

Silencing RNA in vivo (RNAi)

Silencing gene expression using double-stranded RNA (dsRNA), known as RNA interference or RNAi, provides a powerful tool for analyzing gene function. Selectively downregulating (or “knocking down”) the expression of a particular gene allows researchers to determine its function in many cellular processes. Gene knockdown is useful for researchers who want to understand the function of a single gene. Furthermore, knockdown methods benefit the drug development process if they are sufficiently simple and predictable. Gene knockdown experiments are useful for many biological studies including: mapping cellular pathways, selecting suitable targets for pharmaceutical intervention and developing gene therapies. The siCHECK™ Vectors, siLentGene™-2 U6 Hairpin Cloning Systems, siSTRIKE™ U6 Hairpin Cloning Systems and GeneClip™ U1 Hairpin Cloning Systems provide convenient tools for researchers using RNAi to analyze gene function.

RNAi Phenomenon

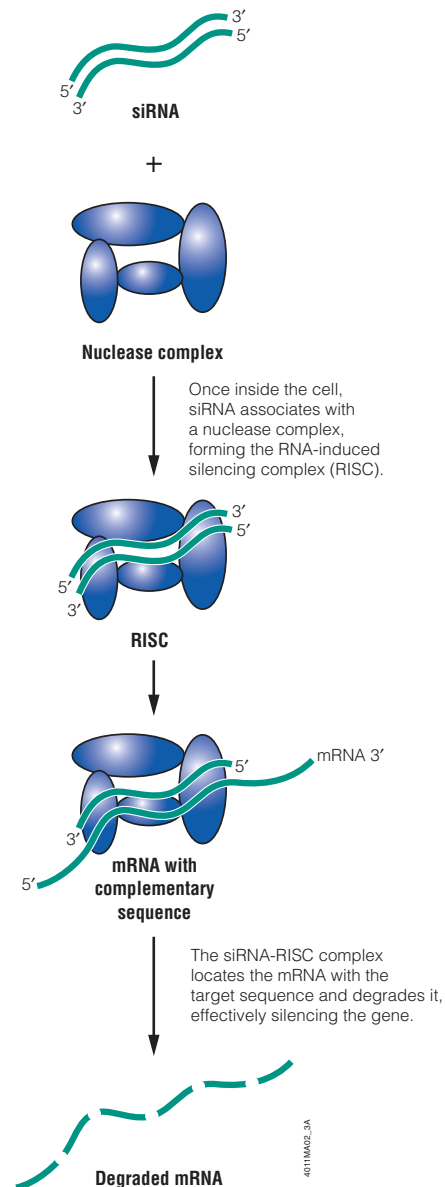
RNAi refers to the cellular process induced by dsRNA in which mRNA that is homologous to the dsRNA is specifically degraded. In some systems, a few copies of the dsRNA can cause total degradation of cognate transcripts in a cell (1). A number of gene products are involved in this process, including the type III RNase “Dicer” from *Drosophila* (2). Dicer processes long dsRNAs or hairpin RNAs (3) into double-stranded, small interfering RNAs (siRNA), which are typically 21–23 nucleotides (nt) long. The resulting siRNAs trigger the formation of RNA-induced silencing complexes (RISC). Helicases in the complex unwind the dsRNA, and the single RNA strand that is most easily unwound in the 5' to 3' direction is used as a guide for substrate selection (4–6). Once the single-stranded RNA (ssRNA) is base-paired with the target mRNA, a nuclease activity within the complex cleaves in the center of the region complementary to the siRNA (7), with the net result being rapid degradation of the target mRNA and decreased protein expression.

Methods for Inducing RNAi in Mammalian Cells

Soon after the discovery of the RNAi phenomenon by Fire *et al.* in 1998 (1), dsRNAs were used successfully as tools to analyze gene function in nematodes, fruit flies and plants (8,9). In mammalian cells, long dsRNAs induce the sequence-specific silencing of genes in mouse embryonal carcinoma cells and embryonic stem cells (10,11). However, introducing long dsRNAs into mammalian somatic cells activates antiviral defense

systems, resulting in nonspecific degradation of RNA transcripts (12) and general loss of host cell protein synthesis (13,14). These two mechanisms effectively shut down mammalian cells and thus override the ability of long dsRNAs to have specific RNAi effects.

Using short (21–23nt) dsRNAs with a 2-nucleotide 3' overhang or short hairpin RNA (shRNA), however, does not trigger these antiviral mechanisms and can mediate gene-specific suppression in mammalian cells. Short dsRNAs can be synthesized in vitro and introduced into mammalian cells. Alternatively DNA vector-based strategies can be used to deliver siRNA into mammalian cells, further expanding the utility of RNAi in mammals.



Proposed mechanism of RNA interference.

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RNAi Target Selection

Design of the siRNA sequence is crucial for effective gene silencing. Rational design strategies for effective siRNAs are being developed based on an understanding of RNAi biochemistry and of naturally occurring microRNA (miRNA) function. Several groups have proposed basic empirical guidelines for designing effective siRNAs that can be applied to the selection of potential target sequences (4, 15–20). Although the existing rules for siRNA selection serve as a reliable guide, they do not ensure that each selected siRNA sequence will reduce gene expression, and the optimal target sequence may need to be determined empirically.



Target Selection made easy!

Use Promega's online siRNA Designer and get the target sequences quickly and easily. Plug in your sequence and all possible siRNA targets are returned. Visit: www.promega.com/siRNA Designer/

The siRNA sequences presented are linked directly to the NCBI BLAST server allowing easy comparison of this siRNA sequence to all sequences in Genbank®.

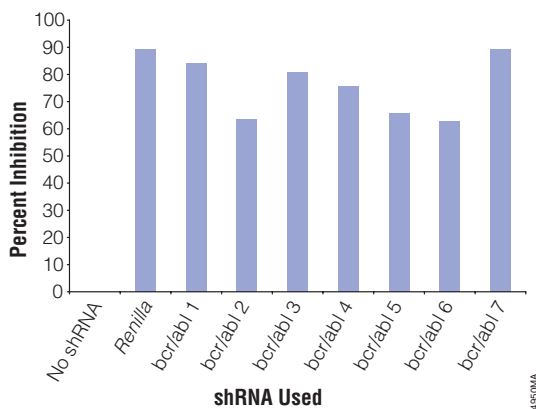
General guidelines for target sequence selection include:

- Avoid regions within 75 bases of a start codon (21).
- Avoid areas with a GC content of >70% or <30% (20,21).
- Avoid potential siRNA sequences with significant homology to other genes (21).
- Avoid stretches of 4 or more of any one nucleotide because these can cause premature transcription termination (21).
- Select siRNA sequences with a thermodynamically unstable 5' end of the antisense strand (4,22).
- Select target sequences that are 19–27 nucleotides long. Historically, siRNA sequences are 19-mers, but longer siRNAs (27-mers) have been reported to mediate RNAi more successfully (23,24).

Promega offers the siRNA Target Designer, available at: www.promega.com/siRNA Designer/, to analyze input DNA or RNA sequences for regions that fit the requirements for design of siRNAs.

Silencing RNA in vivo (RNAi)

Although some algorithms are designed to predict how effective a given siRNA will be at suppressing the expression of its target, selecting optimal regions still remains largely a trial-and-error process. Therefore, multiple regions are generally screened for each target. Recently, several quantifiable procedures that use reporter genes to help rapidly identify effective siRNAs have been developed. In these approaches, the change in expression of a reporter gene fused to a target gene is used as an indicator of the effectiveness of an RNAi methodology. The siCHECK™ Vectors, which contain the bioluminescent *Renilla* luciferase reporter gene, offer several advantages compared to other fusion approaches. Bioluminescent detection of *Renilla* luciferase is sensitive, fast and amenable to high throughput.



Inhibition of *Renilla* luciferase activity by various shRNAs. Full details are given in Betz, N. (2005) Using bioluminescent reporter genes to optimize shRNA target sites for RNAi of the *bcr/abl* gene. *Promega Notes* 90, 26–8.

Effective target sequences can yield robust siRNA-mediated gene-specific inhibition, making the RNAi approach a powerful tool for gene function analysis in mammalian cells. However, screening for effective siRNAs using synthetic RNA can be expensive, so many researchers are turning to DNA-directed RNA interference (ddRNAi). DNA-directed RNA interference (ddRNAi) involves the use of DNA templates to synthesize siRNA or shRNA in vivo. ddRNAi relies on U6, H1 or U1 promoters for the expression of siRNA target sequences that have been transfected into mammalian cells (25–27). siRNA and shRNA target sequences can be generated by PCR, creating “expression cassettes” that can be transfected directly into cells (28,29) or cloned into expression vectors (30–33). PCR generation is recommended when rapid screening of numerous siRNAs is desired. Cloning-based approaches that allow direct ligation of hairpin oligonucleotides into a ddRNAi vector provide another method for quickly and easily screening various targets (34–36). Promega offers a variety of ddRNAi-based systems for RNA interference.

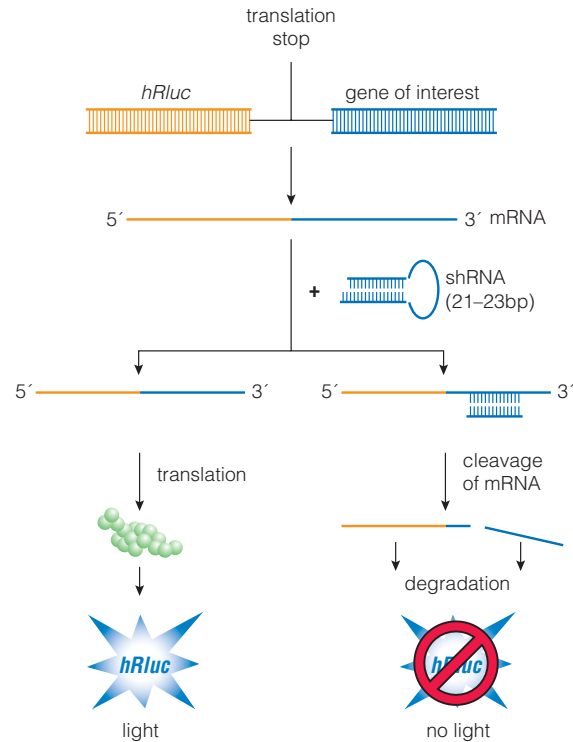
Screen multiple targets rapidly and for less than synthesizing synthetic siRNA duplexes.

Silencing RNA in vivo (RNAi)

Mammalian RNAi: siCHECK™ Vectors

The siCHECK™ Vectors provide a quantitative and rapid approach for the optimization of RNAi site selection. The vectors enable changes in expression of a target gene to be detected by monitoring the activity of *Renilla* luciferase. A target gene of interest is cloned downstream of the stop codon for the *Renilla* luciferase gene, such that a hybrid mRNA is expressed (but no hybrid protein). Initiation of RNAi toward the target gene results in the cleavage and subsequent degradation of the fusion mRNA including the *Renilla* luciferase RNA sequence. This results in a decrease in *Renilla* luciferase activity that can be easily monitored as an indicator of the RNAi effect.

There are two siCHECK™ Vectors, the psiCHECK™-1 Vector and psiCHECK™-2 Vector. Both vectors contain as the primary reporter gene the synthetic version of *Renilla* luciferase, *hRluc*. The psiCHECK™-2 Vector also possesses a secondary firefly reporter expression cassette, which has been specifically designed to be an intraplasmid transfection normalization reporter. Thus when using the psiCHECK™-2 Vector, the *Renilla* luciferase signal can be normalized to the firefly luciferase signal. *Renilla* and firefly luciferase activity can be measured very easily using the Dual-Glo™ Luciferase Assay System.



Mechanism of action of the siCHECK™ Vectors.

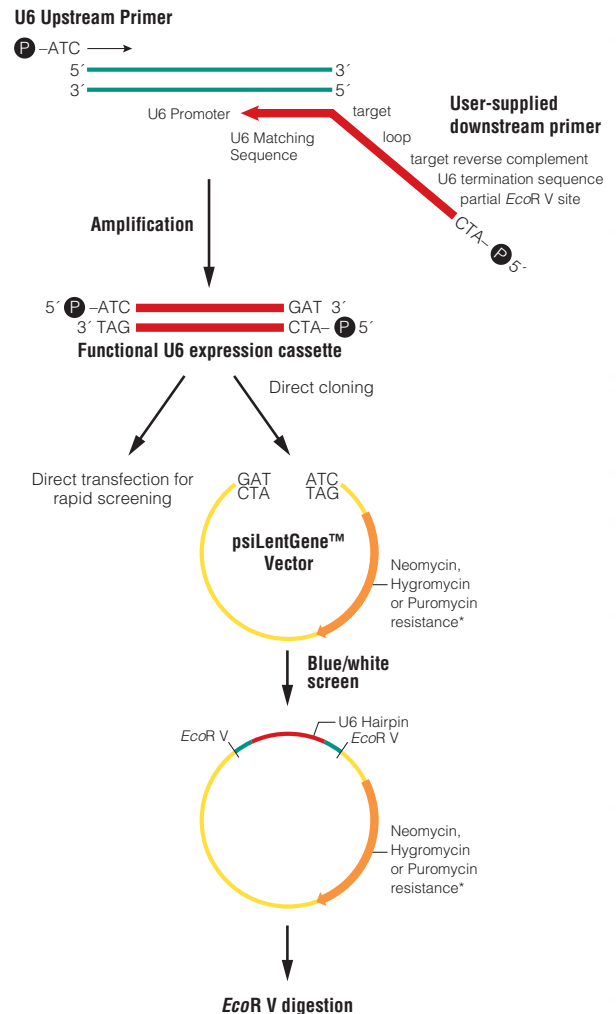
siCHECK™ Vectors Protocol
available at:
www.promega.com/tbs/tm329/tm329.html
Cat.# C8011 and C8021
Featured in Promega Notes 90 available
at: www.promega.com/pnotes/

Silencing RNA in vivo (RNAi)

siLentGene™-2 U6 Hairpin Cloning System

The siLentGene™-2 U6 Hairpin Cloning Systems (Cat.# C7860, C8060, C8070, C8080) are used primarily for screening siRNA targets, which may then be cloned into a vector. A DNA cassette containing a U6 promoter, a hairpin siRNA target sequence and the transcription termination sequence is generated by a single PCR amplification. The resulting PCR product can be directly transfected into human cells for the rapid screening of optimal target sequences.

Once optimal target sequences are identified, the desired PCR products generated using the siLentGene™-2 U6 Hairpin Cloning Systems can be subcloned into plasmid vectors that contain markers for selection of stable transfectants. The vectors are predigested and ready to use for direct subcloning of PCR products, and they also provide blue/white selection, allowing easy identification of recombinants on indicator plates. The psiLentGene™ Vectors contain the β -lactamase (Amp^r) gene, which confers resistance to ampicillin and allows selection in *E. coli*. The vectors are also designed so that the successful ligation of full-length PCR inserts regenerates an *EcoRV* site, providing a convenient method to confirm the presence of the desired insert.



The psiLentGene™ Basic Vector does not contain an antibiotic resistance marker for eukaryotic selection.

Schematic diagram of the siLentGene™-2 U6 Hairpin Cloning System protocol.

siLentGene™-2 U6 Hairpin Cloning System
 Protocol available at:
www.promega.com/tbs/tm247/tm247.html
 Cat.# C7860
 Featured in Promega Notes 87 available at: www.promega.com/pnotes/

Citation of siLentGene™-2 U6 Hairpin Cloning System:

Golubkov, V.S. *et al.* (2005) Membrane type-1 matrix metalloproteinase (MT1-MMP) exhibits an important intracellular cleavage function and causes chromosome instability. *J. Biol. Chem.* **280**, 25079–86.

Golubkov, V.S. *et al.* (2005) Centrosomal pericentrin is a direct cleavage target of membrane type-1 matrix metalloproteinase in humans but not in mice. Potential implications for tumorigenesis. *J. Biol. Chem.* **280**, 42237–41.

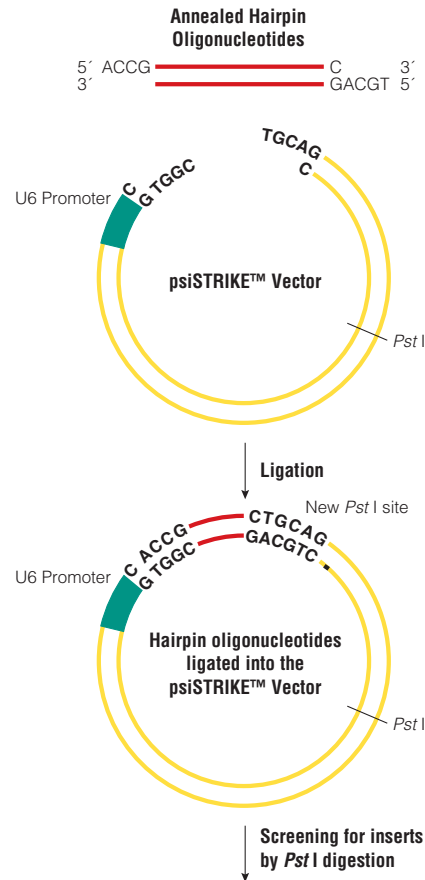
Silencing RNA in vivo (RNAi)

siSTRIKE™ U6 Hairpin Cloning System

When an optimized RNAi target sequence is known or only a few sequences are being evaluated, the siSTRIKE™ U6 Hairpin Cloning Systems (Cat.# C7890, C7900, C7910, C7920) should be used. In the siSTRIKE™ Systems, two short DNA oligonucleotides are synthesized and annealed to form a DNA insert that contains the hairpin siRNA target sequence. Upon annealing, the oligonucleotide forms ends that are compatible with the ends of the linearized psiSTRIKE™ Vector and thus facilitate sticky-end ligation. The linearized plasmids supplied with the system contain the human U6 promoter. Once transfected, RNA polymerase III transcribes the hairpin target sequences to generate hairpin shRNAs in vivo.

All the psiSTRIKE™ Vectors contain the β -lactamase (Amp^r) gene, which confers resistance to ampicillin and allows selection in *E. coli*. Three of the five psiSTRIKE™ Vectors: psiSTRIKE™ Puromycin, psiSTRIKE™ Hygromycin and psiSTRIKE™ Neomycin, also contain selectable markers for use in eukaryotic cells and can be used for both transient and stable expression of shRNA target sequences. The siSTRIKE™ U6 Hairpin Cloning System—Basic and the siSTRIKE™ U6 Hairpin Cloning System—hMGFP are intended for use in transient suppression assays.

The siSTRIKE™ U6 Hairpin Cloning Systems are designed to facilitate easy determination of successful ligation. The hairpin oligonucleotides used in the siSTRIKE™ Systems are less than 60bp in length. Since detection of a 60bp insert is difficult using an agarose gel, the presence of an insert can be detected by a *Pst*I digestion. The psiSTRIKE™ Vectors contain a single *Pst*I site. Successful insertion of a hairpin oligonucleotide creates a second *Pst*I site. Therefore, digestion with *Pst*I will yield two DNA fragments if the insert is present.



Cloning of a hairpin insert into a psiSTRIKE™ Vector. The simple cloning procedure involves ligation of the hairpin insert into the psiSTRIKE™ Vector, which is provided linearized and ready for ligation.

GeneClip™ U1 Hairpin Cloning System

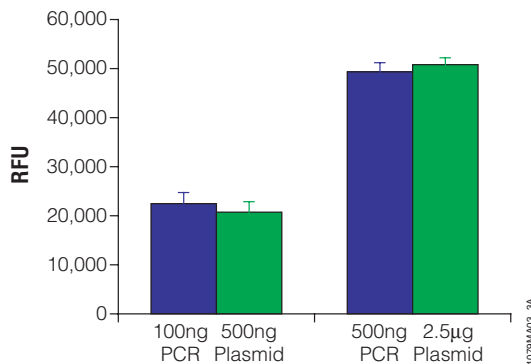
The GeneClip™ U1 Hairpin Cloning Systems are similar to the siSTRIKE™ U6 Hairpin Cloning Systems, except that the siRNAs are transcribed from the U1 promoter. The U1 promoter directs transcription by RNA polymerase II, whereas the U6 promoter is recognized by RNA polymerase III, to transcribe short hairpin RNAs. The GeneClip™ U1 Hairpin Cloning Systems include linearized plasmids (pGeneClip™ Vectors) designed for easy and fast cloning of hairpin target sequences to allow expression of siRNA target sequences in human cells.

siSTRIKE™ U6 Hairpin Cloning System
Protocol available at:
www.promega.com/tbs/tm246/tm246.html
Cat.# C7890
Featured in Promega Notes 87 available
at: www.promega.com/pnotes/

Silencing RNA in vivo (RNAi)

Long double-stranded RNA for nonmammalian RNAi

The T7 RiboMAX™ Express RNAi System is an in vitro transcription system designed to synthesize milligram amounts of RNA in as little as 30 minutes. The buffering system, NTP concentration, T7 RNA polymerase, inorganic pyrophosphatase and magnesium levels are optimized for increased RNA yield compared to standard in vitro transcription reactions (16). The RNA produced is used to generate double-stranded RNA (dsRNA) by simple annealing, nuclease digestion and precipitation steps.



Comparison of yield from PCR or plasmid templates.

In Vitro Transcription.

30 minutes at 37°C.

Annealing to form dsRNA.

10 minutes at 70°C.

20 minutes at room temperature.

DNase and RNase Treatment.

30 minutes at 37°C.

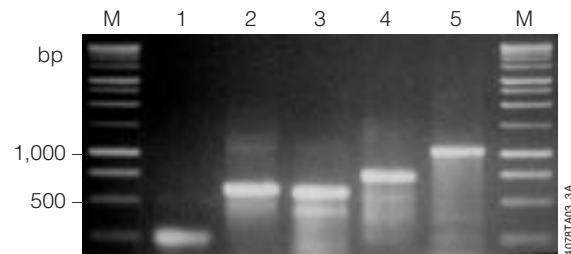
Alcohol Precipitation.

5 minutes on ice.

10 minutes spin in microcentrifuge.

Resuspend. Quantitate. Analyze dsRNA.

Basic protocol for producing double-stranded RNA with the T7 RiboMAX™ Express RNAi System.



Native gel analysis of different sized dsRNA molecules made with the T7 RiboMAX™ Express RNAi System. Approximately 4×10^{11} molecules loaded per lane. Full details in Betz, N. and Worzella, T. (2003) The T7 RiboMAX™ Express RNAi System: Efficient synthesis of dsRNA for RNA interference. *Promega Notes* 84, 7–11.

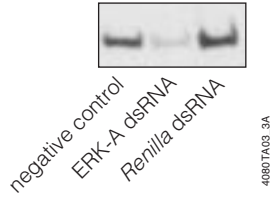
Citations of RiboMAX™ Systems for RNAi:

siStrike

Arcuri, F., *et al.* (2005) The translationally controlled tumor protein is a novel calcium binding protein of the human placenta and regulates calcium handling in trophoblast cells. *Biol. Reprod.* 73, 745–51.

T7 RiboMAX™ Express RNAi System
Protocol available at:
www.promega.com/tbs/tb316/tb316.html
Cat.# P1700
Featured in Promega Notes 84 available
at: www.promega.com/pnotes/

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Western blot analysis of the ERK-A (MAPK) protein from *Drosophila* S2 cells incubated in the presence or absence of ERK-A 778bp dsRNA or Renilla 500bp dsRNA. Full details in Betz, N. and Worzella, T. (2003) The T7 RiboMAX™ Express RNAi System: Efficient synthesis of dsRNA for RNA interference. *Promega Notes* **84**, 7–11.

Works with cloned or PCR-generated templates.

Citations of RiboMAX™ Systems for RNAi:

RiboMAX™ Systems used in RNAi

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Hossain, M.S., *et al.* (2004) DNA topoisomerase II is required for the G0-to-S phase transition in *Drosophila* Schneider cells, but not yeast. *Genes Cells* **9**, 905–17.

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Urban, S., *et al.* (2004) EGF receptor signalling protects smooth-cuticular cells from apoptosis during *Drosophila* ventral epidermis development. *Development* **131**, 1835–45.

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Mammalian RNAi Products	Size	Cat.#
siLentGene™-2 U6 Hairpin Cloning System—Basic ^(q,r)	1 system	C7860
siLentGene™-2 U6 Hairpin Cloning System—Puromycin ^(q,r)	1 system	C8060
siLentGene™-2 U6 Hairpin Cloning System—Hygromycin ^(q,r)	1 system	C8070
siLentGene™-2 U6 Hairpin Cloning System—Neomycin ^(q,r)	1 system	C8080

Each system contains sufficient reagents for 20 reactions.

Mammalian RNAi Products	Size	Cat.#
siSTRIKE™ U6 Hairpin Cloning System—Basic ^(q,r)	1 system	C7890
siSTRIKE™ U6 Hairpin Cloning System—Puromycin ^(q,r)	1 system	C7900
siSTRIKE™ U6 Hairpin Cloning System—Hygromycin ^(q,r)	1 system	C7910
siSTRIKE™ U6 Hairpin Cloning System—Neomycin ^(q,r)	1 system	C7920
siSTRIKE™ U6 Hairpin Cloning System ^(k,q,r,s,x)	1 system	C3550

Each system contains sufficient reagents for 20 reactions.

Mammalian RNAi Products	Size	Cat.#
GeneClip™ U1 Hairpin Cloning System—Basic ^(q,r)	20 reactions	C8750
GeneClip™ U1 Hairpin Cloning System—Puromycin ^(q,r)	20 reactions	C8760
GeneClip™ U1 Hairpin Cloning System—Hygromycin ^(q,r)	20 reactions	C8770
GeneClip™ U1 Hairpin Cloning System—Neomycin ^(q,r)	20 reactions	C8780
GeneClip™ U1 Hairpin Cloning System—hMGFP ^(c,k,q,r,s)	20 reactions	C8790

Each system contains sufficient reagents for 20 ligation reactions.

Mammalian RNAi Products	Size	Cat.#
psiCHECK™-1 Vector ^(s-w)	20µg	C8011
psiCHECK™-2 Vector ^(s-w)	20µg	C8021

Mammalian and Nonmammalian RNAi Product	Size	Cat.#
T7 RiboMAX™ Express RNAi System ^(a,b,g,r)	1 system	P1700

Each system contains sufficient reagents to produce at least 25 dsRNA molecules from 20µl in vitro transcription reactions. Includes DNase to remove DNA template and RNase to remove ssRNA.

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References:

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