p*Silencer*™ 4.1-CMV neo Kit

(Part Number AM5779)

Protocol

P/N 5779M Revision B **Revision Date: May 16, 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. More recent publications feature plasmids that express functional siRNA or hairpin siRNA (also called short hairpin RNA or shRNA) when transfected into mammalian cells (Sui 2002, Lee 2002, Paul 2002, Paddison 2002, Brummelkamp 2002). Using siRNA expression vectors has the advantage that the expression of target genes can be reduced for weeks or even months (Brummelkamp 2002), eclipsing the 6–10 days typically observed with in vitro prepared siRNA used for transient transfection (Byrom 2002).

B. p*Silencer* 4.1-CMV Expression Vectors

The p*Silencer* 4.1-CMV vectors employ a powerful CMV promoter to drive high level expression of cloned hairpin siRNA templates in a wide variety of cell types. They also include an antibiotic resistance gene that provides a mechanism to select for transfected cells that express the introduced DNA.

Mammalian selectable markers

The p*Silencer* 4.1-CMV neo siRNA expression vector contains a neomycin resistance gene to enable antibiotic selection in mammalian cells. Antibiotic selection can be used to enrich for cells that were successfully transfected with p*Silencer* 4.1-CMV neo 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 neomycin resistance gene makes it possible to select cell populations, or clonal cell lines, that stably express the hairpin siRNA.

G418 is an analog of neomycin. We recommend using G418 to select for neomycin resistance. It is an aminoglycoside antibiotic similar in structure to gentamicin B1, produced by *Micromonospora rhodorangea*. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells (Haynes et al. 1995). The neomycin resistance gene from Tn5 encodes an aminoglycoside 3'-phosphotransferase, 3' APH II, that confers resistance to the antibiotic G418. Selection in mammalian cells is usually achieved in 3–7 days with G418 concentrations of 25–4000 µg/mL.

Modified CMV promoter for siRNA expression

Many commonly used systems for expressing siRNA in cells use an RNA polymerase III (RNA pol III) promoter such as U6 or H1. However it has recently been shown that RNA polymerase II (RNA pol II) promoters are capable of expressing high levels of functional siRNA in cells (Xia 2002). The p*Silencer* 4.1-CMV neo System employs a modified Cytomegalomavirus (CMV) promoter to drive expression with RNA pol II, and includes a modified simian virus-40 (SV40) polyadenylation signal downstream of the siRNA template to terminate transcription.

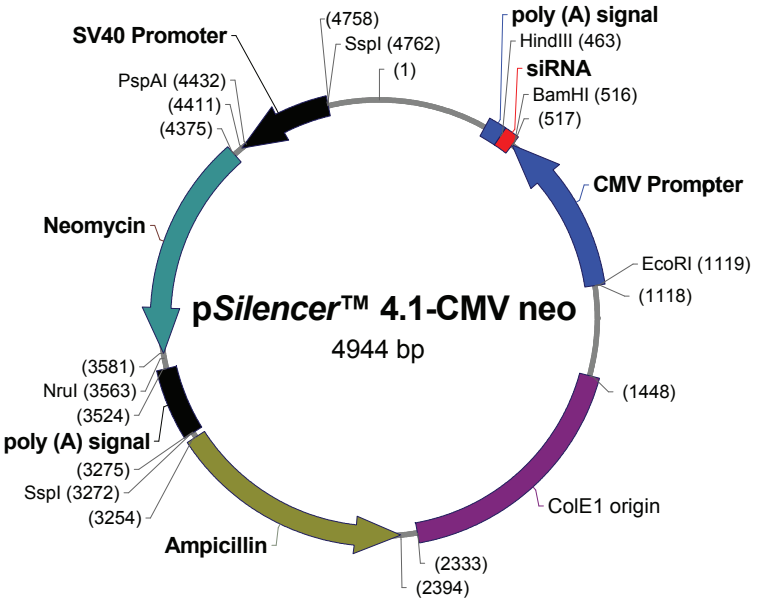
The CMV promoter is considered to be a stronger promoter than the other common RNA pol II promoters used in mammalian expression vectors such as Simian virus-40 (SV40) and Rous sarcoma virus (RSV) (Foecking, 1986). In vivo, RNA pol II is primarily responsible for transcription of mRNA within the cell. The CMV promoter has the advantage of being highly active in a broad range of cell types, and it does not interfere with other transcription events as may be the case with the RNA pol III U6 and H1 promoters in some situations.

p*Silencer* 4.1-CMV plasmids are supplied ligation-ready

The p*Silencer* 4.1-CMV siRNA Expression vectors are linearized with both *Bam*H 1 and *Hin*d 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 hairpin siRNA template insert after ligation, reducing the time and effort required to screen clones. A basic p*Silencer* 4.1-CMV neo vector map is shown in Figure 1 on page 3; more detailed sequence information about the p*Silencer* 4.1-CMV vectors is available on our website by following the links provided at the following web address:

http://www.ambion.com/catalog/CatNum.php?5779

Figure 1. p*Silencer* 4.1-CMV neo vector map



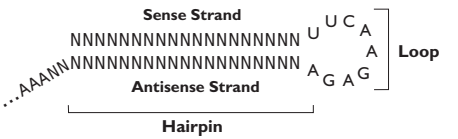
CMV Promoter:	**517–1118**
poly (A) signal:	**400–462**
SV40 Promoter:	**4411–4758**
Neomycin:	**3581–4375**
poly (A) signal:	**3275–3524**
Ampicillin:	**2394–3254**
ColE1 origin:	**1448–2333**

(This map shows the vector containing a typical 55 bp siRNA template cloned into the *Bam*H I and *Hin*d III sites.)

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. 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 overhang (Paddison 2002) (Figure 2). When expressed in mammalian cells, this type of hairpin siRNA or shRNA can 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 plasmids for gene silencing studies. For each target gene, complementary 55–60 mer oligonucleotides with single-stranded overhangs encoding restriction enzyme sites are synthesized, annealed, and ligated into the p*Silencer* 4.1-CMV neo 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 9).

Figure 2. Hairpin siRNA



Strategy for selection of target sites

The susceptibility of mRNA target sites to siRNA-mediated gene silencing appears to be the same for both in vitro prepared siRNA, and siRNA or shRNA expressed in vivo. 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* 4.1-CMV vector. If an siRNA target site has not already been identified, then we recommend that several different target sites be tested per gene. This can be conveniently done using in vitro prepared siRNAs (e.g. made with the Ambion *Silencer* siRNA Construction Kit; P/N AM1620). Once an effective target site is identified, oligonucleotides encoding hairpin siRNAs can be synthesized and ligated into p*Silencer* 4.1-CMV neo 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

Each p*Silencer* 4.1-CMV neo siRNA Expression Vector Kit includes 4 components:

- Linearized p*Silencer* 4.1-CMV neo siRNA Expression Vector ready for ligation
- Circular, negative control p*Silencer* 4.1-CMV neo 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* 4.1-CMV neo vector

Amount	Component
20 µL	p*Silencer* 4.1-CMV neo (0.1 mg/mL)
10 µL	p*Silencer* 4.1-CMV neo Negative Control (0.5 mg/mL)
10 µL	GAPDH Control Insert (80 ng/µL)
1 mL	1X DNA Annealing Solution

Store the p*Silencer* 4.1-CMV neo siRNA Expression Vector Kit at –20°C (if desired the 1X DNA Annealing Solution can be stored at room temp).

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 8)
- 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 reagents

- Small scale plasmid purification reagents are needed to prepare plasmid for restriction and sequence analysis.
- Medium to large scale plasmid purification reagents that yield transfection-grade plasmid are needed to purify p*Silencer* 4.1-CMV after cloning.

Restriction analysis reagents and equipment

For initial clone verification, the restriction enzymes *Bam*H 1 and *Hin*d III are used. The reaction products can be visualized by routine agarose gel electrophoresis.

Sequence analysis services

Putative clones containing p*Silencer* 4.1-CMV neo with your shRNA template should be sequenced before using the plasmid 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 www.ambion.com/prod/transfect for more information.

Cell culture facility and supplies

In addition to routine cell culture media, culture media containing G418 (a neomycin analog) will be needed for selection of p*Silencer* 4.1-CMV neo-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 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.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap* RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution.

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.
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.

II. Planning and Preliminary Experiments

A. Web-Based siRNA Design 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 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" page on our website:

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

B. siRNA Target Site Selection

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

In order to conduct long-term gene knockdown experiments it is important to first 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 or in vitro transcription, or with plasmids transiently expressing shRNA 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.

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 Negative Control supplied with the kit is an siRNA template sequence that lacks significant homology to the mouse, human, and rat genome databases. For many experimental systems, p*Silencer* 4.1-CMV neo containing the Negative Control siRNA template can serve as a negative control.

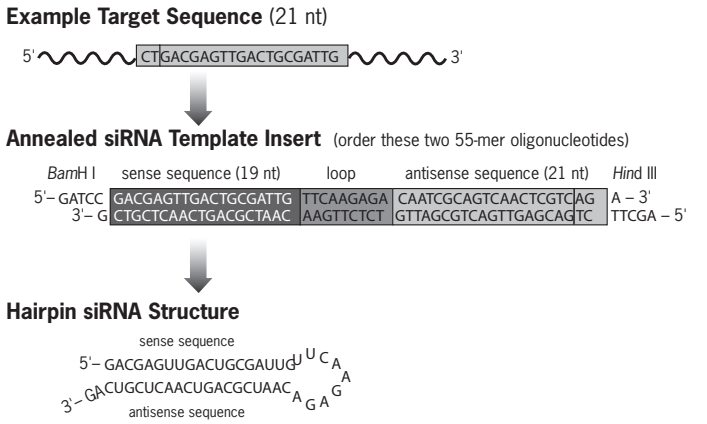
C. Hairpin siRNA Template Oligonucleotide Design & Ordering

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* 4.1-CMV neo for each target gene. These instructions describe how to design the siRNA template oligonucleotides.

siRNA template oligonucleotide design instructions

Figure 3 below shows an example target sequence, and how to design corresponding hairpin siRNA template oligonucleotides that will be annealed to form the DNA insert. The sense and antisense template oligonucleotides should encode a hairpin structure with a 19-mer stem and a 2 nt overhang derived from the 21 nt mRNA target site. Several different loop sequences have been successfully used in hairpin siRNA templates (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 here, 5'-TTCAAGAGA-3', is one that we have used successfully. The 5' ends of the two oligonucleotides form the BamH1 and HindIII restriction site overhangs that facilitate efficient directional cloning into p*Silencer* 4.1-CMV neo.

Figure 3. Example siRNA Template Oligonucleotide Design



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. Ambion can provide a recommendation if you need one—contact our Technical Services Department.

D. Optimizing Antibiotic Selection Conditions

Cell type, culture medium, growth conditions, and cell metabolic rate can all affect the optimal antibiotic concentration for selection of p*Silencer* 4.1-CMV-transfected cells. Identify the lowest level of G418 that kills nontransfected cells within approximately 7 days by testing antibiotic concentrations from 25–4000 µg/mL while keeping all other culture conditions equal. See step *1. G418 titration (kill curve)* below.

Using this optimum G418 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. G418 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 25–4000 µg/mL G418.

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

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

e. Identify the lowest G418 concentration that begins to give massive cell death in approximately 7–9 days, and kills all cells within 2 weeks. Use this G418 concentration to select cells containing the p*Silencer* 4.1-CMV neo 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 G418; use the concentration identified in the previous experiment.

c. Culture the cells for 5–14 days, replacing the antibiotic-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* 4.1-CMV neo clone.

III. Using the p*Silencer* 4.1-CMV siRNA Expression Vector

A. Cloning Hairpin siRNA Inserts into p*Silencer* 4.1-CMV

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

3. Ligate annealed siRNA template insert into the p*Silencer* 4.1-CMV 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* 4.1-CMV 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* 4.1-CMV neo 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 BamHI and HindIII to confirm the presence of the ~55 bp siRNA insert.

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

Sequencing primers
5'-AGGCGATTAAGTTGGGTA-3'
5'-CGGTAGGCGTGTACGGTG-3'

Links to the p*Silencer* 4.1-CMV neo restriction enzyme map and the entire plasmid sequence are provided on our website at this address:
http://www.ambion.com/catalog/CatNum.php?5779

7. Purify p*Silencer* 4.1-CMV plasmid for transfection

p*Silencer* 4.1-CMV 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* 4.1-CMV into Mammalian Cells

We recommend using Ambion siPORT™ *XP-1* transfection agent (P/N AM4506, AM4507) to deliver p*Silencer* 4.1-CMV plasmids 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.2 on page 11, and culture for 24 hr without selection.

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

2. Add medium containing antibiotic

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

C. Selecting Antibiotic-Resistant Transfected Cells

p*Silencer* 4.1-CMV 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 antibiotic selection for enrichment of cells that transiently express the siRNA

In experiments where the transfection efficiency is low, a rapid antibiotic selection can be used to kill cells that were not transfected with the p*Silencer* 4.1-CMV neo 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 antibiotic-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 G418 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 G418 (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 antibiotic 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 antibiotic-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 antibiotic resistance. Often this "minimal level" is about half the antibiotic 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 hairpin siRNA template introduced with p*Silencer* 4.1-CMV. 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 G418-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 4.1-CMV 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* 4.1-CMV-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 screen clones to identify the cells that cause the desired reduction in target gene expression.

a. Culture the cells in medium containing G418 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 G418 antibiotic 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 antibiotic resistance. Often this "minimal level" is about half the antibiotic 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 GAPDH Control Insert

The GAPDH Control Insert (80 ng/µL) is a double-stranded DNA fragment with BamHI and HindIII 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 p*Silencer* 4.1-CMV neo using the standard protocol beginning with step III.A.3 on page 12.

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* 4.1-CMV neo 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. A commonly used negative control insert for expression analysis in a gene silencing experiment is the scrambled sequence of your gene-specific siRNA.

The p*Silencer* 4.1-CMV neo 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* 4.1-CMV neo plasmid into cells. Cells transfected with the p*Silencer* 4.1-CMV neo plasmid expressing your target-specific siRNA should be compared to cells transfected with the p*Silencer* 4.1-CMV neo Negative Control.

Positive control for antibiotic resistance in mammalian cells
The p*Silencer* 4.1-CMV neo Negative Control plasmid can also be used to demonstrate neomycin resistance in mammalian cells conferred by p*Silencer* 4.1-CMV. For this positive control, select transfectants that are antibiotic resistant as described in section II.D on page 10.

Positive Control construct containing the GAPDH Control Insert

The product of the positive control ligation (described in section IV.A above) is a p*Silencer* 4.1-CMV neo plasmid containing a hairpin siRNA template targeting GAPDH. This construct can be used to optimize the p*Silencer* 4.1-CMV neo transfection procedure. Use p*Silencer* 4.1-CMV neo-GAPDH and the p*Silencer* 4.1-CMV neo 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, either of the following assays for assessing siRNA-mediated reduction in GAPDH gene expression can be used:

Quantitate mRNA levels by Northern analysis or RT-PCR.
GAPDH mRNA levels are typically reduced 50–90% 48 hr after transfection.

Analyze protein levels by Western blot, immunohistochemistry, or immunofluorescence.
GAPDH protein levels are typically reduced 50–90% 48 hr 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 12) 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 12) 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 template insert cloning.

c. One or both of the siRNA template oligonucleotides have high levels of non-full-length products.
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 12) 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' *Bam*H 1 and 3' *Hin*d III overhanging sequences for cloning (see Figure 3 on page 10).

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 18.

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* 4.1-CMV neo Negative Control 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. Poor Mammalian Cell Transfection Efficiency

If you suspect that p*Silencer* 4.1-CMV neo 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* 4.1-CMV neo 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.

F. Problems with Neomycin Selection

1. No transfected cells, or only a few transfected cells survive antibiotic 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).

b. TheG418 concentration is too high.
Perform a G418 dose response experiment with the cell line in your study as described in section II.D.1 on page 10. Every cell type responds differently to different antibiotics. Some cells may even be resistant to G418.

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* 4.1-CMV neo 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 antibiotic 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 G418 selection until only ~50% of the cells are killed. Then add fresh medium lacking antibiotic and incubate the culture for 24–48 hr without antibiotic selection. Next add antibiotic-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 G418 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 after adding the antibiotic

The antibiotic may be contaminated. G418 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 G418 concentration is not high enough to kill the cells.
A careful dose response experiment should be performed to determine the concentration that kills cells lacking a neomycin resistance gene. This is described in section *II.D. 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 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 G418 may be inactive.
- At 37°C, G418 is stable for only a few days, therefore antibiotic-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 antibiotic.

V. Appendix

A. References

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B. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

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.

C. Quality Control

Functional Testing

The p*Silencer* 4.1-CMV neo siRNA expression vector is ligated with the GAPDH 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 super-coiled 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.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.