the time and effort required to screen clones. Figures 1 and 2 show basic p*Silencer* 5.1 Retro vector maps; more detailed sequence information about the p*Silencer* 5.1 Retro vectors is available at the following web address:

www.ambion.com/catalog/CatNum.php?5782

C. Hairpin siRNA Template Design

The prototypical siRNA is comprised of two hybridized 21-mer RNA molecules with 19 complementary nucleotides and 3' terminal dinucleotide overhangs. We recommend preparing an insert for expression of a hairpin RNA with a 19-mer stem, a loop, and a 3' terminal overhang (Paddison et al. 2002) (Figure 3). In mammalian cells, this type of hairpin siRNA or shRNA can efficiently induce RNAi of the target gene (Brummelkamp et al. 2002, Paddison et al. 2002, Sui et al. 2002). For each target gene, synthesize two complementary 55–60 mer oligonucleotides with single-stranded overhangs complementary to the overhangs from the restriction enzyme site in the multiple cloning site (MCS): BamH1 and Hind III. These are annealed and ligated into the p*Silencer* 5.1 Retro vector. The oligonucleotide insert should encode a 19-mer hairpin sequence specific to the mRNA target, a loop sequence separating the two complementary domains, and a dinucleotide overhang that can hybridize with the RNA target (this is discussed in section II.C on page 15).

Figure 3. Hairpin siRNA



Strategy for selection of target sites

Both in vitro prepared siRNA and in vivo expressed hairpin siRNA can induce gene silencing. Thus, sequences that have been successfully targeted with a chemically synthesized, in vitro transcribed, or PCR-generated siRNA should also be susceptible to down-regulation with a hairpin siRNA expressed from a p*Silencer* 5.1 Retro vector.

We recommend testing several different siRNAs per gene to identify an effective target site for your hairpin siRNA. This significantly reduces the time and effort required to develop an effective siRNA plasmid specific to a given gene. To select target sites, please see the Online siRNA Target Finder and Ambion Technical Bulletin #506:

www.ambion.com/techlib/misc/siRNA_finder.html

www.ambion.com/techlib/tb/tb_506.html

Once you have a target chosen, use the Insert Design Tool to convert the target sequence into oligonucleotide insert sequences at:

www.ambion.com/techlib/misc/psilencer_converter.html

D. Overview of Recombinant Retroviral Particle Production

Retroviral vectors are used to prepare recombinant infectious viral particles to introduce a nonviral gene into cells in vivo and in vitro. Retroviral vectors are particularly useful for introducing nucleic acids into cells that are not easily transfected, such as primary cells and cells in vivo. Another advantage to using the p*Silencer* 5.1 Retro vectors, compared to nonviral expression vectors, is that antibiotic selection can be used to select cells that have integrated and stably express the introduced genetic material.

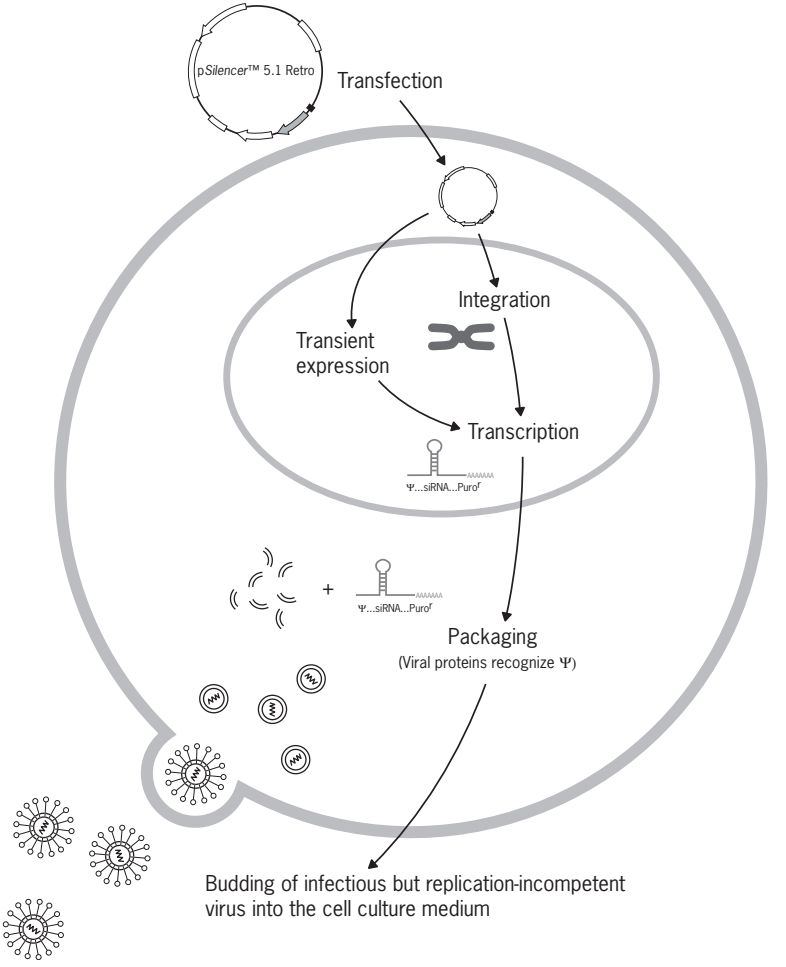
The ability of retroviral particles to infect cells is dependant on the presence of specific receptors on the surface of host cells. Some of these receptors are expressed on nearly every cell type. Immediately after they infect a host cell, retroviruses reverse transcribe their RNA genome to produce a DNA copy by a series of steps involving both retroviral and host factors. The viral DNA between the flanking long terminal repeats (LTRs) then integrates into the host genome where it is referred to as a provirus.

In retroviral vectors like p*Silencer* 5.1 Retro, most of the viral genes encoding virion structural proteins are deleted to make room for inserted genes and to cripple the virus. The cis-acting viral signals needed for transmission, however, remain. These sequences include the Ψ packaging signal which functions in recognition of the viral RNA during the encapsidation process, reverse transcription signals, integration signals, and viral promoter, enhancer, and poly(A) signals. Figure 4 shows an overview of production of recombinant retroviral particles.

Transient or stable production of recombinant retrovirus

In Figure 4, both transient and stable transfection of a retroviral packaging cell are depicted. After transfection with your p*Silencer* 5.1 Retro construct, packaging cell lines will begin to produce infectious, recombinant retroviral particles. Some cells will have retroviral DNA integrated into their genome, and puromycin selection can be applied to obtain a clonal or nonclonal cell line of stably transfected packaging cells. Stably transfected packaging cells provide an ongoing source of your recombinant retrovirus.

Figure 4. Production of Recombinant Viral Particles in a Retroviral Packaging Cell Line.



E. Applications of p*Silencer*™ 5.1 Retro Vectors and Analysis of Gene Silencing

Evaluating gene silencing

Several different approaches can be used to generate valid gene silencing data. Since mRNA recognition and degradation is the basis for RNAi, measuring target mRNA levels is a direct way to document gene silencing; we routinely use reverse transcription followed by real-time PCR (qRT-PCR) to analyze target mRNA levels (see Figures 5, 6, and 7). the Ambion *mir*Vana™ miRNA Isolation Kit and *mir*Vana miRNA Detection Kit provide a way to directly analyze expression of the hairpin siRNA in infected cells (Figures 5 and 7).

It is also common to analyze levels of the target gene product using protein analysis techniques such as western blot and immunohistochemistry. Often the available tools dictate how gene silencing is evaluated.

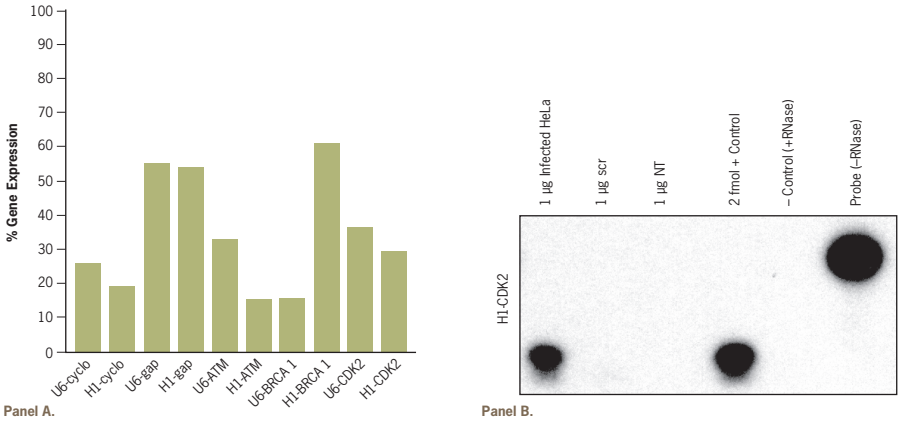


Figure 5. Gene Silencing Using Recombinant Retrovirus Made from p*Silencer* 5.1-U6 Retro and p*Silencer* 5.1-H1 Retro Vectors.

p*Silencer* 5.1-H1 Retro or p*Silencer* 5.1-U6 Retro containing the indicated siRNA template inserts were transfected into a retrovirus packaging cell line. Recombinant retrovirus was then collected and used to infect HeLa cells. (Panel A) Infected HeLa cells were placed under puromycin selection (4 days). RNA was purified from the cells and analyzed by qRT-PCR for target gene expression levels. (Panel B) The expression level of CDK2 siRNA regulated by the H1 promoter (H1CDK2) was monitored using the *mir*Vana™ miRNA Isolation and Detection Kits (Ambion P/N AM1560 and AM1552). Cyclo = cyclophilin A.

Knockdown of gene expression in a primary cell line

As part of the p*Silencer* 5.1 Retro development process, we produced experimental retrovirus bearing an siRNA insert targeting cyclophilin A and a negative control retrovirus expressing a scrambled control siRNA insert. These recombinant retroviruses were used to infect NHDF-neo cells—a primary cell line that is very difficult to transfect, and in parallel it was used to infect HeLa cells. Infected cells were placed under

selection with puromycin until all of the cells infected with a mock viral supernatant were killed. Then the experimental cells were removed from antibiotic selection. Some cells were harvested to analyze cyclophilin knockdown, and some of the cells were propagated for long-term knockdown analysis. In comparing cyclophilin knockdown between HeLa cells and the primary cell line, we found effective reduction of cyclophilin expression in both cell types, indicating that the NHDF-neo cells were effectively infected with the recombinant retrovirus (Figure 6). In addition, the NHDF-neo cells showed more effective reduction in cyclophilin expression than the HeLa cells, suggesting that a given hairpin siRNA template may be more effective in one cell line compared to another.

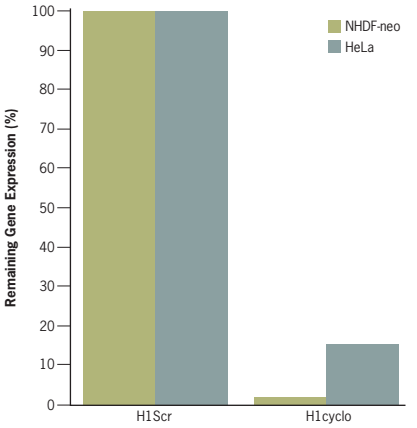


Figure 6. Retroviral Delivery Used to Elicit RNAi in Primary Cells.

p*Silencer* 5.1-H1 Retro containing either a scrambled negative control siRNA template (H1Scr)or the Cyclo Control Insert (H1cyclo) were transfected into a packaging cell line. Three days after transfection, recombinant retrovirus was collected and used to infect either HeLa cells or NHDF-neo cells. The cells were grown under puromycin selection (4 days) and were subsequently harvested for analysis of cyclophilin expression by qRT-PCR.

Stable knockdown of gene expression

Using recombinant retrovirus made from p*Silencer* 5.1 Retro containing the Cyclo Control Insert, we observed knockdown of over 80% as evidenced by qRT-PCR. This level of cyclophilin knockdown was maintained for over 6 months (Figure 7).

Long-term stable reduction of gene expression by siRNAs could mimic natural conditions where gene mutations play a role in specific diseases. To effect long-term gene silencing, we generated recombinant retrovirus targeting a number of genes, including cyclophilin A and BRCA1. After infecting cells and selecting with puromycin, we confirmed gene

silencing by qRT-PCR and passaged cells expressing the hairpin siRNAs for 8 months. We found that morphological changes were not visible when these genes were reduced for 1–2 months; however, long-term stable reduction of cyclophilin A and BRCA1 resulted in a gradual phenotypic change over 3–8 months which caused significant morphological differences compared to a stable cell line expressing the scrambled control siRNA (Figure 7). Although expression of BRCA1 remained at 60% of that seen in the control cells, the 40% reduction in BRCAI gene expression caused pronounced morphological phenotypes. In this case, a relative reduction of gene expression to the nominal cut-off value of 30% or less was not needed to achieve obvious physiological effects. This partial reduction on gene expression may be mimicking the effects of disease causing alleles such as BRCA1, where a disease phenotype is observed when only one of the alleles is mutated for an extended time.

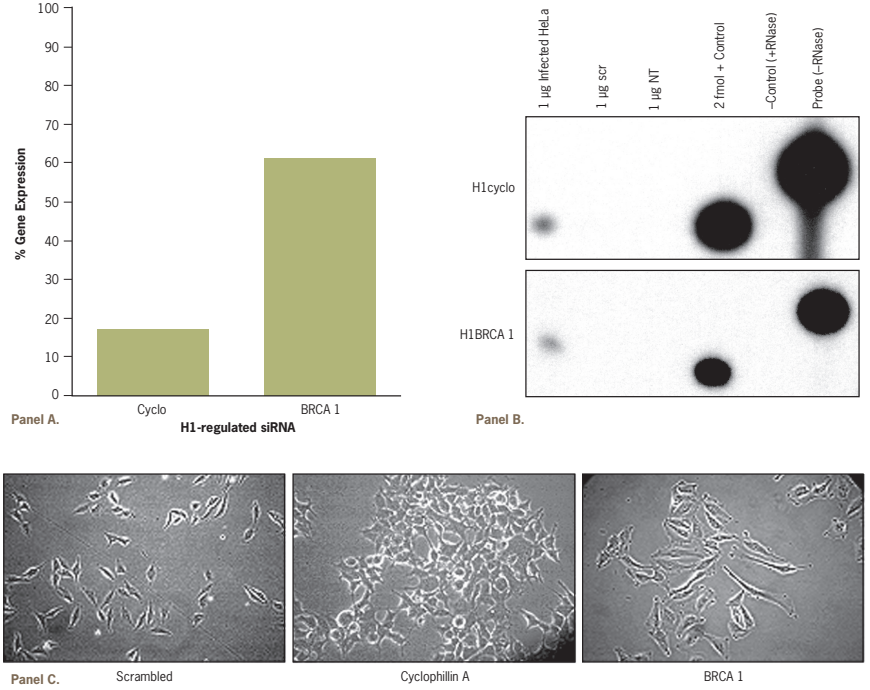


Figure 7. Long-term Reduction in Target Gene Expression Results in Morphological Changes.

p*Silencer* 5.1-H1 Retro vectors containing the indicated inserts were transfected into a retroviral packaging cell line. Three days later virus was collected and used to infect HeLa cells. The HeLa cells were placed under puromycin selection (4 days). When selection was complete, the cells were periodically analyzed for a representative result of gene reduction (Panel A) and for expression of siRNA inserts using the *mir*Vana miRNA Detection Kit (Panel B) Morphological changes were observed after extended growth of cells expressing hairpin siRNAs targeting cyclophilin and BRCA1 (Panel C).

F. Kit Components and Storage Conditions

Each p*Silencer* 5.1 Retro siRNA Expression Vector Kit includes 4 components:

P/N AM5782	P/N AM5784	Component
20 µL	– –	p*Silencer* 5.1-U6 Retro (0.1 mg/mL)
10 µL	– –	p*Silencer* 5.1-U6 Retro Scrambled (0.5 mg/mL)
– –	20 µL	p*Silencer* 5.1-H1 Retro (0.1 mg/mL)
– –	10 µL	p*Silencer* 5.1-H1 Retro Scrambled (0.5 mg/mL)
10 µL	10 µL	Cyclo Control Insert (80 ng/µL)
1 mL	1 mL	1X DNA Annealing Solution

- Linearized p*Silencer* 5.1-U6 Retro or p*Silencer* 5.1-H1 Retro siRNA Expression Vector ready for ligation
- Circular, negative control p*Silencer* 5.1-U6 Retro Scrambled or p*Silencer* 5.1-H1 Retro Scrambled that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes.
- The Cyclo Control Insert codes a cyclophilin-specific, hairpin siRNA insert that can be used as a positive control for ligation. We have used the following PCR primers in real-time RT-PCR to detect knockdown of cyclophilin A in cells infected with recombinant retrovirus made from p*Silencer* 5.1 Retro with the Cyclo Control Insert:
  – Forward: 5'-AGACGCCACCGCCGA-3'
  – Reverse: 5'-CTGACACATAAACCCTGGAATAATTC-3'
  Ambion also offers a cyclophilin specific antibody (P/N AM4309) that can be used in gene silencing studies.
- 1X DNA Annealing Solution to prepare annealed DNA oligonucleotides for ligation into the p*Silencer* 5.1 Retro vector

Storage conditions

Store the p*Silencer* 5.1 Retro Kit at –20°C (if desired the 1X DNA Annealing Solution can be stored at room temp).

Properly stored kits are guaranteed for 6 months from the date received.

G. Safe Handling of Recombinant Retrovirus

CAUTION

The viral supernatants produced from the pSilencer 5.1 Retro retroviral system could contain potentially hazardous recombinant virus. Exercise caution in the production and handling of recombinant retrovirus. The user is advised not to create retrovirus capable of knocking down known tumor suppressors in amphotropic or polytropic host range viruses.

Please refer to the appropriate regional and institutional guidelines. The NIH has classified retrovirus as a Biosafety Level 2 hazard. We strongly recommend that you read the information on safe handling of Biosafety Level 2 hazards in the following CDC/NIH publication: *Biosafety in Microbiological and Biomedical Laboratories, 4th Edition* May 1999.

This publication is available on the internet at these addresses:
http://bmbl.od.nih.gov
http://www.cdc.gov/od/ohs/pdffiles/4th%20BMBL.pdf

H. Other Required Material

Ligation and transformation

- Two complementary oligonucleotides targeting the gene of interest (see section II starting on page 14 for a thorough discussion)
- DNA ligase, ligase reaction buffer, and competent *E. coli* cells are needed to clone your siRNA template insert into p*Silencer* 5.1 Retro.
- Ampicillin or carbenicillin containing plates and liquid bacterial media will be needed to propagate the plasmids.

Plasmid purification reagents

- Small scale plasmid purification reagents are needed to prepare plasmid for restriction and sequence analysis.
- Medium to large scale transfection-grade plasmid purification reagents are needed to purify p*Silencer* 5.1 Retro after cloning.

Restriction analysis reagents and equipment

For initial clone verification, the restriction enzymes BamH 1 and Hind III can be used (see Figure 1 or 2 on page 2). The reaction products can be visualized by routine agarose gel electrophoresis.

Sequence analysis services

We recommend sequencing putative p*Silencer* 5.1 Retro clones containing your hairpin siRNA template before using them for transfection and gene knockdown studies.

Mammalian cell transfection reagents

The optimal mammalian cell transfection conditions including transfection agent and plasmid amount must be determined empirically. See website for more information.
www.ambion.com/prod/transfect

Retroviral packaging cells

Cell lines and systems for production of recombinant retroviruses from retroviral vectors are available from a number of commercial and non-commercial sources such as ATCC, BD Biosciences, Orbigen, Stratagene, and the Nolan Lab at Stanford University (Phi-NX cells).

The p*Silencer* 5.1 Retro system has been tested in the PT67 packaging cell line; however, it is also compatible with other common retroviral packaging systems.

Polybrene

Polybrene is used to maximize infection with retrovirus; one source of polybrene is Sigma-Aldrich (Sequa-brene® Cat #S2667).

Biosafety level 2 cell culture facility

The recombinant retroviruses produced with this kit are classified as a Biosafety Level 2 hazard, please see the previous section (I.G on page 11) for more information.

Cell culture facility and supplies

In addition to routine cell culture media, culture media containing puromycin will be needed for selection of p*Silencer* 5.1 Retro transfected cells.

I. Related Products Available from Applied Biosystems

T4 DNA Ligase P/N AM2130, AM2132, AM2134	T4 DNA Ligase (E.C. 6.5.1.1) catalyzes the formation of phosphodiester bonds between adjacent 3' hydroxyl and 5' phosphate groups in double-stranded DNA. T4 DNA ligase will join both blunt-ended and cohesive-ended DNA and can also be used to repair nicks in duplex DNA. Includes 10X Ligase Reaction Buffer.
siPORT™ *XP-1* DNA Transfection Agent P/N AM4506, AM4507	siPORT *XP-1* is an easy-to-use transfection reagent that efficiently delivers both plasmid DNA and PCR products into a variety of mammalian cell types. Comprised of a proprietary formulation of polyamines, siPORT *XP-1* exhibits low toxicity and can be used either in the presence or absence of serum.
Silencer® siRNAs see our web or print catalog www.ambion.com/siRNA	Ambion *Silencer* Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer* siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer* siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer* siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects.

Silencer® siRNA Controls see our web or print catalog www.ambion.com/siRNA	The *Silencer* siRNA Controls are ready-to-use, chemically synthesized, purified siRNAs targeting mRNAs frequently used as internal controls in RT-PCR, Northern blot, RPA, and other experiments designed to monitor gene expression. Corresponding scrambled siRNA negative controls are included with the gene-specific siRNAs. Also offered are Negative Control siRNAs #1, #2, and #3. They are ideal for use in any siRNA experiment as controls for nonspecific effects on gene expression. *Silencer* siRNA Controls are ideal for developing and optimizing siRNA experiments and have been validated for use in human cell lines. The GAPDH and cyclophilin siRNAs are also validated for use in mouse cell lines.
Antibodies for siRNA Research see our web or print catalog	For select *Silencer* Control and Validated siRNAs, Ambion offers corresponding antibodies for protein detection. These antibodies are ideal for confirming mRNA knockdown results by analyzing concomitant protein levels.
*mir*Vana™ miRNA Isolation Kit P/N AM1560	The *mir*Vana miRNA Isolation Kit (patent pending) is designed especially for the isolation of small RNAs, such as microRNA (miRNA), small interfering RNA (siRNA), and small nuclear RNA (snRNA), from tissues and cells. The kit uses a fast and efficient glass fiber filter (GFF) based procedure to isolate total RNA ranging in size from kilobases down to 10-mers. It also includes a procedure to enrich the population of RNAs that are 200 bases and smaller, which enhances the sensitivity of small RNA detection by solution hybridization and Northern blot analysis.
*mir*Vana™ miRNA Detection Kit P/N AM1552	The *mir*Vana miRNA Detection Kit provides an extremely sensitive solution hybridization assay capable of detecting attomole amounts of RNA. In addition, it can be used to simultaneously detect several small RNAs such as miRNA and siRNA, or both small RNA and long RNA species in the same sample. For a complete solution for small RNA analysis, use this kit in conjunction with the *mir*Vana miRNA Probe Construction Kit and/or the *mir*Vana Probe & Marker Kit.

II. Planning and Preliminary Experiments

A. Web-based siRNA Design Resources

Current, detailed target site selection and hairpin siRNA template design information

Targeted gene silencing using siRNA is a rapidly evolving tool in molecular biology. The instructions in this booklet are based on both the current literature and on empirical data from scientists at Ambion. For an in-depth discussion of information gleaned from the current literature and from experiments performed at Ambion regarding hairpin siRNA stem length and loop design, as well as our most current general recommendations on hairpin siRNA template design, see the "siRNA Design Guidelines" Technical Bulletin on our website:

www.ambion.com/techlib/tb/tb_506.html

To use the Online siRNA Target Finder, go to:

www.ambion.com/techlib/misc/siRNA_finder.html

Web-based target sequence converter

The easiest way to design hairpin siRNA template oligonucleotides is to enter your siRNA target sequence into the web-based Insert Design Tool at the following address:

www.ambion.com/techlib/misc/psilencer_converter.html

B. siRNA Target Site Selection

1. For long-term studies, evaluate whether cells can survive when target gene expression is reduced

For long-term gene knockdown experiments, it is important to determine whether cells can survive and grow when expression of the target gene is eliminated or reduced. We recommend transfecting cells with siRNA generated by chemical synthesis, by in vitro transcription, or with plasmids transiently expressing hairpin siRNA targeting the gene. If a cell population with reduced levels of the target gene product remains viable, then long-term studies will likely be possible. Note that these experiments can be complicated by the limited replication potential of most normal (nontransformed) cell lines. Include a culture where cells are transfected with a negative control siRNA to validate your findings.

2. Find 3–4 potential 21 nt target site sequences in the target mRNA

Scanning downstream from the known start of transcription of your target mRNA, select a few 21 nucleotide sequences as potential siRNA target sites. Research at Ambion has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75–95% reduction. Use the following criteria to help choose target sequences:

- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
- Ambion researchers find that siRNAs with 30–50% GC content are more active than those with a higher GC content.
- Compare the potential target sites to the appropriate species' genome database and eliminate from consideration any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences. For this comparison, we suggest using BLAST, which can be found on the NCBI server at:
  www.ncbi.nlm.nih.gov/BLAST.

3. Negative controls

It is important to plan for negative controls when designing siRNA experiments. One commonly used control is an siRNA with the same base composition as the experimental siRNA, but which lacks sequence identity to genes expressed in the organism under study. Typically, a scrambled siRNA is used for these controls. The p*Silencer* 5.1 Retro Scrambled plasmid supplied with the kit contains an hairpin siRNA template sequence that lacks significant homology to the mouse, human, and rat genome databases. For many experimental systems, the p*Silencer* 5.1-U6 Retro Scrambled plasmid or the p*Silencer* 5.1-H1 Retro Scrambled plasmid can serve as a negative control.

C. Hairpin siRNA Template Oligonucleotide Design & Ordering

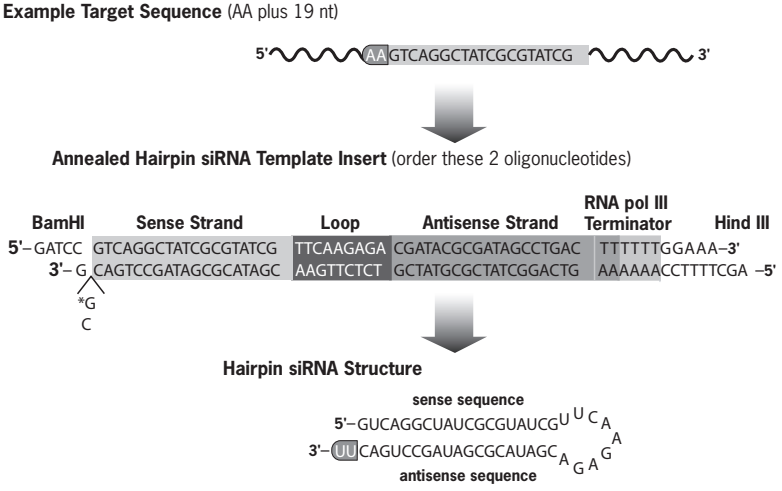
The 21 nt target sequence(s) will serve as a basis for the design of the two complementary 55-mer siRNA template oligonucleotides that are synthesized, annealed, and ligated into p*Silencer* 5.1 Retro for each target gene. The easiest way to design hairpin siRNA template oligonucleotides is to enter your siRNA target sequence into the web-based converter at the following address; otherwise these instructions describe how to design the siRNA template oligonucleotides:
www.ambion.com/techlib/misc/psilencer_converter.html

siRNA template oligonucleotide design instructions

Two complementary oligonucleotides must be synthesized, annealed, and ligated into the linearized p*Silencer* 5.1 Retro vector for each siRNA target site. Figure 8 shows schematically how to convert siRNA target sites into oligonucleotide sequences for use in the p*Silencer* 5.1 Retro vectors. The oligonucleotides encode a hairpin structure with a 19-mer stem derived from the mRNA target site. The loop of the hairpin siRNA is located close to the center of the oligonucleotides; a variety of loop sequences have been successfully used by researchers (Brummelkamp et al. 2002, Paddison et al. 2002, Paul et al. 2002, Lee et al. 2002, Sui et al. 2002), and we have observed no particular benefit in using one or

another. The loop sequence shown in Figure 8, 5'-UUCAAGAGA-3', is one possible sequence. Near the end of the hairpin siRNA template is a 5–6 nucleotide poly(T) tract recognized as a termination signal by RNA pol III that will terminate siRNA synthesis. The function of the 5'-GGAA-3' just downstream of the RNA pol III terminator site is not fully understood, but we recommend that it be included for optimal gene silencing. The 5' ends of the two oligonucleotides are noncomplementary and form the BamH I and Hind III restriction site overhangs that facilitate efficient directional cloning into the p*Silencer* 5.1 Retro vectors. Just downstream of the BamH I site, it is advantageous to have a G or an A residue because RNA pol III prefers to initiate transcription with a purine. For siRNA targets with a C or a U residue at position 1 (the first nucleotide after the AA in the RNA target sequence), add an additional G (shown with an asterisk in Figure 8) to facilitate transcription of the siRNA by RNA pol III.

Figure 8. Example siRNA Template Oligonucleotide Design



*Include an additional GC base pair at this position ***only*** if the downstream base on the top strand (the +1 position of the siRNA) is a T or a C; if the +1 position is a G or an A, as it is in this example sequence, do not include it. This additional base pair provides a G or an A residue as the first nucleotide of the siRNA transcript. Because RNA pol III prefers to initiate transcription with a purine, it helps to facilitate efficient transcription. Note, this additional nucleotide will not be complementary to either the target mRNA or the antisense strand of the hairpin siRNA, but it appears to have no effect on the activity of the hairpin siRNA.

Ordering the siRNA template oligonucleotides

Order a 25–100 nM scale synthesis of each oligonucleotide. Typically we use economical, desalted-only DNA oligonucleotides in this procedure. It is important, however, that the oligonucleotides are mostly full-length. Choose a supplier that is reliable in terms of oligonucleotide sequence, length, and purity.

D. Optimizing Antibiotic Selection Conditions

Negative controls

Using appropriate negative controls for antibiotic selection is required in order to obtain valid and interpretable results. Transfected or retroviral-infected cells will respond differently to antibiotic selection than untreated cells. Basic general information on negative controls for transfection and infection with p*Silencer* 5.1 Retro constructs is provided here.

Negative controls for transfection

When transfecting plasmid DNA, include a mock-transfected culture using either a transfection mixture that does not include DNA or includes a plasmid lacking a puromycin resistance gene instead of the p*Silencer* 5.1 Retro construct. Place the mock-transfected cells under identical puromycin selection as the experimental cultures. When the mock-transfected cells die, the selection is complete.

Negative controls for retroviral infection

As you infect a packaging cell line to prepare retroviral supernatant for your experiments, also prepare a mock viral supernatant as a negative control. Similar to the negative control for transfection, to make a mock viral supernatant, "transfect" the packaging cell line with transfection agent, and either no DNA, or a nonviral DNA plasmid. Collect the media and use it to mock infect cells. Culture both experimental cells, and cells infected with the mock viral supernantant, under identical conditions with puromycin selection. Gauge the puromycin resistance of the experimental cells against the response of cells infected the mock viral supernatant. Mock viral supernatant provides a better negative control for puromycin selection than mock-infecting with untreated packaging cell supernatant.

Overview of optimization of antibiotic selection

Cell type, infection with retrovirus, transfection with plasmid, cell health, culture medium, and growth conditions can all affect the optimal puromycin concentration for selection of p*Silencer* 5.1 Retro-transfected or -infected cells. Identify the lowest level of puromycin that kills mock-transfected or -infected cells within approximately 2–3 days by testing puromycin concentrations from 50–4000 ng/mL while keeping all other culture conditions equal. See "*Puromycin titration (kill curve)*" below.

(optional) Using this optimum puromycin concentration, optimize cell plating density. See "*(optional) Optimal plating density*" below. Plating density can have a strong impact on antibiotic selection because cells growing at higher densities are less effectively killed than cells growing at lower densities. Also, cells that divide more rapidly typically have a lower optimal plating density than cells with a slower doubling rate.

Puromycin titration (kill curve)

a. Plate 20,000 cells into each well of a 24 well dish containing 1 mL of culture medium.

b. After 24 hr, discard the culture media and add 1 mL culture medium containing 50–4000 ng/mL puromycin.

c. Culture the cells for 3–5 days, replacing the puromycin-containing medium every 3 days.

d. Examine the dishes for viable cells every day.

e. Identify the lowest puromycin concentration that begins to give massive cell death in approximately 3–5 days, and kills all cells within 2 weeks. Use this puromycin concentration to select cells that were infected with retrovirus made from p*Silencer* 5.1 Retro (or that were transfected with the plasmid construct).

(optional) Optimal plating density

a. Plate several different amounts of cells into separate wells of a 24 well dish containing 1 mL of culture medium.

b. After 24 hr, discard the culture media and add 1 mL culture medium culture medium containing puromycin; use the concentration identified in the previous experiment.

c. Culture the cells for 3–5 days, replacing the puromycin-containing medium every 3 days.

d. Identify the cell plating density that allows the cells to reach 80% confluency before massive cell death begins and use it to plate cells for puromycin selection.

III. Cloning and Preparation of Recombinant Retrovirus

A. Cloning into p*Silencer* 5.1 Retro Vectors

1. Prepare a 1 µg/µL solution of each oligonucleotide

a. Dissolve the hairpin siRNA template oligonucleotides in approximately 100 µL of nuclease-free water.

b. Dilute 1 µL of each oligonucleotide 1:100 to 1:1000 in TE (10 mM Tris, 1 mM EDTA) and determine the absorbance at 260 nm. Calculate the concentration (in µg/mL) of the hairpin siRNA oligonucleotides by multiplying the A_{260} by the dilution factor and then by the average extinction coefficient for DNA oligonucleotides (~33 µg/mL).

c. Dilute the oligonucleotides to approximately 1 µg/µL in TE.

2. Anneal the hairpin siRNA template oligonucleotides

a. Assemble the 50 µL annealing mixture as follows:

Amount	Component
2 µL	sense siRNA template oligonucleotide
2 µL	antisense siRNA template oligonucleotide
46 µL	1X DNA Annealing Solution

b. Heat the mixture to 90°C for 3 min, then place in a 37°C incubator, and incubate for 1 hr.

c. The annealed hairpin siRNA template insert can either be ligated into a p*Silencer* 5.1 Retro vector immediately or stored at –20°C for future ligation.

3. Ligate annealed siRNA template insert into the p*Silencer* 5.1 Retro vector

a. Dilute 5 µL of the annealed hairpin siRNA template insert with 45 µL nuclease-free water for a final concentration of 8 ng/µL.

b. Set up two 10 µL ligation reactions; a plus-insert ligation, and the minus-insert negative control. To each tube, add the following reagents:

Plus-insert	Minus-insert	Component
1 µL	– –	diluted annealed siRNA insert (from step 3.a)
– –	1 µL	1X DNA Annealing Solution
6 µL	6 µL	Nuclease-free Water
1 µL	1 µL	10X T4 DNA Ligase Buffer
1 µL	1 µL	p*Silencer* 5.1 Retro vector
1 µL	1 µL	T4 DNA ligase (5 U/µL)

c. Using Ambion T4 DNA ligase (P/N AM2134), incubate for 1–3 hr at room temp (the reactions can be incubated overnight at 16°C if very high ligation efficiency is required).
The recommended incubation time and temperature for ligation reactions varies widely among different sources of T4 DNA ligase. Follow the recommendation provided by the manufacturer of your DNA ligase, if using a source other than Ambion.

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

a. Transform an aliquot of cells with the plus-insert ligation products, and transform a second aliquot with the minus-insert ligation products. Use an appropriate amount of ligation product according to how the competent cells were prepared and the transformation method.
(For chemically competent cells, we routinely transform with 3 µL of the ligation reaction.)

b. Plate the transformed cells on LB plates containing 50–200 µg/mL ampicillin or carbenicillin and grow overnight at 37°C. Generally it is a good idea to plate 2–3 different amounts of transformed cells so that at least one of the plates will have distinct colonies.
Always include a nontransformed competent cell control: this negative control is a culture of your competent cells plated at the same density as your transformed cells.

c. Examine each plate and evaluate the number of colonies promptly after overnight growth at 37°C (or store the plates at 4°C until they are evaluated).

5. Expected results

Non-transformed control culture:
The nontransformed control culture should yield no colonies (indicating that the antibiotic in the culture medium is effective at inhibiting the growth of *E. coli* that do not contain the p*Silencer* 5.1 Retro vector).

Plus- and minus-insert ligation transformations
Identify the dilution of plus- and minus-insert ligation transformations that yield well-spaced (countable) colonies, and count the colonies on those plates. The minus-insert ligation will probably result in some ampicillin resistant colonies (background), but ***the plus-insert ligation should yield 2–10 fold more colonies than the minus-insert ligation***. (Remember to take the dilution into account when calculating the proportion of background colonies.)

6. Identify clones with the hairpin siRNA insert

Pick clones, isolate plasmid DNA, and digest the plasmid with BamH I and Hind III to confirm the presence of the ~55 bp siRNA insert.

We strongly recommend sequencing the insert with the following primer to confirm that there are no unwanted mutations:

5'-TTGTACACCCTAAGCCTC -3'

Links to the p*Silencer* 5.1 Retro restriction enzyme maps and the entire plasmid sequences are provided at this address:
www.ambion.com/catalog/CatNum.php?5782

7. Purify p*Silencer* 5.1 Retro plasmid for transfection

p*Silencer* 5.1 Retro plasmid preparations must be free of salts, proteins, and other contaminants to ensure efficient transfection. We routinely purify using commercially available plasmid purification products.

B. Strategies and Controls for Producing Recombinant Retrovirus

Once you have a confirmed p*Silencer* 5.1 Retro clone containing your hairpin siRNA template and you have purified transfection quality plasmid DNA, we recommend transfecting it into a retroviral packaging cell line to prepare infectious recombinant retrovirus (see Figure 4 on page 6). Alternatively, the plasmid can be transfected directly into experimental cells without making retrovirus, this is discussed in section III.G on page 27. Compared to transfecting your p*Silencer* 5.1 Retro clone directly into experimental cells, preparing recombinant retrovirus has the advantages of a higher rate of uptake by a wide range of cell types, and a greater likelihood that the hairpin siRNA template will be stably integrated into the genome of the recipient cells.

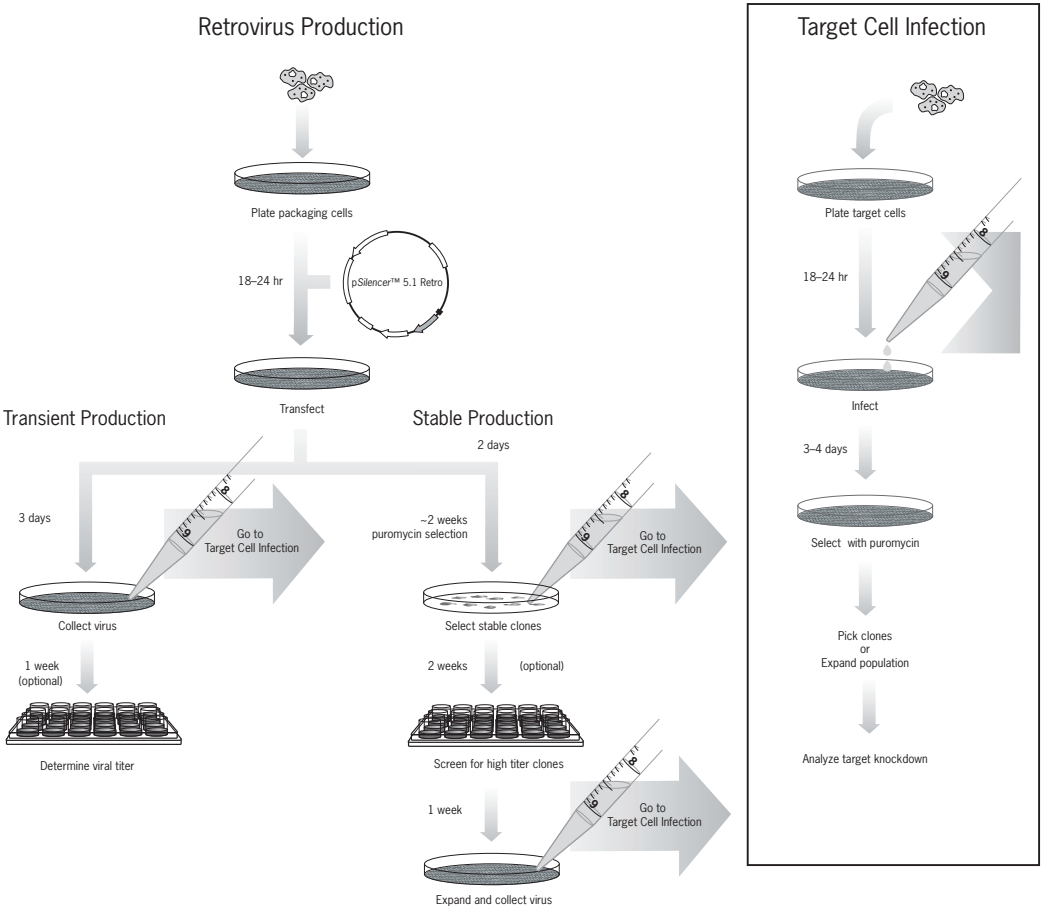
Specifically engineered retroviral packaging cell systems are used to make recombinant retrovirus from retroviral vectors. These systems often include cell lines that contain the viral gag, pol, and env genes needed for viral particle formation and replication. In some retroviral packaging systems, these viral genes are carried on plasmids and are introduced into the packaging cell line by cotransfecting them with the retroviral vector plasmid. Different retroviral packaging systems have been manipulated to viral particles with different tropisms or host ranges. The p*Silencer* 5.1 Retro vectors can be packaged using any typical retroviral packaging system; base your choice on the cell type you want to infect and on convenience.

Transient or stable retrovirus production

Figure 9 shows the typical process of producing recombinant retrovirus transiently, and it also shows how puromycin selection can be used to create a stable packaging cell line that constitutively expresses the recombinant retrovirus. Transient virus production is rapid and reliable, but produces only a limited amount of recombinant retrovirus. Selection of a stable packaging cell line can be very useful for ongoing gene silencing experiments, but is more time consuming, and may be hindered by the insert cloned into the p*Silencer* 5.1 Retro vector. In theory, siRNAs that are active in your target cells should also be active in the packaging cell line and this can impede the production of a stable packaging cell line. One strategy to deal with this problem is to use a pack-

aging cell line from a different species than that targeted by the siRNA so that the hairpin siRNA produced cannot recognize its target in the packaging cell line. For example if a human cell line is the experimental target, then design a hairpin siRNA template insert that cannot target the homologous mouse gene, and use a mouse cell derived packaging cell system to prepare a stable packaging cell line.

Figure 9. Production of Infectious Recombinant Retrovirus



Negative controls

Mock viral supernatant

It is important to include a mock transfection as a negative control for puromycin selection. For this control, transfect packaging cells with either transfection agent only (no DNA) or with a nonviral

plasmid instead of a retroviral vector. This culture provides a negative control for antibiotic selection of packaging cells if you choose to prepare a stable packaging cell line.

It also serves as a source of mock viral supernatant as a negative control for infection of your target cells. Cells infected with the mock viral supernantant serve as a control for puromycin selection after viral infection. When the cells infected with mock viral supernatant are dead, it indicates that the selection with puromycin is complete, and any recombinant retrovirus-infected cells that survive can be expanded and evaluated for gene knockdown.

p*Silencer* 5.1 Retro Scrambled

You will also want to transfect packaging cells with the p*Silencer* 5.1 Retro Scrambled plasmid supplied with the kit; the scrambled negative control retrovirus produced from this transfection will serve as an important control for RNAi studies.

C. Transient Production of Recombinant Retrovirus

Transient virus production is the fastest approach to making replication-incompetent retrovirus and is the method we routinely use at Ambion. Here we provide general instructions for transient production of retrovirus. This transfection protocol typically produces enough viral supernatant for infection of 3 wells of a 24 well plate; it can be scaled up if more viral preparation is needed. You may also need to modify this procedure according to the transfection agent and packaging cell system used.

1. Plate packaging cells, and 1 day later transfect with 2 µg plasmid DNA

a. Plate 4 x 10^5 packaging cells per well in a 6 well dish 1 day before transfection.

b. Transfect retroviral packaging cells with 2 µg of purified p*Silencer* 5.1 Retro per well using standard lipid based transfection (e.g. using Ambion siPORT™ *XP-1* transfection agent). You will also want to transfect separate cultures of packaging cells with the p*Silencer* 5.1 Retro Scrambled plasmid supplied with the kit; the scrambled negative control retrovirus produced from this transfection will serve as an important control for RNAi studies.

2. Add ~3 mL media per well 4 hr after transfection, then grow cells for 3 days

4 hours after transfection, we routinely add ~3 mL of culture medium to the cells in each well. Then, culture the cells for an additional 3 days without changing the media. During this time, your infectious retrovirus will be secreted from the packaging cells into the culture media.

CAUTION

The cells should be handled very carefully as they are now secreting recombinant retrovirus that can infect mammalian cells.

3. Collect and filter the media which contain retrovirus

On day 3, collect the media and sterile filter it to remove cellular debris and contaminating packaging cells. We typically use a sterile 0.45 µm cellulose acetate or polysulfonic (low protein binding) syringe filter. Do not use nitrocellulose filters, because nitrocellulose binds proteins in the retroviral membrane and destroys the virus.

NOTE

We tested supernatants 1, 2, and 3 days after transfection, and we found that supernatants from days 2 and 3 gave the best infectivity of HeLa cells.

- Use the filtered media directly to infect cells
- Store filtered media at 4°C for up to 2 weeks
- Aliquot and store filtered media at –80°C for periods >2 weeks

Note that virus viability is thought to decrease over time so use retroviral supernatants as soon as possible after collecting them for the highest titers. Also, avoid freeze-thawing retroviral preparations, because it will reduce their titer.

D. Production of Cell Lines That Stably Produce Your Recombinant Retrovirus

These instructions are intended to be general guidelines for transfection and selection of packaging cells to produce a cell line that stably produces recombinant retrovirus. You may need to modify this procedure according to the specific transfection agent and packaging cell system used.

1. Plate packaging cells, and 1 day later transfect with 2 µg plasmid DNA

a. Plate 4 x 10^5 packaging cells per well in a 6 well dish 1 day before transfection.

b. Transfect retroviral packaging cells with 2 µg of purified p*Silencer* 5.1 Retro per well using standard lipid based transfection (e.g. using Ambion siPORT™ *XP-1* transfection agent).
See section III.B on page 21 for a discussion on appropriate controls.

2. Incubate overnight

Incubate overnight without changing the media.

CAUTION

This packaging cell line should be handled very carefully as it is now secreting recombinant retrovirus that can infect mammalian cells.

3. Add fresh media and culture cells for 1 day

Remove the media and add fresh normal culture media. Allow the cells to recover by culturing them for another day without puromycin selection.

4. Culture cells under puromycin selection until the mock-transfected cultures are dead

a. Remove the media and replace with media containing the puromycin concentration identified as optimal for the cell line in the experiment described in section II.D. on page 18.

b. Continue to culture the cells under puromycin selection, and check the growth of the mock-transfected cells to determine when the antibiotic selection is complete. Once the cells that did not receive the plasmid are dying or killed, selection of cells that were successfully transfected should be complete. At this point, the retrovirus-producing cells can be expanded to collect recombinant retrovirus. If your experiments demand high titer retrovirus preparations, you can also isolate clones and identify clonal cell lines that produce maximum titer.

5. Passage cells and grow for 2–3 more days without puromycin selection

To collect virus-containing supernatant, plate cells and grow to ~80% confluence. Then remove the culture medium, and replace it with 3 mL (minimal amount) of fresh culture medium lacking puromycin, and incubate for 2–3 days.

NOTE

If the medium needed to grow the packaging cells is different from the medium required by the cells you will infect, you can often collect virus in the medium required by the cells to be infected.

6. Collect and filter the media which contain retrovirus

Following the instructions in step III.C.3 on page 24, collect the culture medium, sterile filter it, and use it immediately or store it for later use.

E. Infecting Experimental Cells with Recombinant Retrovirus

1. Plate 3 wells in a 24 well plate for each infection 1 day before infection

We normally plate 30,000–40,000 cells per well into a 24 well dish 1 day (18–24 hr) before the infection. It is a good idea to have the target cells ready to infect on the day that recombinant retrovirus is collected from the packaging cells.

We recommend preparing 3 wells of a 24 well plate for each retrovirus so that infected cells can be selected using high, medium, and low puromycin concentrations. For example, if your pilot experiment (section II.D. on page 18) identified 2 µg/mL puromycin as the ideal concentration for selection, you might use 1 µg/mL, 1.5 µg/mL, and 2 µg/mL puromycin for selection. This helps to compensate for compromised cell viability from the infection process and variability in the culture and health of the infected cells.

2. Infect cells by replacing their media with 1 mL of viral prep containing 2–4 µg polybrene

On the day of the infection, remove the growth media from the cells and replace it with 1 mL of viral preparation containing 2–4 µg/mL polybrene. Polybrene promotes infection by preventing electrostatic repulsions that can occur between the virus and the cell membrane.


NOTE

If the culture medium for the cells you are infecting is significantly different than the culture medium in which retrovirus was collected, you may want to dilute your retroviral prep 50% with the medium favored by the cells to be infected.

3. Culture cells for 2–3 days

Culture the cells for 2–3 days in the infection media.

4. Change the media and culture the cells for 1 day

Change the culture media, and culture the cells for 1 day (24 hr) to allow them to recover from the infection.

5. Replace the media with puromycin-containing media

Change the culture media again, this time adding media containing a high, medium, or low concentration of puromycin. Grow the cells under puromycin selection until the cells infected with the mock viral supernatant are dying or killed. This indicates that antibiotic selection is complete, and cells that have survived can be grown without selection.

6. Evaluate knockdown and (optional) expand and/or clone cells

After antibiotic selection is complete, the cells can be harvested for knockdown analysis. Many researchers will also want to expand and freeze a portion of their infected cell line or plate the infected cells to isolate clonal cell lines with the desired hairpin siRNA effect.

F. Determining Viral Titer

It may be useful to determine the viral titer of a retrovirus preparation to confirm that the preparation is viable, or to identify specific infection conditions for particular cell types by adjusting the MOI. (MOI is an acronym for multiplicity of infection, it is the number of virus particles per target cell.) Since the hairpin siRNA templates in your experimental p*Silencer* 5.1 Retro constructs may inhibit cell growth, each construct may result in viral preparations with different viral titers.

1. Plate two 6 well plates with 0.5–1 x 10^5 cells per well, and culture for 1 day

Plate two entire 6 well plates (12 wells) of each cell type at a density of 0.5–1 x 10^5 cells per well, and grow for 1 day (18–24 hr).

Standard practice is to use NIH 3T3 cells to determine retroviral titer, but it may be more meaningful to titer the retrovirus using your experimental cells because the infectivity may vary in different cell types. If possible, it is ideal to titer your viral prep with both your experimental cells and 3T3 cells.

2. Prepare five 10-fold dilutions of retrovirus and add polybrene to 4 µg/mL

Prepare five 10-fold dilutions of the filtered virus-containing medium in normal growth medium appropriate for the cells to be infected, and add polybrene to a final concentration of 2–4 µg/mL. You will need 3 mL of each dilution for each well of a 6 well plate to be infected.

3. Infect cells by replacing their media with 3 mL of the diluted retrovirus

On the day of the infection, remove the growth media from the cells and replace it with 3 mL of viral preparation from the previous step.

4. Culture the cells as described in steps 3–5 on page 25

Follow the instructions in steps III.E.3–5 on page 25 to culture the cells after infection with retrovirus.

5. Count colonies on the plate infected with the highest dilution of virus prep to calculate the viral titer

After antibiotic selection, the viral titer corresponds to the number of colonies on the plate infected with the most dilute viral prep that yielded colonies, multiplied by the dilution factor. For example, if the plate that was infected with the 10^5 dilution had no colonies, and the plate infected with a 10^4 dilution had 4 colonies growing on it, this would represent a viral titer of 4 x 10^4 colony forming units (cfu) per mL or 4 x 10^4 cfu/mL.

G. Transfecting p*Silencer* 5.1 Retro Plasmid into Mammalian Cells

p*Silencer* 5.1 Retro vectors can alternatively be used to transfect mammalian cells that are not designed for production of recombinant retrovirus. We recommend using Ambion siPORT *XP-1* transfection agent (P/N AM4506, AM4507) to deliver p*Silencer* 5.1 Retro plasmid constructs into mammalian cells with high efficiency and minimal toxicity. Follow the instructions for using siPORT *XP-1* provided with the product.

1. Transfect cells and culture 24 hr without selection

Transfect the purified plasmid into the desired cell line, plate transfected cells at the plating density identified in step II.D. on page 18, and culture for 24 hr without selection.

It is important to include two non-transfected control cultures. One is subjected to puromycin selection to control for the fraction of cells that survive selection; it will help determine the effectiveness of the transfection and selection. The second control is grown without puromycin selection as a positive control for cell viability.

2. Add medium containing puromycin

Add culture medium containing the concentration of puromycin identified in step II.D. on page 18.

IV. Troubleshooting

A. Positive Control Ligation

1. Description of the Cyclo Control Insert

The Cyclo Control Insert (80 ng/µL) is a double-stranded DNA fragment with BamH I and Hind III sticky ends surrounding an siRNA template that targets the cyclophilin A mRNA. The sequence of the Cyclo Control Insert is perfectly complementary to a region of human cyclophilin mRNA. The hairpin siRNA expressed from this template sequence has been shown to effectively induce silencing of cyclo in human cell lines. The Cyclo Control Insert is provided as a control for the ligation reaction.

2. Ligation instructions

a. Dilute 2 µL of the Cyclo Control Insert with 18 µL nuclease-free water.

b. Ligate 1 µL of the Cyclo Control Insert into p*Silencer* 5.1 Retro using the standard protocol beginning with step III.A.3 on page 19.

3. Expected result of the positive control ligation and *E. coli* transformation

If the ligation reaction and subsequent *E. coli* transformation procedure are functioning properly, then the ligation reaction with the Cyclo Control Insert (the plus-insert reaction) should provide 2–10 times as many colonies as the minus-insert ligation reaction.

B. Using the Positive and Negative Controls

p*Silencer* 5.1 Retro Scrambled

Negative control for RNAi

For any RNAi experiment, it is important to include a culture that is infected with a negative control retrovirus as a basis for analysis of gene knockdown. The p*Silencer* 5.1 Retro Scrambled plasmid supplied with the kit is a circular plasmid encoding a hairpin siRNA whose sequence is not found in the mouse, human, or rat genome databases. It is provided ready-to-transfect at 0.5 µg/µL, and the retrovirus produced, can be used to control for the effects of introducing your recombinant p*Silencer* 5.1 Retro retrovirus into cells.

Positive control for antibiotic resistance in mammalian cells

The p*Silencer* 5.1 Retro Scrambled plasmid, and retrovirus produced with the plasmid can also be used to demonstrate puromycin resistance in mammalian cells conferred by p*Silencer* 5.1 Retro.

Positive Control construct containing the Cyclo Control Insert

The product of the positive control ligation (described in section IV.A above) is a p*Silencer* 5.1 Retro plasmid containing a hairpin siRNA template targeting cyclophilin A. This construct can be used to optimize

conditions for ligation, transfection, and infection. Use p*Silencer* 5.1 Retro-Cyclo and the p*Silencer* 5.1 Retro Scrambled plasmid to transfect packaging cells for production of the corresponding retroviruses.

When successfully infected and expressed, the cyclophilin hairpin siRNA reduces both the mRNA and protein levels of cyclophilin in HeLa, NHDF neo, and A549 cells. To assess whether siRNA-mediated gene silencing is occurring, either of the following assays for assessing siRNA-mediated reduction in cyclophilin gene expression can be used:

Quantitate mRNA levels by Northern analysis or RT-PCR.
Cyclophilin mRNA levels are typically reduced 50–90% after selection with puromycin is complete. We have used the following PCR primers in real-time RT-PCR to detect cyclophilin mRNA:
– Forward: 5'-AGACGCCACCGCCGA-3'
– Reverse: 5'-CTGACACATAAACCCTGGAATAATTC-3'

Analyze protein levels by Western blot, immunohistochemistry, or immunofluorescence.
Cyclophilin protein levels are typically reduced 50–90% 48 hr after selection with puromycin is complete. Ambion cyclophilin specific antibody (P/N AM4309) can be used to evaluate human, mouse, or rat cyclophilin protein levels.

C. Low *E. coli* Transformation Efficiency

1. Low quality competent cells

Cells could either be nonviable or exhibit low transformation competency. This can be tested by transforming a circular plasmid that has been used successfully in the past.

2. Poor ligation efficiency

If the ligation reaction (section III.A.3 on page 19) is inefficient, then there will be relatively few plasmids to transform. Possible causes of poor ligation efficiency include:

a. The concentration of the annealed siRNA template insert is lower than expected.
Evaluate ~5 µL of the insert DNA (from step A.2.c on page 19) using a 12% native polyacrylamide gel and compare its ethidium bromide staining to bands from a molecular weight marker or another standard of known concentration.

b. The ligase or ligase reaction buffer are inactive.
Test your ligation components using another vector and insert or replace your ligation components and retry the siRNA template insert cloning.

c. siRNA template oligonucleotide has high levels of non-full-length product.
If either or both of the oligonucleotides annealed to make the siRNA template insert contain high levels of non-full-length oligonucleotide, it can interfere with ligation. To determine if this is a problem, you can evaluate the size of the oligonucleotides on a 12% native polyacrylamide gel.

d. The oligonucleotide annealing reaction was ineffective.
A low concentration of one of the oligonucleotides or incomplete denaturation of individual oligonucleotides could have reduced the relative amount of dsDNAs.

Compare the annealed siRNA template insert to each of the single-stranded oligonucleotides using native 8–12% polyacrylamide gel electrophoresis. If the annealed siRNA template insert has bands corresponding to the single-stranded oligonucleotides, then adjusting the concentrations of the single-stranded DNA molecules and heat-denaturing at a higher temperature during siRNA insert preparation (step A.2.b on page 19) might improve the percentage of dsDNA products.

e. Ligation inhibitors in the oligonucleotide preparations
EDTA and high concentrations of salts or other small molecules can inhibit ligation efficiency. Ethanol precipitate the oligonucleotides prior to using them in the cloning procedure (either before or after annealing).

f. Incompatible ends on the insert
Verify that the sequences of the hairpin siRNA template oligonucleotides include 5' BamH 1 and 3' Hind III overhanging sequences for cloning (see Figure 8 on page 16).

3. Too much antibiotic or the wrong antibiotic in the media

The plates used for cloning should contain 50–200 µg/mL ampicillin or carbenicillin. Carbenicillin remains active in plates for longer than ampicillin.

4. Cells were handled poorly

Competent cells tend to be fragile, so handle them gently throughout the transformation and plating process.

D. Equal Numbers of *E.coli* Colonies from Minus- and Plus-Insert Ligation Transformations

1. Ligation efficiency for the siRNA insert is low

See section C.2 on page 29.

2. The concentration or activity of the ampicillin is too low or high

If there are large numbers of clones derived from both ligations, then confirm that the ampicillin is active and at 50–200 µg/mL in the medium. If there are low numbers of clones for each, try transforming a

plasmid with an ampicillin resistance gene (the p*Silencer* 5.1 Retro Scrambled plasmid in the kit would be ideal) and confirm that the ampicillin concentration in the plates is not too high to allow the growth of transformed cells.

E. Troubleshooting Retrovirus Production

Problems with transfection of p*Silencer* 5.1 Retro into packaging cells

Good transfection of the packaging cell line is fundamentally important for production of recombinant retrovirus. If you suspect that p*Silencer* 5.1 Retro transfection into your packaging cell line is suboptimal, consider using a mammalian expression plasmid containing a reporter gene such as GFP or β-galactosidase to troubleshoot transfection. Below are listed some general suggestions for troubleshooting mammalian cell transfection.

p*Silencer* 5.1 Retro plasmid is not pure enough

The purity of the siRNA plasmid is vitally important for efficient transfection. Repurify plasmid preparation and transfect again.

Transfection protocol requires optimization

The ratio of transfection agent to cells to plasmid is important. Optimize these three components of the transfection protocol.

Ineffective transfection reagent

If you are using lipofection to facilitate transfection, then test a different transfection agent with your cells. Different cell types respond differently to different transfection reagents.

Problems with puromycin selection of a stable cell line expressing your retrovirus

If no transfected cells, or only a few transfected cells survive puromycin selection, see the troubleshooting suggestions for puromycin selection in the following section.

F. Troubleshooting Infection of Cells With your Retrovirus and Puromycin Selection

The viral titer is low or virus is non functional

If the virus has been treated harshly the titer can drop. For example, avoid freeze thawing retroviral preparations, and always use fresh retrovirus if possible.

- To partially compensate for low viral titer preps, you can infect a single culture multiple times by simply adding more viral prep to cells after they have already been infected. Multiple rounds of infection can increase the number of cells that become infected.
- For some applications, such as infection of cells in vivo (and even infection of cells in vitro), it may be necessary to concentrate retroviral stocks to increase their titer. This is typically necessary because only a limited volume of material can be injected at any one site.

Because viruses are macromolecular structures, they can be concentrated fairly easily by a relatively short centrifugation step. Retroviral particles can either be pelleted or centrifuged using a sucrose density step gradient. Alternatively, viral particles can be precipitated using polyethylene glycol or ammonium sulfate, and the collected by centrifugation. Perhaps most simple of all, small volumes of virus stock can be concentrated by centrifugation through a filter that allows only small molecules to pass. Protocols for several of these procedures can be found in *Current Protocols in Molecular Biology* (Ausubel et al. ed. 1994).

Use polybrene in the infection procedure

Polybrene is required for efficient infection by reducing electrostatic repulsion.

Puromycin selection is too harsh

Set up infections to include a few different concentrations of puromycin as described in section III.E.1 on page 25.

Alternatively, you may consider using the following less stringent antibiotic selection protocol:

- Incubate the culture with puromycin selection until only ~50% of the cells are killed.
- Then add fresh medium lacking puromycin and incubate the culture for 24–48 hr without puromycin selection.
- Next add puromycin-containing culture medium, and culture the cells until ~50% have died a second time.
- Repeat this cycle until colonies are visible. Always include a control where cells that were infected with a mock viral supernatant are grown under the same puromycin selection regimen. Although it occurs at a very low frequency, cells do spontaneously become resistant to antibiotics, so include a negative control culture so that you can determine the effectiveness of the infection and the antibiotic selection.

Packaging cell line was not efficiently transfected with the viral plasmid DNA

Make sure the DNA transfection procedure is working by using a reporter plasmid transfected into the packaging cell line.

The siRNA target may be essential for survival

If the siRNA target is essential for survival, cells infected with retrovirus that express a hairpin siRNA that effectively reduces expression of the target gene may die. To test whether the target gene is essential for survival, infect cells with the p*Silencer* 5.1 Retro virus prep containing your siRNA template, and culture transduced cells without antibiotic selection. If significant cell death occurs, it is likely that the siRNA target is important for cell growth and metabolism.

Cells become contaminated after adding the puromycin

The puromycin may be contaminated. Puromycin solutions can be filter sterilized or purchased as sterile reagents. To prepare antibiotic solutions in the lab, use sterile reagents to resuspend antibiotics.

Cells infected with the mock viral supernantant survive selection

a. The puromycin concentration is not high enough.
A careful dose response experiment should be performed to determine the concentration that kills cells lacking a puromycin resistance gene. This is described in section *II.D. Optimizing Antibiotic Selection Conditions* starting on page 17. The amount of time required to completely kill the cells should also be recorded, and this concentration and time should be used for each experiment.

b. Cell density is too high.
If the cells are too crowded, they may not be killed effectively. Split cultures that are too close to confluency for good antibiotic selection. On the other hand, low cell density cultures typically grow slowly, and may be more sensitive to antibiotics than higher cell density cultures of the same cell line.

c. The puromycin may be inactive.
- At 37°C, puromycin is stable for only a few days, therefore puromycin-containing culture media must be replenished accordingly to apply selective pressure.
- Consider purchasing a new batch of antibiotic or preparing a fresh solution of puromycin.

V. Appendix

A. References

Ausubel, FM, Brent, R., Kingdom RE, Moore DM, Seibman JG, Smith JA, and Struhl K (1994) Current Protocols in Molecular Biology. (Greene publishing Associates, Inc. & John Wiley & Sons, Inc.).

Brummelkamp TR, Bernards R, and Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* ***296***:550–553.

Byrom M, Pallotta V, Brown D, Ford L (2002) Visualizing siRNA in mammalian cells: fluorescence analysis of the RNAi effect. *Ambion TechNotes* **9(3):**6–8.

Coffin JM & Varmus HE, editors (1996) Retroviruses (Cold Spring Harbor Laboratory Press NY).

De la Luna S and Ortin J (1992) Pac gene as efficient dominant marker and reporter gene in mammalian cells. *Meth. Enzymol* **216**:376-385

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494–498.

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001b) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate, *EMBO J* **20(23)**: 6877–88.

Ford LP, Kelnar K, Beauchamp L (2005) Retroviral-mediated Delivery of siRNA: Effective Long-term, Stable Reduction of Gene Expression. www.ambion.com/techlib/tn/121/22.html.

Kunkel GR and Pederson T (1989) Transcription of a human U6 small nuclear RNA gene in vivo withstands deletion of intragenic sequences but not of an upstream TATATA box. *Nucleic Acids Res.* **17**: 7371–7379.

Lee NS, Dohjima T, Bauer G, Li H, Li M-J, Ehsani A, Salvaterra P, Rossi J (2001) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nature Biotechnology* **19**: 500–505.

Paddison PJ, Caudy AA, Bernstein E, Hannon GJ and Conklin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Development* : 948–958.

Paul CP, Good PD, Winer I, Engelke DR (2002) Effective expression of small interfering RNA in human cells. *Nature Biotechnology* : 505–508.

Sui G, Soohoo C, Affar EB, Gay F, Shi Y, Forrester WC, and Shi Y (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* **99(8)**: 5515–5520.

B. Quality Control

Functional testing

The p*Silencer* 5.1-U6 Retro and p*Silencer* 5.1-H1 Retro siRNA expression vectors are ligated with the Cyclo Control Insert according to the instructions in this booklet. Ligation efficiency is then determined.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.