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Promega

I. RNA Interference

A. Introduction

In this chapter, we provide a brief overview of the RNA interference (RNAi) process and discuss technologies and products that can be used for RNAi experiments. A protocol is provided for the enzymatic synthesis of double-stranded RNA in vitro that provides an inexpensive alternative to chemical synthesis of RNAs. We also discuss design and selection of short interfering RNA (siRNA) sequences and describe a vector system, the psiCHECK™ Vectors, that can be used to screen potential siRNA target sequences for effectiveness during RNAi optimization. In addition, the GeneClip™ U1 Hairpin Cloning Systems, a DNA-directed RNAi (ddRNAi) system specifically designed for expression of small hairpin RNAs in mammalian cells, provide a cloning-based approach to allow fast, easy ligation and expression of hairpin oligonucleotides. Various strategies for delivery of siRNA to target cells are discussed, and example protocols for transient and stable transfection of mammalian cells are provided. Finally, methods for quantitating target gene suppression are briefly summarized.

B. Overview and Mechanism of RNAi

RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) suppresses expression of a target protein by stimulating the specific degradation of the target mRNA (for reviews, see Hannon, 2003; Caplen, 2004; Fuchs *et al.* 2004; Betz, 2003a). RNAi has been used to study loss of function for a variety of genes in several organisms including various plants, *Caenorhabditis elegans* and *Drosophila*, and permits loss-of-function genetic screens and rapid tests for genetic interactions in mammalian cells (Hannon, 2002; Williams *et al*. 2003).

RNAi involves a multistep process (Figure 2.1). dsRNA is recognized by an RNase III family member (e.g., Dicer in *Drosophila*) and cleaved into siRNAs of 21–23 nucleotides (Agrawal *et al*. 2003; Elbashir *et al*. 2001b; Bernstein *et al*. 2001; Hammond et al. 2000). These siRNAs are incorporated into an RNAi targeting complex known as RISC (RNA-induced silencing complex), which destroys mRNAs homologous to the integral siRNA (Hammond *et al*. 2000; Bernstein *et al*. 2001). The target mRNA is cleaved in the center of the region complementary to the siRNA (Elbashir *et al*. 2001c), with the net result being rapid degradation of the target mRNA and decreased protein expression.

RNAi has revolutionized the study of gene function, and is being explored as a therapeutic tool (for reviews, see Dorsett and Tuschl, 2004; Hannon and Rossi, 2004). For example, RNAi has been used to identify gene products essential for cell growth (Harborth *et al*. 2001), to cause subtype and species-specific knockdown of various protein kinase C (PKC) isoforms in both human and rat cells (Irie *et al*. 2002), and to specifically target degradation of an oncogene product (Wilda *et al*. 2002). RNAi has also been used to specifically target and prevent viral infections by HIV-1 and HCV in cell culture (Park *et al*. 2002) and intact animals (McCaffrey *et al*. 2002). These observations open the field for further studies toward novel gene therapy approaches for anti-cancer or anti-viral treatments using siRNAs or shRNAs.

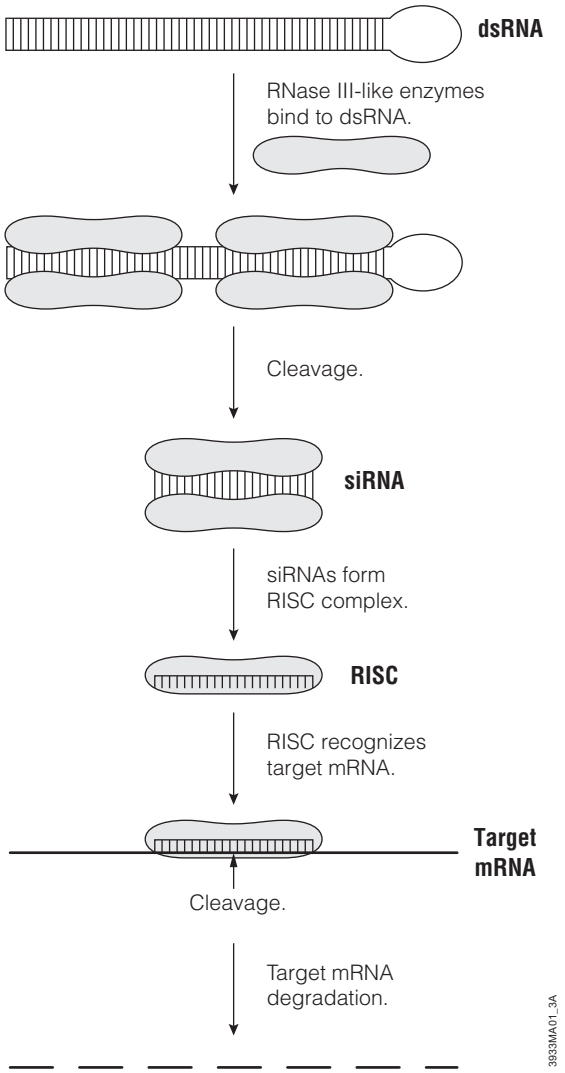

Figure 2.1. Simplified schematic diagram of the proposed RNA interference mechanism. dsRNA processing proteins (RNase III-like enzymes) bind to and cleave dsRNA into siRNA. The siRNA forms a multicomponent nuclease complex, the RNA-induced silencing complex (RISC). The target mRNA recognized by RISC is cleaved in the center of the region complementary to the siRNA and quickly degraded. An animated version of this illustration is also available.

An animated presentation illustrating the entire RNAi process is available on the *Nature* web site.

C. RNAi as a Tool for Targeted Inhibition of Gene Expression

The use of long dsRNAs (>400bp) has been successful in generating RNA interference effects in many organisms including *Drosophila* (Misquitta and Paterson, 1999), zebrafish (Wargelius *et al*. 1999), *Planaria* (Sanchez-Alvarado *et al*. 1999) and numerous plants (Jorgensen, 1990, Fukusaki *et al*. 2004, Jensen *et al*. 2004). In mammalian systems, siRNA

molecules of 21–22 nucleotides or short hairpin RNAs (shRNAs) are used to avoid endogenous nonspecific antiviral responses that target longer dsRNAs (Caplen *et al*. 2001, Elbashir *et al*. 2001a). Yu *et al*. (2002) and others (Brummelkamp *et al*. 2002b; McManus *et al*. 2002; Sui *et al*. 2002; Xia *et al*. 2002; Barton and Medzhitov, 2002) demonstrated that shRNAs bearing a fold-back, stem-loop structure of approximately 19 perfectly matched nucleotides connected by various spacer regions and ending in a 2-nucleotide 3′-overhang can be as efficient as siRNAs at inducing RNA interference. si/shRNAs can induce specific gene silencing in a wide range of mammalian cell lines without leading to global inhibition of mRNA translation (Caplen *et al*. 2001; Elbashir *et al*. 2001a; Paddison *et al*. 2002).

Generation of Short Interfering RNAs

siRNAs are the main effectors of the RNAi process. These molecules can be synthesized chemically or enzymatically in vitro (Micura, 2002; Betz, 2003b; Paddison *et al*. 2002) or endogenously expressed inside the cells in the form of shRNAs (Yu *et al*. 2002; McManus *et al*. 2002). Plasmid-based expression systems using RNA polymerase III U6 or H1, or RNA polymerase II U1, small nuclear RNA promoters, have been used for endogenous expression of shRNAs (Brummelkamp *et al*. 2002b; Sui *et al*. 2002, Novarino *et al*. 2004).

Rational Design of Effective siRNA Probes

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 (Chiu and Rana, 2002; Khvorova *et al*. 2003; Schwarz *et al*. 2003; Hseih *et al*. 2004; Reynolds *et al*. 2004; Ui-Tei *et al*. 2004). In addition, strategies for experimentally screening effective siRNAs from pools of potential siRNAs are being developed (Kumar *et al*. 2003; Vidugiriene *et al*. 2004) and will remain a useful tool until potent siRNAs can be predicted accurately for each target gene.

Delivery of siRNA

The efficient delivery of siRNAs is a vital step in RNAi-based gene silencing experiments. Synthetic siRNAs can be delivered by electroporation or by using lipophilic agents (McManus *et al*. 2002; Kishida *et al*. 2004). siRNAs have been used successfully to silence target genes; however, these approaches are limited by the transient nature of the response. The use of plasmid systems to express small hairpin RNAs helps overcomes this limitation by allowing stable suppression of target genes (Dykxhoorn *et al*. 2003). Various viral delivery systems have also been developed to deliver shRNA-expressing cassettes into cells that are difficult to transfect, creating new possibilities for RNAi usage (Brummelkamp *et al*. 2002a; Rubinson *et al*. 2003). Successful delivery of siRNAs in live animals has also been reported (Hasuwa *et al*. 2002; Carmell *et al*. 2003; Kobayashi *et al*. 2004).

II. siRNA Design and Optimization

A. Design of Target Sequences

Identifying an optimal target sequence is critical to the success of RNA interference experiments. Since it is not possible to predict the optimal siRNA sequence for a given target, multiple siRNAs will usually need to be evaluated. Recommendations for the design of siRNAs are constantly being improved upon as knowledge of the RNAi process continues to expand. At the time of writing this chapter, the recommendations are as follows: siRNA target sequences should be specific to the gene of interest and have ~20–50% GC content (Henshel *et al*. 2004). Ui-Tei *et al*. (2004) report that siRNAs satisfying the following conditions are capable of effective gene silencing in mammalian cells: 1) G/C at the 5′ end of the sense strand; 2) A/U at the 5′ end of the antisense strand; 3) at least 5 A/U residues in the first 7 bases of the 5′ terminal of the antisense strand; 4) no runs of more than 9 G/C residues. Additionally, primer design rules specific to the RNA polymerase used will apply. For example, for RNA polymerase III, the polymerase that transcribes from the U6 promoter, the preferred target sequence is 5′-GN_{18}-3′.

Runs of 4 or more Ts (or As on the other strand) serve as terminator sequences for RNA polymerase III and should be avoided. In addition, regions with a run of any single base should be avoided (Czauderna *et al*. 2003). It is generally recommended that the mRNA target site be at least 50–200 bases downstream of the start codon (Sui *et al*. 2002; Elbashir *et al.* 2002, Duxbury and Whang, 2004) to avoid regions in which regulatory proteins might bind.

B. Use of Reporter Genes for RNAi Optimization

Not all siRNAs directed against a target gene are equally effective in suppressing expression of that target in mammalian cells. Therefore, it is important to identify siRNA sequences that are effective inhibitors of target gene expression. Although rational designs for selection of potential target sequences have been encouraging in generating effective siRNAs, accurate prediction of the most effective siRNAs still remains to be achieved. Current screening technologies are based on semiquantitative, time-consuming methods and are not easily modified to perform rapid, simultaneous screening of multiple siRNA/shRNA sequences. However, as the field of RNAi advances, and more high-throughput applications are adopted, there is a growing need for rapid, quantitative screening to confirm siRNA effectiveness (Kumar *et al*. 2003; Mousses *et al*. 2003).

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. Here, we describe the psiCHECK™ Vector system, which is based on use of the bioluminescent *Renilla* luciferase reporter gene. The psiCHECK™ Vectors offer several advantages compared to other fusion approaches such as green fluorescent protein (GFP)- or Flag-tag-based methods.

Measurement of net fluorescence from GFP in cell culture can be difficult and, in most cases, a flow cytometer is required for quantitation. Flag-tag quantitation requires Western blot analysis, which can be time-consuming. The high sensitivity of bioluminescence detection can readily tolerate lower expression levels, and introduction of a second reporter gene, firefly luciferase, allows normalization of changes in *Renilla* luciferase expression, making the psiCHECK™ Vector approach more robust and giving greater reproducibility of results.

The psiCHECK™-1 and -2 Vectors allow quantitative selection of optimal siRNA target sites and can be adapted for use in high-throughput applications. Figure 2.2 provides a basic illustration of how the psiCHECK™ Vectors are used. Both vectors contain a synthetic version of the *Renilla* luciferase (*hRluc*) reporter gene for monitoring RNAi activity. Several restriction sites are included 3′ of the luciferase translational stop codon, allowing creation of transcriptional fusions between the gene of interest and the *Renilla* luciferase reporter gene. Because of the presence of a stop codon in-frame with the *Renilla* luciferase open reading frame, no fusion protein is produced. Consequently, there is no need to maintain frames when inserting the target gene. Also, toxic genes or gene fragments can be analyzed using this design without the danger of these genes killing the transfected cells.

The psiCHECK™-1 Vector (Cat.# C8011) is recommended for monitoring RNAi effects in live cells. Changes in *Renilla* luciferase activity can be measured with the EnduRen™ Live Cell Substrate (Cat.# E6481). This approach permits continuous monitoring of intracellular luminescence. *Renilla* luciferase expression can be monitored continuously for 2 days without interfering with normal cell physiology.

The psiCHECK™-2 Vector (Cat.# C8021) contains an additional reporter gene, a synthetic firefly luciferase gene (*hluc+*), and is designed for endpoint lytic assays. Inclusion of the firefly luciferase gene permits normalization of changes in *Renilla* luciferase expression to firefly luciferase expression. *Renilla* and firefly luciferase activities can be measured using either the Dual-Luciferase® Reporter Assay System (Cat.# E1910) or the Dual-Glo® Luciferase Assay System (Cat.# E2920).

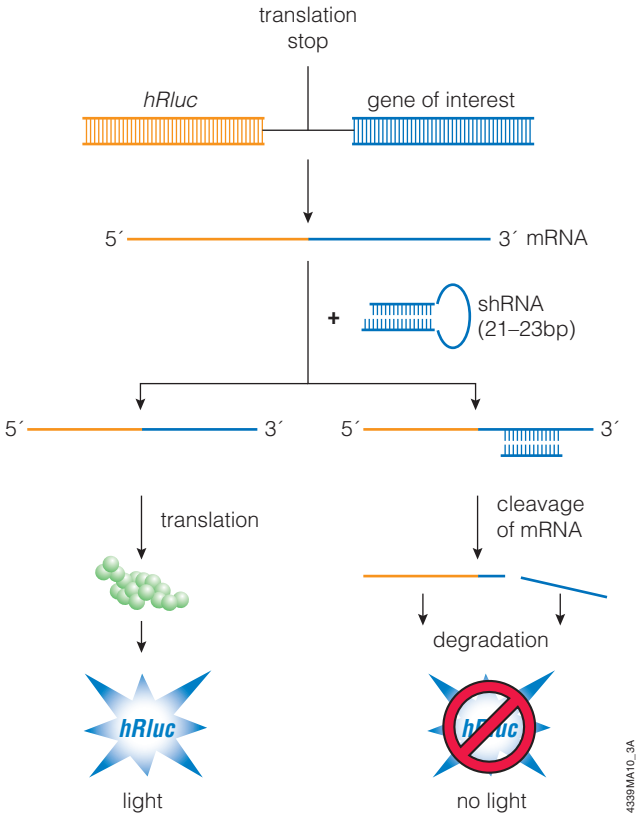

Figure 2.2. Mechanism of action of the psiCHECK™ Vectors.

To use the psiCHECK™ Vectors for screening siRNA targets, the gene of interest is cloned into the multiple cloning region located 3′ to the synthetic *Renilla* luciferase gene and its translational stop codon. After cloning, the vector is transfected into a mammalian cell line, and a fusion of the *Renilla* gene and the target gene is transcribed. Functional *Renilla* luciferase is translated from the intact transcript. Depending on your experimental design, vectors expressing shRNA or synthetic siRNA can be either cotransfected simultaneously or sequentially. If a specific shRNA/siRNA effectively initiates the RNAi process on the target RNA, the fused *Renilla* target gene mRNA sequence will be degraded, resulting in reduced *Renilla* luciferase activity.

Additional Resources for psiCHECK™ Vectors

Technical Bulletins and Manuals

TB329 *psiCHECK™ Vectors Technical Bulletin*

Vector Maps

psiCHECK™ Vectors

Promega Publications

The use of bioluminescent reporter genes for RNAi optimization

III. Enzymatic Synthesis of RNA in Vitro

siRNA synthesis in vitro provides a useful alternative to the potentially expensive chemical synthesis of RNA

(Figure 2.3). The method relies on T7 phage RNA polymerase to produce individual sense and antisense strands that are annealed in vitro prior to delivery into the cells of choice (Fire *et al*. 1998; Donze and Picard, 2002; Yu *et al*. 2002, Shim *et al*. 2002).

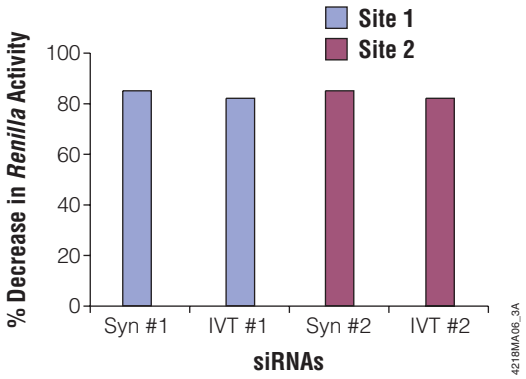

Figure 2.3. Comparison of RNA interference induced by siRNAs synthesized chemically or by in vitro transcription. Two different *Renilla* luciferase siRNA target sequences were synthesized chemically (Syn) or using the T7 RiboMAX™ Express RNAi System (IVT). The target sequences were then evaluated by RNA interference in CHO cells stably expressing *Renilla* luciferase.

The T7 RiboMAX™ Express RNAi System (Cat.# P1700) is an in vitro transcription system designed for rapid production of milligram amounts of double-stranded RNA (dsRNA). The system can be used to synthesize siRNAs for use in mammalian systems (Figure 2.4; Betz, 2003b, Hwang *et al*. 2004) or longer interfering RNAs for nonmammalian systems (Betz and Worzella, 2003; Betz, 2003c). The DNA templates for in vitro transcription of siRNAs are a pair of short, duplex oligonucleotides that contain T7 RNA polymerase promoters upstream of the sense and antisense RNA sequences. Each oligonucleotide of the duplex is a separate template for the synthesis of one strand of the siRNA. The separate short RNA strands that are synthesized are then annealed to form siRNA.

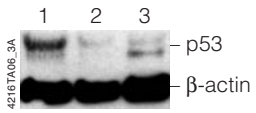

Figure 2.4. Suppression of endogenous p53 protein using siRNA prepared using the T7 RiboMAX™ Express RNAi System. Twenty-four hours after plating in a 12-well plate, 293T cells were transfected with 200ng scrambled siRNA (lane 1), 200ng in vitro synthesized p53 siRNA (lane 2), or 200ng chemically synthesized p53 siRNA (lane 3). Twenty-four hours after transfection, cells were lysed using 1X Reporter Lysis Buffer (Cat.# E3971) containing protease inhibitors, and the protein was quantitated using the BCA Protein Assay (Pierce). Equal amounts of each lysate (10µg) were separated on a 4–12% polyacrylamide Bis-Tris gel (Invitrogen) and transferred to Hybond®-C membrane (Amersham). The blot was probed with both a p53 antibody (Calbiochem) and a β-actin antibody (Abcam). Detection was performed using Goat Anti-Mouse HRP Conjugate (Cat.# W4021) and the Transcend™ Chemiluminescent Non-Radioactive Translation Detection System (Cat.# L5080). The blot was exposed to Kodak X-OMAT® film for approximately 4 minutes. The simultaneous detection of the β-actin protein controlled for loading and transfer. The p53 and β-actin bands are indicated and are of the expected sizes.

A. In Vitro Synthesis of dsRNA for Use in Nonmammalian Systems

RNAi experiments in nonmammalian systems are typically performed with dsRNA of 400bp or larger (Elbashir *et al*. 2001b; Yang *et al*. 2000, Hammond *et al*. 2000). The minimum size of dsRNA recommended for RNAi in these systems is ~200bp. In general, templates for transcription of dsRNA for use in RNAi experiments correspond to most or all of the target message sequence. Data suggests that longer dsRNA molecules are more effective on a molar basis at silencing protein expression, but higher concentrations of smaller dsRNA molecules may have similar silencing effects. Data generated at Promega suggests that smaller dsRNAs can be as effective and efficient at inducing RNAi in nonmammalian systems (Betz, 2003c).

In the T7 RiboMAX™ Express RNAi System, dsRNA production requires a T7 RNA polymerase promoter at the 5′-ends of both DNA target sequence strands. To achieve this, separate DNA templates, each containing the target sequence in a different orientation relative to the T7 promoter, are transcribed in two separate reactions. The resulting transcripts are mixed and annealed post-transcriptionally. DNA templates can be created by PCR or by using two linearized plasmid templates, each containing the T7 polymerase promoter at a different end of the target sequence.

See the *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316 for a protocol for in vitro synthesis of dsRNA for RNAi in nonmammalian systems.

B. In Vitro Synthesis of siRNA for Use in Mammalian Systems

Figure 2.5 outlines the protocol for synthesis of siRNA using the T7 RiboMAX™ Express RNAi System. The initial step is generating the DNA template, which consists of two DNA oligonucleotides annealed to form a duplex. Generally

20pmol of duplex oligonucleotides are required per 20µl in vitro transcription reaction. Using the RiboMAX™ Express T7 Buffer and Enzyme Mix allows efficient synthesis of RNA in as little as 30 minutes. The annealed DNA oligonucleotide template is removed by a DNase digestion step, and the separate small RNA strands (sense and antisense) are annealed to form siRNA. The siRNA is precipitated using sodium acetate and isopropanol, and the resuspended product can be analyzed on polyacrylamide gels for size and integrity. Quantitation of the siRNA can be accomplished by either gel analysis or RiboGreen® analysis (Molecular Probes).

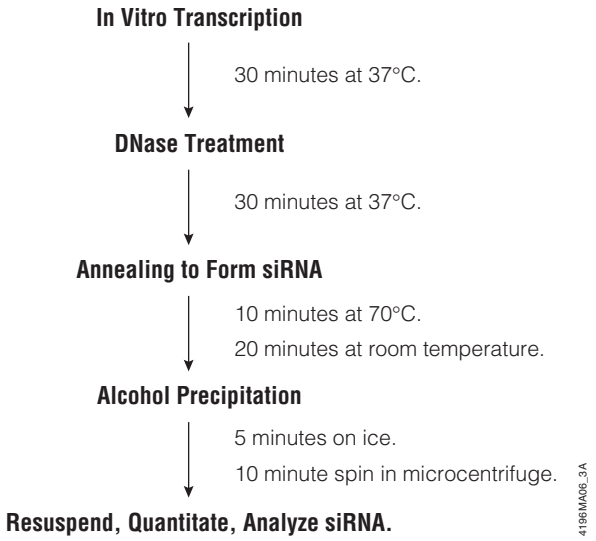

Figure 2.5. The T7 RiboMAX™ Express RNAi System protocol.

Materials Required:

- T7 RiboMAX™ Express RNAi System (Cat.# P1700)
- 2X oligo annealing buffer (20mM Tris-HCl [pH 7.5], 100mM NaCl)
- nuclease-free water
- gene-specific oligonucleotides
- isopropanol
- 70% ethanol

Designing DNA Oligonucleotides

The target mRNA sequence selected must be screened for the sequence 5′-GN_{17}C-3′. The generation of 3–5 different siRNA sequences for a particular target is recommended to allow screening for the optimal target site. The oligonucleotides consist of the target sequence plus the T7 RNA polymerase promoter sequence and 6 extra nucleotides upstream of the minimal promoter sequence to allow for efficient T7 RNA polymerase binding. Details on design of oligonucleotides for use with this system are found in the *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316.

Annealing DNA Oligonucleotides

1. Resuspend DNA oligonucleotides in nuclease-free water to a final concentration of 100pmol/µl.
2. Combine each pair of DNA oligonucleotides to generate either the sense strand RNA or antisense strand RNA templates as follows:

oligonucleotide 1 (100pmol/µl)	10µl
oligonucleotide 2 (100pmol/µl)	10µl
2X oligo annealing buffer	50µl
nuclease-free water	30µl
Final Volume	**100µl**

3. Heat at 90–95°C for 3–5 minutes, then allow the mixture to cool slowly to room temperature. The final concentration of annealed oligonucleotide is 10pmol/µl. Store annealed oligonucleotide DNA template at either 4°C or –20°C.

Synthesizing Large Quantities of siRNA

1. Set up the reaction at room temperature. The 20µl reaction may be scaled as necessary (up to 500µl total volume; use multiple tubes for reaction volumes >500µl). Add the components in the order shown below. For each siRNA, two separate reactions must be assembled as each RNA strand is synthesized separately, and then mixed following transcription.

T7 Reaction Components	Sample Reaction	Control Reaction
RiboMAX™ Express 2X Buffer	10µl	10µl
annealed oligonucleotide template DNA (10pmol/µl)	2.0µl	
pGEM® Express Positive Control Template	—	1.0µl
nuclease-free water	6.0µl	7.0µl
Enzyme Mix, T7 Express	2.0µl	2.0µl
Final Volume	**20µl**	**20µl**

2. Incubate for 30 minutes at 37°C.

Removing the DNA Template and Annealing siRNA

The DNA template can be removed by digestion with DNase following the transcription reaction. RQ1 RNase-Free DNase (Cat.# M6101) has been tested for its ability to degrade DNA while maintaining the integrity of RNA. If accurate RNA concentration determination is desired, the RNA should be DNase-treated and purified to remove potentially inhibitory or interfering components.

1. To each 20µl transcription reaction, add 1µl of RQ1 RNase-Free DNase and incubate for 30 minutes at 37°C.
2. Combine separate sense and antisense reactions and incubate for 10 minutes at 70°C, then allow the tubes to cool to room temperature (approximately 20 minutes). This step anneals the separate short sense and antisense RNA strands, generating siRNA.

Purifying siRNA

1. Add 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol. Mix and place on ice for

5 minutes. The reaction will appear cloudy. Spin at top speed in a microcentrifuge for 10 minutes.

2. Carefully aspirate the supernatant, and wash the pellet with 0.5ml of cold 70% ethanol, removing all ethanol following the wash. Air-dry the pellet for 15 minutes at room temperature, and resuspend the RNA sample in nuclease-free water in a volume 2–5 times the original reaction volume (at least 2 volumes are required for adequate resuspension). Store at –20°C or –70°C.

Additional Resources for T7 RiboMAX™ Express RNAi System

Technical Bulletins and Manuals

TB316 *T7 RiboMAX™ Express RNAi System Technical Bulletin*

Promega Publications

RNAi in *Drosophila* S2 cells: Effect of dsRNA size, concentration, and exposure time

The T7 RiboMAX™ Express RNAi System: Efficient synthesis of dsRNA for RNA interference

Produce functional siRNAs and hairpin siRNAs using the T7 RiboMAX™ Express RNAi System

Citations

Hwang, C.K. *et al.* (2004) Transcriptional regulation of mouse μ opioid receptor gene by PU.1. *J. Biol. Chem.* **279**, 19764–74.

In this article, siRNA was used to reduce the level of the PU.1 transcription factor. The siRNA was generated using the T7 RiboMAX™ Express RNAi System. Annealed siRNA was purified by isopropanol precipitation. Forty-eight hours after transfecting 2.5μg of siRNA into RAW264.7 cells, RNA and protein were isolated from the cells, and the siRNA effect was analyzed by RT-PCR and Western blot.

PubMed Number: 14998994

Kim, C.S. *et al.* (2004) Neuron-restrictive silencer factor (NRSF) functions as a repressor in neuronal cells to regulate μ opioid receptor gene. *J. Biol. Chem.* **279**, 46464–73.

In this article, siRNA was used to silence endogenous mouse and human NRSF expression in NS20Y and HeLa cells. siRNAs were generated using the T7 RiboMAX™ Express RNAi System.

PubMed Number: 15322094

IV. DNA-Directed RNAi

A. Overview of ddRNAi

DNA-directed RNA interference (ddRNAi) involves the use of DNA templates to synthesize si/shRNA in vivo. ddRNAi relies on U6 or H1 [RNA polymerase III], or U1 [RNA polymerase II]) promoters for the expression of siRNA target sequences that have been transfected into mammalian cells (Miyagishi and Taira, 2002; Brummelkamp *et al*. 2002b; Novarino *et al*. 2004). si/shRNA target sequences can be generated by PCR, creating "expression cassettes" that can be transfected directly into cells (Csiszar *et al*. 2004; Castanotto *et al*. 2002) or cloned into expression vectors (Sui *et al*. 2002; Paul *et al*. 2002; Gou *et al.* 2003; Yu *et al*. 2002). 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 (Yeager *et al.* 2005). Screening can also be performed using synthetic RNAs, but this can become expensive for numerous targets.

Vector-based approaches also offer the potential of stable, long-term inhibition of gene expression by providing siRNAs on plasmids that allow selection of transfected cells. Vectors with markers, such as puromycin, neomycin or hygromycin, can be used for suppression of target genes for several weeks or longer. Transfection with synthetic siRNAs allows for only a transient measurement (usually 48–72 hours) of the RNAi effect.

The success of ddRNAi depends on several parameters including generation of vectors containing full-length sh/siRNA sequences, delivery of those vectors into cells, and expression of the si/shRNA constructs. Most strategies for cloning siRNA target sequences into expression vectors utilize the design of a hairpin structure. This design consists of two inverted repeats separated by a short spacer sequence (loop sequence). After transcription by RNA polymerase, the inverted repeats anneal and form a hairpin, which is then cleaved by Dicer to form an siRNA.

The GeneClip™ U1 Hairpin Cloning Systems facilitate easy expression of shRNAs in vivo by ddRNAi-based methods. This system provides a simple, cloning-based approach, allowing ligation of potential shRNA target sequences into vectors that allow transient or stable expression in mammalian cells.

B. GeneClip™ U1 Hairpin Cloning Systems

The GeneClip™ U1 Hairpin Cloning Systems (Cat.# C8750, C8760, C8770, C8780, C8790) are designed for rapid and efficient cloning of hairpin sequences for expression of shRNAs in vivo. The U1 promoter provides the benefit of production of shRNA with a defined termination site, mimicking siRNA produced in vivo when the Dicer complex cleaves the loop off the hairpin. Using the GeneClip™ Systems, complementary oligonucleotides supplied by the user are annealed and cloned into the predigested vector downstream of the U1 promoter. The linearized vectors contain overhangs for increased cloning efficiency. The GeneClip™ Systems are provided in five formats. The GeneClip™ U1 Hairpin Cloning System—Basic is designed for transient suppression of the gene of interest. The GeneClip™ U1 Hairpin Cloning System—hMGFP contains the green fluorescent protein gene from *Montastrea cavernosa* and can therefore be used to determine transfection efficiency; it also allows separation of transfected cells by fluorescent-activated cell sorting (FACS®). The three remaining GeneClip™ U1 Hairpin Cloning Systems provide the ability to stably select

transfected cells using either neomycin, hygromycin or puromycin, so that experimental results do not depend on transfection efficiency.

An overview of the GeneClip™ U1 Hairpin Cloning System protocol is given in Figure 2.6.

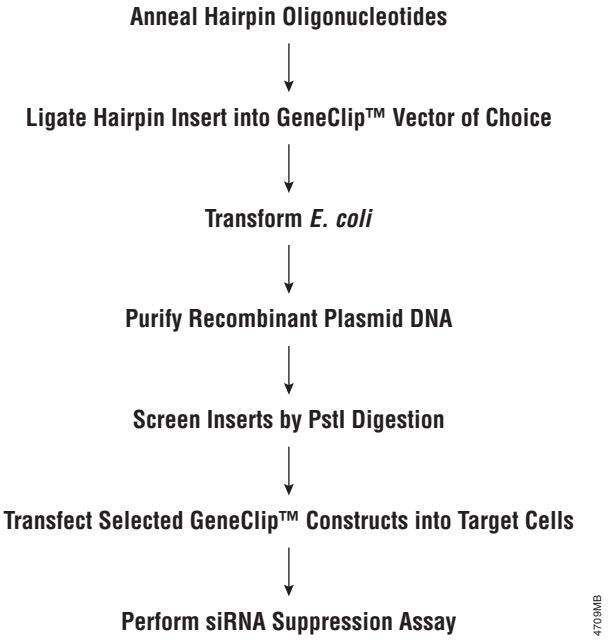

Figure 2.6. Overview of the GeneClip™ U1 Hairpin Cloning System protocol.

Hairpin Cloning

Once target sites have been selected, the hairpin oligonucleotides are annealed and ligated into the pGeneClip™ Vector, which is then screened for the proper insert (Figure 2.7). These two oligonucleotides form a DNA insert that contains a hairpin siRNA target sequence. Standard desalting of the oligonucleotides is necessary, but gel purification and 5′ phosphorylation are not required. Because the oligonucleotides inserted into the vector are short (~60nt), detection by standard agarose gel electrophoresis can be difficult. To make detection of inserts more convenient, a PstI site is engineered into the vector backbone (Figure 2.7). Upon successful ligation of the annealed oligonucleotides into the vector, a second PstI site is created. Digestion with PstI therefore results in two easily separated bands of approximately 3kb and 1kb. If no insert is present, PstI digestion will result in a single band. In our experiments, ligation of the insert typically results in over 50-fold more colonies than control ligations containing vector alone. In addition, the PstI digestion showed over 90% of the colonies contained insert.

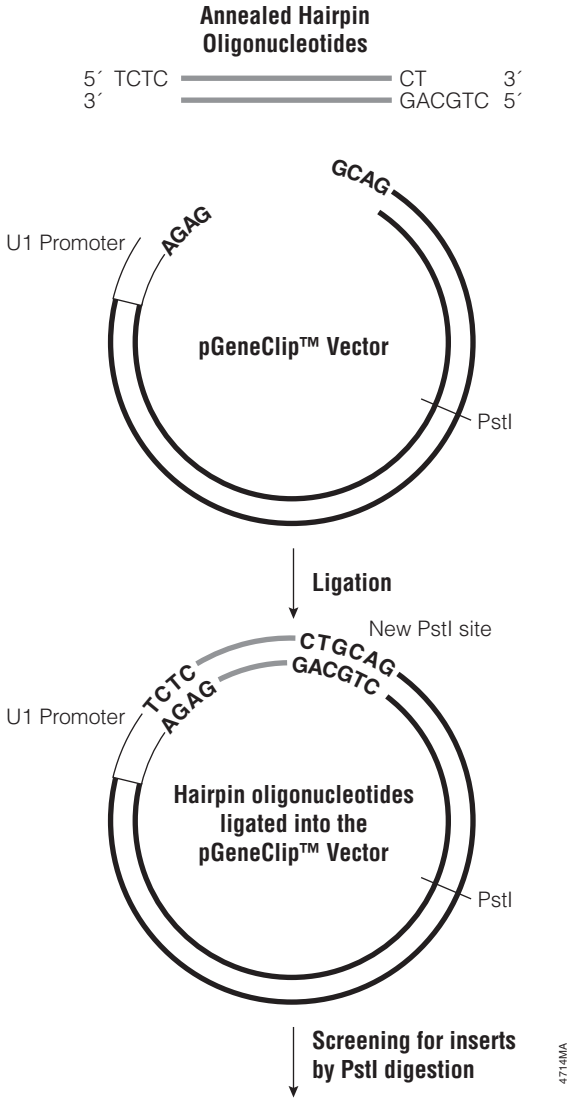

Figure 2.7. Cloning of a hairpin insert into a pGeneClip™ Vector. The simple cloning procedure involves ligation of the hairpin insert into the pGeneClip™ Vector, which is provided linearized and ready for ligation.

Target Site Selection

The introduction of long strands of dsRNA into mammalian cells induces a strong interferon response that can lead to an overall shutdown of protein synthesis and cell death. Short dsRNA molecules (<30nt) can bypass the interferon response and still function in the RNAi pathway (Elbashir *et al.* 2001a). Since the entire mRNA sequence cannot be used in mammalian cells, various target sites must be selected for use in RNAi experiments. Unfortunately not all target sequences in an mRNA will work equally well to suppress target gene expression. Experimental testing of the optimal sequences is required to confirm a high level of inhibition.

Transient and Stable In Vivo Suppression

The pGeneClip™ Basic and pGeneClip™ hMGFP Vectors are recommended for use in transient transfection assays. For many target sequences, transient transfection can quickly yield cells that can be assessed for the effects of gene suppression, including changes in phenotype, protein expression levels or other effects.

One consideration in evaluating transient transfection experiments is transfection efficiency. For example, if only 30% of the cells are transfected, it may be difficult to detect inhibition of expression, since 70% of the cells were not successfully transfected and still express the target mRNA. The GeneClip™ U1 Hairpin Cloning System—hMGFP (Cat.# C8790) provides an shRNA expression vector that contains an internal fluorescent marker for monitoring delivery efficiency of shRNA-expressing constructs. In addition to allowing transcription of hairpin target sequences and generation of siRNAs in vivo, the vector contains an improved, synthetic version of the green fluorescent protein (hMGFP) gene. The hMGFP gene encodes a 26kDa protein that gives improved fluorescence and reduced cytotoxicity compared with other GFP proteins. The presence of GFP allows easy determination of transfection efficiency and allows selection of transfected cells by FACS® (Cormack *et al*. 1996; Sorensen *et al*. 1999; Galbraith *et al*. 1999). Importantly, the expression of hMGFP does not affect gene silencing by shRNA molecules expressed from the same vector.

The pGeneClip™ Puromycin, pGeneClip™ Hygromycin and pGeneClip™ Neomycin Vectors allow selection of stably transfected cells. Thus, the results are no longer dependent on transfection efficiency. If necessary, clonal lines of transfected cells can be generated if the population of selected cells does not show the expected inhibition levels. Because integration of the vector into different positions in the genome can affect expression of the RNAi hairpin, a population of cells may not show suppression. A clonal cell line that sufficiently expresses the RNAi hairpin may be required to demonstrate suppression with the pGeneClip™ Vectors.

To test the effectiveness of the GeneClip™ U1 promoter, we monitored reduction in p53 protein levels after targeting p53 mRNA. p53 expression was targeted using shRNAs cloned into a pGeneClip™ Basic Vector. More than 90% inhibition was observed for the protein compared to a nonspecific control (data not shown). To test p53 reduction in vivo, 293T cells, which contain elevated levels of p53, were transfected with a pGeneClip™ Basic Vector expressing p53 shRNA. Compared to nontransfected cells, the cells containing the pGeneClip™ Vector expressing p53 shRNA exhibited greater than 85% reduction in p53 protein levels (Figure 2.8, Panel A).

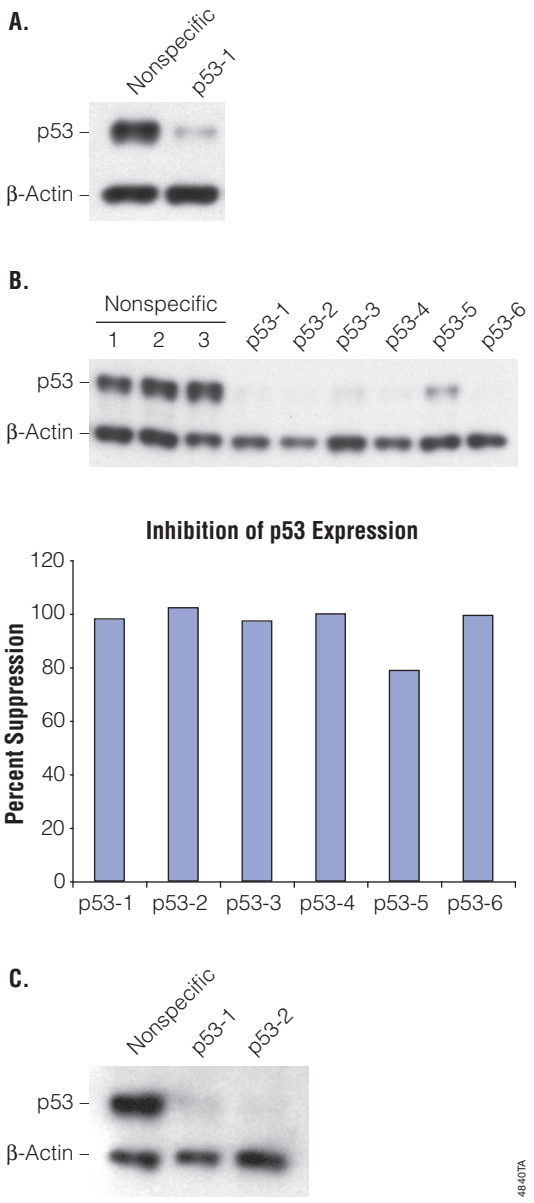

Figure 2.8. Inhibition of p53 expression in transient transfections and in stable clones. 293T cells were transfected with pGeneClip™ Basic Vectors containing either a hairpin target sequence directed against p53 or a nonspecific target sequence. After 48 hours, cells were collected, lysed, and protein levels quantitated by BCA assay (Pierce). For stable transfections, 293T cells were transfected with a pGeneClip™ Puromycin Vector containing either a nonspecific or a p53-specific target sequence. Cells were selected with puromycin for 7 days, and clones were assayed after passage 4 and passage 11. For Western analysis, 2µg protein per lane was loaded on an 8% Tris-Glycine gel and then transferred to nitrocellulose. Blots were probed with monoclonal antibodies to p53 (Oncogene Research Products, Ab-2, 1:1,000 dilution) and β-actin (Abcam, AC-12, 1:5,000 dilution). Detection was performed using ECL™ Plus (Amersham). Protein levels were quantitated by densitometry after exposure to film. **Panel A.** shRNA suppression of p53 expression in transiently transfected cells. **Panel B**. Stable reduction of p53 at passage 4. **Panel C.** Stable reduction of p53 at passage 11.

Additional Resources for the GeneClip™ U1 Hairpin Cloning Systems

Technical Bulletins and Manuals

TM256 *GeneClip™ U1 Hairpin Cloning Systems Technical Manual*

Vector Maps

pGeneClip™ Vectors

Promega Publications

GeneClip™ U1 Hairpin Cloning Systems for expression of short hairpin RNAs in vivo

V. RNA Delivery Strategies

Successful RNAi experiments are dependent on both siRNA design and effective delivery of siRNA duplexes into cells. RNAi delivery strategies vary depending on the target cells or organism. For example, *C. elegans* may be injected (Fire *et al.* 1998; Grishok *et al*. 2000), soaked in (Tabara *et al*. 1998), or fed (Timmons and Fire, 1998; Kamath *et al.* 2001; Fraser *et al*. 2000) dsRNA. Successful delivery of interfering RNA has also been achieved by microinjection of RNA into *Drosophila* embryos (Kennerdell and Carthew, 1998) and mouse oocytes (Wianny and Zernicka-Goetz, 2000). Delivery to *Drosophila* S2 cells in culture can be achieved by incubating the cells with the chosen RNA (Clemens *et al*. 2000; Betz and Worzella, 2003). Use of DNA-based approaches like ddRNAi vectors allows use of standard transfection reagents/methods; for example, cationic lipids, calcium phosphate, DEAE-dextran, polybrene-DMSO or electroporation (Caplen *et al*. 2001; Elbashir *et al*. 2001a).

A. Transfection of ddRNAi Vector Constructs

Once annealed, hairpin oligonucleotides are ligated to the appropriate pGeneClip™ Vector, the resulting constructs can be used for transient or stable transfection. The GeneClip™ U1 Hairpin Cloning Systems provide a choice of vectors containing various selectable markers (neomycin, hygromycin or puromycin) that can be used for stable expression of a pool of cells or individual clones. Transfection of the plasmid DNA into human cells may be mediated by cationic lipids, calcium phosphate, DEAE-Dextran, polybrene-DMSO or electroporation. Transfection conditions will need to be optimized for your particular system. Guidelines for transfection of the pGeneClip™ Vectors are provided in Technical Manual #TM256. General considerations for transient and stable transfection are given below.

Transient Transfection

High transfection efficiency is essential for achieving substantial suppression levels using a transient transfection approach. Prior to testing for suppression of the target protein, optimize the transfection conditions for maximum efficiency in the system to be tested. The optimal conditions will vary with cell type, transfection method used and the amount of DNA. When using the pGeneClip™ Basic, Puromycin, Hygromycin or Neomycin Vectors, optimization can be performed using a GFP reporter such as the Monster Green® Fluorescent Protein phMGFP Vector (Cat.# E6421). The pGeneClip™ hMGFP Vector already contains the GFP reporter. The GFP reporter can also be used to determine transfection efficiency for the assay. To test the effectiveness of the pGeneClip™ Vector constructs (screening various sequences for levels of inhibition), the use of a reporter, such as GFP, is highly recommended. This control can be performed as a separate transfection to determine the percentage of the cell population transfected or as a cotransfection where flow cytometry can be used to sort GFP-positive cells. The level of target RNA suppression in transfected cells can then be determined by taking the transfection efficiency into account.

Obtaining maximum suppression requires optimizing specific assay conditions. We have observed variations in suppression efficiency as a result of the cell line, cell culture conditions, target sequence and transfection conditions. Varying the amount of transfection reagent, amount of DNA and cell density can influence transfection efficiency. Obtaining the highest transfection efficiency with low toxicity is essential for maximizing the siRNA interference (suppression) effect in a transient assay. Additionally, maintaining healthy cell cultures is essential for this application. The key considerations are discussed more fully below.

Cell Density (Confluence) at Transfection: The recommended cell density for most cell types at transfection is approximately 30–50%; this level is lower than standard transfection experiments where cells are plated at 50–70% confluency. The optimal cell density should be determined for each cell type. Continued proliferation and the need to passage cells should be considered when determining the number of cells to plate.

Cell Proliferation: The successful suppression of gene expression requires actively proliferating and dividing cells, so it is essential to maintain healthy cell cultures. It also is important to minimize the decrease in cell growth associated with nonspecific transfection effects and to maintain cell culture under subconfluent conditions to assure rapid cell division. We recommend using the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570) to monitor cell viability and growth.

Time: The optimal time after transfection for analyzing interference effects must be determined empirically by testing a range of incubation times. Typically little inhibition is seen after 24 hours, but the maximal suppression time can vary from 48–96 hours, depending on the cells used and the experimental targets tested.

Stable Transfection

For stable expression, antibiotic selection must be applied following transfection. Cell lines vary in the level of resistance to antibiotics, so the level of resistance of a particular cell line must be tested before attempting stable selection of the cells. A "kill curve" will determine the minimum concentration of the antibiotic needed to kill nontransfected cells. The antibiotic concentration for selection will vary depending on the cell type and the growth rate. In addition, cells that are confluent are more

resistant to antibiotics, so it is important to keep the cells subconfluent. The typical effective ranges and lengths of time needed for selection are given in Table 2.1.

For example, to generate a kill curve for G-418 selection, test G-418 concentrations of 0, 100, 200, 400, 600, 800 and 1,000µg/ml to determine the concentration that is toxic to nontransfected cells. Once the effective concentration of antibiotic has been determined, transfected cells can be selected for resistance.

Once the effective concentration of antibiotic has been determined, transfected cells can be selected for resistance, as outlined in the protocol below.

1. Following transfection, seed cells at a low cell density.
2. Apply antibiotic to the medium at the effective concentration determined from the kill curve.
3. Prepare a control plate for all selection experiments by treating nontransfected cells with antibiotic in medium under the experimental conditions. This control plate will confirm whether the conditions of antibiotic selection were sufficiently stringent to eliminate cells not expressing the resistance gene.
4. Change the medium every 2–3 days until drug-resistant clones appear.
5. Once clones (or pools of cells) are selected, grow the cells in media containing the antibiotic at a reduced antibiotic concentration, typically 25–50% of the level used during selection.

See Figure 2.8, Panels B and C for stably transfected cells that suppressed expression of p53.

B. Delivery of dsRNA to Drosophila S2 Cells in Culture

The protocol outlined below was used to successfully deliver PCR products of various sizes (180bp or 505bp) generated either from the 778bp ERK-A target or from a control plasmid containing the *Renilla* luciferase gene (phRL-null Vector; 500bp or 1,000bp) to *Drosophila* S2 cells in culture (Figure 2.9; Betz and Worzella, 2003). Purified, in vitro-synthesized ERK-A dsRNA was introduced into *Drosophila* S2 cells using the method described by Clemens *et al*. (2000) following the protocol described below.

1. Incubate 1 x 10^6 S2 cells in 1ml of *Drosophila* expression system (DES) serum-free medium (Invitrogen) in triplicate wells of a six-well culture dish in the presence or absence of various amounts (0, 9.5, 38, or 190nM) of the test (ERK-A) dsRNA or a nonspecific (*Renilla* luciferase) dsRNA.
2. Incubate the S2 cells at room temperature with the dsRNA for 1 hour, then add 2ml of complete growth medium.
3. Incubate the cells at room temperature for an additional 3 days to allow for turnover of the target protein.

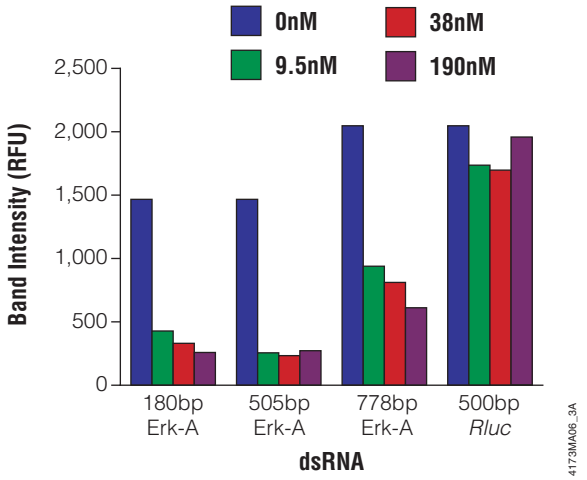

Figure 2.9. Effect of Erk-A dsRNA length and concentration of Erk-A protein levels in S2 cells. Erk-A dsRNAs and a nonspecific control dsRNA (*Renilla* luciferase; *Rluc*) were synthesized, purified, and quantitated using the T7 RiboMAX™ Express RNAi System. The Erk-A dsRNAs were 180bp, 505bp, or 778bp. The *Rluc* negative-control dsRNA was 500bp. *Drosophila* S2 cells were treated with increasing concentrations of each dsRNA (0, 9.5, 38 or 190nM) in triplicate for 3 days. The dsRNA concentration refers to the initial 1ml treatment. Replicate wells were pooled and a cell lysate prepared. The cell lysates were then subjected to Western blot analysis for Erk-A protein levels (Betz and Worzella, 2003). The quantity of Erk-A protein in each sample was quantitated using enhanced chemifluorescent detection reagents (Amersham) and a STORM® fluorescent scanner (blue mode). The basal level of Erk-A in the 180bp and 505bp Erk-A samples is different than in the other two samples because these samples were processed on different blots.

VI. Quantitating siRNA Target Gene Expression

Reduction of the targeted gene expression can be measured by 1) monitoring phenotypic changes of the cell, 2) measuring changes in mRNA levels (e.g., using RT-PCR), or 3) detecting changes in protein levels by immunocytochemistry or Western blot analysis (Huang *et al*. 2003; Kullmann *et al*. 2002; Lang *et al*. 2003). The suppression effect will vary depending on the target, cell line and experimental conditions.

Controlling for nonspecific effects on other targets is very important. As a negative control, cells can be transfected with either a nonspecific or scrambled target sequence. This

Table 2.1. Typical Conditions for Selection of Stable Transfectants.

Vector	Antibiotic	Effective Concentration	Time Needed for Selection
pGeneClip™ Puromycin Vector	Puromycin	1–10µg/ml	2–7 days
pGeneClip™ Hygromycin Vector	Hygromycin	100–1,000µg/ml	3–10 days
pGeneClip™ Neomycin Vector	G-418	100–1,000µg/ml	3–14 days

will show that suppression of gene expression is specific to the expression of the hairpin siRNA target sequences. When suppression is determined by Western analysis, positive controls for other genes (e.g., tubulin or actin) should be included (Huang *et al*. 2003). Additional controls may also be desirable (Editorial (2003) *Nat. Cell Biol*. **5**, 489–90).

A. Confirming the RNAi Effect

Several summary articles are available that suggest various options for controls that should be incorporated into RNAi experimental design to ensure accuracy and correct identification of an RNAi effect (Editorial (2003) *Nat. Cell Biol*. **5**, 489–90; Duxbury and Whang, 2004). The preferred control is to show restoration of functionality of a gene through artificial overexpression of the target gene in a form that is not affected by RNAi. For example, the target gene can be engineered to contain silent mutations that render the mRNA invulnerable to the RNAi effect and introduced into the cell on a plasmid vector. If such constructs "rescue" the original function of the gene, this is a good indication that the observed suppression is mediated by RNAi. Use of siRNAs targeting several different areas of the same gene to suppress expression may also be used to provide evidence that an effect is mediated by RNAi. The observation of the same suppression effect using more that one target RNA can confirm that the observed effect is indeed RNAi.

For experiments using in vitro-synthesized siRNAs, the minimum concentration of RNAi showing an effect should be used to avoid nonspecific effects due to the introduction of large quantities of RNA into the cell. Ideally, any observed suppression should be confirmed at both the mRNA and protein levels. Northern blotting and quantitative, real-time RT-PCR can be used to demonstrate reduction of expression at the RNA level. Quantitative Western blotting, phenotypic and functional assays are some of the options available to show protein knockdown.

B. Negative and Positive Controls

Scrambled siRNAs and siRNAs containing a single mismatch can be used as negative controls. However, the latter are regarded as more informative. Positive controls with RNAs known to exhibit an RNAi effect may also be useful.

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