A Vector System for Expression of Short Interfering RNA

Version: pSUPER.retro (2/01/03)
Format: Linear plasmid
Catalog # VEC-pRT-0001
Protocol

Product: pSUPER.retro Vector
Catalog #: VEC-pRT-0002
Format: Linear Plasmid
Concentration: 1.0 mg/ml
Volume: 12 µl (enough for 10-12 reactions)
Storage: Store at –20°C

PSUPER.retro is typically shipped to customers on dry ice, although it can also be shipped at ambient temperature without affecting product quality and effectiveness.

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

GENERAL INFORMATION

Background
In several organisms, introduction of double-stranded RNA has proven to be a powerful tool to suppress gene expression through a process known as RNA interference (1). However, in most mammalian cells this provokes a strong cytotoxic response (2). This non-specific effect can be circumvented by use of synthetic short [21- to 22-nucleotide (nt)] interfering RNAs (siRNAs), which can mediate strong and specific suppression of gene expression (3). However, this reduction in gene expression is transient, which severely restricts its applications.

To overcome this limitation, the pSUPER RNAi system provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts. The vector uses the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). Most important, the cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3’ overhanging T or U nucleotides (nt).

The pSUPER RNAi System has been used to cause efficient and specific down-regulation of gene expression (4, 5), resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time.

Specific to the pSUPER.retro vector is the increased ability to integrate expression cassettes in the genomes of human cells (5), which mediate RNA interference to induce persistent loss-of-function phenotypes. Vectors like these have at least two potential applications. In gene therapy, the selective downregulation of only the mutant version of a gene allows for highly specific effects on tumor cells, while leaving the normal cells untouched. This feature greatly reduces the need to design viral vectors with tumor-specific infection and/or expression. By designing target sequences that span chromosomal translocation breakpoints found in cancer, these vectors may also be used to specifically inhibit the chimeric transcripts of these translocated chromosomes. The recent demonstration that siRNAs can inhibit gene expression in vivo provides further support for the notion that oncogene-specific RNA interference may be a viable approach to treat cancer (6). In addition, these vectors can be used to efficiently identify the genetic events that are required for cancer cells to manifest a tumorigenic phenotype. Through use of this technology, out of the many genetic alterations present in most human cancer cells, the most effective targets for drug development can be rapidly identified.

References:
**Protocol**

**Oligo Insert Design**

To effect the silencing of a specific gene, the pSUPER.retro vector is used in concert with a pair of 64-nt oligonucleotides, each containing a unique 19-nt sequence derived from the target transcript. These are annealed and ligated by the user into the vector between the BglII/HindIII sites of the vector.

Within the 64-nt oligos, the 19-nt target appears in both sense and antisense orientation, separated by a 9-nt spacer sequence. The resulting transcript is predicted to fold back on itself to form a 19–base pair stem-loop structure. Analysis indicates that the stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA (4). Figure 1 provides an overview of the insert design, and how the oligos are transcribed and process to functional siRNA.

![Fig. 1: Transcription of 64-nt oligo to hairpin RNA, processed to functional siRNA.](image)

It has been shown that a single nucleotide mismatch in the 19-nt targeting sequence abrogates the ability to suppress gene expression (4). Therefore, sequence design is critical.

OligoEngine provides a design tool for the pSUPER RNAi System – as well as for synthetic siRNAs – that analyzes the target transcript and generates candidate sequences. The tool is free to use and can be accessed by clicking on the “Order Now” tab at the top of any page on our Web site, http://www.olgioengine.com.

The design tool automates the target design process recommended by the vector’s inventors (4). Those familiar with siRNA design will note that the design strategy for designing pSUPER oligo sequences is nearly identical; in short, it recommends:

1. Find the start codon of the target transcript and move 75 bases downstream
2. Search for an AA dimer, then analyze the following 19-nt sequence. Optimally, it should have a GC richness of ~50%, or at least greater than 30%.
3. Preferably, the 19 nt target sequence should be flanked in the mRNA with TT at the 3’. However, sequences with flanking 5’ AA only have also given good results.
4. Additionally, the 19 nt must not contain a stretch of four or more Adenines or Thymidines, as this will give premature termination of the transcript.

The OligoEngine design tool also enables users to BLAST their potential target sequence against the NCBI UniGene database to ensure there is no significant homology with other genes in the genome of interest. Of course, if users have successfully generated effective knockdown using synthetic siRNA, it is recommended that they employ the same target sequence with the pSUPER RNAi System.
PROCEDURE

Step One: Anneal Oligos

Obtain two DNA oligonucleotides for hairpin RNA expression. Our experience demonstrates that gel purification of the oligos is not necessary for efficient ligation.

Dissolve the oligos in sterile, nuclease-free H₂O to a concentration of 3 mg/ml. Assemble the annealing reaction by mixing 1 µl of each oligo (forward + reverse) with 48 µl annealing buffer. You may choose to either a) use the Universal Buffer included with your vector, b) use a similar buffer from another manufacturer, or c) create your own buffer stock from 100 mM NaCl and 50 mM HEPES pH 7.4.

Incubate the mixture at 90°C for 4 min, and then at 70°C for 10 minutes. Slowly cool the annealed oligos to 10°C (e.g., step-cool to 37 for 15-20 minutes, then to 10°C or room temperature before using or moving them to refrigerated storage). The annealed oligo inserts can be used immediately in a ligation reaction, or cooled further to 4°C. For longer storage, keep at -20°C until needed.

Lab Tip:
To dilute your lyophilized oligos to a specified concentration, use the following equation to determine how much H₂O (or buffer, etc.) to add to your product on hand:

\[ \text{ml H}_2\text{O required for concentration of } X \text{mg/ml} = \left( \frac{\mu g \text{ oligos} \times 10^{-3}}{X} \right) \]

Thus, if you have 200 µg of oligo*, add ~0.067 ml of H₂O to achieve a concentration of 3mg/ml [(200 x 10⁻³) / 3 = ~0.067]. When diluting your oligos, you may first wish to create a “master stock” of 10 mg/ml, which you can store and dilute further (e.g., to 3mg/ml) as needed.

*If you purchased your oligos through OligoEngine, you can refer to their accompanying Data Sheets for the specific quantity, in µg, of each oligo as delivered. If you do not have this data but instead know the quantity of an oligo in pmoles, you can calculate µg weight by multiplying pmoles x 10⁻⁶ x molecular weight (MW) of the oligo (approx 19710 for each 64mer oligo). Likewise, you can use a UV spectrophotometer at 260 nm to determine the optical density (“OD”) of your oligos and calculate the quantity of each in µg (1 A₂₆₀ OD = 33 µg/ml ssDNA), then adjust your concentration accordingly.

Step Two: Linearize the Vector (Omit this step if you have purchased the linear version of the vector)

Linearize 1 µl of the pSUPER.retro vector with HindIII and BglII restriction enzymes. It is recommended to perform sequential reaction steps rather than simultaneous digestion, as follows: Digest with HindIII for 60 minutes, add BglII and continue reaction for 2 hours, then heat inactivate the reaction (raise the temperature to 65 or 80°C for 20 minutes).

Perform digestion according to the enzyme manufacturer’s instructions; most researchers follow the general rule that 10 units of restriction enzyme is sufficient to overcome variability in DNA source, quantity and purity. Generally, 1µl of enzyme is added to 1 µg of purified DNA in a final volume of 50 µl of the appropriate buffer, followed by incubation for 1 hour at the recommended temperature.

Lab Note:
It is not necessary to CIP-treat the vector because the fully-digested plasmid will contain incompatible ends. However, some users who have failed to detect the presence of an insert have chosen to CIP-treat the vector – and phosphorylate the oligos – and have reported positive results. Again, however, this is only an optional step and is not necessary per se for effective ligation.

Following digestion, we recommend gel purifying the linearized vector on a 1% agarose gel to remove the insert sequence (976 bp stuffer sequence between BglII and HindIII) and to help separate the prep from any undigested circular plasmid and to decrease the background in ligation and transformation.
Step Three: Ligation into pSUPER.retro Vector

Assemble the ligation reaction by adding 2 µl of the annealed oligos to 1 µl of T4 DNA ligase buffer. Add 1 µl pSUPER.retro vector, 5 µl nuclease-free H$_2$O, and 1 µl T4 DNA ligase.

**Lab Note:**

If you are working with the circular version of the pSUPER.retro vector and have performed the BglII / HindIII digestion yourself, you will need to normalize the concentration of vector after the purification process in Step 2. Using a UV spectrophotometer at 260 nm to determine the OD of your vector and calculate the quantity of each in µg, based on the equation $1\ A_{260}\ \text{OD} = 50\ \mu\text{g/ml}\ dsDNA$. Using on this measurement, adjust your concentration of vector to 0.5 mg/ml before proceeding to ligation.

Incubate overnight at room temperature. A negative control ligation should be performed with the linearized vector alone and no insert. After ligation, the BglII site is destroyed. This strategy allows quick screening of the recombinant pSUPER.retro: recombinant plasmids containing the inserts will not be linearized when digested with BglII, where as the wild type vector will be linearized with BglII.

Step Four: Transformation in Bacteria

Recombinant pSUPER.retro should be transformed into competent cells of an appropriate host strain (e.g., DH5α) according to the supplier protocol or the transformation protocol routinely used in your laboratory. In order to monitor the efficiency of the transformation steps, as a negative control, cells should also be transformed either with a vector that has been ligated with a scrambled-base hairpin oligo, or with a circular vector containing no oligo insert.

Grow bacteria in amp-agarose plates overnight (16-24 hrs), then pick and grow colonies in an ampicilin broth for an additional cycle. Pick and miniprep several colonies (it can take many to locate a positive clone) according to supplier’s instructions.

Check for the presence of positive clones (i.e., containing vector with oligo insert) by digesting with EcoRI and HindIII. As mentioned earlier, recombinant pSUPER.retro containing inserts will not be linearized by BglII because the site is destroyed after ligation. Positive clones should have inserts approximately 290 bp in length. An empty vector (i.e., supercoil that was nicked and not fully digested) has an insert of 227 bp.

**Lab Tip:**

On rare occasions, researchers have not been able to identify a positive clone after transformation. Based on extensive evaluation, we believe this is likely due to occasional problems with self-annealing oligos during the initial annealing step. If experience such difficulties, you may wish to alter the anneal procedure as follows:

- Raise the initial annealing reaction temperature to 94°C for 4 min., then cool to 80°C for 4 min.
- Continue by cooling to 75°C for 4 min., then down to 70°C, before proceeding with any further cooling.

If this doesn’t yield any better results, you may want to add even more steps: 94°C for 4 min, then 85°C for 4 min, then 82°C, 80°C, 78°C, 75°C, etc. (If you have access to a PCR block, you may want to use that to perform your annealing reaction for easy and automatic step-cooling.)

Since the melting temperature of the hairpin structure will be less than that of two full-length oligos, this should help to ensure proper annealing if problems arise.

Before transfecting the cells with the construct, the presence of the correct insert can be confirmed by sequencing. Sequences of primers successfully used with pSUPER.retro include:

- 5' - GGAAGCCTTGGCTTTTG - 3' (pos. 1242–1257)
- 5' - CGAACGCTGACGTCATC - 3' (pos. 2645-2629 [complement])

For other sequencing primer options, consult the pSUPER.retro sequence file, available for download from the pSUPER RNAi System section of the OligoEngine Web site (www.oligoengine.com).
Step Five: Transfection of Mammalian Cells

The pSUPER.retro plasmid can be transfected directly into your target cells, or you may wish to utilize a packaging cell line to produce retroviral supernatants (see below). Successful transfection methods utilized for pSUPER.retro include, but are not limited to, electroporation and the use of polyamine reagents.

**Lab Note:**
Because of the various transfection options available, and the continuing evolution of related products, a specific transfection procedure is not specified here. Although the pSUPER.retro vector is unique in its function and capabilities, there is nothing inherently different about the construct of the plasmid that requires any extra-ordinary measures to be taken during transfection. Therefore, it is recommended that each researcher employ the procedure that has produced the best results for him or her in previous applications, and is thus likely to be most familiar, efficient, and effective.

As an example, the procedure used by Drs. Agami and Brummelkamp and referenced in their original publication of the pSUPER system (4) notes that their target cells “…were transfected using an electroporation protocol, which results in more than 90% transfection efficiency.” This protocol is in turn described in detail in a previous paper by Agami and Brummelkamp (Cell 102, 55 (2000)), as follows:

To obtain high transfection efficiency, 3 x 10^5 MCF-7 cells were resuspended in 100 µl of electroporation buffer containing 2 mM HEPES (pH 7.2), 15 mM K2HPO4/KH2PO4, 250 mM manitol, and 1mM MgCl2 at a final pH of 7.2. Either 1 or 2 µg of DNA was added and the cells and DNA were transferred to a 0.1 cm electroporation cuvette (BioRad) and electroporated with Gene Pulser II apparatus and Gene Pulser II RF module (BioRad) at 140 volts, 15 times 1.5 ms burst duration and 1.5 s intervals. Five minutes after electroporation, cells were seeded in either a 10 cm dish or an equivalent area. Cells were washed 16 hr after transfection and the experiment was performed either 24 or 48 hr later.

Again, the above information is provided for example only. Researchers have also reported success utilizing Lipofectamine (Gibco) and the polyamine reagents LT1 and LT2 (from Mirus Corporation, www.genetransfer.com). Refer to these products’ protocols for more specific transfection procedures.

**pSUPER.retro with Packaging Cells**

For a higher rate of stable cell integration, pSUPER.retro can be used with a packaging cell line to produce retroviral supernatants. In particular, OligoEngine recommends use of the Phi-NX cell line, a 293T-based packaging cell line developed by the Nolan Lab at Stanford University (other commercial packaging cell lines are available, and have proven successful in use with the pSUPER.retro vector). Again, standard laboratory protocols can be used for this procedure; the following example (5) is provided as a guide only:

Culture cells in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Transfect Phoenix packaging cells by calcium-phosphate precipitation to produce ecotropic retroviral supernatants. 48 hours post-transfection, filter the tissue culture medium through a 0.45 µm filter, and use the viral supernatant for infection of cells after addition of 4 µg/ml polybrene. Infected cells for at least 6 hr and allow to recover for 24 hr with fresh medium. Select infected cells with puromycin (1–3 µg/ml for 48 hr).

For more information about the Phi-NX packaging cell line, including detailed protocols and an MTA for purchase of Phi-NX from the ATCC, please go online to http://www.stanford.edu/group/nolan/retroviral_systems/phx.html.

Step Six: Selection of Stable Transfectants

Two days after transfection, add puromycin at the appropriate concentration (typically 1 mg/ml, although optimum concentrations can vary widely). Stable transfectants are usually individualized after 1-2 weeks.
RNAi ANALYSIS

Measurement of siRNA–Induced Silencing

The level of suppression of your target gene can be measured by using different techniques:

- To determine the amount of protein expressed by the gene, a Western Blot analysis can be performed.
- For a measurement of the mRNA transcript of your target gene, Northern analysis and quantitative RT-PCR are the most widely used techniques.

Please refer to supplier protocols or standard lab methods handbooks for more information on the appropriate protocol for each technique.

Troubleshooting

Difficulty in achieving detectable levels of gene suppression may be due to problems that can occur at each step of this protocol. Begin by reviewing supplier guides and protocols (e.g., for transformation, transfection, etc.) to eliminate the possibility of error leading beyond the scope of this product/protocol.

Once some of the more basic problems have been ruled out, consider the following:

- **Synthesized oligo may contain incorrect sequence.** Occasional errors in the process of DNA synthesis can cause an incorrect nucleotide to be added within an oligo. Check your data sheet to confirm that the sequence of your synthesized oligo matches what is required for target knockdown (i.e., correct 19-nt target sequence) and for vector ligation (i.e., 5’ and 3’ ends are correct for ligation).

- **Oligo pairs may not have annealed properly.** Self-ligation has been known to occur in rare cases, but can be avoided by following the steps outlined in the “Lab Tip” in Step Four of the procedure.

- **Transfection may be unsuccessful/too low.** To confirm successful transfection of the pSUPER.retro vector, use a positive control plasmid of equivalent size, such as a GFP vector.

- **Mutation may have occurred.** The process of apurination or other factors may lead to a mutation in a nucleotide of an insert oligo. A single mutation is enough to significantly affect gene knockdown. Sequence your plasmid construct using the primers described in Step Four of the procedure to confirm or rule out such problems.

- **Target sequence may not be appropriate for silencing.** This is one of the most common problems for lack of any observable suppression, and to date one of the least understood. Review the target selection strategy described earlier in this protocol, and choose another target sequence from different region of the gene.

Other problems may be more specific to the cell type, target gene, system or species with which you are working. You may wish to consult PubMed for recent articles in the field of RNAi that may provide some insight, or contact OligoEngine technical support (customerservice@oligoengine.com) to discuss your experiment in greater detail.
VECTOR MAP AND FEATURES

This version of pSUPER.retro is a 6349 base-pair vector that has been linearized with BglII and HindIII.

The vector was developed by cloning the pSUPER expression cassette into a self-inactivating pMSCV-puro retroviral vector. This version features a puromycin selection marker driven by a separate promoter.

The 3' LTR was inactivated by an internal (Nhel-Xbal) deletion to generate a self-inactivating virus (∆LTR). Upon integration to the genome of the virus generated from this vector the 3' ∆LTR is duplicated to the 5' LTR to generate a provirus that lacks all LTR's enhancer-promoter activities. BglII and HindIII sites are used for cloning of inserts. Upon ligation, BglII site is destroyed to give an option to better select positive clones after the ligation.

Molecule Features

PLEASE NOTE that this linearized version contains NO STUFFER SEQUENCE as shown above, and that even any “nicked” (i.e., vector not fully digested with both HindIII and BglII) vector will not contain the stuffer: empty clones will contain a 227 bp fragment. The positions below have been adjusted from the map above to account for the lack of stuffer sequence.

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KEY ENZYMES

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