p*Silencer*™ hygro Kit

(Part Number AM5760, AM5766)

Protocol

P/N 5760M Revision B **Revision Date: October 10, 2008**

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I. Product Description and Background

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 2001). Researchers in many disciplines employ RNAi to analyze gene function in mammalian cells. The siRNA used in early studies was typically prepared in vitro and transfected into cells. Later publications feature plasmids that express functional siRNA when transfected into mammalian cells (Sui 2002, Lee 2002, Paul 2002, Paddison 2002, Brummelkamp 2002). Using siRNA expression vectors can reduce the expression of target genes for weeks or even months (Brummelkamp 2002), whereas siRNA prepared in vitro and delivered by transient transfection typically knocks down gene expression for 6–10 days (Byrom 2002).

B. p*Silencer*™ hygro

The p*Silencer* vectors employ RNA polymerase III (pol III) promoters which generate large amounts of small RNA using relatively simple promoter and terminator sequences. They also include an antibiotic resistance gene that provides a mechanism to select for transfected cells that express the introduced DNA.

Mammalian promoters for siRNA expression

Ambion p*Silencer* 2.1-U6 hygro siRNA Expression vector features a human U6 RNA pol III promoter, and p*Silencer* 3.1-H1 hygro contains the H1 RNA pol III promoter. 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 2001).

Based on comparisons of several different RNA pol III promoters, the activities of the two promoters are likely to vary from cell type to cell type (Ilves 1996). The localization of expressed RNA is also likely to vary with cell type and with RNA pol III promoter (Ilves 1996). To optimize siRNA expression, we find it beneficial to clone hairpin siRNAs into both the p*Silencer* 2.1-U6 hygro and p*Silencer* 3.1-H1 hygro vectors and transfect them into the cells being targeted for gene knockdown. The promoter that is more effective for the siRNA and cell type will provide greater levels of gene silencing.

Mammalian Selectable Markers

The p*Silencer* hygro siRNA expression vectors contain a hygromycin resistance gene to enable antibiotic selection in mammalian cells. Antibiotic selection can be used to enrich cultures for cells that were successfully transfected with the siRNA expression vector by killing off cells that lack the plasmid. Short term antibiotic selection is very useful for experiment systems where low transfection efficiency would otherwise preclude detection of a reduction in target gene expression. For long-term gene knockdown studies, the hygromycin resistance gene makes it possible to select cell populations, or clonal cell lines, that stably express the siRNA.

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that inhibits protein synthesis by interfering with translocation and causing mistranslation by the 80S ribosome (Gritz and Davies 1983). The hygromycin resistance gene, hph^r phosphorylates and inactivates the antibiotic, hygromycin B, to confer antibiotic resistance to cells expressing it. A hygromycin B dose of 50–3000 µg/mL is typically optimal for selecting cells containing p*Silencer* hygro. Cells without the resistance gene are normally killed within ~5 days.

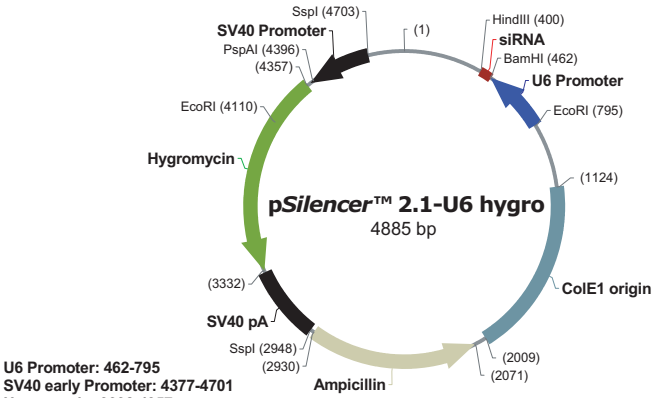
p*Silencer* plasmids are supplied ligation-ready

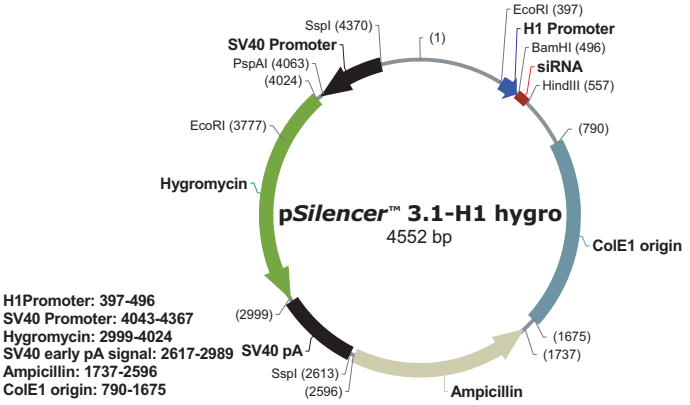
The p*Silencer* siRNA Expression Vectors are linearized with both *Bam*H 1 and *Hin*d III to facilitate directional cloning. They are purified to remove the digested insert so that it cannot re-ligate with the vector. This greatly increases the percentage of clones bearing the hairpin siRNA-coding insert after ligation, reducing the time and effort required to screen clones. Both p*Silencer* 2.1-U6 hygro and p*Silencer* 3.1-H1 hygro are linearized with the same restriction enzymes, so that a given hairpin siRNA insert can be subcloned into either vector using the 5' overhangs left by restriction enzyme digestion. A basic p*Silencer* hygrovector map is shown in Figure 1 on page 3; more detailed sequence information about the p*Silencer* vectors is available at:

http://www.ambion.com/catalog/CatNum.php?5760

Figure 1. p*Silencer* hygro vector map

(These maps show the vectors containing typical siRNA template inserts.)

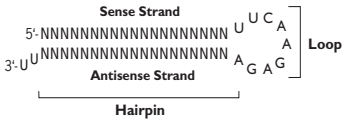




C. siRNA Template Design

The prototypical siRNA comprises two hybridized 21-mer RNA molecules with 19 complementary nucleotides and 3' terminal dinucleotide overhangs. Expression vectors with dual promoters that express the two strands of the siRNA separately can be used (Lee 2002), however, a more efficient scheme is to express a single RNA that is a 19-mer hairpin with a loop and 3' terminal uridine tract (Paddison 2002) (Figure 2). When expressed in mammalian cells, the short hairpin siRNA can

Figure 2. Hairpin siRNA



efficiently induce RNAi of the target gene (Brummelkamp 2002, Sui 2002, Paddison 2002). For cloning into an siRNA expression vector, hairpin siRNA inserts have the advantage that only a single pair of oligonucleotides and a single ligation are needed to generate plasmid for gene silencing studies. For each target gene, design complementary 55–60 mer oligonucleotides with 5' single-stranded overhangs for ligation into the p*Silencer* hygro vectors. The oligonucleotides should encode 19-mer hairpin sequences specific to the mRNA target, a loop sequence separating the two complementary domains, and a polythymidine tract to terminate transcription (this is discussed in section II.B on page 8).

Strategy for selection of siRNA target sites

The susceptibility of siRNA target sites to siRNA-mediated gene silencing appears to be the same for both in vitro prepared siRNAs and RNA pol III-expressed siRNAs. 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 an siRNA expressed from a p*Silencer* vector. If an siRNA target site has not already been identified, then we recommend that several different siRNAs be tested per gene. Once an effective target site is identified, oligonucleotides encoding hairpin siRNAs can be synthesized and ligated into p*Silencer* hygro siRNA Expression Vectors. This significantly reduces the time and effort required to develop an effective siRNA plasmid specific to a given gene.

D. Kit Components and Storage Conditions

Each p*Silencer* hygro siRNA Expression Vector includes 4 components:

- Linearized p*Silencer* hygro siRNA Expression Vector ready for ligation
- Circular, negative control p*Silencer* hygrovector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes
- GFP-specific, hairpin siRNA insert that can be used as a positive control for ligation
- 1X DNA Annealing Solution to prepare annealed DNA oligonucleotides for ligation into the p*Silencer* hygro vector

P/N AM5760	P/N AM5766	Component	
20 µL	– –	p*Silencer* 2.1-U6 hygro	–20°C
10 µL	– –	p*Silencer* 2.1-U6 hygro Negative Control (0.5 µg/µL)	–20°C
– –	20 µL	p*Silencer* 3.1-H1 hygro	–20°C
– –	10 µL	p*Silencer* 3.1-H1 hygro Negative Control (0.5 µg/µL)	–20°C
10 µL	10 µL	GFP Control Insert (80 ng/µL)	–20°C
1 mL	1 mL	1X DNA Annealing Solution	–20°C

E. Other Required Material

Ligation and transformation

- Two complementary oligonucleotides targeting the gene of interest for RNAi (design and ordering is discussed in section II starting on page 7)
- DNA ligase, ligase reaction buffer, and competent *E. coli* cells are needed to subclone the siRNA inserts.
- Ampicillin or carbenicillin containing plates and liquid media will also be needed to propagate the plasmids.

Plasmid purification

For efficient transfection into mammalian cells it is crucial that preparations of p*Silencer* be very pure.

Mammalian cell transfection reagents

The optimal mammalian cell transfection conditions including transfection agent and plasmid amount must be determined empirically.

Cell culture facility and supplies

In addition to routine cell culture media, culture media containing hygromycin will be needed for selection of p*Silencer* hygro-transfected cells.

F. 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 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.
KDalert™ GAPDH Assay Kit P/N AM1639	The KDalert GAPDH Assay Kit is a rapid, convenient, fluorescence-based method for measuring the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured human, mouse, or rat cells. The KDalert GAPDH Assay Kit facilitates identification of optimal siRNA delivery conditions by assessment of GAPDH expression and knockdown at the protein level and integrates seamlessly with the *Silencer*® CellReady siRNA Transfection Optimization Kit (P/N AM86050) and *Silencer* GAPDH Control siRNAs (P/N AM4605, AM4624).
RNase-free Tubes & Tips see our web or print catalog	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.

Silencer® siRNA Construction Kit P/N AM1620	The *Silencer* siRNA Construction Kit (patent pending) synthesizes siRNA by in vitro transcription, producing transfection-ready siRNA at a fraction of the cost of chemical synthesis. The *Silencer* siRNA Construction Kit includes all reagents for transcription, hybridization, nuclease digestion, and clean up of siRNA (except gene specific oligonucleotides for template construction).
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.
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.
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.

II. Planning and Preliminary Experiments

A. siRNA Target Site Selection

Using siRNA for gene silencing is a rapidly evolving tool in molecular biology; these instructions are based on both the current literature, and on empirical observations by scientists at Ambion. Because we are able to modify information on our web site so quickly (compared to printed documents), you may want to check the "siRNA Design" page on our web site for the latest recommendations on siRNA target selection.

http://www.ambion.com/techlib/misc/siRNA_design.html

1. Target gene selection

In order to conduct long term gene knockdown experiments it is important to first determine whether cells can survive and grow when the expression of the target gene is eliminated or reduced. We recommend transfecting cells with siRNA generated by chemical synthesis or in vitro transcription, or with plasmids transiently expressing siRNA targeting the gene. If a cell population with reduced levels of the target gene product remains viable, then long-term studies are likely to be possible.

2. Find 21 nt sequences in the target mRNA that begin with an AA dinucleotide

Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites.

This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. (EMBO 2001) that siRNA with 3' overhanging UU dinucleotides are the most effective. This is compatible with using RNA pol III to transcribe hairpin siRNAs because it terminates transcription at 4–6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail.

3. Select 2–4 target sequences

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. Choose target sites from among the sequences identified in step 2 based on the following guidelines:

- Since a 4–6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, avoid stretches of ≥4 T's or A's in the target sequence.
- 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.

- Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at:
  www.ncbi.nlm.nih.gov/BLAST.
- Ambion researchers find that siRNAs with 30–50% G/C content are more active than those with a higher G/C content.

4. Negative Controls

A complete siRNA experiment should include a nontargeting negative control siRNA with the same nucleotide composition as your siRNA but which lacks significant sequence homology to the genome. To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.

B. Hairpin siRNA Template Oligonucleotide Design & Ordering

Ambion web-based resources

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

Current, detailed hairpin siRNA template design information

Ambion Technical Bulletin #506 includes 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 recommendations on hairpin siRNA template design. Obtain it from the following address, or request it from our Technical Services Department.

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

Oligonucleotide design

Two complementary oligonucleotides must be synthesized, annealed, and ligated into the linearized p*Silencer* vector for each siRNA target site. Figure 3 on page 9 shows schematically how to convert siRNA target sites into oligonucleotide sequences for use in the p*Silencer* 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 (Sui 2002, Lee 2002, Paddsion 2002, Brummelkamp 2002, Paul 2002), and we have observed no particular benefit in using one or another. The loop sequence shown in Figure 3, 5'-UUCAAGAGA-3', is one possible sequence.

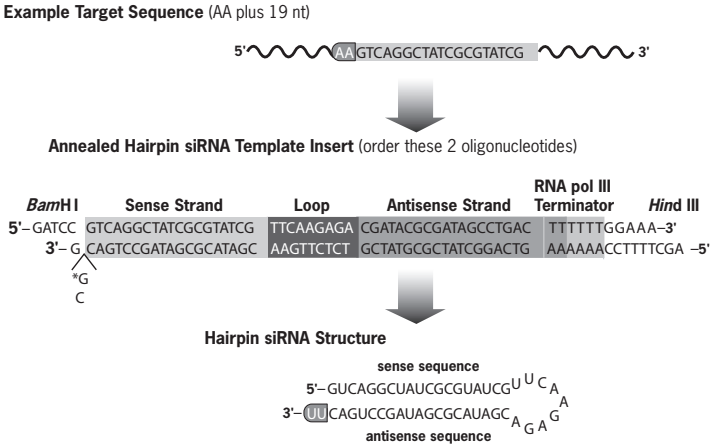
Near the end of the hairpin siRNA template is a 5–6 nucleotide poly(T) tract recognized as a termination signal by RNA pol III that will terminate siRNA synthesis. The function of the 5'-GGAA-3' just downstream of the RNA pol III terminator site is not fully understood, but we recommend that it be included for optimal gene silencing.

The 5' ends of the two oligonucleotides are noncomplementary and form the *Bam*H I and *Hin*d III restriction site overhangs that facilitate efficient directional cloning into the p*Silencer* vectors. Just downstream of the *Bam*H 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 3) to facilitate transcription of the siRNA by RNA pol III.

Synthesis of hairpin siRNA template oligonucleotides for ligation into p*Silencer* vectors

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. Contact Ambion Technical Services Department for an oligonucleotide supplier recommendation if you need one.

Figure 3. Hairpin siRNA Template Design



NOTE

The easiest way to design hairpin siRNA template oligonucleotides is to enter your siRNA target sequence into our web-based target sequence converter.

* 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. The purpose of this additional base pair is to provide 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, thus 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. This extra nucleotide in the sense strand appears to have no effect on the activity of the hairpin siRNA.

C. Optimizing Antibiotic Selection Conditions

Cell type, culture medium, growth conditions, and cell metabolic rate can all affect the optimal hygromycin concentration for selection of p*Silencer*-transfected cells. Identify the lowest level of hygromycin that kills nontransfected cells within approximately 5 days by testing hygromycin concentrations from 50–3000 µg/mL while keeping all other culture conditions equal. See step *1. Hygromycin titration (kill curve)* below.

Using this optimum hygromycin concentration, optimize cell plating density. See step *2. Optimal plating density* below. Plating density can have a strong impact on antibiotic selection because cells growing at higher densities are less effectively killed off than cells growing at lower densities. Also, cells that divide more rapidly typically have a lower optimal plating density than cells that double slowly.

1. Hygromycin 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, add 500 µL culture medium containing 50–3000 µg/mL hygromycin.

c. Culture the cells for 10–14 days, replacing the hygromycin-containing medium every 3 days.

d. Examine the dishes for viable cells every 2 days.

e. Identify the lowest hygromycin concentration that begins to give massive cell death in approximately 5–7 days, and kills all cells within 2 weeks. Use this hygromycinconcentration to select cells containing the p*Silencer* hygro plasmid after transfection.

2. 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, add 500 µL culture medium containing hygromycin; use the concentration identified in the previous experiment.

c. Culture the cells for 5–14 days, replacing the hygromycin-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 transfected with your p*Silencer* hygro clone.

III. Using the p*Silencer* siRNA Expression Vector

A. Cloning Hairpin siRNA Inserts into the p*Silencer* Vector

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* vector immediately or stored at –20°C for future ligation.

3. Ligate annealed siRNA template insert into the p*Silencer* 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* 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* hygro 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 siRNA template insert

Pick clones, isolate plasmid DNA, and digest with *Bam*H I and *Hin*d III to confirm the presence of the ~65 bp siRNA template insert.

At Ambion we sequence the insert to confirm that there are no unwanted mutations. We suggest using the following sequencing primers:

p*Silencer* 2.1-U6 hygro	5'-AGGCGATTAAGTTGGGTA-3'	5'-GTAATACGACTCACTATAGGG-3' T7 sequencing primer
p*Silencer* 3.1-H1 hygro	5'-GTTTTCCCAGTCACGAC-3' M13 forward (–40) sequencing primer	5'-GAGTTAGCTCACTCATTAGGC-3'

Links to the p*Silencer* hygro restriction maps and the entire plasmid sequence are provided at:
http://www.ambion.com/catalog/CatNum.php?5760

7. Purify p*Silencer* plasmid for transfection

p*Silencer* 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. Transfecting p*Silencer* Vectors into Mammalian Cells

We recommend using Ambion siPORT™ *XP-1* transfection agent (P/N AM4507) to deliver p*Silencer* plasmids into mammalian cells with high efficiency and minimal toxicity. Follow the instructions for using siPORT *XP-1* provided with the product. siPORT *XP-1* is a proprietary formulation of polyamines that can be used in the presence or absence of serum in the culture medium. It is suitable for the transfection of a wide variety of cell types.

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.C.2 on page 10, and culture for 24 hr without selection.

It is important to include two non-transfected control cultures. One is subjected to hygromycin 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 hygromycin selection as a positive control for cell viability.

2. Add medium containing hygromycin

Add culture medium containing the concentration of hygromycin identified in step II.C.1 on page 10.

C. Selecting Antibiotic-Resistant Transfected Cells

Once they are prepared, p*Silencer* siRNA expression vectors can be used in transient siRNA expression assays, or to create cell populations or a clonal cell line that stably expresses your siRNA. Note that with normal (nontransformed) and primary cell lines, it may be difficult to obtain clones that stably express siRNA. For these types of cells, we recommend choosing the antibiotic selection strategies outlined in sections 1 and 2 below.

1. Short term hygromycin selection for enrichment of cells that transiently express the siRNA

In experiments where the transfection efficiency is low, a rapid hygromycin selection can be used to kill cells that were not transfected with the p*Silencer* siRNA expression vector. This enrichment for transfected cells can be useful for reducing background when analyzing gene knockdown.

a. Culture the cells for 1–3 days in the hygromycin-containing medium (added in step B.2) to enrich the culture for cells that were successfully transfected.

b. Analyze the population for an expected phenotype and/or the expression of the target gene.

2. Selecting a population of cells that stably express the siRNA

Creating a population of cells stably expressing the siRNA involves treating cells with hygromycin for several days to eliminate cells that were not transfected. The surviving cell population can then be maintained and assessed for reduction of target gene expression.

a. Culture the cells in medium containing hygromycin (added in step B.2) until all of the cells in the non-transfected control culture are killed. At this point, the selection is complete and the cells can be grown without hygromycin until they repopulate the culture vessel.

b. Analyze expression of the target gene at any time after the cells in the non-transfected control culture have been killed.

c. Pool and passage hygromycin-resistant cell cultures as needed. It is a good idea to periodically grow the cells with a minimal level of antibiotic selection, to prevent the accumulation of cells that no longer express hygromycin resistance. Often this "minimal level" is about half the hygromycin concentration used to kill off nontranfected cells, but this value varies widely among different cell types.

3. Selecting for clones that stably express the siRNA

For many researchers, the goal is to create a clonal cell line that expresses the siRNA template introduced with the p*Silencer* vector. Cloning stably expressing cell lines is advantageous because strains that exhibit the desired amount of gene knockdown can be identified and maintained, and clones that are hygromycin-resistant but which do not express the siRNA can be eliminated.


NOTE

It is often difficult to obtain a stably expressing clone from normal (nontransformed) or primary cell lines using pSilencer siRNA expression vectors. If possible choose a transformed or immortal cell line instead.

Typically the levels of siRNA expression and gene knockdown vary widely among cells. In fact p*Silencer*-transfected cells that survive antibiotic selection may not have a significant reduction in expression of the target gene. Instead, they may have found a way to mitigate the effects of a reduction in the target gene expression by compensating in another fashion or by shutting down expression of the siRNA. To avoid this, it can be useful to isolate clones that can be screened to identify the cells that cause the desired reduction in target gene expression.

a. Culture the cells in medium containing hygromycin until all of the cells in the non-transfected control culture are killed. At this point, the selection is complete and the cells can be grown without hygromycin selection.

b. Pick clones:

   i. To pick clones, the cells must be plated at low enough density to grow into colonies without growing into one another. Dip sterilized cloning rings into sterile grease and then place one on top of each colony. Remove the cells that are within the cloning ring and transfer them to a fresh 96-well culture dish.

   ii. When the cells have grown to confluency in a well of a 96 well culture dish, move them to a well in a 24-well culture dish.

   iii. When the cells have grown to confluency in a well of a 24-well culture dish, split them, and grow them with a minimal level of antibiotic selection to prevent the accumulation of cells that no longer express hygromycin resistance. Often this "minimal level" is about half the hygromycin concentration used to kill off nontranfected cells, but this value varies widely among different cell types.

c. Assay individual clones for a reduction in the expression of the target gene.

IV. Troubleshooting

A. Positive Control Ligation

1. Description of the GFP Control Insert

The GFP Control Insert (80 ng/µL) is a double-stranded DNA fragment with *Bam*H I and *Hin*d III sticky ends surrounding an siRNA template that targets the green fluorescent protein (GFP) mRNA. The GFP Control Insert is provided as a control for the ligation reaction.

2. Ligation instructions

a. Dilute 2 µL of the GFP Control Insert with 18 µL nuclease-free water for a final concentration of 8 ng/µL.

b. Ligate 1 µL of the GFP Control Insert into the p*Silencer* hygro vectors using the standard procedure beginning with step III.A.3 on page 11.

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 GFP 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* hygro Negative Control

Negative control for RNAi

For any RNAi experiment, it is important to include a culture that is transfected with a negative control plasmid as a basis for analysis of gene knockdown. The optimal negative control insert for expression analysis in a gene silencing experiment is the scrambled sequence of your gene specific siRNA.

The p*Silencer* hygro Negative Control 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 can be used to control for the effects of introducing the p*Silencer* hygro plasmid into cells. Cells transfected with the p*Silencer* hygro plasmid expressing your target-specific siRNA should be compared to cells transfected with the corresponding p*Silencer* hygro Negative Control.

Positive control for antibiotic resistance in mammalian cells

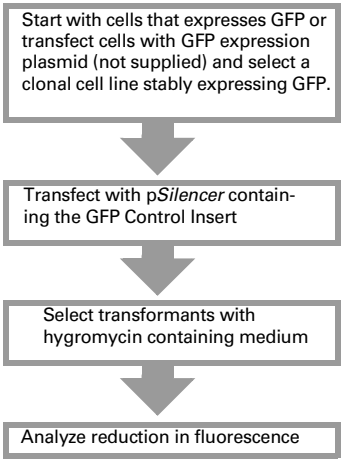
Select transfectants that are hygromycin resistant as described in section II.C on page 10, the p*Silencer* hygro Negative Control plasmid can also be used to demonstrate hygromycin resistance in mammalian cells conferred by p*Silencer*.

Positive Control construct containing the GFP Control Insert

The product of the positive control ligation (described in section IV.A on page 16) is a p*Silencer* hygroplasmid containing an siRNA template targeting GFP. The GFP Control Insert sequence is provided in Figure 4 below.

Figure 4.GFP Control Insert Sequence

```
5'-GATCC GGTTATGTACAGGAACGCA TTCAAGAGA TGCGTTCCTGTACATAACC TTTTTGGAAA-3'
    3'-G CCAATACATGTCCTTGCGT AAGTTCTCT ACGCAAGGACATGTATTGG AAAAACCTTTTCGA-5'
```



In cell lines that stably express a GFP with homology to the siRNA sequence: 5'-GGTTATGTACAGGAACGCA-3', this construct can be used to demonstrate a reduction in GFP expression. Compare the boxed portion of the GFP Control Insert sequence to the sequence of the gene encoding the GFP expressed in your cells to see if they are homologous—if so, p*Silencer* hygro with the GFP Control Insert should be capable of inducing RNAi. Mammalian expression vectors with GFP are available from commercial vendors including Invitrogen and Clontech. At Ambion, a clonal HeLa cell line stably expressing the cycle 3 variant of GFP introduced via Invitrogen's pTracer SV40 Vector (Cat #V871-20) was transfected with p*Silencer* hygro containing the GFP Control Insert, and it did reduce GFP expression.

If you have a clonal cell line that stably expresses the GFP, then transfect the cells with p*Silencer* containing the GFP Control Insert made in the positive control ligation (section IV.A.2 on page 16). As for any RNAi experiment, it is important to include a culture that is transfected with the p*Silencer* hygro Negative Control plasmid as a basis for analysis of gene knockdown. Select transfectants that are hygromycin resistant as described in section III.C.3 on page 14. Finally, analyze GFP expression by fluorescent analysis at regular intervals starting about 24 hr after transfection. Compare reduction of GFP fluorescence caused by the GFP Control Insert with that seen in cells transfected with the p*Silencer* hygro Negative Control plasmid.

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 11) is inefficient, then there will be relatively few plasmids to transform. Possible causes of poor ligation efficiency include the following:

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 11) 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 have become inactive.
Test your ligation components using another vector and insert or replace your ligation components and retry the siRNA insert cloning.

c. One or both of the hairpin siRNA template oligonucleotides have high levels of non-full-length products.
The size of oligonucleotides can be evaluated on an 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 11) might improve the percentage of dsDNA products. Alternatively, in some cases, gel purifying the band corresponding to annealed insert may result in better ligation.

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' *Bam*H 1 and 3' *Hin*d III overhanging sequences for cloning (see Figure 3 on page 9).

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 17.

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

If large numbers of colonies result 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 colonies from both, try transforming a plasmid with an ampicillin resistance gene (e.g., the p*Silencer* hygro Negative Control plasmid in the kit) and confirm that the ampicillin concentration in the plates is not too high to allow the growth of transformed cells.

E. Poor Mammalian Cell Transfection Efficiency

If you suspect that p*Silencer* hygro transfection 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.

1. p*Silencer* hygro plasmid is not pure enough

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

2. Transfection procedure requires optimization

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

3. 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.

4. Ineffective siRNA vector

If you are using siRNA-induced gene knockdown to assess transfection efficiency, consider using a different siRNA.

F. Problems with Hygromycin Selection

1. No transfected cells, or only a few transfected cells survive hygromycin selection

a. Transfection did not work, or the transfection efficiency was poor.
Check transfection efficiency using an expression plasmid that contains a reporter such as GFP or β-galactosidase (this is not supplied with the kit, but it can be prepared using the supplied GFP Control Insert).

b. The hygromycin concentration is too high.
Perform a hygromycin dose response experiment with the cell line in your study as described in section II.C.1 on page 10. Every cell type responds differently to different antibiotics. Some cells may even be resistant to hygromycin.

c. The siRNA target may be essential for survival.
If the siRNA target is essential for survival, cells transfected with plasmids that effectively reduce expression of the target gene may die. To test whether the target gene is essential for survival, transfect cells with the p*Silencer* hygro containing your siRNA template, and culture transformants without antibiotic selection. If significant cell death occurs, it is likely that the siRNA target is important for cell growth and metabolism.

d. Grow the cells that do survive selection (if there are any).
The cells that remain after hygromycin selection can be grown up and subsequently analyzed as a population or can be cloned using cloning rings and analyzed individually.

e. Perform a less stringent antibiotic selection.
Incubate the culture with hygromycin selection until only ~50% of the cells are killed. Then add fresh medium lacking hygromycin and incubate the culture for 24–48 hr without hygromycin selection. Next add hygromycin-containing culture medium again, 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 have not been transfected are grown under the same hygromycin selection regimen. Although it occurs at a very low frequency, cells do spontaneously become resistant to antibiotics and including a non-transfected control culture allows you to determine the effectiveness of the transfection and antibiotic selection.

f. Normal (nontransformed) and primary cell lines may not survive the transfection and/or selection process.
If possible use an immortal or transformed cell line for studies involving stable expression of siRNA.

2. Cells become contaminated following the addition of the hygromycin

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

3. Non-transfected cells survive selection

a. The hygromycin concentration is not high enough to kill cells.

A careful dose response experiment should be performed to determine the concentration that kills cells lacking a hygromycin resistance gene. This is described in section *II.C. Optimizing Antibiotic Selection Conditions* on page 10. The amount of time required to completely kill the cells should also be recorded, and this concentration and time should be used for each transfection experiment.

b. Cell density is too high.

If the cells are too crowded, they may be not be killed very 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 hygromycin may be inactive.

- At 37°C, hygromycin is stable for only a few days, therefore hygromycin-containing culture media must be replenished accordingly in order to apply selection pressure.
- Consider purchasing a new batch of antibiotic, or preparing a fresh solution of hygromycin.

V. Appendix

A. References

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B. Quality Control

Functional testing

The p*Silencer* hygro siRNA expression vector is ligated with the GFP Control Insert according to the instructions in this protocol. 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 glasses, 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.