# pSiEx™-1 Cloning Kit

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# About the Kit

| | | |
|---|---|---|
| pSiEx™-1 Cloning Kit | 20 rxn | 71407-3 |

## Description

The pSiEx-1 vector is designed for DNA-directed gene silencing in mammalian cells by the expression of small interfering RNA (siRNA) driven by a mouse U6 RNA polymerase III promoter. The use of a Pol III promoter and a stop signal of five consecutive T residues provides a mechanism to produce an siRNA hairpin with a defined 3'-terminus (1). For gene silencing, a small DNA insert is cloned into the vector downstream of the Pol III promoter. After transfection into mammalian cells, the insert-containing vector is transcribed and short hairpin RNAs (shRNA) are produced. The shRNA is cleaved by endogenous dicer enzyme to form siRNA, which hybridizes to the target RNA and directs degradation by enzymatic cleavage (2). The pSiEx-1 Cloning Kit includes the linearized vector to facilitate directional cloning. Oligonucleotides encoding siRNA sequences with appropriate overhangs can be readily ligated into the vector and used to transform *E. coli* to generate plasmids for siRNA studies.

The pSiEx-1 Cloning Kit also includes a Control Insert, reagents for insert preparation and ligation, and NovaBlue Competent Cells for transformation. Each kit offers enough components for 20 ligations. We recommend using GeneJuice® Transfection Reagent for efficient transfection into mammalian cells.

## Components

- 1 µg pSiEx™-1 siRNA Expression Vector (linearized, 50 ng/µl)
- 10 µl pSiEx-1 Control Insert (0.05 pmol/µl)
- 250 U T4 Polynucleotide Kinase (10 U/µl)
- 100 µl 10X Kinase Buffer (660 mM potassium acetate, 330 mM Tris-acetate, 100 mM magnesium acetate, 5 mM DTT, pH 7.8)
- 250 µl 10 mM ATP
- 2 × 1.5 ml Nuclease-free Water
- 2 × 55 µl Clonables™ 2X Ligation Premix
- 22 × 50 µl NovaBlue Singles™ Competent Cells
- 4 × 2 ml SOC Medium
- 2 × 10 µl Test Plasmid, 0.2 ng/µl ($Amp^R$)

## Storage

Store pSiEx-1 vector, pSiEx-1 Control Insert, Clonables 2X Ligation Premix, Nuclease-free Water, Competent Cells, SOC Medium, and Test Plasmid at –70°C. Store T4 Polynucleotide Kinase, 10X Kinase Buffer, 10 mM ATP, and Clonables Positive Control at –20°C or –70°C.

## Example of Gene Silencing Procedure Using pSiEx™ Vector

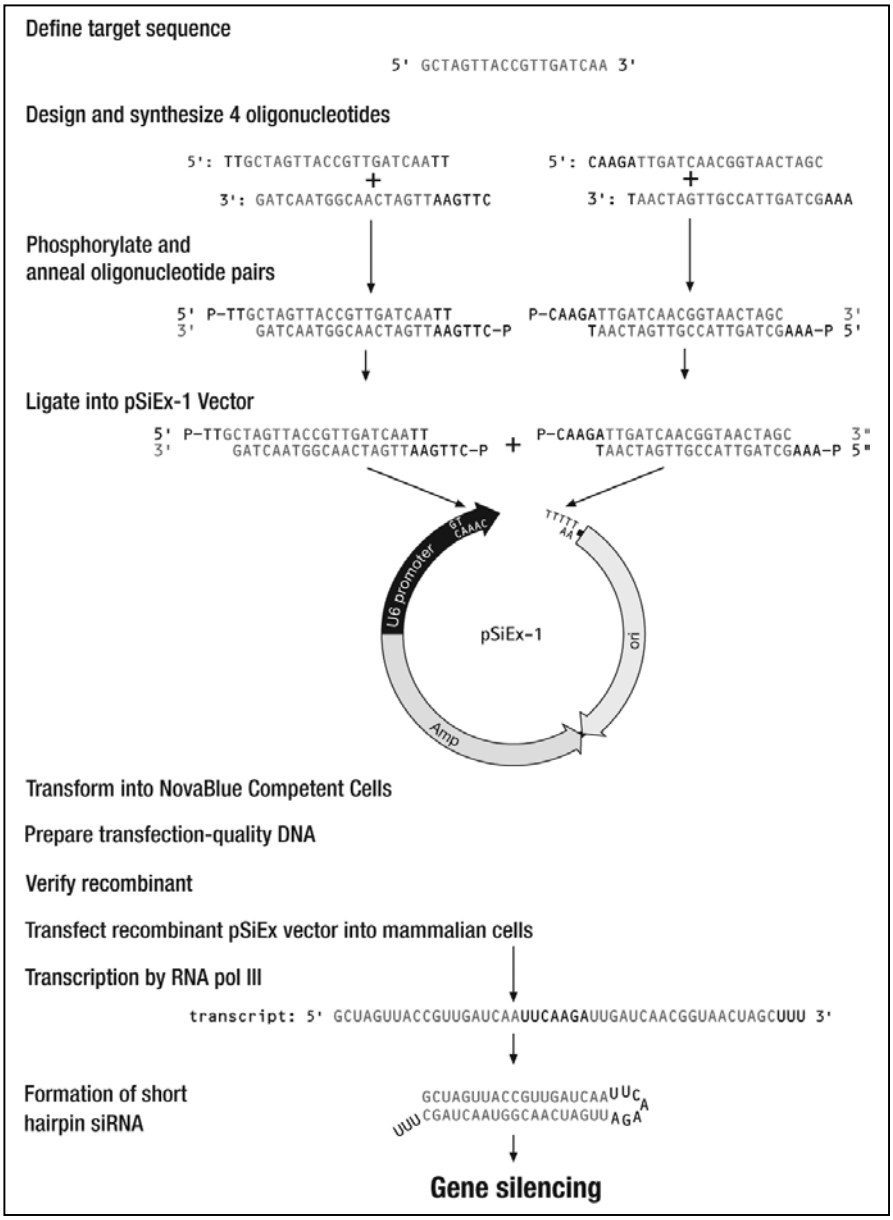


**Diagram of oligonucleotide design, cloning strategy, and hairpin siRNA production.** After the target sequence is defined, the target insert is designed to include the overhangs for cloning, the hairpin sequence, and the antisense sequence of the target sequence. The target insert is divided into two "halves", each consisting of a pair of oligonucleotides. The oligonucleotide pairs are annealed and phosphorylated. The inserts are ligated into the linearized pSiEx™-1 Vector and transformed into NovaBlue Singles™ Competent Cells. After the recombinant is verified, the plasmid is purified and transfected into mammalian cells.

* The target sequence in this example begins with a G, which corresponds to the transcription initiation nucleotide. Its complimentary nucleotide is provided by the vector.

# Oligonucleotide Design

## Selecting the target sequence

In general to avoid regulatory proteins, a target sequence for RNA interference should originate at least 50–100 nucleotides (nt) downstream of the translation start site. Gene silencing has also been effective when targeting 5'- or 3'-untranslated regions. To prevent premature termination of the transcript, the target sequence should be 19–21 nt and have a GC content of 30–50%. The target sequence should not contain four or more consecutive thymidines. A search of the NCBI database should be performed to ensure that the sequence only targets one gene.

## Starting nucleotide/termination signal

The transcriptional start site of the mouse U6 promoter requires the first nucleotide of the shRNA to be a G. This G must be included in the oligonucleotides designed by the user, regardless of what nucleotide is found at the 5'-end of the target sequence. Therefore, it may be helpful to choose a target that begins with a G. Transcription will terminate when four or five consecutive thymidines are reached.

## Hairpin loops

After the target sequence is identified, a hairpin loop sequence should be inserted in between the sense and antisense sequences. Hairpin loops of 3–9 nt are generally effective.

## Cloning strategy

Synthesis of long sequence oligonucleotides increases the chance of sequence errors and decreases the amount of full-length product resulting in poor cloning efficiencies and often incorrect recombinants. Our system uses two pairs of annealed oligonucleotides for each target insert. This strategy ensures that the correct sequence will be cloned efficiently.

The double-stranded target sequence should be divided up into four oligonucleotides (see Diagram, page 3), with a four-base overhang in the hairpin sequence to ensure that both inserts will ligate for efficient cloning.

*Note:* *It is important to divide the double-stranded sequence into four oligonucleotides. If only two oligonucleotides are used, each oligonucleotide will encode a hairpin and will have a tendency to "snap-back" on itself, preventing annealing to the other strand and decreasing cloning efficiency.*

The pSiEx™-1 vector is a linearized, dephosphorylated vector with incompatible overhangs. The 5'-end of the target insert as designed by the user should contain the last three nucleotides of the mouse U6 promoter, including the transcriptional start nucleotide (TTG, see Diagram page 2). The 3'-end of the target insert should include the first three nucleotides of the termination signal (AAA). This allows directional cloning, while adding minimal sequence to the insert.

# Protocol

## Phosphorylation and annealing oligonucleotides

1. Prepare annealing buffer (250 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) for use in Step 5.
2. Resuspend the lyophilized oligonucleotides to 100 pmol/µl in TE or water. Store resuspended oligonucleotides at –20°C.
3. Set up a kinase reaction for each pair of oligonucleotides. Also prepare a negative control, substituting Nuclease-free Water for sense and antisense oligonucleotides. Assemble the following components in a microcentrifuge tube:

   | | |
   |---|---|
   | 1.5 µl | 10X Kinase Buffer |
   | 1.5 µl | 10 mM ATP |
   | 1 µl | Sense oligonucleotide (100 pmol) |
   | 1 µl | Antisense oligonucleotide (100 pmol) |
   | 9.5 µl | Nuclease-free Water |
   | 0.5µl | T4 Polynucleotide Kinase (10 U/µl) |
   | 15 µl | total volume |

   Add T4 Polynucleotide Kinase last. Mix gently by stirring with a pipet tip.
4. Incubate at 37°C for 10 min.
5. Add 20 µl annealing buffer (prepared in Step 1) and 65 µl sterile water to bring the total volume to 100 µl. The concentration of the oligonucleotides will be 1 pmol/µl.
6. Heat the reaction to 95°C for 5 min in a thermal cycler or water bath.
7. Slowly cool the reaction mixture to room temperature.
8. Dilute a sample from each reaction 1:10 in TE. The final concentration of oligonucleotides will be 0.1 pmol/µl.
9. Proceed to ligation protocol or store at –20°C.

## Ligation

For a standard reaction, 1 µl pSiEx™-1 Vector (50 ng; 0.038 pmol) is ligated with 0.1 pmol of each phosphorylated insert in a total volume of 10 µl.

1. Assemble the following components in a microcentrifuge tube:

| | |
|---|---|
| 1 µl | pSiEx-1 Vector (50 ng/µl) |
| 1 µl | Diluted phosphorylated insert A (0.1 pmol/µl)* |
| 1 µl | Diluted phosphorylated insert B (0.1 pmol/µl)* |
| 2 µl | Nuclease-free Water |
| 5 µl | Clonables™ 2X Ligation Premix |
| 10 µl | total volume |

   Add Clonables 2X Ligation Premix last. Mix gently by stirring with a pipet tip.

   * Set up a positive control ligation to test the efficiency of the vector. Substitute 2 µl (0.1 pmol) pSiEx-1 Control Insert instead of the target inserts in the above ligation reaction. The pSiEx-1 Control Insert is a 32mer generated by annealing two phosphorylated oligonucleotides. **The control insert should only be used to monitor the efficiency of the ligation reaction.** It does not target any specific mammalian mRNAs nor would it form a hairpin siRNA.

     Also prepare a negative control ligation, substituting 2 µl from the negative control phosphorylation reaction instead of the target inserts in the above ligation reaction.

2. Incubate the ligation reaction at room temperature for 15 min.

*Note:* *These conditions have been optimized to generate > 2.5 ×$10^5$ recombinants with the pSiEx-1 Control Insert. Incubations longer than 15 min or increasing the molar ratio of insert to vector to greater than 2.5:1 will not necessarily increase the number of recombinants.*

## Transformation of NovaBlue Singles™ Competent Cells

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to NovaBlue Singles™ Competent Cells. Inactivation of the ligase is not required prior to transformation. For transformation, 1 µl of the ligation reaction usually yields sufficient numbers of colonies for screening. Up to 5 µl of the ligation reaction containing high quality reagents can be added to Singles Competent Cells without reducing transformation efficiency.

*Note:* *Upon receipt of competent cells, verify that the cells are frozen and that dry ice is present in the shipping container. Immediately place the competent cells at –70°C or below. For optimal results, do not allow the cells to thaw at any time prior to use. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible. To mix cells, flick the tube 1–3 times. NEVER vortex competent cells.*

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is immersed in ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells. The cells are then ready for the addition of the ligation reaction.
3. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
4. Add 1 µl of a ligation reaction directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is immersed in ice except for the cap. Repeat for additional samples.
5. Incubate the tubes on ice for 5 min.

6. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.

*Note:* *This "heat shock" step is most easily accomplished if the tubes are in a rack that leaves the lower halves of the tubes exposed. Hold the rack in the water bath so that the lower halves of the tubes are submerged for 30 s, and then replace the rack on ice.*

7. Place the tubes on ice for 2 min.
8. Add 250 µl room temperature SOC Medium to each tube.
9. Plate 5–30 µl transformation mixture directly on LB agar medium containing 50 µg/ml carbenicillin. If plating less than 25 µl of the transformation, we recommend plating onto a pool of SOC, which facilitates even colony distribution on the plate surface.

*Notes:* ***When using the Test Plasmid,*** *plate no more than 5 µl of the final transformation mix in a pool of SOC Medium on a LB agar plate containing 50 µg/ml carbenicillin or ampicillin.*

*The antibiotic carbenicillin is recommended over ampicillin. Carbenicillin is less sensitive to the drop in the pH of the growth medium that typically accompanies bacterial growth.*

*The appropriate amount of transformation mixture to plate will vary with efficiency of both the ligation and the competent cells. It is generally recommended to plate two different plates with 5 µl and 30 µl amounts of the transformation mixture to ensure that one of the plates will contain a sufficient number of isolated colonies for screening.*

*ColiRollers™ Plating Beads (Cat. No. 71013-3) are sterile glass beads that eliminate the use of the spreader and alcohol flame while evenly distributing cells without damaging the cells.*

*Using ColiRollers Plating Beads, a sterile bent glass rod, or specialized spreader, spread until the sample is evenly distributed on the plate. If the plates are fairly dry, the sample and cushion will quickly absorb into the plate. After the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed.* ***Do not spread until the sample and cushion have absorbed completely into the plates, because overspreading is lethal to the cells.***

10. Set the plates on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

## DNA Isolation and Sequencing

Novagen Mobius™ and SpinPrep™ Plasmid kits, as well as other standard methods, typically produce plasmid DNA suitable for *E. coli* transformation and routine molecular biology manipulations, including sequence analysis. When isolating plasmid DNA for mammalian cell transfection, however, more rigorous methods are often required (see below).

Recombinants for each construct should be verified for the correct sequence. The hairpin structure of the insert can make sequence analysis of the region difficult. However, we have not found it necessary to linearize the plasmid to overcome this problem. We have used the following sequence for a downstream primer for sequencing: 5' CCT GGT ATC TTT ATA GTC CTG 3'. The binding location of this primer (SiExDOWN primer) is shown on the pSiEx™-1 Vector Map (User Protocol TB409).

The following guidelines may be helpful when using an automated sequencer:

1. Dilute DNA in nuclease-free water to a final concentration of 100 ng/µl. Incubate at 95°C for 5 min. Place on ice. This denatures the plasmid without allowing it to renature.
2. Use 200–300 ng template and 1.6 pmol primer in a 10 µl sequencing reaction.
3. Add 5% DMSO to the reaction.
4. Begin the sequencing reaction with incubation at 95°C for 3 min.

*Note: If the signal strengths of the reactions are too high, the injection times of the samples can be lowered to achieve acceptable levels without having to repeat the sequencing reactions.*

### Preparation of plasmid DNA for mammalian cell transfections

Plasmid DNA preparation intended for transfection of eukaryotic cells must not contain contaminants that interfere with transfection. Although standard plasmid preparation DNA may work for transfection, results are often variable between different plasmids and different preparations of the same plasmid. Mobius and UltraMobius™ Plasmid Kits produce DNA of consistent quality for transfection. Alternatively, transfection quality plasmid DNA may be prepared by using a CsCl/EtBr protocol.

| Plasmid Preparation Kit | Scale | DNA Yield | Cat. No. | Size |
|---|---|---|---|---|
| Mobius 1000 Plasmid Kit | 100 ml culture | > 1 mg | 70854-3 | 2 rxn |
| | | | 70853-3 | 10 rxn |
| | | | 70853-4 | 25 rxn |
| UltraMobius 1000 Plasmid Kit | 100 ml culture | > 1 mg | 70907-3 | 2 rxn |
| | | | 70906-3 | 10 rxn |
| | | | 70906-4 | 25 rxn |
| Mobius 200 Plasmid Kit | 35 ml culture | > 200 µg | 70970-3 | 25 rxn |
| UltraMobius 200 Plasmid Kit | 35 ml culture | > 200 µg | 71090-3 | 25 rxn |

## Transfection and Analysis in Mammalian Cells

After the recombinants have been verified and high quality plasmid DNA has been isolated, the vectors can be transfected into mammalian cells and then analyzed for gene silencing.

### Transfection

The pSiEx™-1 vector can be transfected into mammalian cells with a variety of methods and reagents. GeneJuice® Transfection Reagent (Cat. No. 70967-3), is a proprietary formulation of a nontoxic cellular protein and a small amount of a novel polyamine optimized for maximal transfection efficiency, ease of use, and minimal cytotoxicity. GeneJuice has been demonstrated to provide excellent performance in both stable and transient transfections of eukaryotic cells. See User Protocol TB289 for more information.

The pSiEx-1 vector can be used in conjunction with the Novagen pTK-neo Vector (Cat. No. 71284-3). G418 (Cat. No. 345812) can be used to select for stably transfected cells. A minimal thymidine kinase promoter controls expression of the neomycin resistance gene. This promoter facilitates selection for stable integration of both the selection plasmid and a cotransfected expression plasmids, such as the pSiEx-1 vector, into the genome. See User Protocol TB379 for more information.

### Analysis of gene silencing

After the pSiEx-1 DNA has been transfected into the cell, cells can be harvested for analysis of shRNA effect or reporter assays. Reportasol™ Extraction Buffer (Cat. No. 70909) efficiently extracts soluble reporter enzymes (e.g., *Renilla* luciferase and β-galactosidase) from mammalian cells while maintaining maximal activity. CytoBuster™ Protein Extraction Reagent (Cat. No. 71009) extracts protein from mammalian cells and has been specifically formulated for use with Western blotting protocols, immunoprecipitations, and kinase/phosphatase assays.

## References

1. Yu, J. Y., DeRuiter, S. L., and Turner, D. L. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 6047–6052.
2. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002) *Genes Dev.* **16**, 948–958.