TECHNICAL BULLETIN

T7 RiboMAX™ Express RNAi System

Instructions for use of Product
P1700

Revised 9/16
TB316

T7 RiboMAX™ Express RNAi System

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Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA
Toll Free in USA 800-356-9526 · Phone 608-274-4330 · Fax 608-277-2516 · **www.promega.com**
Printed in USA. Part# TB316
Revised 9/16

1. Description

The T7 RiboMAX™ Express RNAi System[a] is an in vitro transcription system designed for producing milligram amounts of double-stranded RNA (dsRNA) in a short amount of time. The dsRNA is free of protein and other contaminants and is suitable for use in RNA interference (RNAi). The T7 RiboMAX™ Express RNAi System is designed for the synthesis of dsRNA molecules approximately 200bp and larger (see Figure 1) and can be used with plasmid or PCR templates. Yields of >2mg dsRNA per milliliter of reaction have been observed with the T7 RiboMAX™ Express RNAi System. For most RNAi applications in nonmammalian systems dsRNAs of 400bp or larger are used (1,2).

The T7 RiboMAX™ Express RNAi System can also be used to synthesize short interfering RNAs (siRNAs) of 21bp (see Figure 2) for use in mammalian systems. The DNA template for synthesis of siRNAs consists of annealed DNA oligonucleotides containing a T7 RNA polymerase promoter. Yields equal to or greater than 500µg siRNA per milliliter of reaction are generally observed.

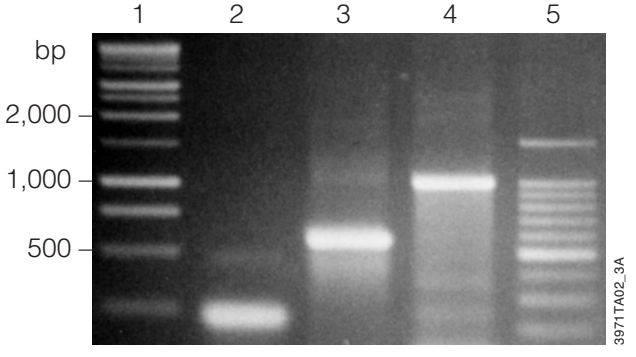

Figure 1. Native agarose gel analysis of different sized dsRNA molecules generated using the T7 RiboMAX™ Express RNAi System. Approximately 4×10^{11} molecules of each dsRNA were separated on a 1.8% agarose/1X TAE gel and visualized by staining the gel with 0.5µg/ml ethidium bromide. Lane designations: lane 1, 1kb DNA Ladder (Cat.# G5711); lane 2, 74ng 180bp dsRNA; lane 3, 200ng 500bp dsRNA; lane 4, 400ng 1,000bp dsRNA; lane 5, 100bp DNA Ladder (Cat.# G2101). **Note:** dsRNA migrates more slowly than dsDNA.

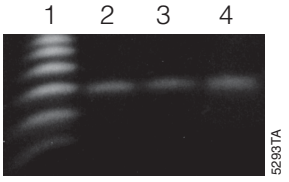

Figure 2. Native polyacrylamide gel analysis of different siRNA molecules generated using the T7 RiboMAX™ Express RNAi System. Approximately 50ng of each siRNA was analyzed on a 4–20% TBE PAGE gel with TBE running buffer. Following electrophoresis, the gel was stained with 0.5µg/ml ethidium bromide. Lane designations: lane 1, 10bp DNA Ladder (Cat.# G4470); lane 2, 21bp chemically synthesized *Renilla* siRNA (Dharmacon); lane 3, 21bp *Renilla* siRNA synthesized using the T7 RiboMAX™ Express RNAi System; lane 4, 21pb p53 siRNA synthesized using the T7 RiboMAX™ Express RNAi System. **Note:** siRNA migrate more slowly than double-stranded DNA.

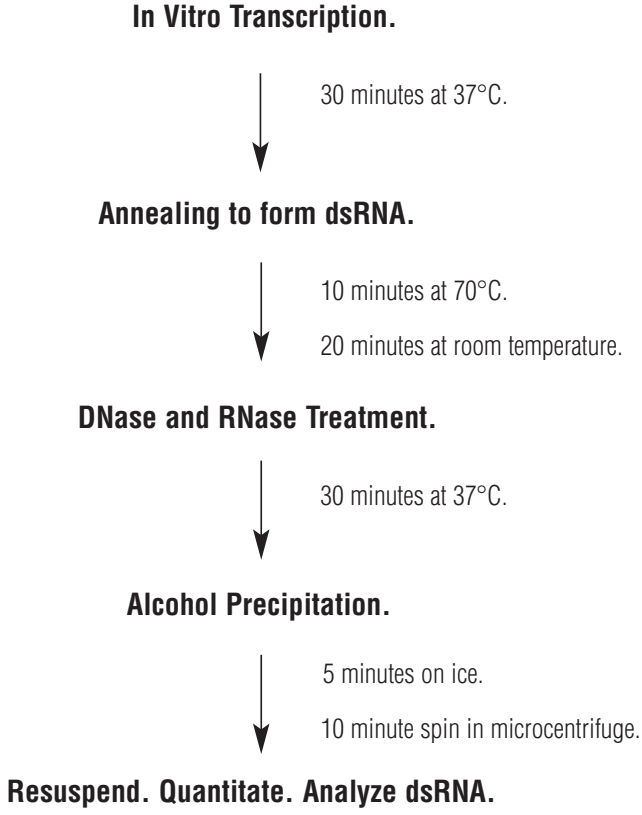

Figure 3. Outline of protocol for the production and purification of dsRNA using the T7 RiboMAX™ Express RNAi System.

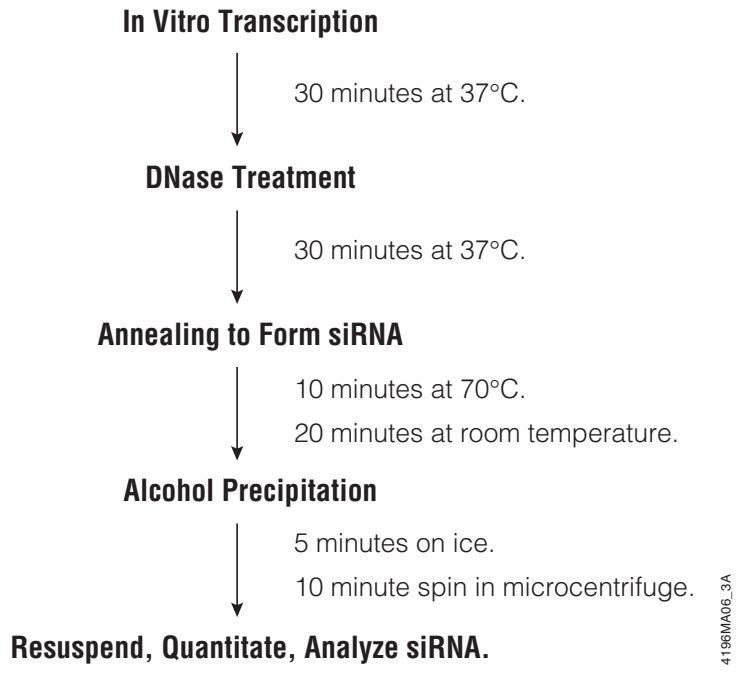

Figure 4. Outline of protocol for the production and purification of siRNA using the T7 RiboMAX™ Express RNAi System.

Using this system, two complementary RNA strands are synthesized from DNA templates supplied by the user. The resulting RNA strands are annealed after the transcription reaction to form dsRNA or siRNA. In the case of dsRNA, any remaining single-stranded RNA and DNA template are removed with a nuclease digestion step following annealing. For siRNA production, template DNA is removed, and the separate RNA strands are annealed to form siRNA. The dsRNA or siRNA is then purified by isopropanol precipitation and can be introduced into the organism of choice for RNAi applications (see Figures 3 and 4 for protocol outlines).

RNA interference, in which long dsRNA molecules specifically suppress expression of a target gene, was originally discovered in *C. elegans* (3) but has now been observed in numerous organisms including *Drosophila* (4), Trypanosomes (5), *Planaria* (6), *Hydra* (7) and Zebrafish (8). The RNAi mechanism appears to be mediated by smaller dsRNA intermediates. The parent, larger dsRNA is processed by a ribonuclease III-like enzyme (i.e., Dicer) into smaller fragments in vivo, and the resulting small interfering RNAs (siRNAs) direct post-transcriptional, but pretranslational, degradation of the targeted mRNA (1,9). The RNA interference process seems to act nonstoichiometrically and has been observed to spread between cells (3).

The introduction of longer dsRNA into most mammalian systems induces global and nonspecific mRNA degradation and inhibition of protein synthesis, referred to as the interferon response (10). Studies by Tuschl and coworkers and Fire and colleagues demonstrated that chemically synthesized siRNAs could induce specific gene silencing in a wide range of mammalian cell lines without inducing the potent antiviral interferon response (11,12). Subsequently, siRNAs synthesized in vitro were shown to be equally as effective as chemically synthesized siRNAs for inducing RNAi in mammalian systems (13). The most potent siRNA duplexes have been shown to be 21 nucleotides long, comprising a 19bp duplex with a 2-uridine 3´ overhang at each end (1). RNAi is a powerful tool for investigating gene function through the specific suppression of a particular mRNA and thus "knockout/knockdown" phenotypes for a specific protein. For a general review on RNA interference refer to reference 14.

This system may also be used for the synthesis of large amounts of single-stranded RNA by omitting the annealing and RNase A digestion steps (see the *T7 RiboMAX™ Express Large-Scale RNA Production System Technical Bulletin* #TB298 for details).

The T7 RiboMAX™ Express RNAi System differs from the standard RiboMAX™ System in two ways:

- **Saves Time:** The T7 RiboMAX™ Express RNAi System produces milligram amounts of RNA in as little as 30 minutes rather than the 2–4 hours required by other commercially available systems, including the original RiboMAX™ System.

- **Offers Convenience:** The rNTPs and Transcription Buffer have been combined, minimizing pipetting errors and setup time.

2. Product Components and Storage Conditions

Product	Size	Cat.#
T7 RiboMAX™ Express RNAi System	50 × 20µl reactions	P1700

Each system contains sufficient reagents for 50 standard 20µl reactions, which can synthesize 25–50 dsRNAs or siRNAs. Includes:

- 100µl Enzyme Mix, T7 Express (T7 RNA Polymerase, Recombinant RNasin® Ribonuclease Inhibitor and Recombinant Inorganic Pyrophosphatase)
- 500µl RiboMAX™ Express T7 2X Buffer
- 110 units RQ1 RNase-Free DNase, 1u/µl
- 5µg pGEM® Express Positive Control Template, 1mg/ml
- 1ml 3M Sodium Acetate (pH 5.2)
- 1.25ml Nuclease-Free Water
- 200µl RNase A Solution

Storage and Stability: Store all components at –20°C. The RNase A Solution should be stored at 25°C after the initial thaw and is stable for 12 months if stored and handled properly.

Note: The pGEM® Express Positive Control Template serves as a control for the transcription reaction only. It is not used as a control for subsequent steps in the procedure.

3. Producing Longer Double-Stranded RNAs (dsRNAs)

Materials to Be Supplied by the User

(Solution compositions are provided in Section 7.)

- chloroform:isoamyl alcohol (24:1)
- TE-saturated (pH 8.0) phenol:chloroform:isoamyl alcohol (25:24:1)
- 70% ethanol
- gene-specific amplification primers or templates
- isopropanol or 95% ethanol

RNAi experiments in nonmammalian targets are typically performed with dsRNA of 400bp or larger (1,2,15). The minimum size of dsRNA recommended for RNAi 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, and data generated at Promega suggests that smaller dsRNAs can be as effective and efficient at inducing RNAi in nonmammalian systems (16).

In this system, dsRNA production requires a T7 RNA polymerase promoter at the 5´-ends of both DNA target sequence strands. Separate DNA templates, each containing the target sequence in a different orientation relative to the T7 promoter should be used. The resulting transcripts are mixed and hybridized post-transcriptionally.

3.A. Producing PCR Product Templates

A T7 RNA polymerase promoter can be added to any DNA sequence using PCR by including the T7 promoter sequence at the 5´-end of either of the amplification primers.

The minimal T7 RNA polymerase promoter sequence requirement is:

5´-TAATACGACTCACTATA**G**$GN_{(17-22)}$-3´.

The +1 base in bold (**G**) is the first base incorporated into the RNA strand, and the second G should then be followed by 17–22 gene-specific nucleotides. The addition of extra bases upstream of the minimal T7 RNA polymerase promoter (boldface) sequence may increase yield by allowing more efficient polymerase binding and initiation (i.e., 5´ GGATCC-**TAATACGACTCACTATAGG**$N_{(17-22)}$ 3´). Thus use of the extended T7 promoter sequence is recommended.

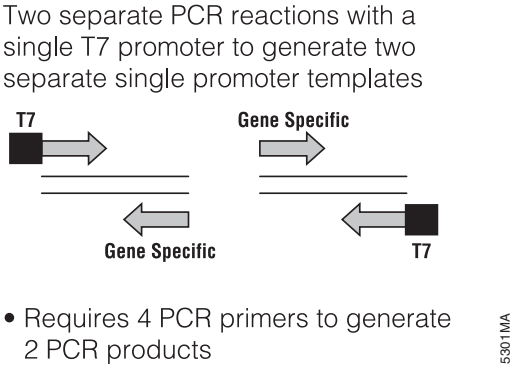

Figure 5. Strategy for adding T7 promoters to DNA templates by PCR.

Generating the necessary two DNA templates requires four PCR primers and two PCR amplifications (see Figure 5). Amplification strategies using primers containing T7 promoter sequences may include an initial 5–10 cycles at an annealing temperature approximately 5°C above the melting temperature of the gene-specific sequences, followed by 20–35 cycles of annealing approximately 5°C above the melting temperature of the entire primer, including the T7 promoter.

PCR products should be examined by agarose gel electrophoresis before transcription to verify that a single PCR product of the expected size is generated. We recommend purifying the PCR product using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281), followed by quantitation by absorbance at 260nm. This system allows for both purification and concentration, and if necessary, gel purification of PCR products. Small volumes of unpurified PCR product may be used as template DNA (1–2µl per 20µl reaction), but in general, higher yields are obtained with purified products.

3.B. Linearizing Plasmid Templates

Using plasmid templates to generate dsRNA requires generation of two separate clones having the same target sequence in opposite orientations. Each clone should contain the T7 RNA polymerase promoter at a different end of the target sequence. PCR products may be cloned directly into the pGEM®-T (Cat.# A3600) or pGEM®-T Easy (Cat.# A1360) Vectors. These vectors contain a single T7 promoter sequence upstream of the multiple cloning region. The clones may then be screened for orientation by PCR using a vector-specific primer and an internal primer that will generate different fragment sizes for each orientation, or by restriction enzyme digestion such that clones in each orientation are selected.

Optimal RNA yields depend on starting with a high-quality DNA template. Both cesium chloride purification and the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330) yield DNA suitable for transcription reactions. It is important that no RNase be present in the DNA. If the presence of RNase is suspected, treat the DNA with proteinase K (100µg/ml) and SDS (0.5%) in 50mM Tris-HCl (pH 7.5) and 5mM $CaCl_2$ for 30 minutes at 37°C (5). Purify the DNA further by extraction with TE-saturated (pH 8.0) phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitate.

DNA templates should be linearized prior to in vitro transcription to produce RNA transcripts of defined length. Linearize the DNA by digestion with an appropriate restriction endonuclease that cuts downstream of the target sequence, then perform the appropriate clean-up procedure, such as phenol extraction followed by ethanol precipitation. Alternatively, the Wizard® DNA Clean-Up System (Cat.# A7280) can be used. Dissolve the DNA template in nuclease-free water before adding it to the transcription reaction. It is useful to start with at least 30% more DNA than required for the transcription reaction to allow for DNA loss during purification and for visualization by gel electrophoresis.

It is important to avoid the use of restriction enzymes that produce 3´ overhangs (see Table 1). Extraneous transcripts have been reported, in addition to the expected transcript, when such templates are transcribed (17). These extraneous transcripts can contain sequences complementary to the expected transcript as well as sequences corresponding to the vector DNA. If these enzymes must be used, the ends of the linearized template can be made blunt prior to transcription using DNA Polymerase I Large (Klenow) Fragment or T4 DNA Polymerase.

Table 1. Commonly Used Restriction Enzymes That Generate 3´ Overhangs.

Aat II	*Bsp*1286 I	*Hha* I	*Sac* I	*Sph* I
Apa I	*Bst*X I	*Kpn* I	*Sac* II	
Ban II	*Cfo* I	*Pst* I	*Sfi* I	
Bgl I	*Hae* II	*Pvu* I	*Sgf* I	

The purified linear DNA should be examined by agarose gel electrophoresis prior to transcription to verify complete linearization and to ensure the presence of a clean (nondegraded) DNA fragment of the expected size.

3.C. Synthesizing Large Quantities of dsRNA

The linear control DNA supplied with the system produces single-stranded transcripts that are 1.1kb and 2.3kb in length. The transcripts produced from the pGEM® Express Positive Control Template are not complementary and thus will not form double-stranded RNA. They are to be used as a positive control for transcriptional activity of the system and RNA integrity following transcription.

1. Set up the appropriate reaction size 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.

T7 Reaction Components	**Sample Reaction**	**Control Reaction**
RiboMAX™ Express T7 2X Buffer*	10.0µl	10.0µl
linear DNA template (~1µg total; see Note 1)	1–8µl	—
pGEM® Express Positive Control Template	—	1.0µl
Nuclease-Free Water	0–7µl	7.0µl
Enzyme Mix, T7 Express	2.0µl	2.0µl
Final Volume	**20.0µl**	**20.0µl**

*Frozen RiboMAX™ Express T7 2X Buffer will contain a precipitate that can be dissolved by warming the buffer at 37°C and mixing well. Note that the buffer contains spermidine, which can precipitate DNA at temperatures colder than room temperature.

2. Mix gently and incubate at 37°C for 30 minutes (see Notes 1–3).

Notes:

1. For plasmid templates, up to 3µg of purified template may be included per 20µl reaction.

2. To maximize yield, incubation at 37°C may proceed for up to 2–6 hours. In general, however, no dramatic increase in yield is observed beyond the 30-minute incubation period, except for smaller transcripts (~200 bases). A time-course experiment may be performed to determine the optimal incubation time for maximal RNA synthesis.

3. Incubation at 42°C may improve the yield of dsRNA for transcripts that contain secondary structure. If sufficient yield is not obtained at 37°C and the template is GC-rich, incubate the reaction at 42°C instead.

3.D. Removing the DNA Template, Annealing dsRNA and Removing ssRNA

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 anneal the RNA strands, mix equal volumes of complementary RNA reactions together and incubate at 70°C for 10 minutes, then slowly cool to room temperature (~20 minutes). This allows annealing of the double-stranded RNA.

2. Dilute the supplied RNase Solution 1:200 by adding 1µl RNase Solution to 199µl Nuclease-Free Water. Add 1µl freshly diluted RNase Solution and 1µl RQ1 RNase-Free DNase per 20µl reaction volume, and incubate for 30 minutes at 37°C. This will remove any remaining single-stranded RNA and the template DNA, leaving double-stranded RNA. **Do not save or reuse the diluted RNase Solution.**

3.E. Purifying Double-Stranded RNA

1. Add 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol or 2.5 volumes of 95% ethanol. Mix and place on ice for 5 minutes. The reaction will appear cloudy at this stage. Spin at top speed in a microcentrifuge for 10 minutes.

2. A white pellet should be visible at the bottom of the microcentrifuge tube. Carefully pour off or 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.

Do not over-dry the RNA pellet, as it may become difficult to resuspend completely.

3. The dsRNA may be further purified following precipitation using a G25 micro spin column following the manufacturer's instructions (Amersham Biosciences, Cat.# 27-5325-01). This will remove any remaining rNTPs and allow accurate quantitation by absorbance at 260nm. We do not recommend processing more than an initial 40µl reaction volume per spin column. A loss of yield can be expected following G25 purification (approximately 66% recovery).

3.F. Determining RNA Concentration and Visualizing by Gel Electrophoresis

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.)

- Blue/Orange Loading Dye, 6X (Cat.# G1881)
- RNA sample buffer
- RNA loading buffer

After removing the DNA template, unincorporated nucleotides (by G25 chromatography) and any remaining single-stranded RNA, the dsRNA concentration can be quantitated by ultraviolet light absorbance. Prepare a 1:100 to 1:300 dilution of the RNA and read the absorbance at a wavelength of 260 nanometers. One A_{260} unit equals ~40µg/ml of dsRNA.

The DNase-treated dsRNA transcript can be examined by native gel electrophoresis using 1X Blue/Orange Loading Dye to determine the accuracy of the A_{260} quantitation and/or the integrity of the full-length dsRNA transcript. Including 1kb DNA Ladder (Cat.# G5711) on the gel can help determine the size of the dsRNA sample. Note that double-stranded RNA may migrate more slowly than double-stranded DNA. Use 1–5µl of diluted dsRNA per lane (dilute at least 1:50 with Nuclease-Free Water) or use 50–500ng per lane. The dsRNA may be gel quantitated by comparison to known amounts of double-stranded DNA.

Prepare either an agarose gel in 1X TAE or an acrylamide minigel, depending upon the length of the transcript involved (0.7–2.0% agarose for transcripts from 200 to several thousand nucleotides). The separated dsRNA may be visualized by staining the gel in 0.5mg/ml ethidium bromide or 1:10,000 SYBR® Green II stain (Molecular Probes). We do not recommend incorporating the stain into the gel as it can alter the migration rate of the dsRNA, making accurate size determination difficult.

To assess single-stranded RNA integrity, denaturing gel electrophoresis can be performed using RNA sample buffer and RNA loading buffer. Including RNA Markers (Cat.# G3191) on the gel can help determine the size and integrity of the RNA sample.

While denaturing gels (containing formaldehyde, glyoxal or 8M urea) provide the greatest resolution of the denatured RNA, we have found that acceptable results can usually be obtained using nondenaturing gels loaded with denatured RNA. Add 1–5µl of diluted RNA (diluted at least 1:50 with Nuclease-Free Water) to 10–20µl of RNA sample buffer. Add 2–5µl of RNA loading buffer, and heat the sample for 5–10 minutes at 65–70°C prior to loading on the gel. Perform electrophoresis under standard conditions used for the analysis of DNA samples, and visualize RNA with either ethidium bromide or SYBR® Green II stain following electrophoresis. The denatured dsRNA may be gel quantitated by comparison to known amounts of ssRNA.

The linear control DNA produces two single-stranded RNA transcripts approximately 2.3kb and 1.1kb in length. These strands are not complementary and will not anneal to form dsRNA. They will appear rather diffuse on a native gel.

4. Producing Short Interfering RNAs (siRNAs)

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.)

- 2X Oligo Annealing Buffer
- nuclease-free water
- gene-specific DNA oligonucleotides
- isopropanol
- 70% ethanol

4.A. Designing DNA Oligonucleotides

The DNA template for the in vitro transcription of siRNAs is a short duplex oligonucleotide that contains a T7 RNA Polymerase promoter upstream of either the sense or antisense mRNA sequence. Two oligonucleotides are annealed to generate separate templates for synthesis of each strand of the siRNA. The separate short RNA strands that are synthesized are then annealed to form siRNA.

In order to synthesize siRNAs in vitro using T7 RNA Polymerase, 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 for screening for the optimal target site, as the efficiency of silencing by an siRNA can vary greatly between different sites within the same target. Currently, predicting siRNA effectiveness is impossible, thus the need to screen multiple siRNAs against a particular target mRNA is required for optimal suppression.

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 more efficient T7 RNA Polymerase binding. The oligonucleotide structures are as follows: (T7 promoter sequence underlined):

mRNA sequence:

5´-XXXXX$G_1N_{2\text{-}18}C_{19}$XXXXX-3´

complement:

3´-XXXXX$C_1N_{2\text{-}18}G_{19}$XXXXX-5´

Oligo 1 (top strand for sense):

5´-<u>GGATCCTAATACGACTCACTATA</u>-**$G_1N_{2\text{-}18}C_{19}$**-3´
($G_1N_{2\text{-}18}C_{19}$ = sense mRNA sequence)

Oligo 2 (bottom strand for sense):

3´-<u>CCTAGGATTATGCTGAGTGATAT</u>-**$C_1N_{2\text{-}18}G_{19}$**-AA-5´

Oligo 2 to order:

5´-AA-**$G_{19}N_{18\text{-}2}C_1$**-TATAGTGAGTCGTATTAGGATCC-3´

Oligo 3 (top strand for antisense):

5´-GGATCCTAATACGACTCACTATA-$\mathbf{G_{19}N_{18\text{-}2}C_1}$-3´
($G_{19}N_{18\text{-}2}C_1$ = antisense mRNA sequence)

Oligo 4 (bottom strand for antisense):

3´-CCTAGGATTATGCTGAGTGATAT-$\mathbf{C_{19}N_{18\text{-}2}G_1}$-AA-5´

Oligo 4 to order:
5´-AA-$\mathbf{G_1N_{2\text{-}18}C_{19}}$-TATAGTGAGTCGTATTAGGATCC-3´

An additional two adenine nucleotides are added to the 5´ end of oligo 2 and oligo 4 to allow for addition of the two uridine 3´ overhangs in the siRNA strands.

A universal minimal T7 RNA polymerase promoter top strand oligo may be used for the generation of all siRNA strands (i.e., 5´-GGATCCTAATAGC-ACTCACTATAG-3´) to minimize the number of oligonucleotides necessary for DNA template preparation. However, we have observed optimal yields for siRNAs using completely double-stranded oligonucleotides (as pictured above). Some templates however, produce similar amounts of RNA with either a minimal top-strand oligo or a full-length, top-strand oligo; the effect appears to be template dependent.

Table 2. Comparison of RNA yield, using either a universal minimal T7 RNA Polymerase promoter top-strand oligo or a longer top-strand T7 oligo for 6 different DNA templates that generate 6 different short RNAs.

RNA Produced	Universal Minimal Oligo (arbitrarily set to 100%)	Longer Top-Strand Oligo
Template 1	100 %	86 %
Template 2	100 %	132 %
Template 3	100 %	20 %
Template 4	100 %	90 %
Template 5	100 %	258 %
Template 6	100 %	147 %

4.B. Annealing DNA Oligonucleotides

Resuspend DNA oligonucleotides in nuclease-free water to a final concentration of 100 pmol/µl.

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**

Heat oligonucleotide mixture 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° or –20°C.

Note: Incubating the oligonucleotide mixture in a beaker of 90–95°C water for 3–5 minutes, then placing this beaker of water in a room temperature water bath to allow for slow cooling has worked well for scientists at Promega. This process takes approximately 2 hours. Alternately, the heated beaker of water may be allowed to cool slowly to room temperature overnight.

4.C. Synthesizing Large Quantities of siRNA

The linear control DNA supplied with the system produces single-stranded transcripts that are 1.1kb and 2.3kb in length. The transcripts produced from the pGEM® Express Positive Control Template are not complementary and thus will not form double-stranded RNA or siRNA. They are to be used as a positive control for transcriptional activity of the system and RNA integrity following transcription only.

1. Set up the appropriate reaction size 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. Two separate reactions must be assembled for each siRNA, as each short RNA strand is synthesized separately, and then mixed following transcription.

T7 Reaction Components	Sample Reaction	Control Reaction
RiboMAX™ Express T7 2X Buffer*	10.0µl	10.0µl
Annealed Oligonucleotide Template DNA (from above; 10pmol/µl; see Note 1)	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	**20.0µl**	**20.0µl**

*Frozen RiboMAX™ Express T7 2X Buffer will contain a precipitate that can be dissolved by warming the buffer at 37°C and mixing well. Note that the buffer contains spermidine, which can precipitate DNA at temperatures colder than room temperature.

2. Incubate for 30 minutes at 37°C (see Notes 2–3).

Notes:

1. Up to 6µl of annealed DNA oligonucleotide template may be added to a single 20µl in vitro transcription reaction without inhibition due to the annealing buffer. Optimal yields of RNA has been observed using approximately 20pmol annealed DNA oligonucleotide template per 20µl in vitro transcription reaction.

2. To maximize yield, incubation at 37°C may proceed for up to 2 hours. Generally, no dramatic increase in yield is observed beyond the 30-minute incubation period. However, template-dependent increase is sometimes observed. A time-course experiment may be performed to determine the optimal incubation time for maximal RNA synthesis.

3. Incubation at 42°C may improve the yield of siRNA for transcripts that contain secondary structure and may be useful for a GC-rich substrate if sufficient yield is not obtained at 37°C.

4.D. 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.

Do not add diluted RNase A Solution to reactions generating siRNA. RNase A will cleave the required 2 uracil 3´ overhang. The overhang is not required for RNAi induced by longer RNA molecules in nonmammalian systems.

1. To each 20µl transcription reaction, add 1µl RQ1 RNase-free DNase and incubate for 30 minutes at 37°C.

2. Combine separate sense and antisense reactions together 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.

4.E. Purifying siRNA

1. Add 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol (see Note). Mix and place on ice for 5 minutes. The reaction will appear cloudy at this stage. Spin at top speed in a microcentrifuge for 10 minutes.

2. A white pellet should be visible at the bottom of the microcentrifuge tube. 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). Do not over-dry the pellet, because it may become difficult to resuspend completely. Store at –20°C or –70°C.

   Note: Adding glycogen during the isopropanol precipitation step may increase yield. We recommend using 50ng glycogen from Roche Applied Science (Cat.# 901-393) per microliter of transcription reaction. The presence of glycogen in the purified siRNA does not interfere with subsequent quantitation or functionality.

4.F. Determining siRNA Concentration and Visualizing by Gel Electrophoresis

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.)
- Blue/Orange Loading Dye, 6X (Cat.# G1881)

The DNase-treated siRNA transcript can be examined by native gel electrophoresis to determine the integrity of the full-length siRNA transcript. Including 10bp DNA Ladder (Cat.# G4471) on the gel can help determine the size of the siRNA sample. Note that siRNA may migrate more slowly than double-stranded DNA. Use 1–5µl of diluted siRNA per lane (dilute at least 1:20 with nuclease-free water) or use 50–500ng per lane. The siRNA may be gel quantitated by comparison to known amounts of the annealed DNA oligonucleotide templates used to generate the siRNA strands. For absolute quantitation, gel analysis using a purified, chemically synthesized siRNA should be performed using SYBR® Green II stain and a fluorescent scanner.

Prepare either a 2–3% agarose gel or a polyacrylamide minigel, depending upon the length of the transcript involved (10–20% PAGE for siRNAs). The separated siRNA may be visualized by staining the gel in 0.5mg/ml ethidium bromide or 1:10,000 SYBR® Green II stain (Molecular Probes). We do not recommend incorporating the stain into the gel as it can alter the migration rate of the siRNA, making accurate size determination more difficult.

Alternately, the purified siRNA may be quantitated using RiboGreen® dye with the ribosomal RNA standard included in the RiboGreen® kit using the high-range standard curve and diluting siRNA samples in serial 1:10 dilutions (Molecular Probes; Cat.# R-11490).

We do not recommend quantitation using absorbance at 260nm. The carryover of ribonucleotides does not allow for accurate quantitation using this method, and G-25 column purification results in much reduced yields of siRNAs.

5. RNAi and siRNA Applications

The following references are meant to serve as a guide. Protocols for performing RNAi in nonmammalian and mammalian systems are constantly being modified and optimized. Consult recent literature for the most current techniques.

Use in *C. elegans*

- Injecting Worms: References 1 and 18 include guidelines for using dsRNA for the injection of intact worms.
- Soaking Worms: Soaking worms in a solution containing dsRNA has been described in reference 19.
- Feeding Worms: Protocols for the introduction of dsRNA into worms by feeding bacteria that express dsRNA from a feeding vector are described in references 20–22.

Use in Insect Cell Culture

- RNAi studies have been performed using cultured *Drosophila* S2 cells. The protocol described in reference 16 has been successfully used by Promega scientists with dsRNA synthesized by the T7 RiboMAX™ Express RNAi System.
- The introduction of dsRNA into insect cells using transfection reagents is described in reference 24. Procedures for performing RNAi in cultured mosquito cells (25) or cultured *Drosophila* organs (26) have also been described.

Use in Mammalian Cell Systems

- Transfection of in vitro synthesized siRNAs or hairpin siRNAs into mammalian cell lines can be accomplished using a number of different protocols (12,13,27).

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low amounts of RNA synthesized using standard transcription protocol	Precipitation of the DNA template by the spermidine in the Transcription 2X Buffer. Make sure the reaction components are assembled at room temperature and in the order listed.
	Inhibitors present in template DNA. Typical inhibitors include residual SDS, salts, EDTA and RNase. Residual NaCl used to precipitate the template DNA may inhibit RNA polymerase activity by as much as 50%. The template DNA may be desalted by column chromatography and precipitated in the presence of another salt. Wash the resulting pellet 1–2 times with 70% ethanol.
	Nucleases present in template DNA. The use of Recombinant RNasin® Ribonuclease Inhibitor is recommended for all in vitro transcription reactions. RNasin® Ribonuclease Inhibitor is included in the Enzyme Mix. Any solutions not provided should be made up in 0.1% DEPC-treated water.
	Proteinase K treatment may improve template quality if nucleases are present. Treat the template DNA with proteinase K (100–200µg/ml) and SDS (0.5%) for 30 minutes at 50°C, followed by phenol/chloroform extraction and ethanol precipitation. Carryover of SDS can be minimized by diluting the nucleic acid several-fold before ethanol precipitation. Vigorously wash the pellet with 70% ethanol before resuspension.
	Inefficient template or presence of secondary structure. Clone the DNA template so that a different portion of the insert is adjacent to the transcriptional start site. Moving the transcription start point of the T7 promoter in relation to the transcript sequence can overcome transcription problems.

6. Troubleshooting (continued)

Symptom	Causes and Comments
Low amounts of RNA synthesized using standard transcription protocol (continued)	Incubation at 42°C instead of 37°C may increase transcription of templates containing secondary structure.
	Inactive RNA polymerase. The activity of the RNA polymerase may be evaluated by in vitro transcription of the control template.
Presence of incomplete transcripts	Premature termination of RNA synthesis. Lower the incubation temperature from 37°C to 30°C. This has been shown to increase the proportion of full-length transcripts in some cases (28).
Presence of transcripts larger than expected	Protruding 3´ termini on the DNA template. If the DNA template has been linearized with a restriction enzyme that generates a protruding 3´-end, transcription results in synthesis of significant amounts of long RNA molecules that are initiated at the terminus of the template (17). If it is impossible to avoid using a restriction enzyme of this type, make the ends of the linear DNA blunt with DNA Polymerase I Large (Klenow) Fragment before using in a transcription reaction.
	Nonlinearized plasmid is present in the sample. Analyze the sample by gel electrophoresis. If undigested vector is noted, redigest with the appropriate restriction enzyme.

7. Composition of Buffers and Solutions

2X Oligo Annealing Buffer

20mM Tris-HCl (pH 7.5)
100mM NaCl

Use nuclease-free water for preparing the buffer. Store at room temperature.

RNA loading buffer

50% glycerol
1mM EDTA
0.4% bromophenol blue

Use a high-grade glycerol. Lower grades of glycerol contain ribonuclease activity. Dispense RNA loading buffer into aliquots and store at –20°C.

RNA sample buffer

10.0ml deionized formamide
3.5ml 37% formaldehyde
2.0ml MOPS buffer

Dispense into aliquots and store at –20°C for up to 6 months. Do not freeze-thaw more than twice.

TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA

TE-saturated phenol:chloroform: isoamyl alcohol (25:24:1)

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part chloroform:isoamyl alcohol (24:1). **Note:** For removal of the DNA template following transcription, use TE buffer at pH 4.5 rather than TE buffer at pH 8.0.

MOPS buffer (10X)

0.2M MOPS (pH 7.0)
50mM sodium acetate
5mM EDTA (pH 8.0)

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9. Related Products

RNA Production

Product	Cat.#
Riboprobe® System—SP6	P1420
Riboprobe® System—T3	P1430
Riboprobe® System—T7	P1440
Riboprobe® System Buffers	P1121
RiboMAX™ Large Scale RNA Production System—SP6	P1280
RiboMAX™ Large Scale RNA Production System—T7	P1300
T7 RiboMAX™ Express Large Scale RNA Production Systems	P1320

For Laboratory Use.

DNA Purification

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System	50 preps	A1330
	250 preps	A1460
Wizard® *Plus* Minipreps DNA Purification System	50 preps	A7100
	100 preps	A7500
	250 preps	A7510
Wizard® *Plus* Midipreps DNA Purification System	25 preps	A7640
Wizard® *Plus* Maxipreps DNA Purification System	10 preps	A7270
Wizard® *Plus* Megapreps DNA Purification System	5 preps	A7300
Wizard® DNA Clean-Up System	100 preps	A7280
Wizard® SV Gel and PCR Clean-Up System	50 preps	A9281
	250 preps	A9282

For Laboratory Use.

Single-Stranded RNA Markers

Product	Size	Cat.#
RNA Markers	50µl	G3191

For Laboratory Use.

Amplification Products

Product	Size	Cat.#
PCR Master Mix	10 reactions	M7501
	100 reactions	M7501
	1,000 reactions	M7505

For Laboratory Use.

PCR Cloning Products

Product	Size	Cat.#
pGEM®-T Vector System I	20 reactions	A3600
pGEM®-T Vector System II	20 reactions	A3610
pGEM®-T Easy Vector System I	20 reactions	A1360
pGEM®-T Easy Vector System II	20 reactions	A1380

For Laboratory Use.

10. Summary of Changes

The following changes were made to the 9/16 revision of this document:

1. Removed reference to the siRNA Target Designer software, which is no longer available.
2. Updated Related Products.

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