User Manual

Knockout™ RNAi Systems User Manual

Clontech

United States/Canada
800.662.2566
Asia Pacific
+1.650.919.7300
Europe
+33.(0)1.3904.6880
Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

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Customer Service/Ordering:	**Technical Support:**
Telephone: 800.662.2566 (toll-free)	Telephone: 800.662.2566 (toll-free)
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Web: www.clontech.com	Web: www.clontech.com
E-mail: orders@clontech.com	E-mail: tech@clontech.com

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I. Introduction

A. Summary

The human genome project has generated the sequences of thousands of genes (Aaronson *et al.*, 1996; Hillier *et al.*, 1996), allowing researchers to focus on the question of gene function in biology. A key approach to determining gene function has been the targeted "knockout" of specific genes; gene inactivation is accomplished by disruption of the target gene's coding sequence and then introducing the altered gene into embryonic stem cells. Animal models carrying heterozygous and homozygous gene knockouts provide the ability to determine whether a particular gene is essential and what functions are perturbed by its loss. However, the amount of time and labor required to generate animal knockout models is quite extensive. Achieving such models in somatic cell lines has also proven difficult (Sedivy & Dutriaux, 1999).

Another method for eliminating gene expression takes advantage of the phenomenon of post-transcriptional gene silencing (PTGS). Specifically, the cellular process of RNA interference (RNAi) has been used to effectively silence gene expression (Figure 1). RNAi is activated by introducing a double-stranded (ds) RNA whose sequence is homologous to the target gene transcript. The exogenous RNA is digested into 21–23 nucleotide (nt) small interfering RNAs (siRNAs), which bind a nuclease complex to form an RNA-induced silencing complex (RISC). The RISC then targets the endogenous gene transcripts by base-pairing and cleaves the mRNA (Hammond *et al.*, 2001; Sharp, 2001; Huntvagner & Zamore, 2002; and Nykanen *et al.*, 2001). In contrast to traditional knockout methods, specific gene silencing is achieved quickly and easily in both animal and cell line models.

The Knockout RNAi Systems allow you to quickly express functional hairpin siRNA molecules in mammalian cells for the purpose of silencing target genes. These systems include RNAi-Ready pSIREN Vectors that use the cell's own RNA Polymerase III (Pol III) to transcribe a specifically designed small hairpin RNA (shRNA) using the human U6 promoter. The human U6 promoter provides a high level of expression in many cell types (Kunkel & Pederson, 1989), resulting in target gene suppression.

For maps and detailed information on the RNAi-Ready pSIREN Vectors, see the Appendix or the Vector Information Packet(s) provided with your product.

B. Study and mechanism of RNAi

PTGS was initially described in plants, when attempts to augment the expression of chalcone synthase in petunias resulted in the effective suppression of both the endogenous gene as well as the introduced gene; the phenomenon was coined "cosuppression" (Napoli *et al.*, 1990). Later, the use of antisense RNA, a single-stranded (ss) RNA sequence

I. Introduction *continued*

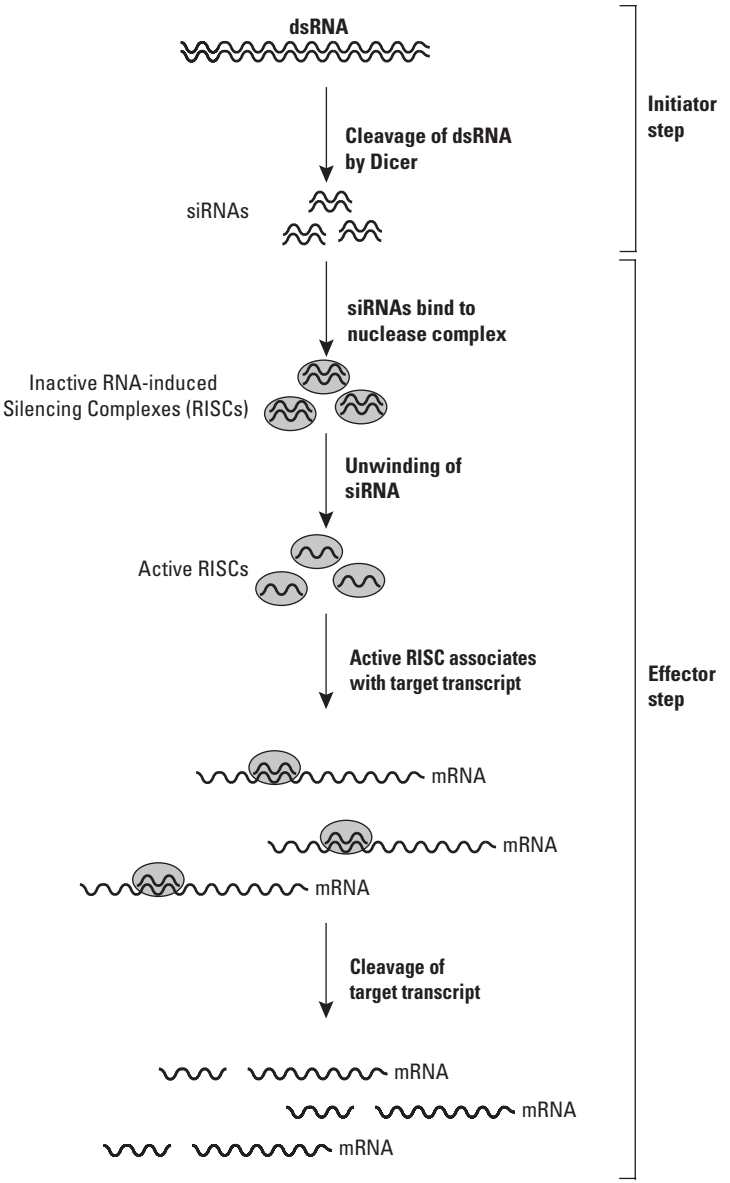


Figure 1. Mechanism of RNA interference (RNAi).

I. Introduction *continued*

that is complementary to a particular mRNA, to suppress the expression of the par-1 gene in *C. elegans* yielded intriguing results: injecting sense strand RNA (essentially duplicating the par-1 gene transcript) also resulted in silencing expression (Guo & Kempheus, 1995). It was finally determined that dsRNA was even more effective than ssRNA in gene silencing; the phenomenon was called "RNA interference" (Fire *et al.*, 1998).

The current model of the RNAi mechanism includes both initiator and effector steps (for reviews see Hutvagner & Zamore, 2002; Hammond e*t al.*, 2001; and Sharp, 2001). The initiator step involves the digestion of the input dsRNA into siRNAs 21–23 nt in length. These siRNAs are produced by the action of an enzyme known as Dicer, which belongs to the RNAse III family of dsRNA-specific ribonucleases and is evolutionarily conserved in worms, plants, fungi, and mammals (Bernstein *et al.*, 2001). The cleavage of input dsRNA by Dicer is accomplished in a processive, ATP-dependent manner, eventually generating 19–21 bp siRNA duplexes with a 3' overhang of 2 nt (Figure 1).

The effector step occurs when these siRNA duplexes bind to a nuclease complex and form the RISC (Figure 1). RISC is activated by the ATP-dependent unwinding of the siRNA duplex. Active RISC then targets the native, homologous transcript by base pairing and subsequently cleaves the mRNA at approximately 12 nt from the 3' end of the siRNA (Hammond *et al.*, 2001; Sharp, 2001; Huntvagner & Zamore, 2002; and Nykanen *et al.*, 2001). An amplification step has also been proposed to explain the potency of the RNAi process; the exogenous RNA is copied many times either before or after the generation of the siRNAs (Hammond *et al.*, 2001; Sharp, 2001; and Huntvagner & Zamore, 2002).

RNAi, then, can serve as a powerful tool in the field of functional genomics. By simply designing and introducing a dsRNA sequence that is complementary to a region of a target gene transcript, loss-of-function phenotypes can be generated quickly and easily.

C. Establishing RNAi in mammalian cells

RNAi as a technique for gene function analysis has been well-established in plants, nematodes, protozoa, and *Drosophila*. In particular, studies in *C. elegans* and *Drosophila* have shown that using dsRNA to induce RNAi is effective in generating models for the analysis of genes involved in cell division or development (Schumacher *et al.*, 1998; Gonczy *et al.*, 1999; Moore *et al.*, 1999; Jantsch-Plunger & Glotzer, 1999; Chase *et al.*, 2000; Kennerdell & Carthew, 1998).

In mammalian cells, establishing RNAi is complicated by nonspecific gene silencing. Transfecting long dsRNAs (>30 nt) activates an antiviral response, which can be mediated by either protein kinase R (PKR) or

I. Introduction *continued*

RNase L. PKR activity leads to a global repression of translation (Manche *et al.*, 1992), while RNase L catalyzes nonspecific RNA degradation (Minks *et al.*, 1979).

RNAi was finally achieved in mammalian cells by the introduction of siRNAs (<30 nt), mimicking the initiator step of the RNAi mechanism (Elbashir *et al.*, 2001). The introduction of siRNAs bypasses the antiviral response in mammalian cells, allowing functional gene suppression. Thus, siRNAs can be used to establish RNAi in mammalian cells and to study specific gene function.

Available technologies for generating siRNAs include chemical synthesis and *in vitro* transcription. Early experiments in mammalian cells used chemically synthesized siRNAs, which was an effective but expensive means to induce RNAi. To reduce the cost, a method for generating siRNA using *in vitro* transcription was employed to produce multiple different siRNAs that could be introduced into cells using transient transfection; however, RNAi achieved in this way eventually diminishes.

A number of groups have designed plasmid expression vectors to generate sustained production of siRNAs by transient or stable transfection. Some of these vectors have been engineered to express small hairpin RNAs (shRNAs), which are processed *in vivo* into siRNA-like molecules capable of carrying out gene-specific silencing (Brummelkamp e*t al.*, 2002; Paddison e*t al.*, 2002; Paul e*t al.*, 2002; and Yu e*t al.*, 2002). After construction is complete, these vectors contain a DNA sequence that encodes the shRNA cloned between a Pol III promoter and a transcription termination site comprising 4–5 thymidine residues. The transcript is terminated at position 2 of the termination site and then folds into a stem-loop structure with 3' UU-overhangs (Figure 2). The ends of the shRNAs are processed *in vivo*, converting the shRNA into ~21 nt siRNA-like molecules, which in turn initiate RNAi (Brummelkamp e*t al.*, 2002). These vectors represent a definite improvement in initiating RNAi in cells; however not all cell types are easy to transfect using these vectors.

D. Knockout RNAi Systems

Clontech® has addressed shRNA delivery problems by offering expression vectors that allow efficient, cost-effective shRNA delivery to many cell types.

The **RNAi-Ready pSIREN-RetroQ Vector**, **RNAi-Ready pSIREN-RetroQ-ZsGreen1 Vector**, and **RNAi-Ready pSIREN-RetroQ-DsRed-Express Vector** are retroviral shRNA expression vectors. The vectors are provided linearized and ready for ligation with a ds DNA oligonucleotide encoding an shRNA. pSIREN-RetroQ contains a puromycin resistance gene for the selection of stable transfectants. For the direct detection of cells containing your gene silencing construct, pSIREN-RetroQ-ZsGreen1 and

I. Introduction *continued*

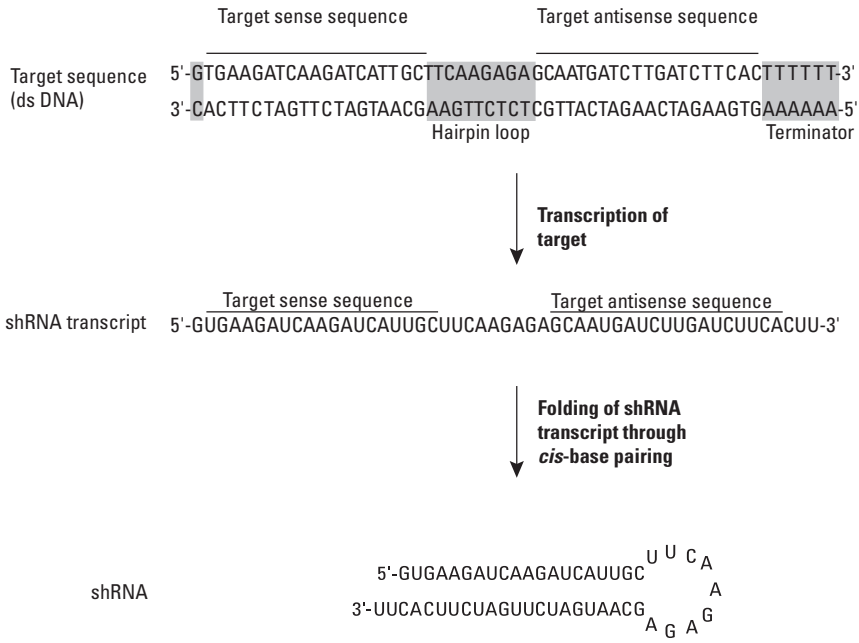


Figure 2. Small hairpin RNAs (shRNAs) generated using an oligonucleotide DNA sequence. This example shows a target sequence derived from the coding region of the β-actin gene (Harborth e*t al.*, 2001). This target sequence is cloned downstream of a Pol III promoter in an expression vector for gene silencing in mammalian cells. A hairpin loop sequence is located between the sense and antisense sequences on each complementary strand. The shRNA behaves as an siRNA-like molecule capable of carrying out gene-specific silencing. This mechanism is employed by the pSIREN vector family.

pSIREN-RetroQ-DsRed-Express constitutively express the ZsGreen1 and the DsRed-Express fluorescent protein, respectively (Matz *et al.*, 1999). Each vector is self-inactivating and optimized to eliminate promoter interference from the upstream LTR in the integrated provirus (Julius *et al.*, 2000). The CMV Promoter in the 5' LTR produces high viral titers in standard HEK 293-based packaging cell lines by 48 hr after transfection. See the Appendix or the provided Vector Information Packet for the vector map. The Retroviral GeneTransfer and Expression User Manual (PT3132-1) provides protocols for packaging recombinant pSIREN-RetroQ, pSIREN-RetroQ-ZsGreen1, or pSIREN-RetroQ-DsRed-Express into infectious, replication-incompetent particles. You then simply apply virus-containing media to dividing target cells. Retroviral infection allows you to introduce your shRNA into virtually any mitotically active cell with high efficiency and moderate shRNA expression.

I. Introduction *continued*

The **RNAi-Ready pSIREN-Shuttle Vector** is a plasmid shRNA expression vector that is also linearized and ready for ligation. pSIREN-Shuttle can be used directly in transient transfection experiments. pSIREN-Shuttle can also be used with the Adeno-X™ Expression System 1 (Cat. No. 631513) to introduce the shRNA sequence into an adenoviral genome. Standard ligation techniques are used to transfer the shRNA expression cassette (SEC) from pSIREN-Shuttle to a replication-deficient, Ad5 genome (Mizuguchi & Kay, 1999). See the Appendix or the provided Vector Information Packet for the vector map. The Adeno-X™ Expression System 1 User Manual (PT3414-1, included with the vector) provides protocols for transferring the SEC from pSIREN-Shuttle into the adenoviral genome, producing recombinant adenovirus, and infecting target cells. Adenoviral gene transfer is effective at infecting both dividing and nondividing mammalian cells for transient and high shRNA expression.

The **Knockout Adenoviral RNAi System 1** is a complete kit for constructing recombinant gene silencing adenovirus. The kit is based on the Adeno-X Expression System 1 and uses a routine *in vitro* ligation method to transfer a functional SEC from pSIREN-Shuttle (included in the kit) into adenoviral DNA.

See Table I for a guide to choosing the appropriate vector or system for your needs.

This User Manual provides protocols for generating your own shRNA construct. Protocols in this User Manual include shRNA oligonucleotide sequence design, annealing of shRNA oligonucleotides, ligation of annealed oligonucleotides into RNAi-Ready pSIREN vectors, and transformation and transfection of pSIREN constructs. Protocols for recombinant pSIREN viral packaging, production, and infection are described in the secondary User Manual(s) that accompany your Knockout RNAi System (see Section VIII). For viral delivery of your pSIREN constructs, we strongly recommend that you thoroughly read the secondary User Manual(s) that accompany your Knockout RNAi System before beginning the procedure. The secondary User Manuals include important background information, tips, and additional troubleshooting suggestions for using a specific viral system.

I. Introduction *continued*

TABLE I. DELIVERY OPTIONS FOR KNOCKOUT RNAi VECTORS AND SYSTEMS[a]

Vector/System	Delivery option(s)
RNAi-Ready pSIREN-Shuttle	Transient transfection[b]
RNAi-Ready pSIREN-RetroQ RNAi-Ready pSIREN-RetroQ-ZsGreen1 RNAi-Ready pSIREN-RetroQ-DsRed-Express	Transient/stable transfection and retroviral infection
Knockout Adenoviral RNAi System 1 (ligation-based cloning)	Transient transfection and adenoviral infection

[a] Use this table as a guide for deciding which Knockout RNAi vector or system to use.

[b] You can also transfer your functional shRNA expression cassette (SEC) from pSIREN-Shuttle into an adenoviral genome by purchasing the Adeno-X System 1 Viral DNA (linear; Cat. No. 631026) and the Adeno-X Accessory Kit (Cat. No. 631027).

II. List of Components

Store all components at –20°C.

The following reagents are suitable for 20 ligations into the RNAi-Ready pSIREN Vector. In addition, each Knockout Adenoviral System provides sufficient reagents to generate 5 recombinant adenoviruses.

The gene-specific sequences of the Luciferase shRNA Annealed Oligonucleotide and the Negative Control shRNA Annealed Oligonucleotide derive from GenBank Accession No. M15077

RNAi-Ready pSIREN-RetroQ Vector (Cat. No. 631526)

- 1 µg RNAi-Ready pSIREN-RetroQ Vector (linearized, 25 ng/µl)
- 20 µl Luciferase shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- 20 µl Negative Control shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- pSIREN-RetroQ Vector Information Packet (PT3737-5)
- Retroviral GeneTransfer and Expression User Manual (PT3132-1)

RNAi-Ready pSIREN-RetroQ-ZsGreen1 Vector (Cat. No. 632455)

- 1 µg RNAi-Ready pSIREN-RetroQ-ZsGreen1 Vector (linearized, 25 ng/µl)
- 20 µl Luciferase shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- 20 µl Negative Control shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- pSIREN-RetroQ-ZsGreen1 Vector Information Packet (PT3777-5)
- Retroviral GeneTransfer and Expression User Manual (PT3132-1)

RNAi-Ready pSIREN-RetroQ-DsRed-Express Vector (Cat. No. 632487)

- 1 µg RNAi-Ready pSIREN-RetroQ-DsRed-Express Vector (linearized, 25 ng/µl)
- 20 µl Luciferase shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- 20 µl Negative Control shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- pSIREN-RetroQ-DsRed-Express Vector Information Packet (PT3830-5)
- Retroviral GeneTransfer and Expression User Manual (PT3132-1)

II. List of Components *continued*

RNAi-Ready pSIREN-Shuttle Vector (Cat. No. 631527)

- 1 µg RNAi-Ready pSIREN-Shuttle Vector (linearized, 25 ng/µl)
- 20 µl Luciferase shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- 20 µl Negative Control shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- pSIREN-Shuttle Vector Information Packet (PT3736-5)
- Adeno-X Expression System 1 User Manual (PT3414-1)

Knockout Adenoviral RNAi System 1 (Cat. No. 631528)

- 1 µg RNAi-Ready pSIREN-Shuttle Vector (linearized, 25 ng/µl)
- 20 µl Luciferase shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- 20 µl Negative Control shRNA Annealed Oligonucleotide (0.5 pmol/µl)
- 20 µl Adeno-X Viral DNA (PI-*Sce* I/I-*Ceu* I digested; 250 ng/µl)
- 25 µl I-*Ceu* I (5 units/µl)
- 100 µl PI-*Sce* I (1 unit/µl)
- 250 µl 10X Double Digestion Buffer
- 100 µl 100X BSA
- 100 µl Adeno-X Forward PCR Primer (100 ng/µl)
- 100 µl Adeno-X Reverse PCR Primer (100 ng/µl)
- Adeno-X PCR Screening Primer Set Protocol-at-a-Glance (PT3507-2)
- pSIREN-Shuttle Vector Information Packet (PT3736-5)
- Adeno-X Expression System 1 User Manual (PT3414-1)

III. Additional Materials Required

The following materials are required but not supplied:

- **Stellar™ Competent Cells** (Cat. No. 636763)
- **Xfect™ Transfection Reagent** (Cat. No. 631317) or **CalPhos™ Mammalian Transfection Kit** (Cat. No. 631312)
- **Falcon 6-well cell culture plates** (Cat. No. 353046)
- **Falcon 12 x 75-mm sterile polystyrene tubes** (Cat. No. 352003)
- **NucleoBond Xtra Maxi EF** (Cat. No. 740424.10)
- **NucleoBond Xtra Midi EF** (Cat. No. 740420.10)
- **NucleoSpin Plasmid Kit** (Cat. No. 740588.250)
- **Thermal cycler**
- **Bovine serum albumin (BSA),** 10 mg/ml
- **T4 DNA ligase** (New England Biolabs, Cat. No. M0202S). 10X T4 DNA Ligase Buffer is provided with the enzyme.
- **Luciferase expression vector.** We recommend the pGL2 Control Vector from Promega (Cat. No. E1611).
- **Cell culture medium** (with and without Fetal Bovine Serum)
- **Phosphate buffered saline (PBS) (pH 7.4)**

	Final Conc.	To prepare 2 L
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve components in 1.8 L of distilled H_2O. Adjust to pH 7.4 with 0.1 N NaOH. Add ddH_2O to final volume of 2 L. Autoclave. Store at room temperature.

- **Trypsin/EDTA** (VWR/Hyclone No. 16777-166)

IV. General Considerations

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Figure 3 shows an overview of the procedure described in this User Manual, which provides protocols for shRNA oligonucleotide sequence design, annealing of shRNA oligonucleotides, ligation of annealed oligonucleotides into RNAi-Ready pSIREN, and transformation and transfection of pSIREN constructs.

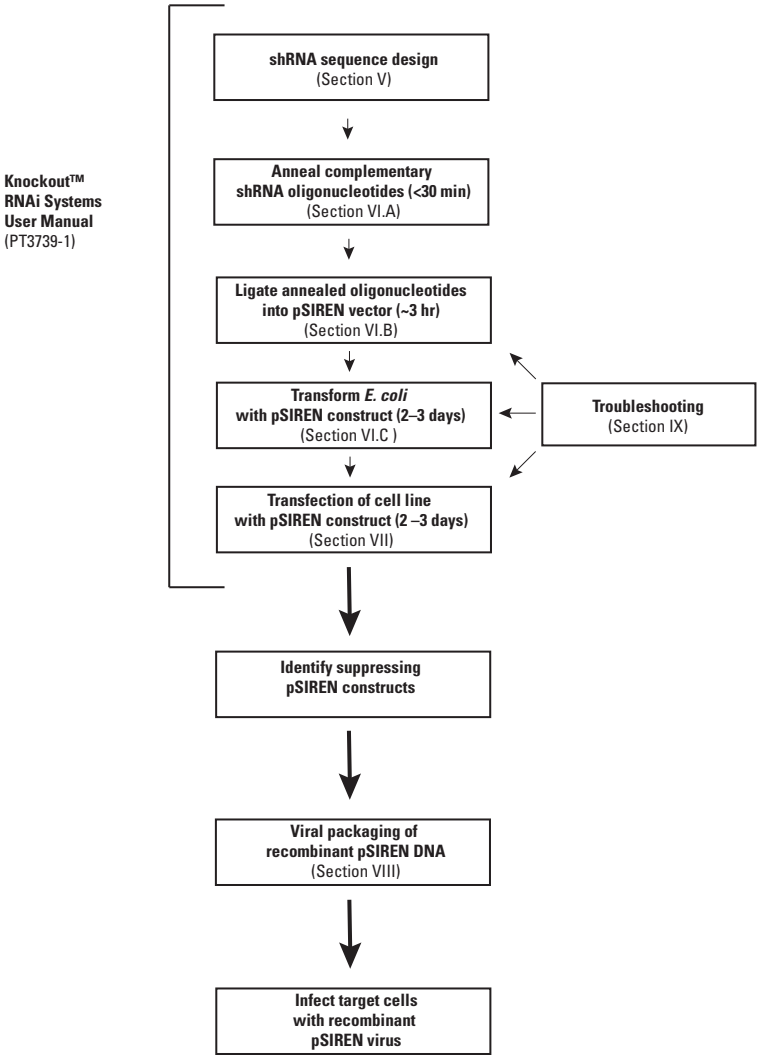


Figure 3. Overview of the Knockout RNAi Systems procedure.

IV. General Considerations *continued*

Protocols for recombinant pSIREN viral packaging, production, and infection are described in the secondary User Manual(s) that accompany your Knockout RNAi System (see Section VIII). We strongly recommend that you thoroughly read *all* User Manuals before beginning the procedure.

shRNA Oligonucleotide Design (Section V)

- The success of your experiment depends on choosing the proper target sequence within your gene of interest and the proper design of the shRNA oligonucleotides. In addition, we highly recommend that you test more than one shRNA sequence for a gene of interest.
- PAGE purification of your designed oligonucleotides ensures that a higher percentage of the oligonucleotides will be full-length and increases the chance of cloning a complete and functional insert. When using PAGE-purified oligonucleotides, we typically achieve 80–90% of clones with the right insert.
- When testing your pSIREN construct for functionality, you will need a gene-specific assay to test for the suppression of Gene X. Examples of gene-specific assays that can be used include:
    - Western blot with an antibody to Protein X
    - RT-PCR using Gene X primers. Be sure you can discriminate PCR products generated from genomic DNA from true RT-PCR products.
    - Northern blot with Gene X probe
    - Functional assay for Protein X
        - ProLabel Screening Kits. Our screening kits allow fast and quantititative chemiluminescent measurement of expression levels of any gene fused to the ProLabel tag. For more details, please see the ProLabel Screening Kit User Manual (PT3789-1).

Transfection of Recombinant pSIREN Vectors (Section VII)

- The transfection protocol included in this User Manual is intended for the screening of functional shRNA constructs and gene silencing experiments using transfection. For performing gene silencing experiments using viral infection, please refer to the secondary User Manuals included with your Knockout RNAi System. The secondary User Manuals describe the protocols for viral packaging and infection using pSIREN constructs.
- If a transfection method is already established for your cell line model, proceed with those conditions. It is important to keep optimized parameters constant to obtain reproducible results.
- To ensure the purity of the DNA, isolate all plasmids for transfection using a NucleoBond Xtra Maxi EF Kit (Cat. No. 740424.10) or by CsCl density gradient purification (Sambrook & Russell, 2001).

V. shRNA Oligonucleotide Design

This section describes the process of identifying target sequences within a gene of interest and designing the corresponding oligonucleotides to generate the shRNA.

When possible, avoid selecting target sense or antisense sequences that contain a consecutive run of 3 or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination the shRNA transcript.

A. Selecting Target Sequences

1. Choose a region of 19 nucleotides. Do not select sequences within the 5' and 3' untranslated regions (UTRs) nor regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites (Elbashir e*t al.*, 2001). UTR-binding proteins and/or translation initiation complexes may interfere with binding of the RISC.
2. Calculate the GC content of the selected 19-base oligonucleotide sequence. The GC content should be between 40% and 60%; a GC content of approximately 45% is ideal.
3. Sequences that have at least 3 A or T residues in positions 15–19 of the sense sequence appear to have increased knockdown activity.
4. Check the 19-base oligonucleotide for secondary structure and long base runs, both of which can interfere with proper annealing. Eliminate candidate sequences that display these characteristics.
5. Compare the remaining candidate sequences to an appropriate genome database to identify sequences that are specific for the gene of interest and show no significant homology to other genes. Candidate sequences that meet these criteria are potential shRNA target sites.

   To optimize gene silencing, we highly recommend that you test more than one shRNA target sequence for a gene. We provide enough RNAi-Ready pSIREN vector to perform 20 ligations, which allows you to screen for functional shRNA sequences within your gene of interest. You should test at least 4 shRNAs per gene. It may help to choose shRNA targets that are positioned all along the length of the gene sequence to reduce the chance of targeting a region that is either highly structured or bound by regulatory proteins.

   Note: You will need to design a gene-specific assay to test for the suppression of Gene X, if you have not already done so. See Section IV for additional information.

V. shRNA Oligonucleotide Design *continued*

B. Designing Oligonucleotides

It is necessary to synthesize two complementary oligonucleotides (a top strand and a bottom strand) for each shRNA target site. Figure 4 illustrates the overall structure of the prototypical oligonucleotide sequences for use in pSIREN. The sequences of the oligonucleotides should include:

- A 5'-*Bam*H I restriction site overhang on the top strand and a 5'-*Eco*R I restriction site overhang on the bottom strand. These restriction sites will enable directional cloning of the annealed oligonucleotides into the RNAi-Ready pSIREN vector
- A guanine (G) residue added upstream of the 5'-end of the shRNA sense strand, if the target sequence does not start with a purine (preferred as Pol III transcription start site).
- The 19-base oligonucleotide sense sequence (target sense sequence) of the shRNA target site.
- A 7–9 nucleotide hairpin loop sequence (We typically use 5'-TTCAAGAGA- 3'; see Sui *et al.*, 2002; Lee *et al.*, 2002; Paddsion *et al.*, 2002; Brummelkamp *et al.*, 2002; and Paul *et al.*, 2002 for other effective loop sequences.)
- The 19-base oligonucleotide antisense sequence (target antisense sequence) of the shRNA target site; ensure proper orientation for correct formation of the hairpin structure (see Figure 2).
- A RNA Pol III terminator sequence consisting of a 5–6 nucleotide poly(T) tract
- (Optional, but recommended) A unique restriction site immediately downstream of the terminator sequence for restriction digest analysis to confirm the presence of the cloned insert

  Note: The *Mlu* I restriction site has been engineered into the Luciferase shRNA Annealed Oligonucleotide as well as the Negative Control shRNA Annealed Oligonucleotide so that a simple diagnostic digest can be used to verify that the pSIREN vector contains the shRNA oligo. You can engineer this same restriction site into your gene-specific shRNA oligonucleotides as long as *Mlu* I does not cut within the oligonucleotide sequences.

A typical oligonucleotide has 5 bases for the restriction site at the 5' end, 19 bases of sense strand, 7–9 bases of hairpin loop, 19 bases of antisense strand, 6 bases of terminator, 6 bases of a unique restriction site, and 1 base for the restriction site at the 3' end (when digested with *Eco*R I), resulting in an oligonucleotide of 63–65 bases. See Table II for examples of sense and antisense sequences designed for certain genes. Our shRNA Sequence Designer tool at **www.clontech.com/US/Support/Online_Tools** can design the required oligonucleotides for any sequence input.

V. shRNA Oligonucleotide Design *continued*

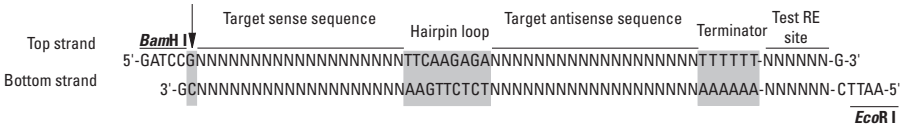


Figure 4. shRNA oligonucleotide sequence design. Arrow denotes the purine residue required for RNA Pol III to initiate transcription. The hairpin loop sequence shown is one of many functional loop sequences used to generate shRNAs. Termination is signaled using a poly(T) tract. Including a unique restriction site (Test RE site) allows confirmation of the cloned insert after the ligation and transformation reactions. 5' *Bam*H I and 3' *Eco*R I overhangs are necessary for directional cloning into RNAi-Ready pSIREN vectors. See Table II for examples of target sense and antisense sequences for certain genes.

TABLE II. EXAMPLES OF PUBLISHED TARGET SEQUENCES[a]

Gene	Target sequence[b]	Sense sequence	Antisense sequence	Reference
β-actin	AATGAAGATCAAGATCATTGC	TGAAGATCAAGATCATTGC	GCAATGATCTTGATCTTCA	Harborth e*t al.*, 2001
Bcr-abl	AAGCAGAGTTCAAAGCCCTT	GCAGAGTTCAAAGCCCTT	AAGGGCTTTGAACTCTGC	Scherr e*t al.*, 2002
hRad9	AAGTCTTTCCTGTCTGTCTTT	GTCTTTCCTGTCTGTCTTT	AAAGACAGACAGGAAAGAC	Hirai & Wang, 2002

[a] Sequences are shown for top strand oligo design. All sequences shown 5' to 3'. Bottom strand oligo design (not shown) is the complementary sequence to the top strand.
[b] Identified from gene coding sequence.

See Section IV for our recommendation to use PAGE-purified oligonucleotides. It is possible to clone without PAGE purification, but it is likely that the overall ligation efficiency and the number of correct clones will decrease due to the impact of incomplete oligonucleotide extensions. If the oligonucleotides are PAGE purified, order at the 200 nmol scale.

There is no need to order phosphorylated oligonucleotides. RNAi-Ready pSIREN Vectors have not been dephosphorylated after linearization; thus ligation will proceed smoothly using unphosphorylated oligonucleotides.

VI. Cloning into RNAi-Ready pSIREN Vectors

A. Annealing the Oligonucleotides

For convenience, Steps 3–6 can be done in a thermal cycler.

1. Resuspend each purified oligonucleotide in TE buffer to a concentration of 100 µM.
2. Mix the oligos for the top strand and the bottom strand at a 1:1 ratio. This will ultimately give 50 µM of ds oligo (assuming 100% theoretical annealing).
3. Heat the mixture to 95°C for 30 sec to remove all secondary structure.

   Note: Heating to 95°C ensures that the internal hairpin of each oligonucleotide is disrupted and promotes intermolecular annealing.
4. Heat at 72°C for 2 min.
5. Heat at 37°C for 2 min.
6. Heat at 25°C for 2 min.
7. Store on ice.

The annealed oligonucleotide is now ready for ligation into an RNAi-Ready pSIREN vector. Alternatively, the ds oligonucleotide can be stored at –20°C until ready to use.

B. Ligating ds Oligonucleotide into RNAi-Ready pSIREN

1. Dilute the annealed oligo (from Section VI.A.7) with TE buffer to obtain a concentration of 0.5 µM.

   Note: To ensure good ligation efficiency it is necessary to dilute the oligo so that it is only in moderate excess. Using an excess of the oligo will block ligation.
2. Assemble a ligation reaction for each experimental annealed oligonucleotide. Also set up ligations using the Luciferase shRNA and Negative Control shRNA Annealed Oligonucleotides.

   a. For each ligation, combine the following reagents in an microfuge tube:

2	µl	Linearized pSIREN vector (25 ng/µl)
1	µl	Diluted, annealed oligonucleotide (0.5 µM)
1.5	µl	10X T4 DNA Ligase Buffer
0.5	µl	BSA (10 mg/ml)
9.5	µl	Nuclease-free H_2O
0.5	µl	T4 DNA ligase (400 U/µl)
15	µl	Total volume

   b. Set up separate ligations using 1 µl of the Luciferase shRNA Annealed Oligonucleotide or 1 µl of the Negative Control shRNA Annealed Oligonucleotide. If desired, an additional control ligation (vector only plus ligase) can be assembled using 1 µl Nuclease-free H_2O instead of annealed oligonucleotide.

VI. Cloning into RNAi-Ready pSIREN Vectors *continued*

Note: The "vector only plus ligase" control plate often yields numbers of colonies similar to those obtained from the test reactions that combine vector with a shRNA-annealed oligonucleotide in the presence of ligase. DO NOT BE ALARMED. We believe that the excess of shRNA oligo present in the test reactions inhibits religation of single cut vector. Since no shRNA oligo is present in the control reactions, single cut vector can religate. Even it you observe many colonies on the "vector only plus ligase" plate, proceed to screen the colonies on the test reaction plates, since approximately 80% of these should contain an insert.

3. Incubate the reaction mixture for 3 hr at room temperature.

   Note: Do not let the ligation reaction go longer than 3 hr. If you are unable to immediately perform the transformation after this step, store the completed ligation at –20°C until ready to use.

C. Transforming Stellar™ Competent Cells with recombinant pSIREN

Stellar Competent Cells are an *E. coli* K-1 strain that provides high transformation efficiency. The strain carries recA and endA mutations that make it a good host for obtaining high yields of plasmid DNA. We routinely use this strain for all our shRNA cloning.

1. Thaw the required number of tubes of cells on ice for 10 min. Tap gently to ensure that the cells are suspended.
2. Add 2 µl of the ligation mixture (from Section VI.B.3) directly to 50 µl of cell suspension. Mix gently to ensure even distribution of the DNA solution.
3. Incubate the transformation mixture (DNA + cells) on ice for 5 min.
4. Heat the tubes for precisely 30 sec in a water bath at 42°C without shaking.
5. Remove the tubes from the water bath and place them directly on ice for 2 min.
6. Add 250 µl room-temperature SOC medium to each tube. Incubate at 37°C for 60 min while shaking at 250 rpm.
7. Plate 30 µl (1/10 of the transformation volume) from each transformation on selective medium containing the appropriate concentration of antibiotic. Incubate at 37°C.

   Notes

   - Both cell competency and ligation efficiency affect the outcome of the transformation. We suggest plating different amounts on separate plates to identify the optimal volume for determining transformation efficiency and isolating colonies.
   - Plating is accomplished by spreading cells on selective medium [e.g., LB agar + Ampicillin (50–100 µg/ml)]. See the Certificate of Analysis that accompanies the RNAi-Ready pSIREN vector.
   - We have observed that recombinant pSIREN DNA usually generates smaller and slower-growing colonies.
   - If the plating density is too high, dilute the transformation mixture 1:10 with room- temperature SOC and spread plates with 30 µl of the 1:10 dilution (1/100 of the original transformation volume).

VI. Cloning into RNAi-Ready pSIREN Vectors *continued*

8. Inoculate a small-scale liquid culture with a single, well-isolated colony. We recommend you set up 4–8 such cultures to ensure you obtain at least one positive clone. After overnight incubation, isolate plasmid DNA using any standard method. For small-scale purification (20 µg plasmid DNA), we recommend our NucleoSpin Plasmid Kit (Cat. No. 740588.250).
9. Identify the desired recombinant plasmid by restriction analysis using the unique restriction site within the shRNA oligonucleotide sequence. If desired, verify your insert by sequencing.

   Notes: The *Mlu* I restriction site has been engineered into the Luciferase shRNA Annealed Oligonucleotide as well as the Negative Control shRNA Annealed Oligonucleotide so that a simple diagnostic digest can be used to verify that the pSIREN vector contains the shRNA oligo.

   Since there is always a chance for mutations in the oligo due to synthesis errors, we strongly recommend that you sequence at least two clones to verify the correct oligo sequence. Because hairpin sequences are difficult to sequence, inform your sequencing facility so that sequencing conditions can be adjusted accordingly.
10. Once a positive clone has been identified, inoculate a large-scale liquid culture to prepare greater quantities of your recombinant pSIREN vector. To ensure optimal purity of the DNA, isolate all plasmids for transfection using a NucleoBond Xtra Maxi EF Kit (Cat. No. 740424.10) or by CsCl density gradient purification (Sambrook & Russell, 2001).

VII. Transfection of Recombinant pSIREN Vectors

Follow the transfection protocol that comes with your reagent for both the screening for functional shRNA constructs and gene silencing experiments using transfection. If you will perform experiments using viral delivery, first use the transfection protocol for your reagent to screen constructs, then proceed to the secondary User Manuals included with your Knockout RNAi System. The secondary User Manuals describe the protocols for viral packaging and infection using pSIREN constructs. If your target cells cannot be transfected, then viral delivery should be tried for both functional shRNA screening and gene silencing experiments.

For further information on cell culture techniques, see Freshney (2000).

The efficiency of a mammalian transfection procedure is primarily dependent on the host cell line. Therefore, when working with a cell line for the first time, we recommend you compare the efficiencies of several transfection protocols. After choosing a method of transfection, optimize cell density (usually 60–80% confluency), the amount and purity of the DNA, media conditions, and transfection time.

If a transfection method is already established for your cell line model, proceed with those conditions. It is important to keep optimized parameters constant to obtain reproducible results.

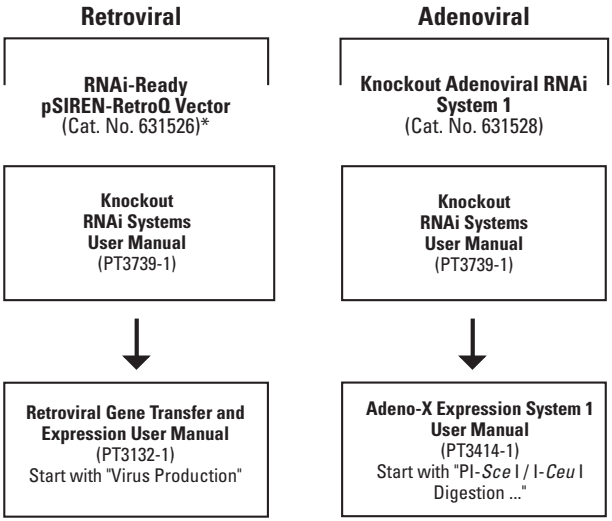
For our transfections, we have been successful using the CalPhos™ Mammalian Transfection Kit (Cat. No. 631312; Protocol PT3025-1) and the Xfect™ Transfection Reagent (Cat. No. 631317; Protocol PT5003-2). Protocols for Clontech products are available for download at **www.clontech.com/manuals**.

Please refer to the protocol for your particular reagent to transfect your cells. Then, assay for transient gene suppression 48 hr post-transfection.

Note: If you have transfected a pSIREN-RetroQ construct, you can start selection for stable transformants 48–72 hr post-transfection.

VIII. Viral Delivery of pSIREN Constructs

Once you have identified functional pSIREN constructs for specific gene silencing, you can proceed with viral packaging of the constructs. For this purpose, secondary User Manuals are supplied with your RNAi System. Figure 5 illustrates the procedural steps for retroviral and adenoviral delivery.



* The **RNAi-Ready pSIREN-RetroQ-ZsGreen1 Vector** (Cat. No. 632455) or **RNAi-Ready pSIREN-RetroQ-DsRed-Express Vector** (Cat. No. 632487) may be used instead of the pSIREN-RetroQ Vector, for the production of recombinant pSIREN retroviral particles expressing a fluorescent marker.

Figure 5. Procedures provided for viral delivery of recombinant pSIREN. After generating functional pSIREN constructs using the procedure detailed in this User Manual, proceed with the protocols described in the provided viral expression User Manuals to produce recombinant virus for efficient delivery of recombinant pSIREN to many cell types.

IX. Analysis of Results and Troubleshooting Guide

A. Poor transformation efficiency

Low transformation efficiency can be the result of a problem with the oligonucleotides, ligation, and/or transformation.

Incompatible ends on the insert	Confirm that the ends of the annealed oligonucleotide contain 5' *Bam*H I and 3' *Eco*R I overhangs for proper ligation into pSIREN.
Ineffective oligo annealing	Verify that the top and bottom strand sequences are correct. To ensure a high amount of dsDNA in the annealing reaction, mix an equal ratio of top and bottom strands. It may be necessary to increase the denaturation temperature (Section VI.A.3.) to increase the yield of annealed oligonucleotide.
Oligos are not full-length	Verify oligonucleotide size using a 12% native polyacrylamide gel. Order PAGE-purified oligonucleotides to ensure a higher percentage of full-length oligonucleotides and increase the chance of cloning a complete and functional insert.
Suboptimal oligo concentration in ligation	Verify the concentration of the annealed oligo nucleotide used for ligation. Too little or too much oligonucleotide can affect ligation. To improve ligation efficiency, perform a range of 5- or 10-fold dilutions of the annealed oligonucleotide for use in ligation.
Inactive ligase and/or ligase buffer	Check your ligase and ligase buffer for activity using a different vector and insert. Replace the ligation reagents if they prove inactive.
Suboptimal competent cells	Transform Stellar Competent Cells using the provided Test Plasmid. Calculate the number of cfu/µg to determine the cells' competency. Handle competent cells gently during transformation and plating. Perform the heat shock step (Step VI.C.4.) for precisely 30 sec. Extending this time will drastically reduce cell viability.
Wrong antibiotic or suboptimal antibiotic concentration	Verify the correct antibiotic and its concentration by checking the Certificate of Analysis that accompanies the RNAi-Ready pSIREN vector.

IX. Analysis of Results and Troubleshooting... *cont.*

B. Poor transfection efficiency

Transfection efficiency can be affected by plasmid purity or transfection conditions. Alternatively, an ineffective pSIREN construct can be misinterpreted as low transfection efficiency.

Poor purity of the pSIREN construct	Ensure the purity of recombinant pSIREN DNA by isolating all plasmids for transfection using a NucleoBond Xtra Midi EF Kit (Cat. No. 740420.10) or by CsCl gradient.
Ineffective transfection	The efficiency of a mammalian cell transfection depends primarily on the host cell line. Optimizing the transfection parameters for each cell type is crucial to obtaining consistently successful transfections. Therefore, for each cell type you plan to use, perform preliminary experiments to determine the optimal: 1) amount of transfection reagent; 2) amount and purity of DNA; 3) ratio of transfection reagent to DNA; 4) cell density; 5) transfection incubation time; and 6) media conditions.
No detectable gene silencing	You should test at least 3-4 pSIREN constructs per gene to optimize gene silencing. We provide enough RNAi-Ready pSIREN vector in each kit to perform 20 ligations, which allows you to screen for functional shRNA sequences within your gene of interest. We have not observed loss or mutation of the annealed oligonucleotides when cloned into RNAi-Ready pSIREN vectors and propagated using the recommended conditions. To ensure integrity do not overgrow transformed cultures. If planning an overnight culture, inoculate as late as possible in the day using a 1:1000 dilution of freshly grown stock. Incubate with sufficient shaking to ensure good aeration (250 rpm) and harvest the culture as early as possible the next day to prevent culture overgrowth. Do not serially passage your cultures. In addition to keeping glycerol stocks of transformed cells, we highly recommend keeping DNA stocks of your pSIREN constructs.

X. References

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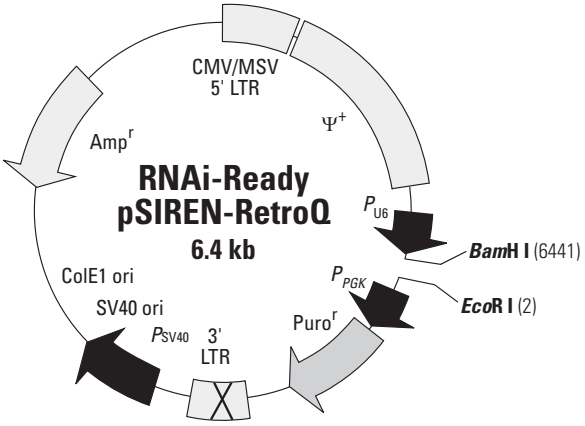
Appendix: Vector Information



6430 6440 10 20 30 40

5'-TGTGGAAAGGACGAGGGATCC[...shRNA oligo cloning site...]GAATTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGT-3'
*Bam*H I *Eco*R I
3'-ACACCTTTCCTGCTCCTAGG[...shRNA oligo cloning site...]CTTAAGATGGCCCATCCCCTCCGCGAAAAGGGTTCCGTCA-5'

Figure 6. Restriction Map and Cloning Site of the RNAi-Ready pSIREN-RetroQ Retroviral Vector. Unique restriction sites are in bold. RNAi-Ready pSIREN-RetroQ is provided as a linearized vector digested with *Bam*H I and *Eco*R I. Nucleotides in gray were removed during linearization. This linearized vector is ready for ligation of a ds DNA oligonucleotide containing *Bam*H I and *Eco*R I overhangs. RNAi-Ready pSIREN-RetroQ is a self-inactivating retroviral expression vector designed to express a small hairpin RNA (shRNA) using the human U6 promoter (P_{U6}; RNA Pol III-dependent). This vector is used for targeted gene silencing when a ds DNA oligonucleotide encoding an appropriate shRNA is ligated into the vector. You can transfect your pSIREN-RetroQ construct as a plasmid expression vector, or upon transfection into a packaging cell line, this vector can transiently express, or integrate and stably express a viral genomic transcript containing the human U6 promoter and the shRNA. The vector contains a puromycin resistance gene for the selection of stable transfectants. This retroviral vector is optimized to eliminate promoter interference through self-inactivation. The hybrid 5' LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter. This construct drives high levels of transcription in HEK 293-based packaging cell lines due, in part, to the presence of adenoviral E1A (Kinsella & Nolan, 1996; Ory *et al.*, 1996; Pear *et al.*, 1993; and Yang *et al.*, 1999) in these cells. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. This may reduce the phenomenon known as promoter interference (Emerman & Temin, 1984) and allow more efficient expression. Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (Psi^+), and tRNA primer binding site. pSIREN-RetroQ also contains a bacterial origin of replication and *E. coli* Amp^r gene for propagation and selection in bacteria.

RNAi-Ready pSIREN-RetroQ-ZsGreen1 and **RNAi-Ready pSIREN-RetroQ-DsRed-Express** constitutively express a *Zoanthus* sp. green fluorescent protein (ZsGreen1) and a variant of *Discosoma* sp. red fluorescent protein (DsRed-Express), respectively (Matz *et al.*, 1999). The fluorescent marker allows you to directly monitor the delivery efficiency of your gene silencing construct using either fluorescence microscopy or flow cytometry. Complete sequence information is provided in the RNAi-Ready pSIREN-RetroQ-ZsGreen1 Vector Information Packet (PT3777-5) and the RNAi-Ready pSIREN-RetroQ-DsRed-Express Vector Information Packet (PT3830-5).

Appendix: Vector Information *continued*

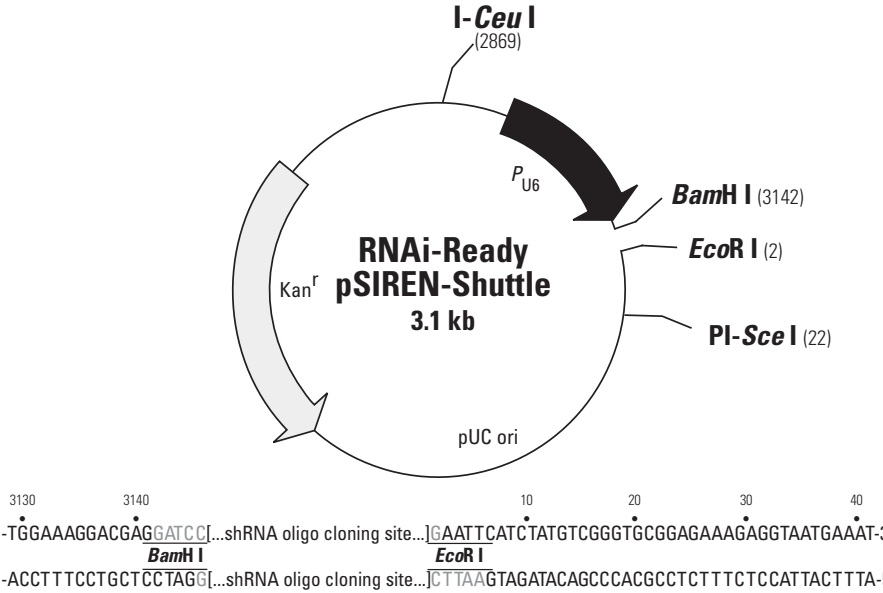


Figure 7. Restriction Map and Cloning Site of the RNAi-Ready pSIREN-Shuttle Vector. Unique restriction sites are in bold. RNAi-Ready pSIREN-Shuttle is provided as a linearized vector digested with *Bam*H I and *Eco*R I. Nucleotides in gray were removed during linearization. This linearized vector is ready for ligation of a ds DNA oligonucleotide containing *Bam*H I and *Eco*R I overhangs. RNAi-Ready pSIREN-Shuttle is available separately, and as a component of the Knockout Adenoviral RNAi System 1 (Cat. No. 631528). RNAi-Ready pSIREN-Shuttle is an expression vector designed to express an small hairpin RNA (shRNA) using the human U6 promoter (P_{U6}; RNA Pol III-dependent). This vector is used for targeted gene silencing when a ds DNA oligonucleotide encoding an appropriate shRNA is ligated into the vector. The pSIREN-Shuttle expression cassette consists of the human U6 promoter(P_{U6}) and a cloning site. The entire cassette is flanked by unique I-*Ceu* I and PI-*Sce* I restriction sites so that it can be excised and directly ligated to Adeno-X Viral DNA, the adenoviral genome. The vector backbone also contains the pUC origin (pUC ori) and a kanamycin resistance gene (Kan^r) for propagation and selection in *E. coli*. You can transfect your pSIREN-Shuttle construct as a plasmid expression vector, or in conjunction with the Adeno-X Expression System 1 (Cat. No. 631513) or its Accessory Kits (Cat. Nos. 631026 & 631027); this vector can transiently express a viral genomic transcript containing the human U6 promoter and the shRNA. In the Adeno-X System, standard ligation techniques are used to transfer an expression cassette from pSIREN-Shuttle to a replication-deficient, Ad5 genome (Mizuguchi & Kay, 1999).

Notes

This document has been reviewed and approved by the Clontech Quality Assurance Department.