



pSUPER RNAi System™

pSUPER.retro.puro : Manual

A Vector System for Expression of Short Interfering RNA

The following manual contains information about and
Instructions for the following pSUPER.retro vectors:

<u>Vector Name</u>	<u>Catalog#</u>
pSUPER.retro.puro	VEC-PRT-0001 (linear) VEC-PRT-0002 (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:	<i>pSUPER.retro. is shipped to customers at ambient temperature to reduce shipping and handling costs without affecting product quality and effectiveness.</i>

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.

OLIGO INSERT DESIGN

To effect the silencing of a specific gene, the pSUPER.retro 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 BamHI, 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.

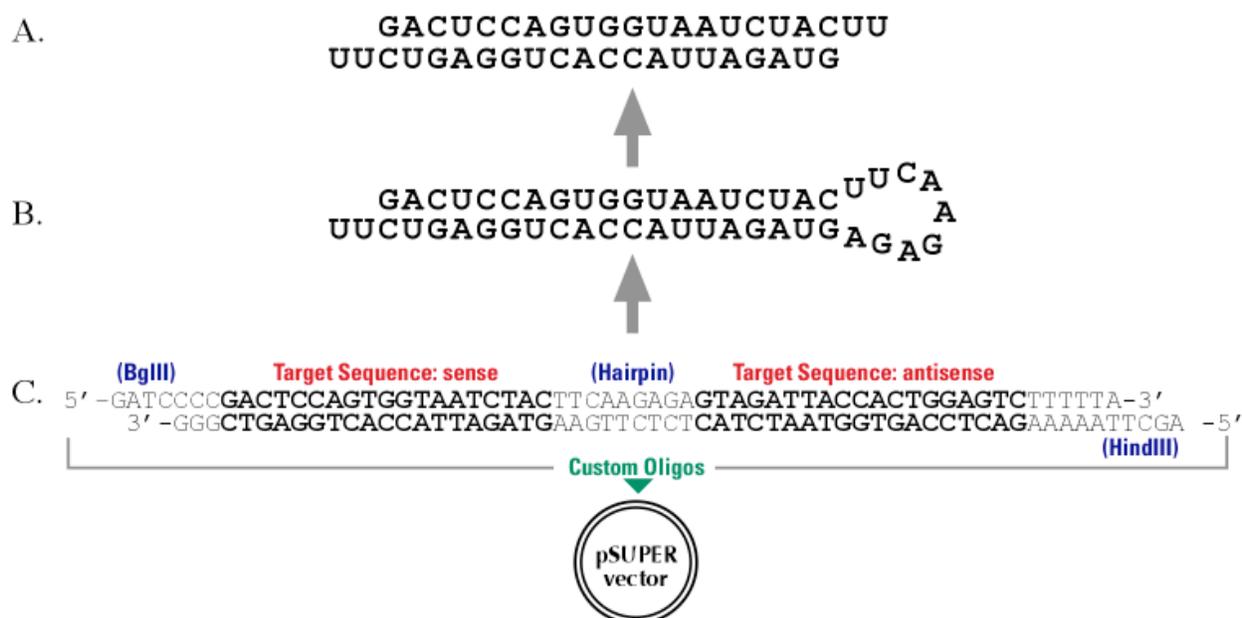


Fig. 1: Transcription of 60-nt oligo to hairpin RNA, processed to functional siRNA.

NEW: BglIII / XhoI INSERT OLIGO DESIGN

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

- BglIII / HindIII (original format)
- BglIII / 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 BglIII / 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 is critical.

OligoEngine provides a design tool for the pSUPER RNAi System – as well as for the SI² 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.oligoengine.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 BglIII (BamHI) and HindIII OR BglIII (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 pSUPER.retro 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 pSUPER.retro 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 pSUPER.retro vector into packaging cell line and introduce retrovirus to mammalian cells; -OR- transiently transfect pSUPER.retro vector directly into mammalian cells
6. Select with puromycin or neomycin to establish a stable cell line for siRNA expression (.neo or .puro versions)
7. Assay the effects on protein expression and/or mRNA levels

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 H₂O 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 pSUPER.retro 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, 1 µl 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.

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.

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.

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 pSUPER.retro Vector

Assemble the cloning 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 H₂O, 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 pSUPER.retro 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	Approx 227	Approx 248

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

In addition, the presence of the correct insert within your recombinant pSUPER.retro vector can be confirmed by sequencing prior to transfection in mammalian cells. For sequencing primer options, consult the appropriate pSUPER 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: 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). 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.

pSUPER.retro with Packaging Cells

The pSUPER.retro vectors can be transfected per the above procedure (i.e., for transient transfection) – or, for a higher rate of stable cell integration, pSUPER.retro vectors can be transfected into a packaging cell line by these same methods 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ for 48 hr).

Again, it should be emphasized that your selection of packaging cell line is likely to be dependent on the organism and cell type with which you are working; therefore, please refer to the procedures accompanying your packaging cell line for specific methods and materials information.

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.

Since every cell line is different and may require a different method of transfection, some experimentation may be needed to determine the optimal conditions. 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).

>> 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 pSUPER vectors. If possible choose a transformed or immortal cell line instead.

Moreover, pSUPER-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 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.

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 H₂O (or buffer, etc.) to add to your product on hand:

$$\text{ml H}_2\text{O required for concentration of Xmg/ml} = (\mu\text{g oligos} \times 10^{-3}) / \text{X}$$

Thus, if you have 200 μg of oligo*, add ~0.067 ml of H₂O to achieve a concentration of 3mg/ml [(200 $\times 10^{-3}$) / 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.

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 $\times 10^{-6} \times$ 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 $\mu\text{g/ml}$ ssDNA), then adjust your concentration accordingly.*

Normalization of Plasmid Concentration

If you are working with the circular version of the pSUPER.retro vector and have performed the BglIII / 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 $\mu\text{g/ml}$ 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 oligonucleotides:

- 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.)

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.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 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: pSUPER.retro.puro
CATALOG#: VEC-PRT-0001/0002

Length: 6349 bp

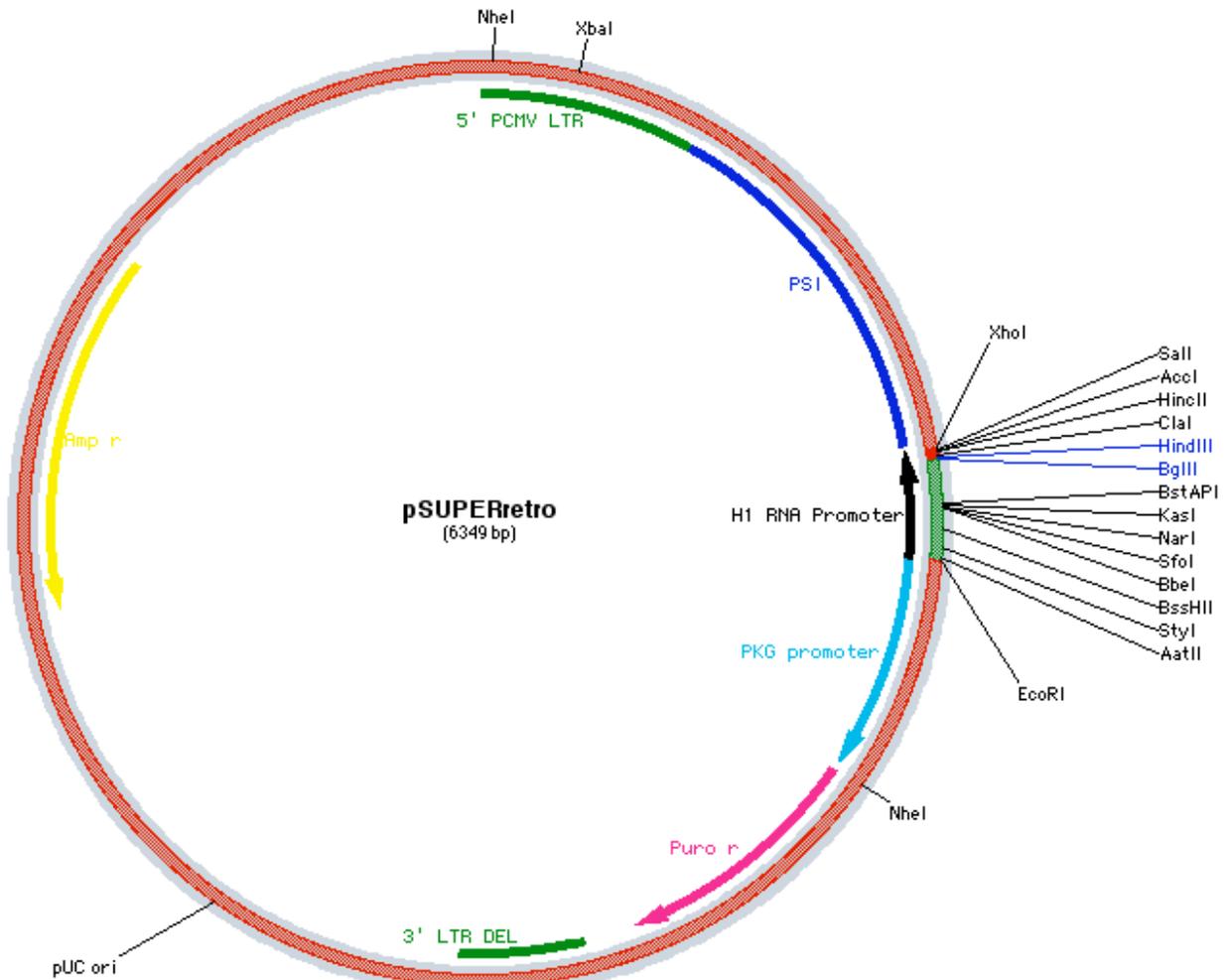
Key Sites

BglII: 1447
HindIII: 1441
EcoRI: 1668
Sall: 1426
XhoI: 1420

Vector Features

PGK promoter: 1669-2177
Puro ORF: 2202-2801
H1 promoter: 1420-1668
Ampicillin resistance ORF: 4558-5415
3' delta LTR: 2931-3224
5' LTR: 1-515 (homologous to other MSCV LTR)

Sequencing primer 5'-GGAAGCCTTGGCTTTTG-3' binding site: 1241-1257



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