Do It Yourself siRNA Synthesis

Produce Functional siRNAs and Hairpin siRNAs using the T7 RiboMAX™ Express RNAi System

By Natalie Betz, Ph.D., Promega Corporation

Abstract

We have developed an optimized protocol for generating short interfering RNAs (siRNAs) or hairpin siRNAs in vitro using T7 RNA Polymerase and annealed DNA oligonucleotide templates. Two RNA interference studies in different mammalian model systems demonstrate the functionality of the synthesized siRNAs. The efficient and inexpensive synthesis of siRNAs in vitro allows for quick screening of multiple target sites to identify the siRNA that produces the most potent silencing effect.

The T7 RiboMAX™ Express RNAi System is designed to quickly and efficiently synthesize siRNAs or hairpin siRNAs in vitro for use in mammalian RNAi studies.

Introduction

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 complementary target mRNA (for a general review see reference 1 and references therein). RNAi is a powerful technique that allows researchers to investigate gene function by knocking out or knocking down the level of a particular protein.

RNAi involves a multistep process in which the dsRNA is recognized by an RNase III family member (e.g., Dicer in *Drosophila*) and is cleaved into siRNAs of 21–23 nucleotides (2–4). The siRNAs are incorporated into an RNAi targeting complex known as RISC (RNA-induced silencing complex), which destroys mRNAs homologous to the siRNA (3,4) contained in the complex.

In most mammalian systems, introducing longer dsRNAs (>30bp) induces a potent antiviral response that results in generalized mRNA degradation and inhibition of protein synthesis (5). However studies have shown that chemically synthesized siRNAs can induce specific gene silencing in a wide range of mammalian cell lines without causing the nonspecific antiviral response (2,6). The most potent siRNA duplexes are 21 nucleotides in length, comprising a 19bp duplex sequence with a 2-uridine 3′ overhang at each end (7).

Studies have demonstrated the successful synthesis of functional siRNAs and hairpin siRNAs in vitro using T7 RNA Polymerase (8,9). The only sequence requirement for generating siRNAs using T7 RNA Polymerase is the presence of a "$GN_{17}C$" sequence in the target mRNA; T7 RNA Polymerase highly prefers a G nucleotide for transcription initiation (10).

The T7 RiboMAX™ Express RNAi System[a,b,c] is designed to quickly and efficiently synthesize siRNAs or hairpin siRNAs in vitro for use in mammalian RNAi studies. In addition, this system can also synthesize longer dsRNAs used in most nonmammalian RNAi systems (11). 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 (12).

Synthesizing siRNA with the RiboMAX™ System

Figure 1 outlines the protocol for synthesis of siRNA using the T7 RiboMAX™ Express RNAi System. The initial step is generating the DNA template, in which two DNA oligonucleotides are 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 are annealed to form siRNA. In the case of hairpin siRNA, the single small RNA is allowed to anneal to itself to form the hairpin. 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).

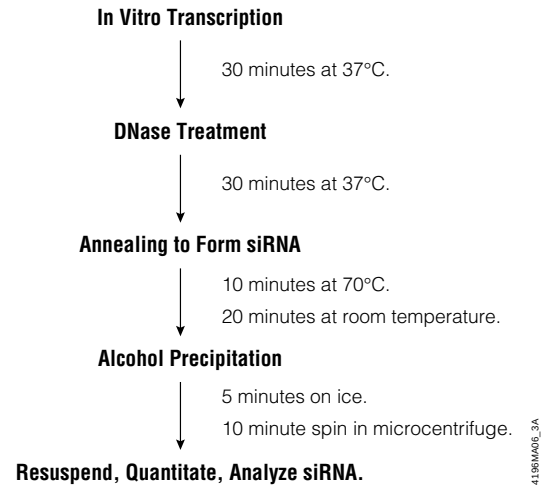

Figure 1. Protocol for producing and purifying siRNA using the T7 RiboMAX™ Express RNAi System.

We used the T7 RiboMAX™ Express RNAi System to generate 21bp siRNAs for several targets, as well as a hairpin siRNA. Following in vitro transcription and purification, aliquots of each siRNA were analyzed on a 4–20% native polyacrylamide gel and compared to a chemically synthesized siRNA of the same size. As seen in Figure 2, the in vitro synthesized siRNAs migrated comparably to the chemically synthesized siRNA. The larger hairpin siRNA migrated more slowly, as expected.

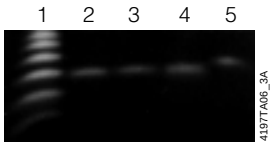

Figure 2. Native polyacrylamide gel analysis of different siRNA molecules generated using the T7 RiboMAX™ Express RNAi System. Approximately 50ng of each siRNA was analyzed on a 4–20% TBE PAGE gel with TBE running buffer. Following electrophoresis the gel was stained with 0.5µg/ml ethidium bromide. Lane designations: lane 1, 10bp DNA Step Ladder (Cat.# G4471); lane 2, 21bp chemically synthesized *Renilla* siRNA (Dharmacon); lane 3, 21bp *Renilla* siRNA synthesized using the T7 RiboMAX™ Express RNAi System; lane 4, 21bp p53 siRNA synthesized using the T7 RiboMAX™ Express RNAi System; lane 5, 49-nucleotide long *Renilla* shRNA (19bp duplex with 9 nucleotide loop and 2-uridine 3´ overhang) synthesized with the T7 RiboMAX™ Express RNAi System. **Note:** siRNA migrates more slowly than double-stranded DNA.

The yield of siRNA or hairpin siRNA generated from different annealed DNA oligonucletide templates are shown in Table 1. Yields of >500µg siRNA per milliliter of reaction or >1mg hairpin siRNA per milliliter of reaction are typical. Users can maximize yield for any particular template by increasing the incubation time from 30 minutes to 2 hours or by incubating the transcription reaction at 42°C instead of 37°C; this can increase the transcription efficiency of GC-rich templates or those with secondary structure.

Template Design and Considerations

The template DNA for generating small RNAs using in vitro transcription consists of annealed DNA oligonucleotide duplexes. A T7 RNA Polymerase promoter sequence is engineered into the top- and bottom-strand oligos to allow transcription. The target sequence must contain the sequence 5′-GN$_{17}$C-3′ to allow efficient transcription initiation by T7 RNA Polymerase.

Each siRNA requires two separate, annealed DNA oligonucleotide templates, and thus a total of four oligonucleotides are necessary. One template transcribes the sense RNA strand, and the other template transcribes the antisense RNA strand. The sequence requirements for these oligonucleotides are shown in Figure 3. Following separate transcription reactions of these two annealed DNA oligonucleotide templates, the sense and antisense RNA strands are annealed to form the siRNA.

A single DNA template containing two annealed oligonucleotides may be used to generate a single hairpin siRNA. Several studies have demonstrated

Table 1. Yields of Six Different siRNA Templates Synthesized Using the T7 RiboMAX™ Express RNAi System (average of triplicates per template).

siRNA Sample	Yield (mg siRNA/ml reaction)
Renilla Site 1 siRNA	0.85
Renilla Site 1 hairpin siRNA	4.20
Renilla Site 2 siRNA	1.50
Renilla Site 2 hairpin siRNA	2.10
Renilla Site 3 siRNA	1.20
Renilla Site 3 hairpin siRNA	1.70

that short hairpin RNAs (shRNAs) that contain ~19 nucleotides of perfectly matched base pairing, are connected by various spacer regions and end in a 2-nucleotide 3′-overhang can be as efficient as siRNAs at inducing RNA interference (8, 12–17). Synthesis of a shRNA requires two long oligonucleotides, which when annealed, serve as the template for synthesizing a single RNA molecule. This RNA anneals to itself to form the hairpin structure. The loop length and sequence shown in Figure 3 were obtained from reference 13.

Each shRNA requires one single, annealed DNA oligonucleotide template, and thus a total of two oligonucleotides are necessary. The sequence requirements for these oligonucleotides are also shown in Figure 3.

siRNA and Hairpin siRNA are Functional in RNAi

To verify that the siRNAs or shRNAs produced using the T7 RiboMAX™ Express RNAi System were functional for RNA interference in mammalian cells, we tested two different model systems. One system targeted a stably integrated exogenous reporter gene, while the second system targeted an endogenous transcription factor gene.

CHO cells stably expressing *Renilla* luciferase were transfected with either siRNAs or shRNAs generated using the T7 RiboMAX™ System. Each siRNA was either synthesized chemically or in vitro. An siRNA molecule that contained nucleotides found in the target site "scrambled" in a random manner was transfected as a negative control. The average inhibition of *Renilla* luciferase activity compared to the scrambled siRNA control is shown in Figure 4. The in vitro synthesized siRNAs against the three different target sites functioned comparably to the chemically synthesized siRNA of the same sequence. In addition, the shRNAs synthesized in vitro performed comparably to the other siRNAs. Thus siRNAs or shRNAs synthesized in vitro using the T7 RiboMAX™ System are functionally equivalent to chemically synthesized siRNAs for inducing RNAi.

A second mammalian model system involved inhibiting the expression of an endogenous target. We chose the transcription factor p53, the most commonly mutated tumor suppressor gene in human cancers (18). The p53 protein has a short half-life but can be stabilized with either point mutation of the gene or interaction with

mRNA Target: 5´-$G_1N_{2–18}C_{19}$-3´

Complement: 3´-$C_1N_{2-18}G_{19}$-5´

Oligonucleotides for siRNA Production:

Oligo 1 (top strand for sense): 5´-GGATCCTAATACGACTCACTAATA-**$G_1N_{2–18}C_{19}$-3´ ($G_1N_{2–18}C_{19}$ = sense mRNA sequence)**

Oligo 2 (bottom strand for sense): 3´-CCTAGGATTATGCTGAGTGATAT-$C_1N_{2–18}G_{19}$-AA-5´

Oligo 3 (top strand for antisense): 5´-GGATCCTAATACGACTCACTAATA-**$G_{19}N_{18–2}C_1$-3´ ($G_{19}N_{18–2}C_1$ = antisense mRNA sequence)**

Oligo 4 (bottom strand for antisense): 3´-CCTAGGATTATGCTGAGTGATAT-$C_{19}N_{18–2}G_1$-AA-5´

Oligonucleotides for Hairpin siRNA Production:

Oligo 1 (top strand for hairpin): 5´-GGATCCTAATACGACTCACTAATA-**$G_1N_{2–18}C_{19}$**(sense)-TTCAAGAGA(loop)-**$G_{19}N_{18–2}C_1$**(antisense)-3´

Oligo 2 (bottom strand for hairpin): 3´-CCTAGGATTATGCTGAGTGATAT-**$C_1N_{2–18}G_{19}$**(sense)-AAGTTCTCT(loop)-**$C_{19}N_{18–2}G_1$**(antisense)-AA-5´

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Figure 3. Required DNA oligonucleotides to generate templates for making siRNA or hairpin siRNA. The sense sequence of the target mRNA is assumed to be the protein coding sequence.

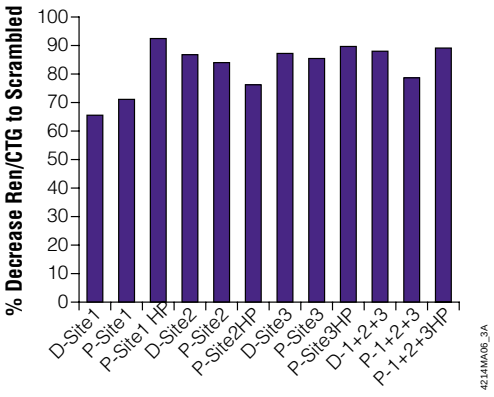

Figure 4. Comparing three different *Renilla* siRNAs generated either chemically or in vitro using the T7 RiboMAX™ Express RNAi System to reduce the expression of *Renilla* luciferase. CHO cells stably expressing the codon-optimized *Renilla* luciferase (hR*luc*) gene were transfected with 20ng of siRNA to three different sites in the hR*luc* mRNA. In addition, in vitro synthesized shRNAs to the same 3 sites were also tested. As a negative control a scrambled siRNA was used; as a positive control chemically synthesized siRNAs were used (Dharmacon). Transfections were performed in replicates of 8 in 96-well plates using the siLentGene™ Transfection Reagent. Following transfection (24 hours), quadruplicate wells were processed for either hR*luc* activity using the *Renilla* Luciferase Assay System[d,e] or cell number using the CellTiter-Glo® Luminescent Cell Viability Assay[e,f]. The ratio of the the average hR*luc* signal compared to the average CellTiter-Glo® signal for each siRNA transfection was calculated, and the decrease in this ratio relative to the scrambled siRNA control was determined. The data is presented as the percent decrease in the *Renilla*/CellTiter-Glo® Assay ratio as compared to a transfected scrambled siRNA. D = chemically synthesized siRNA from Dharmacon; P = in vitro transcribed using the T7 RiboMAX™ Express RNAi System; HP = shRNA in vitro transcribed using the T7 RiboMAX™ Express RNAi System. All in vitro generated siRNAs and shRNAs were synthesized purified as described in Technical Bulletin #TB316. The siRNAs and shRNAs were quantitated by 2.5% agarose/1X TAE gel analysis and compared to known amounts of a chemically synthesized siRNA. Following electrophoresis, the gel was stained with 1:10,000 SYBR® Green II stain (Molecular Probes) for 20 minutes and the gel scanned and quantitated using a Molecular Dynamics STORM® fluorescent scanner (blue mode; PMT = 1,000).

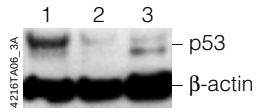

Figure 5. Suppression of endogenous p53 protein. Twenty-four hours after plating in a 12-well plate, 293T cells were transfected with 200ng scrambled siRNA (lane 1), 200ng in vitro synthesized p53 siRNA (lane 2), or 200ng chemically synthesized p53 siRNA (lane 3) using the siLentGene™ Transfection Reagent. Twenty-four hours following transfection cells were lysed using 1X Reporter Lysis Buffer (Cat.# E3971) containing protease inhibitors and the protein 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 done using a goat anti-mouse HRP conjugate (Cat.# W4021) and the chemiluminescent detection reagents in the Transcend™ Chemiluminescent Non-Radioactive Translation Detection System (Cat.# L5080). The blot was exposed to Kodak X-OMAT® film for approximately 4 minutes. In addition, 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.

certain DNA tumor virus proteins, such as the SV40 large T antigen. We used 293T cells to test for inhibition of the p53 target because they contain the SV40 large T antigen that allows for a high level of p53 accumulation. The cells were transfected with either scrambled siRNA, chemically synthesized p53 siRNA, or p53 siRNA generated using the T7 RiboMAX™ Express RNAi System.

The results shown in Figure 5 demonstrate that both the chemically synthesized and in vitro transcribed p53 siRNAs were capable of reducing p53 protein levels. The p53 siRNA generated using the T7 RiboMAX™ Express RNAi System is functionally comparable to the chemically synthesized p53 siRNA of the same sequence.

Conclusions

The T7 RiboMAX™ Express RNAi System allows the quick and efficient in vitro synthesis of siRNAs or shRNAs that are functional in mammalian RNA interference studies. Yields of ~1–2mg of siRNA or shRNAs per milliliter of transcription reaction are typical, and the only requirement is annealed dsDNA oligonucleotide templates that contain the target sequence of interest. The ease of synthesis allows for the rapid generation of multiple siRNAs to a single target to enhance the ability to quickly generate and screen for the optimal siRNA that exhibits the highest inhibition of target protein production.

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Protocols

- *T7 RiboMAX™ Express RNAi System* Technical Bulletin #TB316, Promega Corporation. (www.promega.com/tbs/tb316/tb316.html)

Natalie Betz, Ph.D.
Applications Scientist

Ordering Information

Product	Size	Cat.#
T7 RiboMAX™ Express RNAi System[a,b,c]	50 reactions	P1700
siLentGene™ U6 Cassette RNA Interference System[c,g,h,i]	20 reactions	C7800

[a] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[b] U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

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