pSUPER RNAi System™

pSUPERIOR : Manual

A Vector System for Inducible Expression of Short Interfering RNA

The following manual contains information about and Instructions for the following pSUPERIOR vectors:

Vector Name	Catalog#
pSUPERIOR	VEC-IND-0001 (linear) VEC-IND-0002 (circular)
pSUPERIOR.neo	VEC-IND-0003 (linear) VEC-IND-0004 (circular)
pSUPERIOR.puro	VEC-IND-0005 (linear) VEC-IND-0006 (circular)
pSUPERIOR.neo+gfp	VEC-IND-0007 (linear) VEC-IND-0008 (circular)

NEW : BglII / XhoI Oligo Insert Design Option – See Inside...

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v. 2

PRODUCT INFORMATION

Concentration: 0.5 mg/ml

Volume: 12 µl

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

Storage: Store at 4°C

Shipping: *pSUPERIOR is shipped to customers at ambient temperature to reduce shipping and handling costs without affecting product quality and effectiveness.*

OVERVIEW

The pSUPERIOR vectors are inducible versions of the widely-used pSUPER suite of vectors for siRNA expression in mammalian cells. pSUPERIOR vectors are tetracycline-regulated expression vectors that utilize regulatory elements from the *E. Coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994; Hillen *et al.*, 1983). Tetracycline regulation in pSUPERIOR vectors is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (Yao *et al.*, 1998).

In its silencing function, each pSUPERIOR vector is identical to its pSUPER (i.e., non-inducible) counterpart. All designated features – selection markers, vector backbone, etc. – are identical as well. For example:

Vector Name	***Vector Features***	***Inducible Vector Name***
pSUPER	Basic siRNA expression vector with no mammalian selection marker	pSUPERIOR
pSUPER.puro	Expression vector with puromycin resistance gene expressed under control of separate PGK promoter	pSUPERIOR.puro
pSUPER.retro.neo+gfp	Retroviral vector for siRNA expression/retrovirus production, with neo-GFP fusion protein expressed under control of separate PGK promoter	pSUPERIOR.retro.neo+gfp

REQUIRED COMPONENTS FOR REGULATION OF TRANSCRIPTION

In addition to the pSUPERIOR vector, the two critical items required for controlled regulation of transcription are 1) a TetR-expressing vector (or a cell line that stably expresses the TetR protein); and 2) tetracycline.

Currently, OligoEngine is not licensed to provide these products directly to our customers. However, both are readily available from Invitrogen. Invitrogen is the exclusive source of the pcDNA6/TR© regulatory vector; this vector expresses high levels of the TetR gene (Postle et al., 1984) under the control of the human CMV promoter. The pcDNA6/TR© vector can be introduced into mammalian host cells by standard transfection methods to generate stable expression of the Tet repressor in your target cell line. Alternately, three cell lines which stably express the TetR repressor are available from Invitrogen: HeLa, 293, and U2OS cells.

Tetracycline is also available from Invitrogen. Doxycycline may be used as an alternative inducing agent with pSUPERIOR; Dox is similar to Tet in its mechanism of action, and exhibits similar dose response and induction characteristics as tetracycline when used with pSUPER. Doxycycline has been shown to have a longer half-life than tetracycline (48 hours vs. 24 hours, respectively). Doxycycline may be obtained from Sigma-Aldrich.

BACKGROUND: THE pSUPER RNAi SYSTEM

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.

References:

1. P. A. Sharp, Genes Dev. 13, 139 (1999).
2. T. Hunter, T. Hunt, R. J. Jackson, H. D. Robertson, J. Biol. Chem. 250, 409 (1975).
3. S. M. Elbashir et al., Nature 411, 494 (2001).
4. T.R. Brummelkamp, R. Bernards, and R Agami, Science 296, 550 (2002).
5. T.R. Brummelkamp, R. Bernards, and R Agami, Cancer Cell Published online Aug. 22, 2002.

pSUPERIOR INDUCIBLE SYSTEM

With pSUPERIOR vectors, expression of your siRNA is repressed in the absence of tetracycline and induced in the presence of tetracycline. When induced, transcription of the siRNA-precursor RNA hairpin occurs in the same manner as in the "standard" pSUPER vectors.

In fact, the siRNA expression cassettes in all pSUPER and pSUPERIOR vectors are completely identical, except for one key feature: ***a sequence modification of the H1 promoter between the TATA box and the RNA hairpin transcription start site***.

The following two sequences illustrate the difference:

- ***H1 promoter (from 35nt upstream of BglII / HindIII cloning site AGATCTaagctt):***

  5′…GAATCT**TATA**AGTTCTGTATGAGACCACAGATCTaagctt…3′

- ***Inducible H1 promoter (from 35nt upstream of BglII / HindIII cloning site AGATCTaagctt):***

  5′…GAATCT**TATA**AGTTCCCTATCAGTGATAGAGATCTaagctt…3′

The underlined 19-nt region of the second sequence indicates the modification, which corresponds to the tetracycline operator 2 ($TetO_2$) site. The $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor, and the change of the H1 promoter sequence in this manner does not in itself affect the transcription activity of the vector.

Unlike other tetracycline-regulated systems that use hybrid regulatory molecules and viral transactivation domains (Gossen and Bujard, 1992), pSUPERIOR vectors use only regulatory elements from the native Tet operon (Yao et al., 1998). This method more closely resembles the regulation of the native bacterial tet operon (Hillen and Berens, 1994; Hillen et al., 1983) and – importantly for RNAi research – avoids the potentially toxic effects of viral transactivation domains observed in some mammalian cell lines.

MECHANISM OF REPRESSION

In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to the $TetO_2$ sequence (Hillen and Berens, 1994) in the H1 promoter of the pSUPERIOR vector. The affinity of the Tet repressor for the tet operator is $K_B = 2 \times 10^{11}\ M^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994). Binding of the Tet repressor homodimer to the $TetO_2$ sequence represses transcription of the RNA hairpin precursor of the siRNA duplex targeted to silence your gene of interest.

Upon addition, tetracycline binds with high affinity to the Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The association constant, K_A, of tetracycline for the Tet repressor is $3 \times 10^{9}\ M^{-1}$ (Hillen and Berens, 1994). The Tet repressor tetracycline complex then dissociates from the Tet operator and allows transcription of the RNA hairpin precursor of the siRNA duplex.

OLIGO INSERT DESIGN

To effect the silencing of a specific gene, the pSUPERIOR vector is used in concert with a pair of custom oligonucleotides that contain, among other features, a unique 19-nt sequence derived from the mRNA transcript of the gene targeted for suppression (the "N-19 target sequence").

The N-19 target sequence corresponds to the sense strand of the pSUPER-generated siRNA, which in turn corresponds to a 19-nt sequence within the mRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridizes to this region of the mRNA to mediate cleavage of the molecule.

These forward and reverse oligos are annealed and cloned by the user into the vector, between the unique BglII and HindIII enzyme sites. This positions the forward oligo at the correct position downstream from the H1 promoter's TATA box to generate the desired siRNA duplex.

The sequence of this forward oligo includes the unique N-19 target in both sense and antisense orientation, separated by a 9-nt spacer sequence. The 5' end corresponds to the BglII site, while the 3' end contains the T5 sequence and any HindIII-corresponding nucleotides. *NOTE that while the 5' overhang of the oligo corresponds to the 3' BglII overhang of the plasmid, the overhang sequence of the oligo actually corresponds to the BamH1, and thus destroys the BglII site upon ligation to enable more efficient screening of positive clones.*

The resulting transcript of the recombinant vector 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.

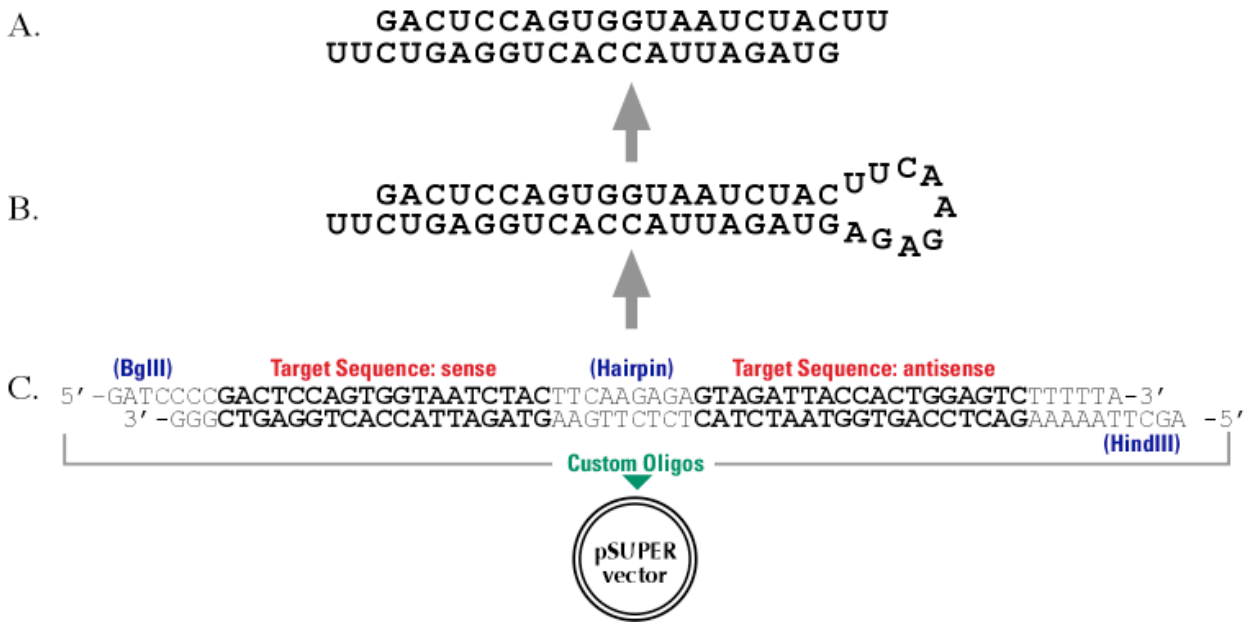


Fig. 1: *Transcription of 60-nt oligo to hairpin RNA, processed to functional siRNA.*

Many researchers have used oligos with this 60-nt design in pSUPERIOR vectors for effective gene suppression, as this is the configuration used for "non-inducible" versions (pSUPER and pSUPER.retro) of this vector. Others use a 59-nt configuration to account for the single base added in altering the H1 sequence to correspond to the $TetO_2$ site (see "pSUPERIOR Inducible System" above). While the RNA hairpin transcript is identical in either case, the 59-nt sequence reads as follows:

NEW: BglII / XhoI INSERT OLIGO DESIGN

To facilitate easier linearization of the pSUPER vector, OligoEngine now offers the option to purchase oligo inserts with the following 5' / 3' ends:

- BglII / HindIII (original format)
- BglII / XhoI (new format)

When designing and/or purchasing oligos, the OligoEngine workstation gives users the option to select either configuration. See below for more information about using the workstation in conjunction with pSUPER, and refer to the Procedure section for instructions on using BglII / XhoI oligos in pSUPER vectors.

OLIGOENGINE RNAi DESIGN TOOLS

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 in critical.

OligoEngine provides a design tool for the pSUPER RNAi System – as well as for the SI^2 Silencing Duplex – that generates N-19 target sequences for any gene of interest. The tool can be accessed by clicking on the "Order Now" tab at the top of any page on our Web site, http://www.olgioengine.com. Or, you can click the "Download Workstation" link from the Web site home page to run this tool as a stand-alone application.

The RNAi Design Tool automates the target design process recommended based on the most recent published research on RNAi mechanisms, as well as our own proprietary design algorithms. It helps users choose and configure these oligos by analyzing a their gene sequence and applying various algorithms according to the chosen design method and user parameters.

Once the design is complete you can order your oligos right from the Design Tool. These are synthesized with BglII (BamHI) and HindIII OR BglII (BamHI) and XhoI ends, so no digestion is required prior to cloning.

For more information and instructions, visit www.oligoengine.com.

PROCEDURE

Outline

Here are the general steps for an experiment utilizing a pSUPERIOR vector:

1. Anneal the forward and reverse strands of the oligos that contain the siRNA-expressing sequence targeting your gene of interest.
2. Linearize the pSUPERIOR vector with BglII and HindIII ***(OMIT this step if you have the linear vector)***
3. Clone the annealed oligos into the vector
4. Transform the vector in bacteria
5. Transfect pSUPERIOR vector into mammalian cells - OR - Co-transfect the pSUPERIOR vector and the TetR expressing vector into mammalian cells. *
6. Monitor EGFP fluorescence (for "+GFP" versions only)
7. Select with puromycin or neomycin to establish a stable cell line for siRNA expression (.neo or .puro versions)
8. Treat cells with tetracycline to induce transcription of the siRNA
9. Assay the effects on protein expression and/or mRNA levels

**Alternately, you can establish a stable cell line that constitutively expresses the Tet repressor and then transfect with the pSUPERIOR vector.*

General Molecular Biology Techniques

For many of the steps described below you may use the method of choice for your lab or level of experience. For assistance with transformations, restriction enzyme digestion and analysis, DNA purification, sequence and biochemistry, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994). See the "Lab Notes" and "Lab Tips" for recommendations based on the experience of the vector's inventors, and on feedback from our research customers.

>> 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 H_2O to a concentration of 3 mg/ml. If you need assistance to determine how much H2O (or buffer, etc.) to add to your product on hand, see the Lab Tips section in this manual following the procedure.

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 a similar buffer from another manufacturer, or b) 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.

>> Step Two: Linearize the Vector (Omit if you have the "Linear" version of this vector)

Linearize 1 µl of the pSUPERIOR vector with BglII and either HindIII OR XhoI restriction enzymes, depending on the configuration of your oligos as previously discussed. It is recommended to perform sequential reaction steps rather than simultaneous digestion, as follows: Digest with HindIII OR XhoI 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 fragment, and to help separate the prep from any undigested circular plasmid and to decrease the background in ligation and transformation.

Prior to the cloning reaction in Step Three, normalize the concentration of your digested plasmid to between 0.2 and 0.5 mg/ml. If you need assistance in adjusting your concentration, see the Lab Tips section of this manual following the protocol.

>> Step Three: Ligation into pSUPERIOR Vector

Assemble the cloning reaction by adding 2 µl of the annealed oligos to 1 µl of T4 DNA ligase buffer. Add 1 µl pSUPERIOR vector, 5 µl nuclease-free H_2O, and 1 µl T4 DNA ligase.

Incubate overnight at room temperature. A negative control cloning reaction should be performed with the linearized vector alone and no insert.

After cloning and prior to transformation, plasmids should be treated with BglII to reduce the level of background in your transformation.

To perform this reaction:

1. Add 1.0 µl of BglII to your plasmid
2. Incubate for 30 minutes at 37°

Remember, the BglII site is destroyed upon successful cloning of the oligo pair, so those vectors cut by the enzyme will NOT contain the insert fragment.

>>Step Four: Transformation in Bacteria

Recombinant pSUPERIOR vector 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 either HindIII or XhoI, depending on the configuration of your oligos as previously discussed. (Again, BglII can also be used since positive clones will not cut with BglII.) After digestion, determine your results as follows:

	Cut with EcoRI & HindIII	Cut with EcoRI & XhoI
Positive clone: vector with insert	281 bp	281 bp
Negative clone*: no insert	227 bp	248 bp

**e.g., supercoil that was nicked and not fully linearized with both enzymens) has a fragment of 227 or 248bp.*

In addition, the presence of the correct insert within your recombinant pSUPERIOR vector can be confirmed by sequencing prior to transfection in mammalian cells. For sequencing primer options, consult the appropriate pSUPERIOR vector map included in this manual, or consult the sequence file, which is available for download from the pSUPER RNAi System section of the OligoEngine Web site (www.oligoengine.com).

>> Step Five: Co-transfection of Mammalian Cells

The pSUPERIOR plasmid can now be transfected directly into your target cells along with a Tet repressor-expressing vector (pcDNA6/TR, Invitrogen).

> ***Lab Note:***
>
> Before co-transfection you must first obtain clean plasmid preparations of the regulator vector as well. Please refer to the instructions that accompany the regulator vector for details on preparing this plasmid.

Methods for transfection include, but are not limited to, calcium phosphate (Chen and Okayama, 1987; Wigler et al., 1977), lipid-mediated (Felgner et al., 1989; Felgner and Ringold, 1989) and electroporation (Chu et al., 1987; Shigekawa and Dower, 1988). Researchers have also reported success utilizing Lipofectamine (Invitrogen) and the polyamine reagents LT1 and LT2 (from Mirus Corporation, www.genetransfer.com). Refer to these products' protocols for more specific transfection procedures. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

> ***Lab Note:***
>
> When culturing cells in medium containing fetal bovine serum (FBS), please note that many lots of FBS contain tetracycline as FBS is generally isolated from cows that have been fed a diet containing tetracycline. If you culture your cells in medium containing FBS that is not reduced in tetracycline, you may observe low basal expression of your gene of interest in the absence of tetracycline. If your gene of interest produces a toxic protein, you may wish to culture your cells in tetracycline-reduced FBS. For more information, please consult the supplier of your serum.

As an example, the following procedure provides guidelines to co-transfect your pSUPERIOR vector with the pcDNA6/TR TetR-expressing regulator vector. Because tetracycline-regulated expression utilizing the pSUPERIOR is based on a repression/ derepression mechanism, the amount of Tet repressor that is expressed in the host cell line from the regulator vector will determine the level of transcriptional repression of the Tet operator sequence in the pSUPERIOR vector. Tet repressor levels should be sufficiently high to suitably repress basal level transcription. Based on information from extensive use of the pcDNA6/TR vector in other inducible systems, we recommend that you co-transfect your mammalian host cell line with a ratio of at least 1:6 (w/w) pSUPERIOR vector : TetR regulator vector.

Co-transfection Example:

1. Use cells that are approximately 60% confluent for transfection.

2. Cotransfect the pSUPERIOR vector and the regulator plasmid (in this case, pcDNA6/TR) at a ratio of 1:6 (w:w) into the cell line of choice using your preferred method. Absolute amounts of plasmid will vary depending on the method of transfection and the cell line used.

3. After transfection, add fresh medium and allow the cells to recover for 24 hours before induction.

Since every cell line is different and may require a different method of transfection, some experimentation may be needed to determine the optimal conditions for inducible expression. For established cell lines (e.g. HeLa, COS-1), please consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line (pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells).

Using Stable Cells

You may wish to establish a stable cell line that constitutively expresses the Tet repressor and inducibly expresses your siRNA. We recommend that you first create a stable cell line that expresses only the Tet repressor, then use that cell line to create a second cell line that will express your siRNA from the pSUPERIOR vector. Alternatively, you can transfect with both plasmids (pcDNA6/TR and pSUPERIOR) and dual-select with to isolate a single stable cell line expressing both the Tet repressor and your gene of interest.

Remember, when generating a stable cell line expressing the Tet repressor, you will want to select for clones that express the highest levels of Tet repressor to use as hosts for your inducible expression construct. Those clones which express the highest levels of Tet repressor should exhibit the most complete repression of basal transcription of your gene of interest. Again, please refer to the instructions that accompany the regulator vector for additional details.

>> Step Six: Detection of EGFP Fluorescence

A fluorescent inverted microscope provides the easiest and most recommended method for detection of EGFP expression, and enables analysis of live cells.

If you do not have access to an inverted microscope, fluorescence can also be detected with either a photographic (35-mm) or digital camera (which frequently harbors a cooled charge-coupled device, a CCD), fitted with the appropriate filter. You can achieve excellent results using standard filter sets, such as FITC filters to detect EGFP, although optimized filter sets for detecting GFP are also available. Refer to the “Lab Tips” section of this manual following the protocol for this method of analyzing fixed cells.

>> Step Seven: Selection of Stable Transfectants

The levels of siRNA expression and gene knockdown will typically vary widely among cells. In particular, transfection efficiency may be lower for primary cells; it is often difficult to obtain a stably expressing clone from normal (non-transformed) or primary cell lines using pSUPERIOR vectors. If possible choose a transformed or immortal cell line instead.

Moreover, pSUPERIOR-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. Therefore, it can be useful to isolate clones that can be screened to identify the cells that cause the desired reduction in target gene expression.

Permanent cell lines may be created by growing the transfected cells under antibiotic selection. Begin by splitting the cells at 1:10-20 dilution.

> ***Lab Note:***
>
> When selecting for positive clones, be sure to establish a kill curve for each lot of antibiotic to determine optimal effective dose. For puro selection, identify the lowest level of antibiotic that kills non-transfected cells within approximately 5 days by testing antibiotic concentrations from 1–10 µg/ml while keeping all other culture conditions equal. For neo/G418 selection, identify the lowest level that kills non-transfected cells within approximately 7 days by testing antibiotic concentrations from 25–4000 µg/ml

Note that the selection method described herein is optimized for 293 cells. For other cells, follow manufacturer's directions, or refer to the following procedure, which is provided as a reference method for stable cell selection (your standard laboratory procedure may work best for your specific conditions):

a. Plate transfected cells at an optimized cell plating density: plating density can have a strong impact on antibiotic selection because cells at higher densities are less effectively killed off than cells at lower concentration. Also, cells that divide more rapidly typically have a lower optimal plating density than cells that double slowly.

   It is important to include two non-transfected control cultures. One is subjected to antibiotic selection to control for cells that spontaneously become antibiotic resistant or are already antibiotic resistant; 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.

b. After 24 hours, add culture medium containing the optimum antibiotic concentration:

   - ***For .neo vectors***: Grow the cells in medium containing 500 µg/ml of neomycin or G418 (Invitrogen). Untransfected cells should die within 5-15 days.

   - ***For .puro vectors***: Grow the cells in medium containing 1-10 µg/ml of puromycin. Untransfected cells should die within 5-10 days.

c. Culture the cells in medium containing antibiotic 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 selection.

>> Step Eight: Induction with Tetracycline

Remove medium and add fresh medium containing the appropriate concentration of tetracycline to the cells; it is recommended to take the cells off antibiotic to avoid potential uncontrollable effects on the tetracycline-inducible system by the antibiotic. In general, we recommend that you add tetracycline to a final concentration of 1 µg/ml (5 µl of a 1 mg/ml stock per 5 ml of medium) to the cells and incubate the cells for 24 hours at 37°C. To prepare a stock solution from tetracycline please consult the protocol supplied by the manufacturer. The following is provided as an example for a stock of tetracycline salt:

1. Weigh out 10 mg of tetracycline and transfer to a sterile 15 ml conical polypropylene tube.
2. Resuspend 10 mg of tetracycline in 10 ml of water to produce a 1 mg/ml stock solution that is yellow in color. (Note: If you are using a different form of tetracycline (i.e. free base form), resuspend in 100% ethanol rather than water.)
3. Store the stock solution at -20°C protected from exposure to light.

Lab Notes:

Tetracycline (MW = 444.4) is commonly used as a broad spectrum antibiotic and acts to inhibit translation by blocking polypeptide chain elongation in bacteria. With the pSUPERIOR vector, tetracycline is used as an inducing agent to induce transcription of the gene of interest from the inducible expression vector. Tetracycline induces transcription by binding to the Tet repressor homodimer and causing the repressor to undergo a conformational change that renders it unable to bind to the Tet operator. The association constant of tetracycline to the Tet repressor is 3×10^9 M^{-1} (Takahashi et al., 1991). Please note that the concentrations of tetracycline used to induce gene expression in this procedure are generally not high enough to be toxic to mammalian cells. Always remember:

- Tetracycline is light sensitive. Store the powdered drug at +4°C in the dark. Prepare medium containing tetracycline immediately before use.
- Tetracycline is toxic. Do not ingest or inhale the powder or solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling tetracycline and tetracycline-containing solutions.

Harvest the cells and assay for expression of your gene. To optimize the level of siRNA transcription by pSUPER, you may want to vary the concentration of tetracycline (0.1 to 1 µg/ml) and time of exposure to tetracycline (8 to 24 hours) to optimize or modulate expression for your cell line.

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.

Lab Tips

Diluting Oligonucleotides

To dilute your lyophilized oligos to a specified concentration, use the following equation to determine how much H_2O (or buffer, etc.) to add to your product on hand:

$$\text{ml } H_2O \text{ required for concentration of } \mathbf{X}\text{mg/ml} = (\mu\text{g oligos} \times 10^{-3}) / \mathbf{X}$$

Thus, if you have 200 µg of oligo*, add ~0.067 ml of H_2O to achieve a concentration of 3mg/ml [$(200 \times 10^{-3}) / 3 = \sim 0.067$]. 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.

You can access OligoEngine's "Concentration calculator" online at www.oligoengine.com/calculator.html.

> **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. 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.*

Normalization of Plasmid Concentration

If you are working with the circular version of the pSUPERIOR vector and have performed the BglII / HindIII digestion yourself, you may wish 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 A260 OD = 50 µg/ml dsDNA.
- Using on this measurement, adjust your concentration of vector to between 0.2 and 0.5 mg/ml before proceeding to ligation (ethanol precipitation and re-dilution may be required).

Step-Cooling Suggestions for Annealing Oligos

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 to include more steps in the cooling process. 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.

The following procedure has proven to reduce the occurrence of self-hybridizing oligonculeotides:

- 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.)

Standard Microscopy Procedure for Fluorescence Detection

The protocol provided below is one possible microscopy procedure for detection of the EGFP expression by pSUPERIOR vectors. Other equally suitable and more detailed microscopy procedures may be found elsewhere (e.g., Ausubel et al., 1995 et seq.)

Materials required

- 70% Ethanol
- Dulbecco's Phosphate buffered saline (DPBS; pH 7.4)
- DPBS/4% paraformaldehyde (pH 7.4–7.6)
  Add 4 g of paraformaldehyde to 80 ml of DPBS. Heat to dissolve. Once the solution has cooled, readjust the pH if necessary, then dilute to a final volume of 100 ml. Store at –20°C.
- Rubber cement, molten agarose, or commercial mounting medium
  (e.g., ProLong® Antifade Kit, Molecular Probes)

Procedure

In a tissue culture hood:

a. Sterilize a glass coverslip with 70% ethanol.

b. Place the coverslip in a sterile tissue culture dish.

c. Plate and transfect cells in the tissue-culture dish containing the coverslip.
   Note: Some cell types may not adhere to the glass coverslip. In these cases, you may need to pre-treat the glass coverslip with a substrate that promotes cell adhesion (e.g., lamin, or poly-D-lysine, or both).

d. At the end of the culture period, remove the tissue culture media and wash once with DPBS.

e. Fixing cells:

   i. After cells have been washed with DPBS, add freshly made DPBS/4% paraformaldehyde directly to the coverslip.
   ii. Incubate cells in solution at room temperature for 30 min.
   iii. Wash cells twice with DPBS. Allow cells to soak in DPBS for 10 min during each wash.

f. Mounting the coverslip onto a glass microscope slide:

   i. Carefully remove the coverslip from the plate with forceps. Pay close attention to which side of the coverslip contains the cells.
   ii. Place a tiny drop of commercial mounting solution (e.g., ProLong® Antifade Kit, Molecular Probes) on the slide, and allow the coverslip to slowly contact the solution and to lie down on the slide, cell side down.
   iii. Carefully aspirate the excess solution around the edge of the coverslip using a Pasteur pipette connected to a vacuum pump.
   iv. If desired, seal the coverslip to the microscope slide using molten agarose, rubber cement, or black nail polish.
   v. Allow to dry. The drying time may vary depending on the mounting solution used.
   vi. Examine slides by fluorescence microscopy. Once fixed, cells can be stored in the dark at 4°C.

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 proper ligation and effective target knockdown.

- ***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 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 the appropriate vector map or sequence file 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.

pSUPER RNAi System™

VECTOR: pSUPERIOR.basic
CATALOG#: VEC-IND-0001/0002

Length: 3177 bp

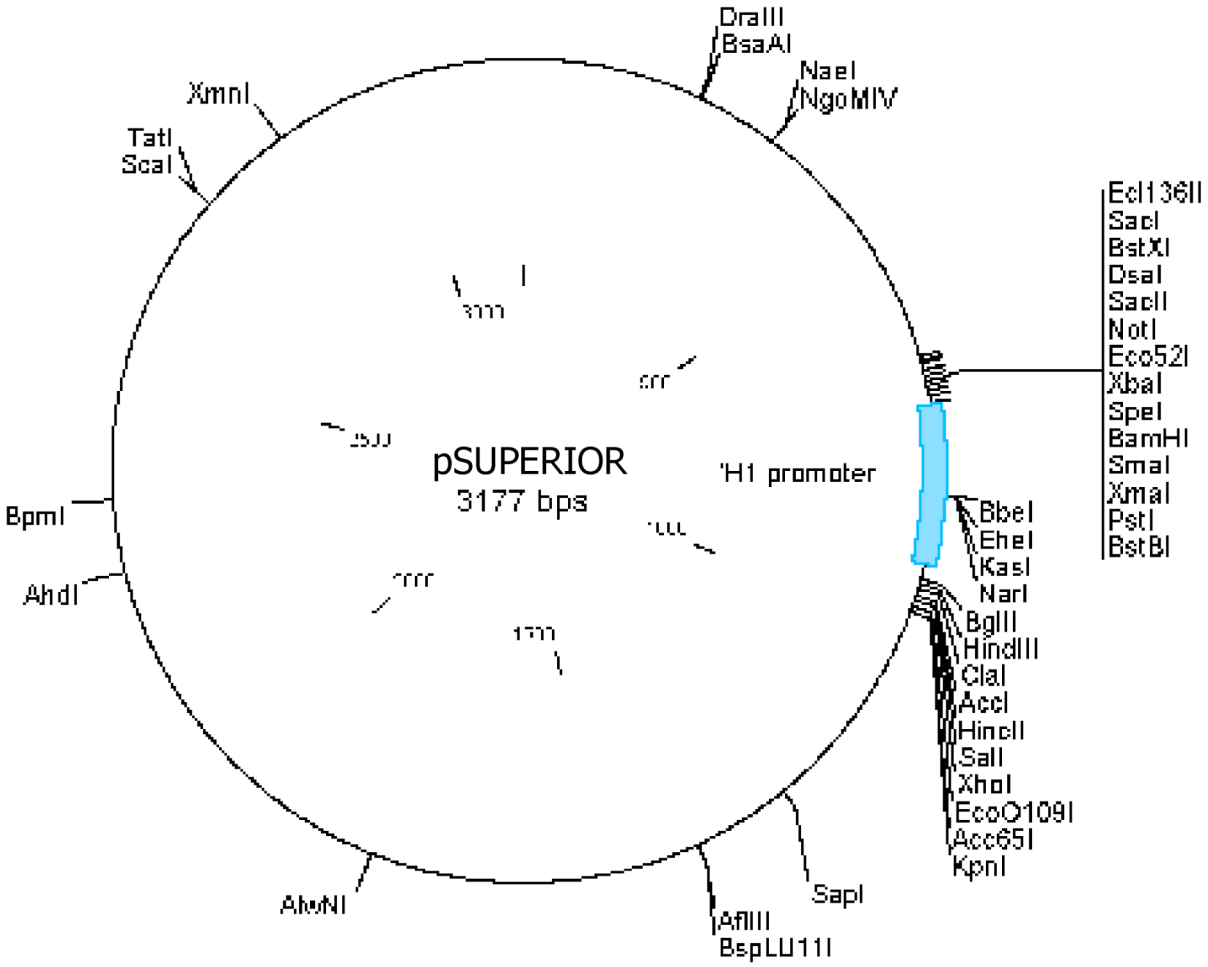
Key Sites

BglII: 929
HindIII: 935
EcoRI: 708
SalI: 950
XhoI: 956

Vector Features

f1(+) origin: 441-135
H1 promoter: 708 - 935
pUC origin: 1374-2041
Ampicillin resistance ORF: 3049-2192

T7 primer (AATACGACTCACTATAG): 627-643
T3 primer (ATTAACCCTCACTAAAG): 1006-990
M13 (-20) primer (GTAAAACGACGGCCAGT): 600-616
M13 reverse primer (AACAGCTATGACCATG): 1039-1024



pSUPER RNAi System™

VECTOR: pSUPERIOR.neo
CATALOG#: VEC-IND-0003/0004

Length: 4700 bp

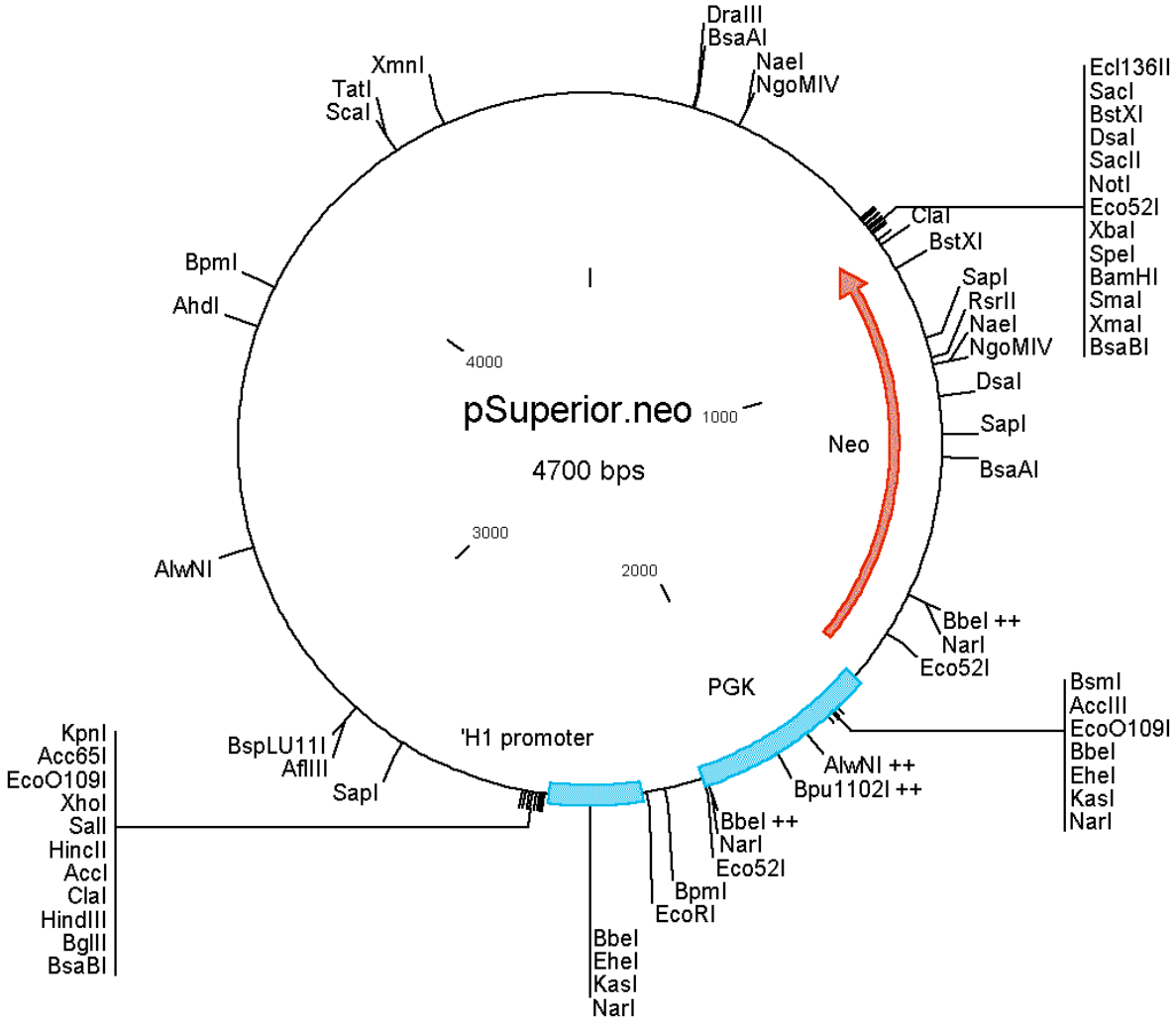
Key Sites

BglII: 2452
HindIII: 2458
EcoRI: 2230
SalI: 2473
XhoI: 2479

Vector Features

PGK promoter: 2110-1712
Neo ORF: 1684-715
H1 promoter: 2235-2437
Ampicillin resistance ORF: 3369-4226

T3 primer (ATTAACCCTCACTAAAG): 2529-2513
M13 reverse primer (AACAGCTATGACCATG): 2562-2547



pSUPER RNAi System™

VECTOR: pSUPERIOR.puro
CATALOG#: VEC-IND-0005/0006

Length: 4354 bp

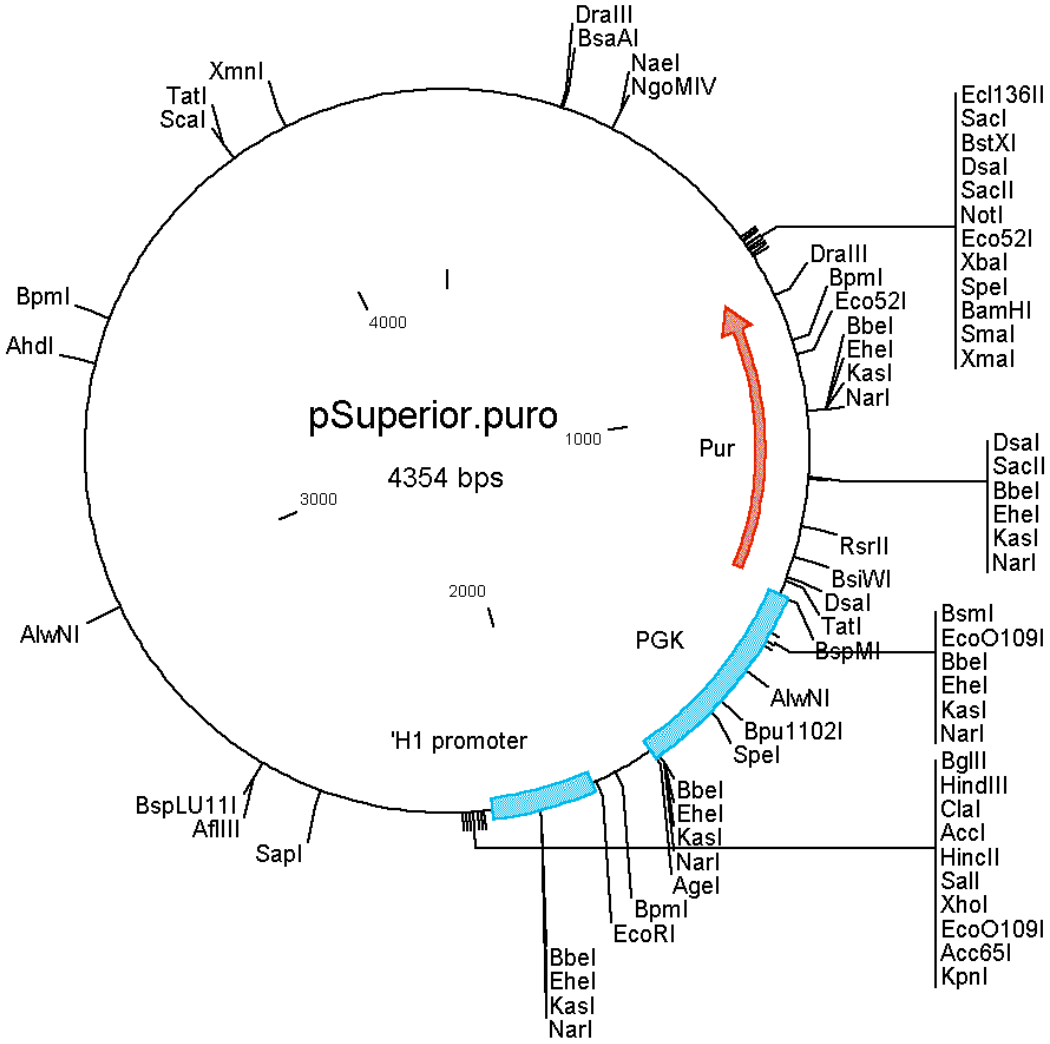
Key Sites

BglII: 2106
HindIII: 2112
EcoRI: 1884
SalI: 2127
XhoI: 2133

Vector Features

f1(+) origin: 135-441
PGK promoter: 1768-1370
Puro ORF: 1355-756
H1 promoter: 1889-2091
Ampicillin resistance ORF: 3369-4226

T3 primer (ATTAACCCTCACTAAAG): 2183-2167
M13 reverse primer (AACAGCTATGACCATG): 2216-2201



pSUPER RNAi System™

VECTOR: pSUPERIOR.neo+GFP
CATALOG#: VEC-IND-0007/0008

Length: 5430 bp

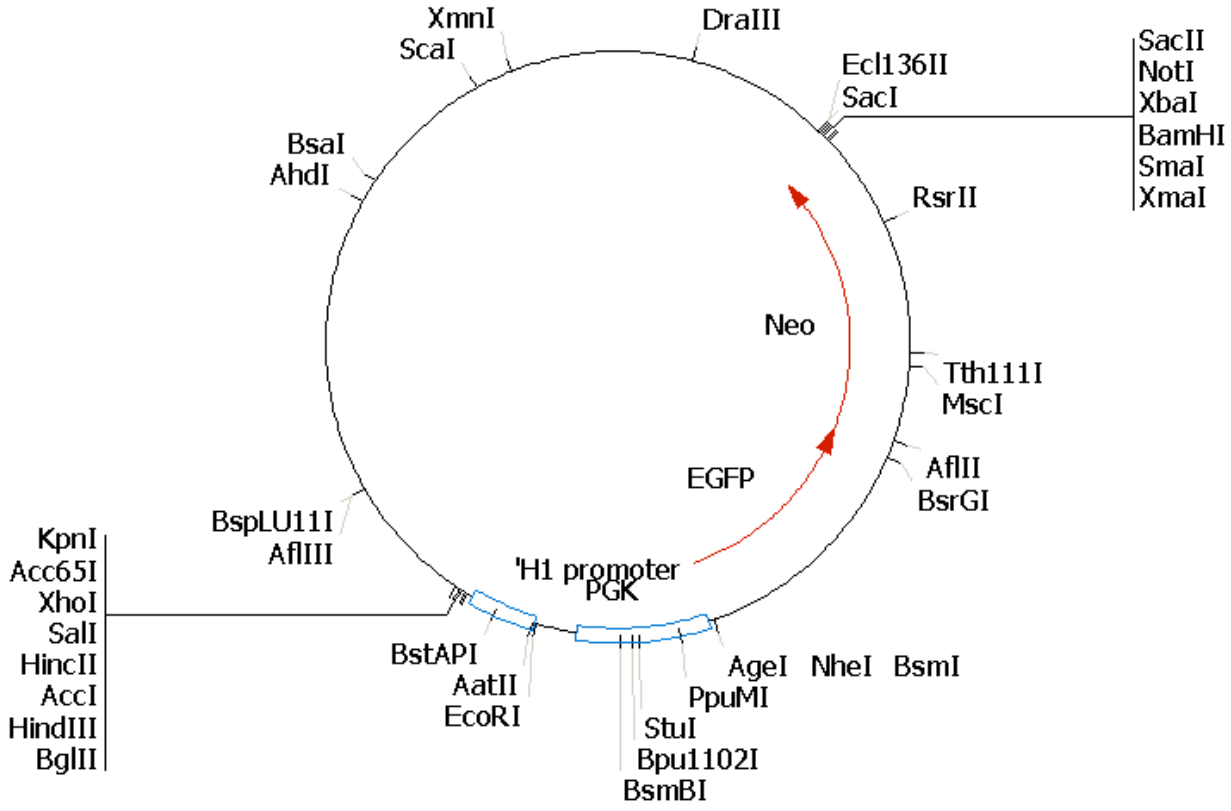
Key Sites

BglII: 3182
HindIII: 3188
EcoRI: 2960
SalI: 3203
XhoI: 3209

Vector Features

f1(+) origin: 135-441
PGK promoter: 2840-2442
Neo ORF: 1684-715
EGFP ORF: 2424-1691
H1 promoter: 2965-3167
Ampicillin resistance ORF: 5302-4445

T3 primer (ATTAACCCTCACTAAAG): 3259-3243
M13 reverse primer (AACAGCTATGACCATG): 3292-3277



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