p*Silencer*™ siRNA Expression Vectors

(Cat #AM7209, AM7210)

Instruction Manual

Manual 7209M Revision B **Revision Date: November 2, 2006**

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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*™ siRNA Expression Vectors

Mammalian promoters for siRNA expression

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. Ambion's p*Silencer* 2.0-U6 siRNA Expression vector features a human U6 RNA pol III promoter, and p*Silencer* 3.0-H1 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* 2.0-U6 and p*Silencer* 3.0-H1 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.

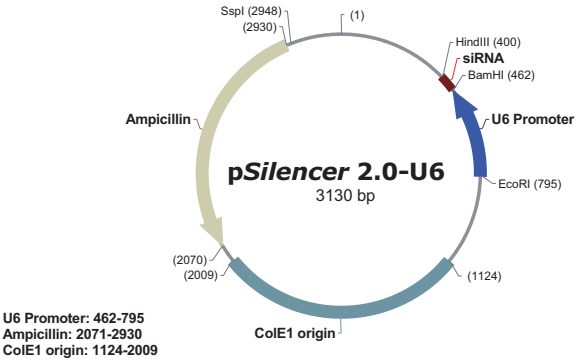
p*Silencer* plasmids are supplied ligation-ready

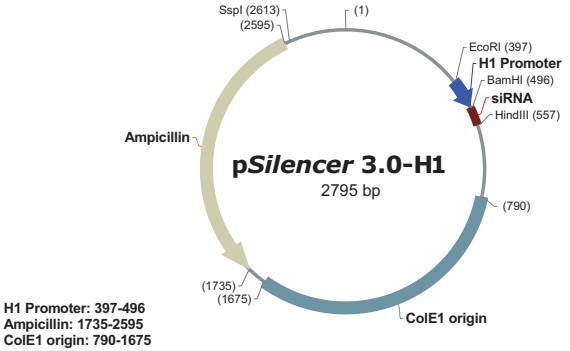
The p*Silencer* siRNA Expression Vectors are linearized with both *Bam*H 1 and *Hin*d III to facilitate directional cloning. They are purified to remove the digested insert so that it cannot re-ligate with the vector. This greatly increases the percentage of clones bearing the hairpin siRNA-coding insert after ligation, reducing the time and effort required to screen clones. Both p*Silencer* 2.0-U6 and p*Silencer* 3.0-H1 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* vector map is shown in Figure 1 on page 2; more detailed sequence information about the p*Silencer* vectors is available on Ambion's website. Follow the links at the following web address:

http://www.ambion.com/catalog/CatNum.php?7209

Figure 1. p*Silencer* Vector Map

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

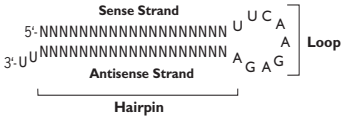




C. siRNA Template Design

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 is apparently recognized by Dicer, the nuclease responsible for activating dsRNAs for the RNAi pathway, and cleaved to form a functional siRNA (Brummelkamp 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* 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 7).

Figure 2. Hairpin siRNA



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* 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* siRNA Expression Vector includes 4 components:

- Linearized p*Silencer* siRNA Expression Vector ready for ligation
- Circular, negative control p*Silencer* vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes
- GAPDH-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* vector

Cat #AM7209	Cat #AM7210	Component	Storage
20 µL	– –	p*Silencer* 2.0-U6	–20°C
10 µL	– –	p*Silencer* 2.0-U6 Negative Control (0.5 µg/µL)	–20°C
– –	20 µL	p*Silencer* 3.0-H1	–20°C
– –	10 µL	p*Silencer* 3.0-H1 Negative Control (0.5 µg/µL)	–20°C
10 µL	10 µL	GAPDH Control Insert (80 ng/µL)	–20°C
1 mL	1 mL	1X DNA Annealing Solution	–20°C

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

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 6)
- 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.

F. Related Products Available from Ambion

T4 DNA Ligase Cat #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 Cat #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.
KDalert™ GAPDH Assay Kit Cat #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 (Cat #AM86050) and *Silencer* GAPDH Control siRNAs (Cat #AM4605, 4624).
RNase-free Tubes & Tips see our web or print catalog	Ambion's 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) for specific information.
***Silencer*® siRNA Construction Kit** Cat #AM1620	The *Silencer* siRNA Construction Kit 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's *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** Cat #AM4250–AM4639 see our web or print catalog www.ambion.com/siRNA	*Silencer* siRNA Controls are chemically synthesized siRNAs for genes commonly used as controls. Validated control siRNAs are available for genes such as GAPDH, β-actin, cyclophilin, KIF11 (Eg5), GFP, and luciferase. These siRNAs are ideal for developing and optimizing siRNA experiments and have been validated for use in human cells; many are also validated in mouse and rat cells.

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. 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.

2. 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 1 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.

3. 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's 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 our web site at the following address, or request it from our Technical Services Department (contact information is on the back cover of this booklet).

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

Oligonucleotide design

Two complementary oligonucleotides must be synthesized, annealed, and ligated into the p*Silencer* vector for each siRNA target site. Figure 3 on page 8 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.

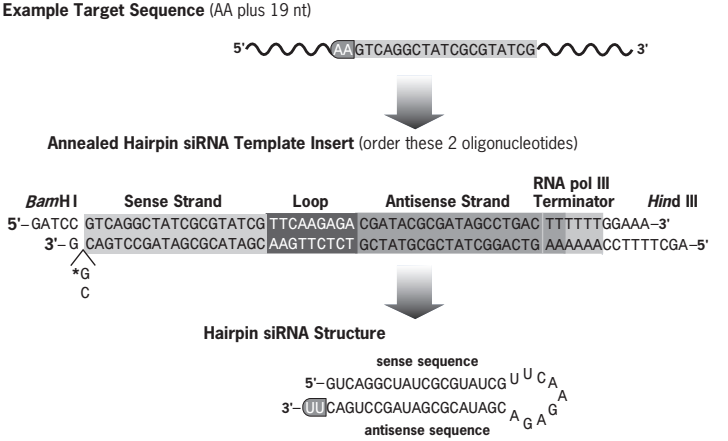
Near the end of the hairpin siRNA template is a 5–6 nucleotide poly(T) tract recognized as a termination signal by RNA pol III that will terminate siRNA synthesis. The function of the 5'-GGAA-3' just downstream of the RNA pol III terminator site is not fully understood, but we recommend that it be included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

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's T4 DNA ligase (Cat #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* 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 sequence with the primers shown below to verify that the clone contains the insert, and that it is the desired sequence. A link to the entire p*Silencer* sequence is provided on Ambion's web site, at:

http://www.ambion.com/catalog/CatNum.php?7209

p*Silencer* 2.0-U6	5'-AGGCGATTAAGTTGGGTA-3'	5'-TAATACGACTCACTATAGGG-3' (T7 sequencing primer)
p*Silencer* 3.0-H1	5'-GTTTTCCCAGTCACGAC-3' (M13 forward (–40) sequencing primer)	5'-GAGTTAGCTCACTCATTAGGC-3'

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's siPORT™ *XP-1* transfection agent (Cat #AM4506, 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. Check our website for more information on siPORT *XP-1*.

www.ambion.com/catalog/CatNum.php?4506

IV. Troubleshooting

A. Positive Control Ligation

1. Description of the GAPDH Control Insert

The GAPDH 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 GAPDH mRNA. The sequence of the GAPDH Control Insert is perfectly complementary to a region of human GAPDH mRNA. The siRNA expressed from this template sequence has been shown to effectively induce silencing of GAPDH in human cell lines. The GAPDH Control Insert is provided as a control for the ligation reaction.

2. Ligation instructions

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

b. Ligate 1 µL of the GAPDH Control Insert into the p*Silencer* vectors using the standard protocol beginning with step III.A.3 on page 9.

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 GAPDH 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* Negative Control

The p*Silencer* Negative Control plasmid supplied with the kit is a circular plasmid encoding a nontargeting 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* plasmid into cells. Cells transfected with the p*Silencer* plasmid expressing your target-specific siRNA should be compared to cells transfected with the corresponding p*Silencer* Negative Control.

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.

Positive Control construct containing the GAPDH Control Insert

The product of the positive control ligation (described in section IV.A on page 12) is a p*Silencer* plasmid containing an siRNA template targeting GAPDH. This construct can be used to optimize the p*Silencer*

transfection procedure. Use p*Silencer* -GAPDH and the p*Silencer* Negative Control to transfect cells, and monitor cell viability and gene silencing of GAPDH to identify optimal transfection conditions.

When successfully transfected and expressed, the GAPDH siRNA reduces both the mRNA and protein levels of GAPDH in human cell lines. This slows the growth rate of the cells and reduces the rate of cell proliferation of most cell types. To assess whether siRNA-mediated gene silencing is occurring, levels of GAPDH RNA, levels of GAPDH protein, and/or cell proliferation can be monitored.

Any of the following assays for assessing siRNA-mediated reduction in GAPDH gene expression can be used:

a. Quantitate mRNA levels by Northern analysis or RT-PCR.

GAPDH mRNA levels are typically reduced 50–90% 48 hr after transfection.

b. Analyze protein levels using Ambion's KDalert™ GAPDH Assay Kit (Cat #AM1639) or by Western blot, immunohistochemistry, or immunofluorescence.

GAPDH protein levels are typically reduced 50–90% 48 hr after transfection.

c. Look for a reduction in cell proliferation caused by GAPDH knock down.

Although it is less direct than looking at GAPDH mRNA or protein levels, a reduction in GAPDH activity can be assessed by measuring cell proliferation. Depending on cell type, there should be a >40% reduction in cell number 48–72 hours after transfection.

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 9) 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 9) 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 9) 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 8).

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 13.

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* 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* 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* 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 protocol requires optimization

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

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. The GAPDH positive control insert supplied with the kit can be used to prepare a vector that has been shown to reduce GAPDH mRNA and protein levels in a variety of cell types.

V. Appendix

A. References

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B. p*Silencer*™ siRNA Expression Vectors Specifications

Components and storage conditions

Cat #7209	Cat #7210	Component	Storage
20 µL	– –	p*Silencer* 2.0-U6 Vector	–20°C
10 µL	– –	p*Silencer* 2.0-U6 Negative Control	–20°C
– –	20 µL	p*Silencer* 3.0-H1 Vector	–20°C
– –	10 µL	p*Silencer* 3.0-H1 Negative Control	–20°C
10 µL	10 µL	GAPDH Control Insert	–20°C
1 mL	1 mL	1X DNA Annealing Solution	–20°C

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from our website by going to the following address and clicking on the link for the p*Silencer*™ siRNA Expression Vectors:
  www.ambion.com/techlib/msds
- Alternatively, e-mail us at MSDS@ambion.com to request MSDSs by e-mail, fax, or ground mail. Specify the Ambion catalog number of the kit(s) for which you want MSDSs and whether you want to receive the information by e-mail, fax, or ground mail. Be sure to include your fax number or mailing address as appropriate. If the mode of receipt is not specified, we will e-mail the MSDSs.
- Customers without internet access can contact our technical service department by telephone, fax, or mail to request MSDSs (contact information on the back of this booklet).

C. Quality Control

Functional testing

The p*Silencer* siRNA expression vector is ligated with the GAPDH Control Insert according to the instructions in this booklet. Ligation efficiency is then determined.

Nuclease testing

Each component is tested in Ambion's rigorous nuclease assays.

RNase activity

None detected after incubation with ^{32}P-labeled RNA; analyzed by PAGE.

Non-specific endonuclease/nickase activity
None detected after incubation with supercoiled plasmid DNA; analyzed on 1% agarose.

Exonuclease activity
None detected after incubation with ^{32}P-labeled *Sau*3A fragments of pUC19; analyzed by PAGE.