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Favorgen Biosettia™ Single Oligonucleotide RNAi Technology for Gene Silencing

**pRNAi vector system for transient and stable
shRNA expression**

Catalog number: FA-SORT-A##

August 2008

Amount: 20 reactions

Storage Temperature: -20°C upon arrival

User Manual

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Product Description

RNA interference (RNAi) refers to a mechanism by which double-strand RNAs (e.g. siRNA, shRNA, and miRNA) inhibit gene expression via nucleotide sequences complementary to the targeted messenger RNA, leading to mRNA degradation or translational repression (for reviews, see [1-7]). The discovery that small interfering RNA (siRNA) can trigger knockdown of gene expression in mammalian systems in 2001 has made RNAi a standard tool for sequence-specific gene silencing [8]. Instead of the time-consuming and labor-intensive knockout procedures in animal models, the RNAi-based gene knockdown method provides an alternative approach to functional genomic studies in both animal and cell line models.

Available technologies for generating small double-strand RNAs (i.e. siRNA and shRNA) for RNAi include chemical synthesis (for siRNA) and vector-based expression (for shRNA) [8-12]. While effective in triggering RNAi, the synthetic siRNAs are expensive and only mediate a transient knockdown effect. In contrast, the promoter-driven expression of short hairpin RNAs (shRNAs) in cells is more cost-effective, and the effect of gene silencing can possibly persist for a long period of time by stable selection of the antibiotic-resistant gene encoded on the vector.

The pRNAi vector system is designed to facilitate the cloning of the double-strand DNA oligonucleotide encoding a stem-loop sequence, which is transcribed into shRNA by RNA polymerase III promoter (e.g. H1, human and mouse U6 promoters) once expressed in cells (Fig. 1). Our single oligonucleotide RNAi technology (SORT) for gene silencing delivers a unique feature in the construction of shRNA expression vectors. Distinct from any other shRNA expression vectors currently available, only one DNA oligonucleotide is required for cloning a shRNA sequence into the pRNAi vector (see “Advantages of the product” below and Fig. 2). The novelty of the pRNAi vector system benefits the users with lower cost, higher efficiency, and less complexity.

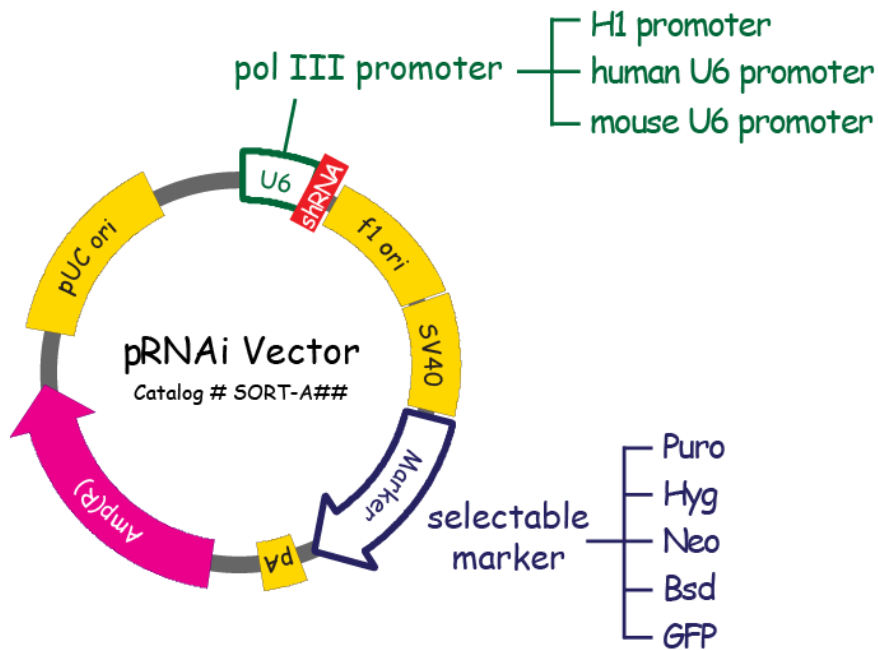


Figure 1. Structure map of pRNAi vector system.

Product Components

Each pRNAi kit provides a sufficient amount of reagents for 20 ligation reactions:

20µl	pRNAi vector (linearized, 10ng/µl/rxn, 10µl/tube)
100µl	10× annealing buffer (store at room temperature)
20µl	pRNAi with shRNA insertion as negative control* (1ng/µl)
30µl	Sequencing primer** (10µM)

*control shRNA sequence: GTGACCAGCGAATACCTGTCTTGGATCCAAGAACAGGTATTTCGCTGGTCAC

**sequencing primer: H1, AACGCTGACGTCATCAACCCGC; hU6, ATTTGCATATACGATACAAGGC; mU6, GAAGCTCGGCTACTCCCCTGCC.

Advantages of the Product

1. The pRNAi vector is ready-to-use. No restriction digestion or vector purification is required.
2. Less cloning complexity. The single-strand DNA oligo encoding shRNA sequence is a perfect palindrome, and the same (two) palindromic oligos can anneal to each other to form a double-strand oligo. This eliminates the need to mix and anneal two different DNA oligos and reduces operational mistakes during the cloning process. The overall chance of mutations introduced during DNA oligo synthesis is also reduced by 50% (Fig. 2A).
3. Lower cost. Only one DNA oligo is required to generate an shRNA expression clone. This setup is the most economical, and is distinguishable from all the other shRNA vectors commonly used in academia and industry, for which two different oligos are needed to make a shRNA expression clone. Also, the length of the DNA oligo used for pRNAi (52-56 nt) is shorter than the length of DNA oligos required for most of the other commercially available shRNA vectors.
4. High efficiency and low background in shRNA cloning. The 5'-AAAA overhangs on annealed double-strand oligo can only be ligated to the 5'-TTTT overhangs on both ends of the linear pRNAi vector. Unlike the overhangs generated by restriction digestion in other available shRNA expression vectors, these 5'-TTTT overhangs in pRNAi will not self-ligate. As a result, the ligation of the double-strand oligo into the pRNAi vector is highly efficient, and the contamination of empty, self-ligated pRNAi vectors is greatly reduced (Fig. 2B).
5. The choice of three promoters for shRNA expression. The shRNAs can be expressed from human H1 (H1), human U6 (hU6), or mouse U6 (mU6) promoters in the pRNAi system. Researchers are able to choose the pol III promoter suitable to their model system.

6. Long-term suppression. The pRNAi vectors encode antibiotics resistance markers for puromycin (Puro), hygromycin (Hyg), neomycin (Neo), or blasticidin (Bsd), allowing researchers to generate stable cell lines with long-term suppression of genes of interest.

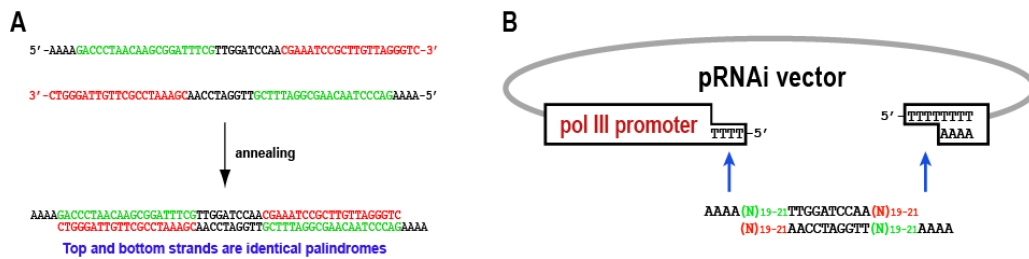


Figure 2. Overview of pRNAi cloning system. (A) Example of single oligonucleotide RNAi technology: two identical DNA oligos anneal to each other to form a double-strand oligo with 5'-AAAA overhangs. The single-strand oligo encodes 21-nt sense target sequence (in green), 10-nt loop sequence (TTGGATCCAA), and 21-nt antisense target sequence (in red). (B) The 5'-AAAA overhangs of an annealed double-strand oligo can only ligate with the 5'-TTTT overhangs on the pRNAi vector, which greatly increases the ligation efficiency and reduces the background of cloning.

Protocol

Designing oligonucleotide encoding shRNA (sense-loop-antisense) sequence

The features of single-strand oligonucleotide encoding shRNA sequence include the following (Fig. 3):

1. Stem-loop sequence. Consist of 19-21 nucleotides (nt) target (or sense) sequence, 10 nt loop sequence (TTGGATCCAA), and 19-21 nt target antisense sequence. This sense-loop-antisense sequence is a perfect palindrome with 48-52 nt in length. Therefore, only one oligonucleotide has to be ordered since both strands in the annealed double-strand oligonucleotide are identical (Fig. 2A).
2. Overhang sequence. The 5'-AAAA sequence is complementary to the overhang of the pRNAi vectors and restores the promoter and terminator sequences in the vector (Fig. 2B).

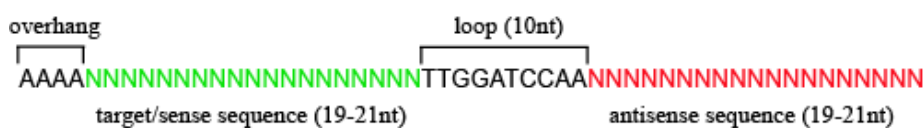


Figure 3. Structure of oligonucleotide encoding shRNA

There are no standard but only general guidelines for selecting the shRNA targeting sequences [13-17]. However, following these guidelines does not guarantee efficient knockdown. The effectiveness of shRNA sequence has to be determined by gene suppression analysis. In general, we suggest selecting 3 to 5 target sequences per gene of interest to identify at least two functional shRNAs with over 70% knockdown efficiency in target gene expression.

Listed below are a few rules we recommend for selecting shRNA sequences:

- Unique 19-21 nt target sequence with 30%-70% GC content; A 35%-55% GC content is preferred.
- Avoid target sequences with significant homology to other genes, unless the shRNA is intended to knockdown a gene family.
- A target sequence within an open reading frame is preferred; targeting 3' UTR is ok.
- No more than 4 consecutive T in the shRNA sequence to cause early transcription termination.
- GC-rich in the 5' end and AT-rich in the 3' end of target sequences are preferred.
- Choose a target sequence that starts with guanosine (G) if the U6 promoter is used.

The target sequence can be designed from the following web tools.

- <http://www.promega.com/siRNADesigner/program/>
- <https://rnaidesigner.invitrogen.com/rnaiexpress/index.jsp>
- <http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA>
- <http://www.genelink.com/sirna/shRNAi.asp>
- <http://www.dkfz.de/signaling2/e-rnai/>
- <http://alps3.gi.k.u-tokyo.ac.jp/~yamada/sidirect2/index.php>
- <http://side.bioinfo.ocha.fib.es/>
- <http://i.cs.hku.hk/~sirna/software/sirna.php>
- <http://sfold.wadsworth.org/index.pl>

Note: Please make sure the 5' overhang sequence is AAAA and the loop sequence is TTGGATCCAA.

Suggestion: Ordering oligonucleotide with gel purification and 5' phosphorylation is not required.

As a negative control for knockdown analysis, use a nonspecific target sequence or a scrambled sequence. For example, shRNA oligonucleotide targeting luciferase or β -galactosidase gene could be used as negative control.

Cloning double-strand oligo into pRNAi vector: annealing, ligation, transformation and screening for insertions.

1. In a 1.5 ml microcentrifuge tube, set up the annealing reaction in total 20µl mixture as following:
 - 10 µl 100 µM single-strand oligo
 - 2 µl 10× annealing buffer
 - 8 µl distilled water
2. Incubate the DNA oligo at 95°C for 5 minutes.
3. Remove the tube from the water bath and allow the reaction mixture to cool to room temperature for 10 minutes.
4. Dilute 1 µl annealed double-strand oligo in 499 µl distilled water, vortex for 5sec.
 Suggestion: Load 20µl of diluted oligo onto 4% agarose gel (FMC Bioproducts, NuSieve GTG Agarose Catalog No. 50082) to determine annealing efficiency (example in Fig. 4).

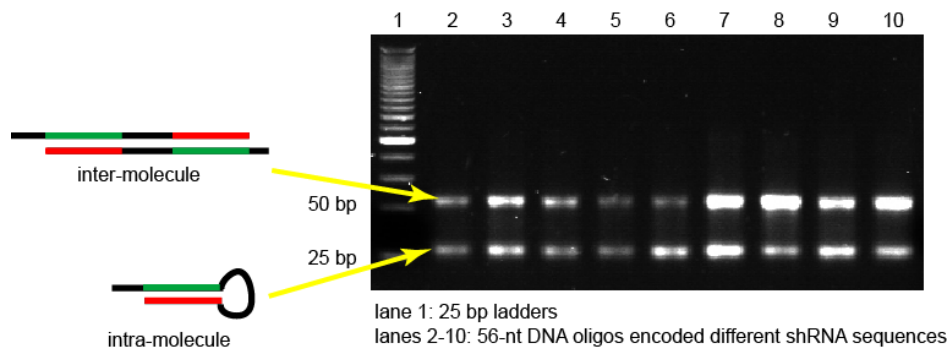
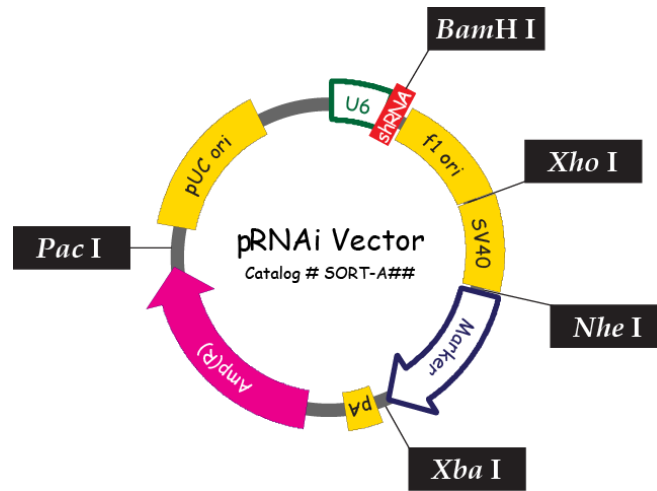


Figure 4. Example of annealing DNA oligonucleotides encoding shRNA sequences.

5. Store the remainder of the annealed oligo mixture at -20°C.
6. Mix 1 µl (10ng) pRNAi vector with 2 µl 500× diluted (50nM final) double-strand oligo.
 Note: Make sure to mix pRNAi vector with annealed oligo before adding other ligation components.
7. Add ligase and other ligation components to total 20µl.
8. Incubate the ligation reactions at room temperature for ≥3 hours or incubate the reactions overnight at 4°C.
9. Transfer 2-5 µl ligation reaction mixtures to competent cells (e.g. DH5α) with a transformation efficiency $\geq 1 \times 10^8$ pfu/µg DNA according to the supplier protocol or the transformation protocol routinely used in your laboratory.
 Suggestion: Keep the rest of ligation mixture at 4°C for overnight to obtain higher number of colonies in another transformation if necessary.
10. Plate all transformation reaction on LB plate with 50-100 µg/ml ampicillin and incubate at 37°C for overnight.
11. Inoculate ≥ 2 colonies from each pRNAi-shRNA clone to prepare plasmids for further experiments.
12. The shRNA sequence containing a *BamH* I (GGATCC) site inside the loop sequence. Therefore, restriction digestions by *BamH* I plus another enzyme (e.g. *Nhe* I, *Xba* I, or *Xho* I) can be used to confirm the insertion of double-strand oligo in the pRNAi vector (example in Fig. 5).

Suggestion: DNA sequencing is recommended to ensure no mutations are present in the oligo insertion



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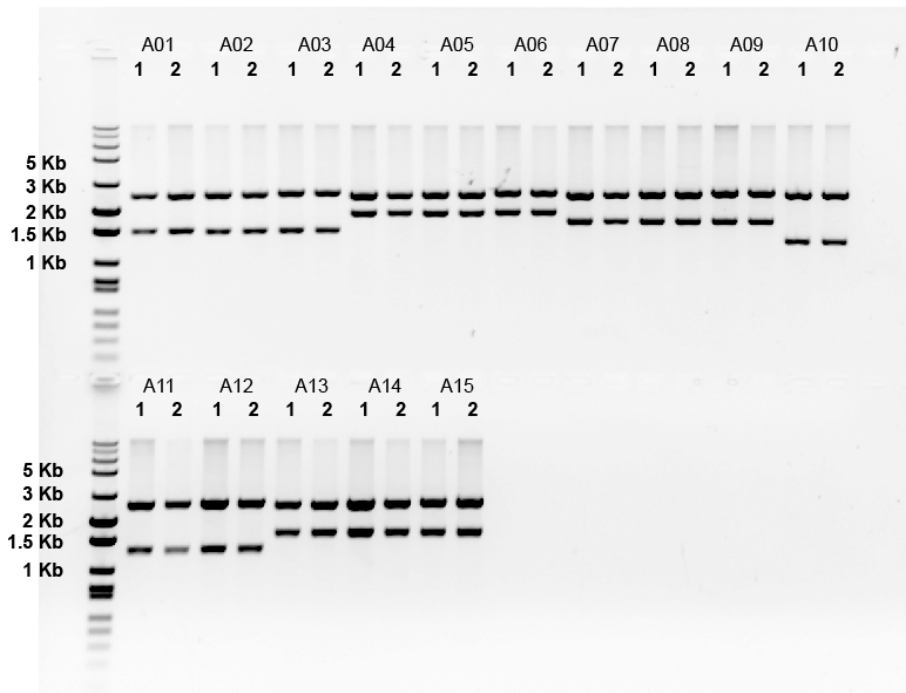
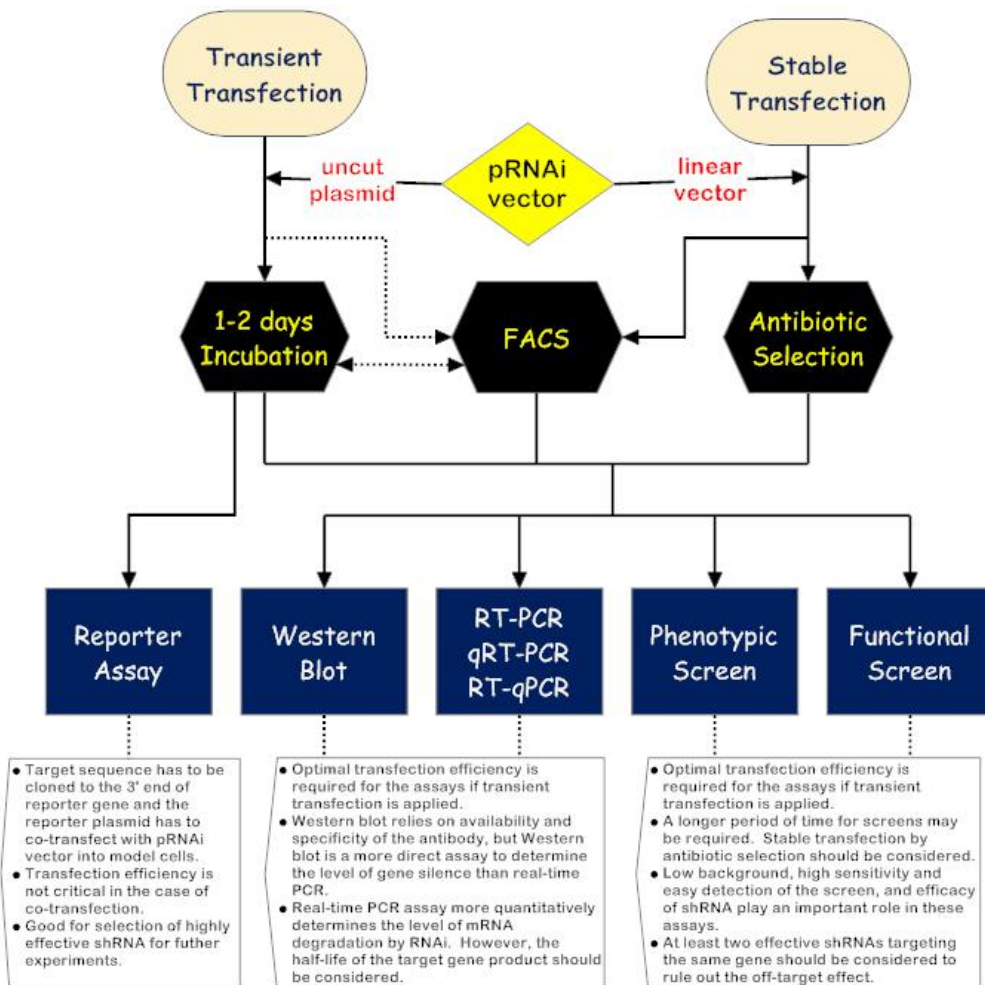


Figure 5. Patterns of fragments from *Bam*HI + *Xba* I restriction digestion of the pRNAi vectors with a 52-bp shRNA insertion. The 56-nt oligo (AAAAGTGACCAGCGAATACCTGTTCTTGGATCCAAGAACAGGTATTCGCTGGTAC) as negative control was annealed and cloned into different pRNAi vectors available from Favorgen Biosettia™ (catalog # FA-SORT-A01 to A15) as the protocol described above. Two colonies from each clone were inoculated and grown in LB medium containing ampicillin, and then the recombinant plasmids were digested with restriction enzymes *Bam*HI + *Xba* I. 0.3 µg of digested DNA was separated on 1% agarose gel and detected by staining with ethidium bromide.

Confirmation of knockdown and RNAi analysis

Confirmation of knockdown of the specific target gene is an essential step to ensure the success of an RNAi experiment. The approaches to validating effective shRNAs include the demonstration that the expression level of the target mRNA and/or gene product is substantially reduced while a negative control shRNA shows no effect. Alternatively, a reporter assay can also be applied to screen potent shRNAs (see the example described below).

The flow chart below illustrates an overview of the options and strategies to analyze RNAi efficacy with the pRNAi vector.



Transient and stable transfection for RNAi analysis

The transfection efficiency can be affected by many factors including cell types and density, transfection reagents and methods, quality and quantity of DNA, and etc. To achieve substantial gene silencing, high (or maximum) transfection efficiency is especially important for transient transfection. Prior to testing the inhibition by RNAi, transfection conditions should be optimized for the cell line of interest (e.g. by transfecting a GFP reporter plasmid). If the efficiency of transient transfection is lower than 70% under an optimized condition, we would suggest generating cell lines stably expressing shRNA, instead of using transient transfection, for RNAi analysis.

Transient transfection is most efficient with supercoiled plasmid DNA. However, in stable transfection the supercoiled plasmid may not efficiently facilitate recombination of the DNA encoding both shRNA expression and antibiotic selection with the chromosomes of the cells of interest. The pRNAi vector contains a *Pac I* restriction site in its non-essential location (Fig. 6A). For the generation of stable cell lines, it is recommended to linearize the pRNAi plasmid with *Pac I* enzyme before transfection to yield optimal integration of the DNA into the host genome (Fig. 6B).

Follow the protocol below to select stable transfected cells.

1. Transfect the linearized pRNAi vector under the optimized transfection condition for the cell line of your choice.

2. Two days post transfection, replace medium with fresh medium containing antibiotic to select for stable transfected cells.

Note: The appropriate antibiotics should be used based on the pRNAi vector you choose, and the pRNAi-green vectors are not suitable for antibiotic selection. The optimal antibiotic concentration varies from cell line to cell line, and the minimum concentration of antibiotics required to kill untransfected cells should be determined before attempting stable selection.

Suggestion: Leave one untransfected well or plate for mock control

3. Replace medium containing antibiotic every 2 days, split cells if it is necessary.

4. Start RNAi analysis when no live cells are growing in the mock well or plate.

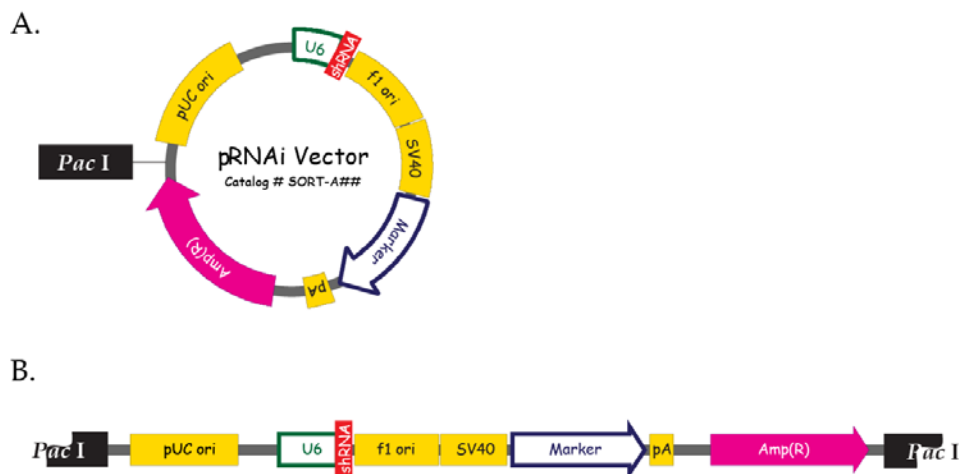
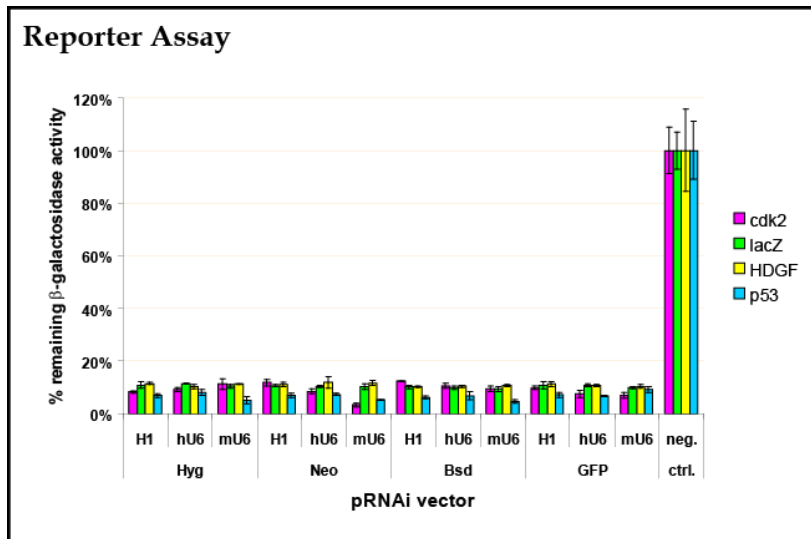


Figure 6. (A) The map for the pRNAi vector and the location of the *Pac I* site. (B) The map for the pRNAi vector linearized by *Pac I*

Examples of Knockdown of Gene Expression with pRNAi Vectors

Determination of Knockdown Efficiency by a Reporter Assay.

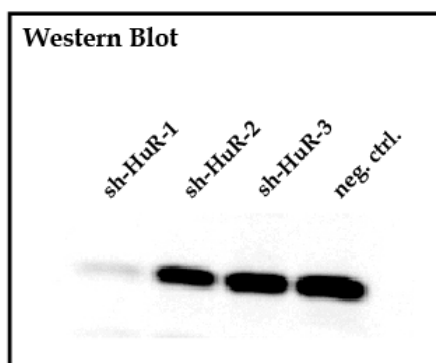
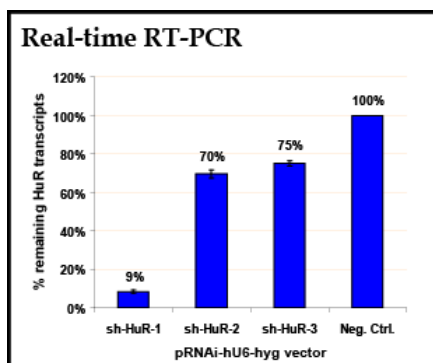
The shRNA target gene (CDK2, LacZ, HDGF or p53) was cloned into the 3' end of the β -galactosidase gene to generate the reporter:target construct. The reporter:target construct was cotransfected into HEK 293 cells with the appropriate pRNAi vector, and the β -galactosidase activity was measured. The level of suppression of the β -galactosidase fusion activity by a specific shRNA indicates the efficacy of this shRNA. The advantage of the reporter assay is all experiments can be carried out in HEK 293 cells regardless the tissue and cell specificity of target gene expression.



sh-cdk2 oligo sequence: AAAAGACCCTAACAAGCGGATTTCGTGGATCCAACGAAATCCGCTTGTAGGGTC
 sh-lacZ oligo sequence: AAAAGCAGTTATCTGGAAGATCAGGTTGGATCCAACCTGATCTCCAGATAACTGC
 sh-HDGF oligo sequence: AAAAGGCTGTGGGAGATCGAGAACATTGGATCCAATGTTCTCGATCTCCACAGCC
 sh-p53 oligo sequence: AAAAGCATCTTATCCGAGTGAAGGTTGGATCCAACCTCCACTCGGATAAGATGC
 neg. ctrl. oligo sequence: AAAAGCACATATCGAGGTGGACATCTTGGATCCAAGATGTCCACCTCGATATGTGC

Determination of Knockdown Efficiency by Real-time PCR and Western Blot

The DNA oligos encoding shRNA sequence targeting RNA-binding protein HuR were annealed and cloned into pRNAi-hU6-hyg vector (catalog # SORT-A05), linearized, and transfected into MCF-7 cells. The transfected cells were grown in medium containing hygromycin for 5 days, then total RNAs and proteins were extracted to perform real-time RT-PCR assay and Western Blot to determine RNAi efficacy.



sh-HuR-1 oligo sequence: AAAAGGTTTGGCTTGTGACCATGATTGGATCCAATCATGGTCACAAAGCCAAACC
 sh-HuR-2 oligo sequence: AAAAGGGTTGCGTTTATCCGGTTTGTGGATCCAACAAACCGGATAAAGCGCAACCC
 sh-HuR-3 oligo sequence: AAAAGGTTTGGGCGGATCATCAACTTGGATCCAAGTTGATGATCCGCCCAAAACC
 neg. ctrl. oligo sequence: AAAAGCACATATCGAGGTGGACATCTTGGATCCAAGATGTCCACCTCGATATGTGC

References

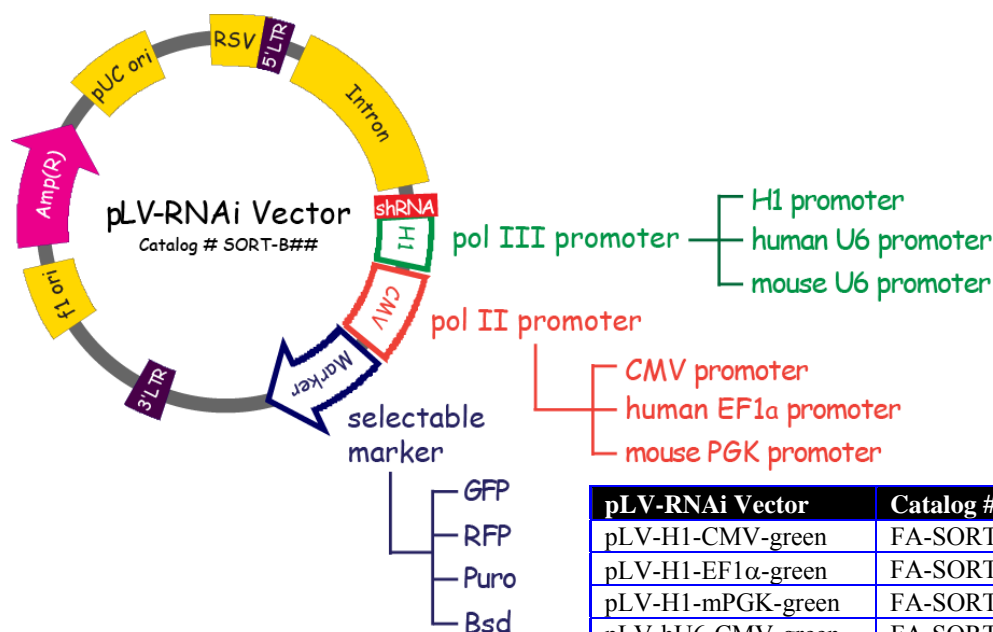
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pRNAi Vector Maps



Related Products

1. FAVMA 001/002 Favor Vectormat™ Plasmid DNA Transfection Reagent 0.5ml/1.5ml
2. pLV-RNAi is a lentiviral-based RNAi vector system that allows efficient delivery of RNAi into cells via lentiviral transduction in some model systems where transfection is inadequate. Same as the pRNAi vectors, the pLV-RNAi system provides options for different pol III promoters for shRNA expression and for different selectable markers for establishing stable cell lines. Moreover, the pLV-RNAi system offers choices of various pol II promoters, which drive different expression levels of the selectable makers in the model system of you choice.



pLV-RNAi Vector	Catalog #
pLV-H1-CMV-green	FA-SORT-B01
pLV-H1-EF1α-green	FA-SORT-B02
pLV-H1-mPGK-green	FA-SORT-B03
pLV-hU6-CMV-green	FA-SORT-B04
pLV-hU6-EF1α-green	FA-SORT-B05
pLV-hU6-mPGK-green	FA-SORT-B06
pLV-mU6-CMV-green	FA-SORT-B07
pLV-mU6-EF1α-green	FA-SORT-B08
pLV-mU6-mPGK-green	FA-SORT-B09
pLV-H1-CMV-red	FA-SORT-B10
pLV-H1-EF1α-red	FA-SORT-B11
pLV-H1-mPGK-red	FA-SORT-B12
pLV-hU6-CMV-red	FA-SORT-B13
pLV-hU6-EF1α-red	FA-SORT-B14
pLV-hU6-mPGK-red	FA-SORT-B15
pLV-mU6-CMV-red	FA-SORT-B16
pLV-mU6-EF1α-red	FA-SORT-B17
pLV-mU6-mPGK-red	FA-SORT-B18
pLV-H1-EF1α-puro	FA-SORT-B19
pLV-hU6-EF1α-puro	FA-SORT-B20
pLV-mU6-EF1α-puro	FA-SORT-B21
pLV-H1-EF1α-bsd	FA-SORT-B22
pLV-hU6-EF1α-bsd	FA-SORT-B23
pLV-mU6-EF1α-bsd	FA-SORT-B24

User Notification

The pRNAi vector system is shipped to customers at ambient temperature to reduce shipping and handling costs without affecting product quality.

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Biosettia is Favorgen's partner in RNAi technology.