

**pSilencer[®] 1.0–U6
siRNA Expression
Vector**



Note: Commercial use of this vector requires a license from Ambion.

Catalog #: 7207 – 20 µg
Concentration: 0.5 mg/ml
Volume: 40 µl
Storage Conditions: Store at –20°C or 4°C.

All Ambion plasmids are shipped at ambient temperature for convenience and cost. This in no way affects the high quality performance of this plasmid. Upon receipt, store the plasmid at 4°C for frequent use or –20°C for long term storage.

Storage Buffer: 10 mM Tris-HCl pH 7.4, 1 mM EDTA.

Quality Control: The sequence of the polylinker region of pSilencer™ 1.0–U6 siRNA Expression Vector has been confirmed by dideoxynucleotide sequencing.

The vector is linearized with *EcoRI* and *Apa I* for 1 hr, re-ligated at room temperature for 3 – 18 hr then tested for transformation, and antibiotic resistance.

USER INFORMATION

RNA interference (RNAi) is now being used routinely in mammalian cells to study the functional consequences of reducing the expression of specific genes. RNAi in mammalian cells is induced by transfecting small interfering RNAs (siRNAs), comprising double-stranded RNA molecules ~21 nt in length with 2 nt 3' overhangs (1), or hairpin-forming 45–50mer RNA molecules (2), that are complementary to the gene of interest. In addition to direct introduction of siRNAs into cells, RNAi can also be induced by transfecting expression plasmids that produce siRNAs within cells (3–9). Stable transfection of siRNA expression vectors (when cotransfecting a selectable marker) enables studies of long-term reduction in target gene expression (3). When transfected into mammalian cells, siRNA expression plasmids have been shown to reduce the levels of both exogenous and endogenous gene products (3–9).

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pSilencer[®] 1.0–U6 siRNA Expression Vector

Ambion's pSilencer 1.0-U6 siRNA Expression Vector is designed for plasmid-based siRNA experiments. The pSilencer 1.0-U6 is a 3.24 Kb vector, containing sequence elements for cloning and bacterial replication, i.e. f1 origin, ColE1 origin, Ampicillin resistance gene, and multiple cloning site (Figure 1). A U6 RNA Polymerase III (Pol III) promoter (-315 to +1) is cloned into the *Kpn* I and *Apa* I sites to generate small RNA transcripts. The U6 Pol III promoter was chosen because it has a well-defined transcription initiation site and Pol III termination sequence at a string of 4–5 uridines. This vector was developed by Sui and colleagues at Harvard Medical School and has been successfully used to knock down expression of *cdk-2* and *lamin A/C* in HeLa, H1299, U-2 OS and C-33A (*cdk-2* only) cells (5). The complete sequence of the pSilencer™ 1.0-U6 siRNA Expression Vector is available on Ambion's web site at <http://www.ambion.com/catalog/Sequence.html?306>

The following recommendations for insert design and cloning strategy are made based both on current literature (3–9) and research by Ambion scientists. The loop sequence and length, as well as the length of the siRNA stem, can be varied. The insert can be cloned into the pSilencer™ vector by using either a two step method (5) or the one step method used by Ambion, and described below. Consult the literature for alternate insert design strategies (3-9).

siRNA Insert Design

Labs that initially reported the use of siRNA expression vectors to induce RNAi had different design criteria for their inserts. Most of the designs have two inverted repeats separated by a short spacer sequence and end with a string of Tsthat serves as a transcription termination site. The length of the inverted repeats, which encode the stem of a putative hairpin, and the length and composition of the spacer sequence, which encodes the loop of the hairpin, vary among the different reports (3–10).

Sui and colleagues designed their inserts with an inverted repeat containing two 21 nt coding sequences interrupted by a 6 nt loop sequence, which was ligated into the vector using a two step method. The insert also contained a 5 T transcription termination sequence at the 3' end of the repeat. The coding sequence started with G, which was chosen because U6 promoter transcripts start with a conserved G (5).

Paddison and colleagues have investigated the importance of stem and loop length, sequence specificity, and presence of overhangs in determining siRNA activity. In short, they showed that stem lengths could range anywhere from 25 to 29 nt and loop size could range between 4 to 23 nt without adversely affecting silencing activity (10).

The first step in designing an appropriate insert is to choose the siRNA target site. Before the introduction of siRNA expression vectors, siRNA target sites were typically chosen by scanning an mRNA sequence for AA dinculeotides, recording the 19 nucleotides immediately downstream of the AA, and then comparing the potential siRNA target sequences with an appropriate genome database to eliminate any sequences with significant homology to other genes. Ambion provides a "siRNA Target Finder and Design Tool" to facilitate selection of siRNA sequences: http://www.ambion.com/techlib/misc/siRNA_finder.html . In general, we find that a little more than half of the siRNAs designed using this approach provide at least a 50% reduction in target mRNA levels and approximately 1 out of 4 siRNAs provide a 75–95% reduction (11).

For screening, we typically test four siRNAs per target, spacing the siRNAs down the length of the gene sequence to reduce the chances that we are targeting a region of the mRNA that is either highly structured or bound by regulatory proteins. Because constructing and testing four siRNA expression plasmids per target can be time-consuming, it may be preferable to screen potential siRNAs prepared by in vitro transcription or chemical synthesis. The sequences that result in the highest level of gene knockdown can then be incorporated into the pSilencer vector. Ambion scientists have determined that sequences that function well as transfected siRNAs also function well as siRNAs that are expressed in vivo. The only exception is that siRNA sequences to be expressed in vivo should not contain a run of 4 or 5 As or Ts, as these can act as termination sites for Polymerase III.

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Based on the chosen siRNA sequence, two DNA oligonucleotides ~55 nt in size are designed for insertion into the pSilencer 1.0-U6 vector (Figure 2). In the forward oligonucleotide, the 19-nucleotide sense siRNA sequence is linked to the reverse complementary antisense siRNA sequence by a short spacer. Ambion scientists have successfully used a 9-nucleotide spacer (TTCAAGAGA), although other spacers can be designed. 5–6 Ts are added to the 3' end of the oligonucleotide. In the reverse oligonucleotide, 4-nucleotide overhangs to the *EcoR* I (AATT) and *Apa* I (G GCC) restriction sites are added to the 5' and 3' end of the 54 nt sequence complementary to the forward oligonucleotide, respectively. The resulting RNA transcript is likely to fold back and form a stem-loop structure comprising 19 bp stem and 9 nt loop with 2-3 Us at the 3' end.

siRNA Insert Preparation

Order the two oligonucleotides for the siRNA insert. Gel purification of the oligonucleotides is not necessary for efficient ligation. Dissolve the oligonucleotides at a concentration of 1 $\mu\text{g}/\mu\text{l}$. Assemble the annealing reaction by mixing 2 μl of each oligonucleotide with 46 μl annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM Mg-acetate). Incubate the mixture at 90°C for 3 min, and then at 37°C for 1 hr. The annealed siRNA insert can be used directly in a ligation reaction or stored at -20°C until needed.

Digestion of pSilencer[®] 1.0-U6 and Ligation of the siRNA Insert with the Vector

Sui and colleagues used a two step method to add the inserts into the vector, in which the first repeat motif was cloned into the vector digested with *Apa* I (blunted) and *Xho* I. The second repeat motif, along with the spacer and 5 Ts, was subsequently subcloned into the *Xho* I an *EcoR* I sites (5).

Alternatively, a one step method has been used and is recommended by Ambion scientists to simplify the cloning procedure. In the following one step sub-cloning protocol, *Apa* I and *EcoR* I restriction enzymes are used to linearize the pSilencer 1.0-U6 vector and create a directional cloning site. This method generates similar constructs to those generated by the two step method. The *Apa* I site is used because it is adjacent to the U6 promoter and the second G of the *Apa* I site is predicted to be the transcription start site. Downstream restriction sites other than *EcoR* I can also be used.

Linearize 2-4 μl pSilencer[™] 1.0-U6 Vector with *Apa* I and *EcoR* I restriction enzymes in a 10 μl reaction according to the manufacturer's instructions. Following digestion, we recommend gel purifying the linearized vector on a 1% agarose gel to remove any undigested circular plasmid and to decrease the background in ligation and transformation. Dilute the purified DNA to 0.1 $\mu\text{g}/\mu\text{l}$.

Bring the double-stranded siRNA insert to a concentration of 8 ng/ μl for the ligation. For a 10 μl ligation reaction, mix 1 μl of the insert (8 ng) with 1 μl of the linearized vector (100 ng), 1 μl of 10X T4 DNA Ligase Buffer, 1 μl of T4 DNA Ligase (Ambion Cat #2130), and 5 μl of Nuclease-Free Water (Ambion Cat #9915G). Incubate the ligation reaction at room temperature over night. A negative control ligation should be performed with linearized vector alone and no insert.

Transformation

DNA from the ligation reactions can be transformed into competent *E. coli* cells by heat shock or electroporation following the manufacturer's protocol or published protocols such as those available in *Molecular Cloning* (Cold Spring Harbor Laboratory Press). 5 μl of the ligation reaction can be used for transformation of 100 μl of competent cells. Competent cells such as DH5 α , DH10b, and HB101, have been used successfully at Ambion (Follow Manufacturer's recommendation). The negative control ligation reaction should produce at least 2X less colonies than the ligation reaction containing the vector and the siRNA-encoding insert.

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To select positive clones that contain the siRNA insert, first digest purified plasmid DNA from different clones with *Hind* III. Clones with inserts will not be cleaved by *Hind* III, whereas clones from pSilencer 1.0-U6 vectors without inserts will be linearized. The positive clones (those that don't cut with *Hind* III) can be further confirmed by sequencing the cloning site using a T3 promoter primer, or by PCR amplifying the insert portion of the clones with a gene-specific forward primer and the T3 promoter primer (5' AATTAACCCTCACTAAAGGG 3').

Transfection and Analysis of siRNA Effect

Reduction of gene expression by a specific pSilencer 1.0-U6 construct can be investigated by transfecting the plasmid DNA into experimental cells using standard DNA transfection techniques, such as calcium phosphate- and cationic lipid-mediated methods (3-10). In all cases, we recommend that the concentration of cultured cells and plasmid be varied to optimize transfection efficiency. The siRNA effect can be assessed at both RNA and protein levels by Northern analysis, RT-PCR, Western analysis and immunofluorescence.

For additional information on the procedure and protocol, please contact Ambion's Technical Services Department (1-800-888-8804, option 2 or TechServ@ambion.com).

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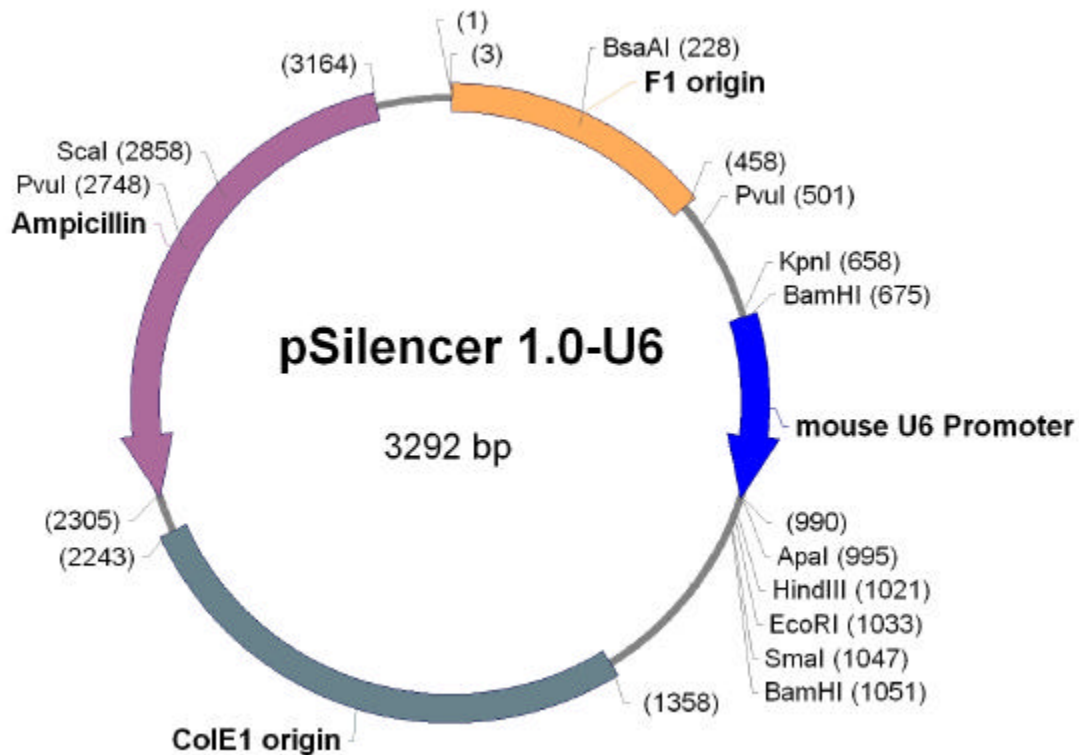


Figure 1. Schematics of the pSilencer[®] 1.0-U6 siRNA Expression Vector.

Sense	Loop	Antisense	
5'-N(19)	TTCAAGAGA	N(19)	TTTTTT-3' (53 bases)
3'-CCGG N(19)	AAGTTCTCT	N(19)	AAAAAATTAA-5' (61 bases)
Apal			EcoR I

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**Figure 2. Sequence and Structure of siRNA insert.
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