USER GUIDE

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Silencer® siRNA Construction Kit

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Silencer® siRNA Construction Kit

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Introduction

Background

Post-Transcriptional Gene Silencing and RNA interference (RNAi) are terms describing the specific suppression of genes by complementary dsRNA (for a recent review, see Sharp 2001). Although the mechanism by which dsRNA suppresses gene expression is not entirely understood, experimental data provide important insights. In non-mammalian systems such as *Drosophila*, it appears that longer dsRNA is processed into 21–23 nt dsRNA (called small interfering RNA or siRNA) by an enzyme containing RNase III motifs (Bernstein et al., 2001, Grishok et al., 2001, Hamilton and Baulcombe 1999, Knight and Bass 2001, Zamore et al., 2000). The siRNA apparently then acts as a guide sequence within a multicomponent nuclease complex to target complementary mRNA for degradation (Hammond et al., 2001).

Long dsRNA is routinely used in non-mammalian cells and organisms to effect gene silencing. Mammalian cells, however, have a potent antiviral response pathway that induces global changes in gene expression when dsRNA molecules longer than 30 nt are introduced into cells (Stark et al., 1998, Manche et al., 1992, Minks et al., 1979). The antiviral response makes it difficult to distinguish target-specific effects of long dsRNA from the general antiviral response. siRNAs, comprised of 21-mer dsRNAs, do not trigger the antiviral response, making it possible to perform gene silencing experiments in mammalian cells (Elbashir et al., 2001, Caplen et al., 2001, Hutvagner et al., 2001, Jarvis and Ford 2001).

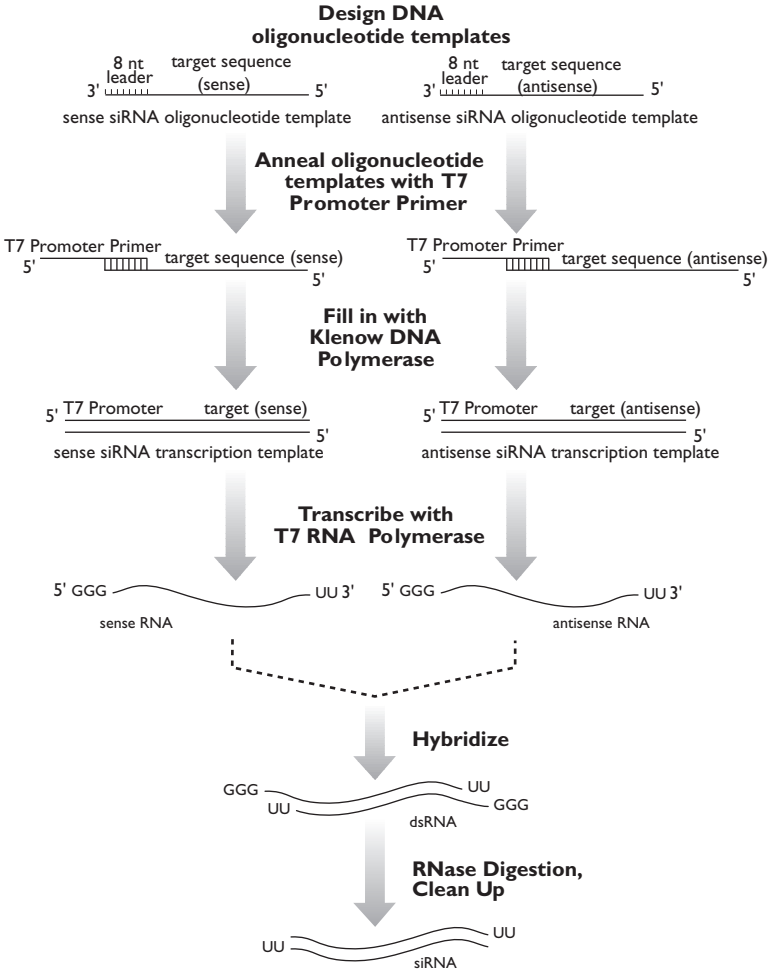
siRNA synthesis by in vitro transcription

Whereas most RNAi experiments in *Drosophila*, *C. elegans*, and other non-mammalian cells have used in vitro transcribed RNA molecules, early siRNA experiments in mammalian cells used chemically synthesized RNA oligomers. Chemically synthesized siRNAs were probably used because there are no sequence limitations for chemical synthesis. In contrast, in vitro transcription using T7 RNA polymerase requires that the first 2 nucleotides of the RNA transcript be GG or GA to ensure efficient synthesis (Milligan 1987). Requiring a GG or GA at the 5' ends of both the sense and antisense strands of an siRNA in addition to the required 3' terminal UU greatly reduces the number of potential target sites for siRNA experiments. This constraint essentially eliminates in vitro transcription as a viable option for preparing siRNAs.

The *Silencer*® siRNA Construction Kit overcomes the sequence requirements of traditional in vitro transcription strategies by using siRNA template oligonucleotides containing a "leader" sequence that is complementary to the T7 Promoter Primer included in the kit (Figure 2). Inclusion of leader sequences provides two benefits:

- The 8 nt leader sequence is optimized for maximal RNA yield.
- After transcription and hybridization of the sense and antisense strands of the siRNA, the leader sequences are efficiently removed from the dsRNA preparation, eliminating the need to select target mRNA sequences that are compatible with T7 transcription.

Figure 1 *Silencer*® siRNA Construction Kit procedure overview



a. Two 29-mer DNA oligonucleotides (template oligonucleotides) with 21 nt encoding the siRNA and 8 nt complementary to the T7 Promoter Primer are synthesized and desalted.

b. In separate reactions, the 2 template oligonucleotides are hybridized to a T7 Promoter Primer (an oligonucleotide provided with the kit that contains a T7 promoter sequence and 8 nt complementary to the template oligonucleotides).

c. The 3' ends of the hybridized DNA oligonucleotides are extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates.

d. The sense and antisense siRNA templates are transcribed by T7 RNA polymerase and the resulting RNA transcripts are hybridized to create dsRNA. The dsRNA consists of 5' terminal single-stranded leader sequences, a 19 nt target specific dsRNA, and 3' terminal UUs.

e. The leader sequences are removed by digesting the dsRNA with a single-strand specific ribonuclease. Overhanging UU dinucleotides will remain on the siRNA because the RNase does not cleave U residues. The DNA template is removed at the same time by a deoxyribonuclease.

f. The resulting siRNA is purified by glass fiber filter binding and elution which removes excess nucleotides, short oligomers, proteins, and salts in the reaction.

The end product is a double-stranded 21-mer siRNA with 3' terminal uridine dimers that can effectively reduce the expression of target mRNA when transfected into mammalian cells.

Reagents provided with the kit and storage

The *Silencer*® siRNA Construction Kit provides reagents for construction of 15 different siRNAs.

Template Preparation

Amount	Component	Storage
200 µL	DNA Hybridization Buffer	–20°C
60 µL	T7 Promoter Primer	–20°C
60 µL	10X Klenow Reaction Buffer	–20°C
60 µL	10X dNTP Mix	–20°C
60 µL	Exo– Klenow	–20°C

Transcription Reagents

Amount	Component	Storage
60 µL	T7 Enzyme Mix	–20°C
60 µL	10X T7 Reaction Buffer	–20°C
300 µL	2X NTP Mix†	–20°C

† Kit life can be prolonged by storing the 2X NTP Mix at –80°C

siRNA Purification

Amount	Component	Storage
90 µL	Digestion Buffer	–20°C
37.5 µL	DNase	–20°C
45 µL	RNase	–20°C
7.8 mL	siRNA Binding Buffer Add 5.3 mL 100% ethanol before use	room temp
20 mL	siRNA Wash Buffer Add 11 mL 100% ethanol before use	room temp
15	Filter Cartridges	room temp
30	Collection Tubes	room temp
3.5 mL	Nuclease-free Water	any temp†

† Store Nuclease-free Water at –20°C, 4°C, or room temp

Positive Control Reagents

Amount	Component	Storage
10 µL	Sense Control DNA (100 µM)	–20°C
10 µL	Antisense Control DNA (100 µM)	–20°C

Materials not supplied with the kit

Template oligonucleotides (DNA)

Template oligonucleotides (DNA) are 29 nt in length. The 8 nucleotides at the 3' end of each oligonucleotide must be 5'-CCTGTCTC-3'. See section "siRNA design" on page 8 for complete instructions on designing template oligonucleotides.

Order the smallest scale synthesis of the oligonucleotides. Desalting is typically sufficient purification.

(optional) Radiolabeled NTP

Radiolabeled NTP, for example [a-^{32}P]GTP, ATP, or CTP, can be included in the reaction as a tracer to aid in the quantitation and assessment of the siRNA synthesized. Any specific activity of radiolabeled nucleotide is acceptable.

IMPORTANT! [a-^{32}P]UTP should not be used to trace label the siRNA because a modified UTP is present in the NTP mixture to enhance siRNA potency.

Silencer® siRNA Construction Kit instructions

siRNA design

Using siRNA for gene silencing is a rapidly evolving tool in molecular biology; these instructions are based on both the current literature, and on empirical observations by scientists at Life Technologies.

1. Find 21 nt sequences in the target mRNA that begin with an AA dinucleotide

   Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites.

   This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. (EMBO 2001) that siRNA with 3' overhanging UU dinucleotides are the most effective. Since then, however, siRNA with other 3' terminal dinucleotide overhangs have been transfected into cells and shown to induce RNAi. If desired, you may modify this target site selection strategy to produce siRNA with other dinucleotide overhangs, but it is essential to avoid G residues in the overhang because the siRNA will be cleaved by RNase at single-stranded G residues.

2. Select 2–4 target sequences

   Research at Life Technologies has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75–95% reduction. Choose target sites from among the sequences identified in step 1 based on the following guidelines:

   - Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
   - Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at:
     www.ncbi.nlm.nih.gov/BLAST.
   - Life Technologies researchers find that siRNAs with 30–50% GC content are more active than those with a higher G/C content.

3. Negative Controls

   siRNA experiments should include a negative control siRNA with the same nucleotide composition as the experimental siRNA but which lacks significant sequence homology to the genome. To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.

4. Design template oligonucleotides (DNA)

   a. See Figure 2 on page 10 for an example of template oligonucleotide design.
      - The antisense template oligonucleotide should have 21 nt at the 5' end that is the DNA counterpart of the target mRNA sequence chosen, i.e. the same sequence as the target RNA except that U residues are replaced with T's.
      - The sense template oligonucleotide should start with an AA dinucleotide at the 5' end followed by 19 nt that are complementary to the target sequence identified in step 2.
      - The 8 nt at the 3' end of both oligonucleotides should be the following sequence: 5'-CCTGTCTC-3'.

This 8 nt sequence is complementary to the T7 Promoter Primer provided with the *Silencer*® siRNA Construction Kit. Hybridization of the template oligonucleotides to the T7 Promoter Primer will add the T7 promoter sequence to the 5' ends of the template oligonucleotide so that after the fill-in reaction, they can be efficiently transcribed.

Figure 2 Example of Template Oligonucleotide Design

Target mRNA sequence
5'- AACGAUUGACAGCGGAUUGCC-3'

Order these oligonucleotides to make an siRNA that targets the mRNA sequence shown above:

Antisense template oligonucleotide (DNA)
5'-AACGATTGACAGCGGATTGCCCCTGTCTC-3'

Sense template oligonucleotide (DNA)
5'-AAGGCAATCCGCTGTCAATCGCCTGTCTC-3'

b. Check your design

Note that transcription of the antisense oligonucleotide will generate RNA that is complementary to the target mRNA.

Also note that transcription of the sense template generates a 3' terminal UU that is not complementary to the antisense strand of the siRNA. This UU sequence does not need to be part of the mRNA sequence because the sense strand of the siRNA appears to have no function in targeting mRNAs for degradation.

c. Order the oligonucleotides

Order the sense and antisense template DNA oligonucleotides for each siRNA. The smallest scale synthesis (40 nmol or less) is sufficient for hundreds of transcription reactions. Desalting is typically sufficient purification for generating efficient transcription templates.

Transcription template preparation

To make an efficient transcription template, the sense and antisense template oligonucleotides (DNA) for each siRNA must be converted to dsDNA with a T7 promoter at the 5' end. This is accomplished by hybridizing the 2 oligonucleotides to the T7 Promoter Primer provided with the *Silencer*® siRNA Construction Kit and extending the T7 Promoter Primer and template oligonucleotides using a DNA polymerization reaction.

1. Resuspend the template oligonucleotides to 200 µM in nuclease-free water

   Oligonucleotides are usually supplied dry; tap the tubes containing the oligonucleotides on the bench to force the powder to the bottom of the tubes. Check the specification sheet supplied with the oligonucleotides to see how much was synthesized, and dissolve the sense and antisense template oligonucleotides in nuclease-free water to approximately 200 µM.

2. Determine the template oligonucleotide concentration by A_{260}

   a. Measure the A_{260} of a 1:250 dilution of the DNA template oligonucleotides

Dilute a small sample of the sense and antisense template oligonucleotides 1:250 into TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and read the absorbance at 260 nm in a spectrophotometer. Be sure to blank the spectrophotometer with the same TE that was used for sample dilution.

b. Determine the oligonucleotide concentration in µg/mL

Multiply the absorbance reading by 5000 to determine the concentration of the oligonucleotides in µg/mL. (See the explanation below.)

5000 = 250-fold dilution X 20 µg oligo/mL per absorbance unit*

*20 µg/mL is used to compensate for the non-full length oligonucleotide that is typically present in chemically synthesized oligonucleotide preps.

c. Determine the molar concentration of the oligonucleotides

The molar concentration of the oligonucleotides in µM can be determined by dividing the µg/mL concentration by 9.7. (See the explanation below.)

- There are 9.7 µg of DNA in 1 nmole of an average 29-mer:

  29 nt X 0.333 µg/nmol for each nt = 9.7 µg/nmol

- Dividing the µg/mL concentration by 9.7 yields the µM concentration as shown below:

$$\frac{\frac{X\ \mu g}{mL}}{\frac{9.7\ \mu g}{nmol}} = \frac{X\ \mu g}{mL} \times \frac{nmol}{9.7\ \mu g} = \frac{X\ nmol}{mL\ (9.7)} = \frac{X\ \mu mol}{L\ (9.7)} = \frac{X\ \mu M}{9.7}$$

Therefore µM = X ÷ 9.7

d. Example calculation

A 1:250 dilution of an oligonucleotide solution has an A_{260} of 0.4. The molar concentration is determined as follows:

0.4 x 5000 µg/mL per A_{260} = 2000 µg/mL

2000 µg/mL divided by 9.7 µg/nmol = ~206 µM

3. Make a 100 µM solution of each oligonucleotide

Dilute an aliquot of each template oligonucleotide to 100 µM using nuclease-free water or TE (10 mM Tris-HCl pH 8, 1 mM EDTA). Prepare ~20 µL of 100 µM oligonucleotide solutions.

4. Thaw the frozen template preparation reagents

Thaw the following kit components at room temperature, then briefly vortex each before use.

- T7 Promoter Primer
- 10X Klenow Reaction Buffer
- 10X dNTP Mix
- Nuclease-free Water

IMPORTANT! Keep the tube of Exo– Klenow at –20°C and do not vortex it.

5. Hybridize each template oligonucleotide to the T7 Promoter Primer
   a. In separate tubes mix the following:

Amount	Component
2 µL	T7 Promoter Primer
6 µL	DNA Hyb Buffer
2 µL	either sense or antisense template oligonucleotide

   b. Heat the mixture to 70°C for 5 min, then leave at room temp for 5 min.

6. Fill in with Klenow DNA polymerase
   a. Add the following to the hybridized oligonucleotides:

Amount	Component
2 µL	10X Klenow Reaction Buffer
2 µL	10X dNTP Mix
4 µL	Nuclease-free Water
2 µL	Exo– Klenow

   b. Gently mix by pipetting or slow vortexing. Centrifuge briefly to collect the mixture at the bottom of the tube.
   c. Transfer to 37°C incubator and incubate for 30 min.

7. Proceed to dsRNA synthesis, or store the templates at –20°C

   The siRNA templates can be used directly in a transcription reaction (see next section below) or stored at –20°C until they are needed for transcription.

dsRNA synthesis

The sense and antisense siRNA templates are transcribed for 2 hours in separate reactions. The reactions are then mixed, and the combined reaction is incubated overnight. Transcribing the templates separately eliminates potential competition between templates for transcription reagents that might limit the synthesis of 1 of the 2 strands of the siRNA duplex. Mixing the transcription reactions facilitates hybridization of the 2 siRNA strands and enables continued RNA synthesis to maximize the dsRNA yield.

1. Thaw the 2X NTP Mix and 10X T7 Reaction Buffer

   Thaw the 2X NTP Mix and 10X T7 Reaction Buffer at room temperature. After they have thawed, vortex each tube. Check the 10X T7 Reaction Buffer to see if a precipitate is visible, and if so, vortex the tube until the solution is completely resuspended. Briefly spin both tubes prior to using to ensure that no solution is lost when the tubes are opened.

   Keep the tube of T7 Enzyme Mix at –20°C and do not vortex it.

2. Assemble the transcription reactions and mix gently

   a. For each siRNA, assemble 2 transcription reactions at room temperature to synthesize the sense and antisense RNA strands of the siRNA. For each transcription reaction, mix the following components in the order shown:

Amount	Component
2 µL	sense or antisense siRNA template (from step 7. on page 12)
4 µL	Nuclease-free Water
10 µL	2X NTP Mix
2 µL	10X T7 Reaction Buffer
2 µL	T7 Enzyme Mix

   b. Gently mix contents thoroughly by flicking or brief vortexing and then microfuge briefly to collect the reaction mixture at the bottom of the tube.

3. Incubate reactions 2 hr at 37°C

   Incubate transcription reactions for 2 hr at 37°C, preferably in a cabinet incubator. (This will prevent condensation, which may occur if the tube is incubated in a heat block.)

4. Combine the sense and antisense transcription reactions and incubate at 37°C overnight

   Combine the sense and antisense transcription reactions into a single tube and continue incubation at 37°C overnight. The overnight incubation will maximize the yield of RNA and facilitate hybridization of the sense and antisense strands of the siRNA.

siRNA preparation/ purification

The dsRNA made by in vitro transcription has 5' overhanging leader sequences that must be removed prior to transfection. The leader sequence is digested by a single-strand specific ribonuclease. In the same digestion reaction, the DNA template is eliminated by DNase digestion. The resulting siRNA is recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification. The purified siRNA is eluted from the column into Nuclease-free Water, providing siRNA that is ready for transfection.

1. Digest the siRNA with RNase and DNase

   a. Thaw the Digestion Buffer at room temperature and vortex the tube to mix the contents thoroughly.

   b. To the tube of dsRNA from step 4. above, add the following reagents in the indicated order:

Amount	Component
6 µL	Digestion Buffer
48.5 µL	Nuclease-free Water
3 µL	RNase
2.5 µL	DNase

   c. Mix gently, and incubate for 2 hr at 37°C.

2. Before their first use, add 100% ethanol to the siRNA Binding and Wash Buffers

   Before using the siRNA Binding and Wash Buffers for the first time, add 100% ethanol as shown in the table below, and mix well. The prepared siRNA Binding and Wash Buffers can be stored at room temperature for the life of the kit.

	Amount of 100% ethanol to add:
siRNA Binding Buffer	5.3 mL
siRNA Wash Buffer	11 mL

3. Add 400 µL siRNA Binding Buffer and incubate 2–5 min at room temp

   Add 400 µL of siRNA Binding Buffer to the nuclease digestion reaction and incubate for 2–5 min at room temperature.

4. Heat Nuclease-free Water to 75°C

   Preheated Nuclease-free Water will be used to elute the siRNA from the Filter Cartridge in step 7. on page 14.

5. Prewet a Filter Cartridge with 100 µL siRNA Wash Buffer and bind the siRNA
   a. For each siRNA preparation, place a Filter Cartridge in a 2 mL Tube (provided with the kit).
   b. Apply 100 µL of siRNA Wash Buffer to the filter of the Filter Cartridge.
   c. Add the siRNA in the siRNA Binding Buffer to a prewet Filter Cartridge and spin at ~10,000 rpm in a microcentrifuge for 1 min.
   d. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the 2 mL Tube.

6. Wash the Filter Cartridge with 2 x 500 µL of siRNA Wash Buffer
   a. Apply 500 µL of siRNA Wash Buffer to the filter of the Filter Cartridge and spin at 10,000 rpm for 1 min. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the 2 mL Tube.
   b. Repeat the wash with a second 500 µL of siRNA Wash Buffer.
   c. Transfer the Filter Cartridge to a new 2 mL Tube.

7. Elute the siRNA in 100 µL of 75°C Nuclease-free Water
   a. Add 100 µL of the preheated Nuclease-free Water to the filter of the Filter Cartridge and incubate at room temperature for 2 min.
   b. Spin the Filter Cartridge at 12,000 rpm for 2 min. The purified siRNA will be in the eluate (in the 2 mL Tube).

8. Store siRNA at –20°C or –80°C

   siRNAs should be stored at –20°C or –80°C until they are prepared for transfection.

siRNA quantification

The siRNA concentration used for transfection is critical to the success of gene silencing experiments. Transfecting too much siRNA causes nonspecific reductions in gene expression and toxicity to the transfected cells. Transfecting too little siRNA does not change the expression of the target gene. Assuming that the UV spectrophotometer is accurate, measuring the absorbance of the siRNA sample at 260 nm is the simplest method to assess the concentration of the siRNA preparation.

1. Measure the A_{260} of a 1:25 dilution of the siRNA

   Dilute a small sample of the siRNA 1:25 into TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and read the absorbance at 260 nm in a spectrophotometer. Be sure to blank the spectrophotometer with the same TE that was used for sample dilution.

2. Determine the concentration of the siRNA in µg/mL

   Multiply the absorbance reading by 1,000 to determine the concentration of the purified siRNA in µg/mL (explanation below).

   1,000 = 25-fold dilution x 40 µg siRNA/mL per absorbance unit

3. Determine the molar concentration of the siRNA

   The molar concentration of the siRNA in µM can be determined by dividing the µg/mL concentration of the siRNA by 14 (explanation below).

   - There are 14 µg of RNA in 1 nmol of an average 21-mer dsRNA:

     21 nt x 2 strands = 42 nt x 0.333 µg/nmol for each nt = 14 µg/nmol
   - Dividing the µg/mL concentration by 14 yields the µM concentration as shown below:

$$\frac{\frac{X\ \mu g}{mL}}{\frac{14\ \mu g}{nmol}} = \frac{X\ \mu g}{mL} \times \frac{nmol}{14\ \mu g} = \frac{X\ nmol}{mL} = \frac{X\ \mu mol}{L\ (14)} = \frac{X\ \mu M}{14}$$

   Therefore µM = X ÷ 14

4. Example calculation

   A 1:25 dilution of purified siRNA has an A_{260} = 0.4. The molar concentration is determined as follows:

   0.4 x 1,000 µg siRNA/mL per A_{260} = 400 µg/mL

   400 µg/mL divided by 14 µg siRNA/nmol siRNA = ~29 µM siRNA

Transfecting mammalian cells

The efficiency with which mammalian cells are transfected with siRNAs will vary according to cell type and the transfection agent used. This means that the optimal siRNA concentration used for transfections should be determined empirically. We have found that siRNAs generated with the *Silencer*® siRNA Construction Kit typically work best when present in cell culture medium at 0.1–10 nM.

Most protocols recommend maintaining mammalian cells in the medium used for transfection. This is to avoid diluting or removing the siRNAs from the cells by adding medium or washing the cells with new medium. We have found that mammalian cells diluted 2 fold with fresh medium 24 hours after transfection typically exhibit greater viability than those left in the medium used for transfection. Furthermore, adding fresh medium does not appear to have a detrimental effect on the activity of the transfected siRNAs.

Troubleshooting

Using the Control DNA supplied with the kit

The Sense and Antisense Control DNA templates supplied with the siRNA Construction Kit can be used to generate an siRNA specific to GAPDH. This GAPDH siRNA has been used successfully in human, mouse, and monkey cell lines, thus it can be used both as a control to confirm that the kit is working properly and as a positive control for many siRNA experiments. GAPDH mRNA and protein are expressed at levels in most mammalian cells that can be readily detected by Northern and Western analysis.

1. Instructions for the positive control reaction

   Use 2 µL of the Sense and Antisense Control DNA in siRNA synthesis following the procedure in this Protocol. Start at step 4. on page 11, and continue to the end of the section.

   Analyze the outcome of the reaction by measuring the A_{260} of the purified siRNA, and determine the yield in µg as described in section "siRNA quantification" on page 14. Check the size of the siRNA by running 10 µL of the purified siRNA on a 2% agarose gel following the instructions in section "Gel analysis of siRNA" on page 25.

2. Expected result of the positive control reaction

   The positive control reaction should yield ≥10 µg of siRNA, the majority of which is 21–22 bp. There may also be a minor band on the gel corresponding to an RNA duplex containing 1 incompletely digested 27 nt strand. This secondary band will be ≤30% as intense as the primary band.

3. Troubleshooting low yield from the positive control reaction

   If the positive control does not yield at least 10 µg of siRNA, then the digestion reaction, column purification, or quantification could be faulty. Distinguish between these 3 possibilities by doing the following experiment.

   a. Reaction setup and analysis (see also Figure 3)

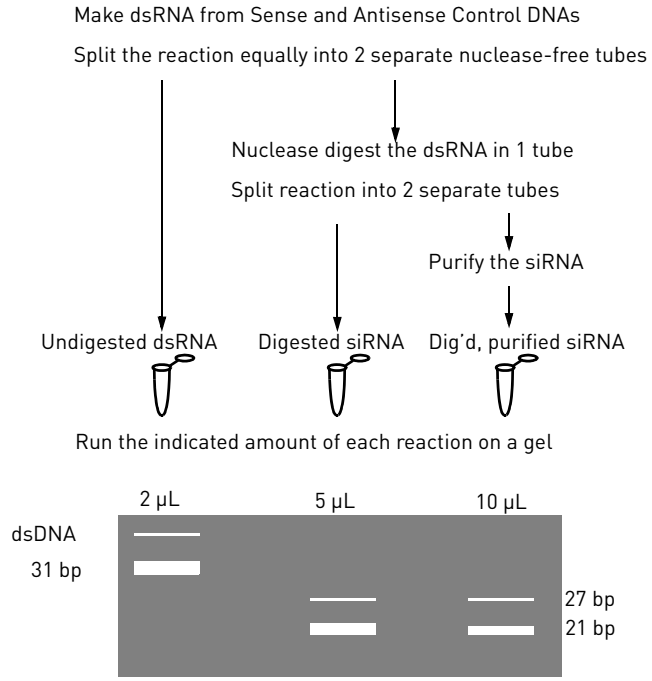
      Set up the positive control reaction, and allow it to progress through step 4. on page 13. At this point, split the dsRNA into 2 equal portions (20 µL each) in 2 separate nuclease-free tubes.

      i. Set aside 1 tube for gel analysis; this is undigested dsRNA.

      ii. Nuclease digest the other tube as follows:

Amount	Component
20 µL	dsRNA
3 µL	Digestion Buffer
24.25 µL	Nuclease-free Water
1.5 µL	RNase
1.25 µL	DNase

Figure 3 Positive Control Troubleshooting Experiment



iii. Split the digested siRNA into 2 equal portions (25 µL each) in 2 separate nuclease-free tubes.
Set aside 1 tube for gel analysis; this is digested siRNA.
Column purify the contents of the other tube, following the instructions in steps 3.–7. on page 14. This is digested, purified siRNA.

iv. Run the amounts indicated below of each reaction on a 2% agarose gel (see section "Gel analysis of siRNA" on page 25).

Reaction	Amount to run on the gel
undigested dsRNA	2 µL
digested siRNA	5 µL
digested, purified siRNA	10 µL

b. Expected result of the positive control troubleshooting reaction

The undigested reaction should have a primary band at 31 bp. The digested reaction should yield a band at 21 bp that is slightly less intense than the undigested product. The column purified product should provide the same 21 bp product with at least 50% the intensity of the unpurified, digested RNA.

c. If all 3 reactions generate the expected products and they are easily visible on the gel (but yield in the initial positive control experiment was <10 µg):

Confirm that the absorbance reading for quantitation was accurate by measuring another RNA whose concentration is known.

d. If the yield from all 3 reactions is less than expected, then consider the following possible reasons for low yield:
   - The spermidine in the 10X T7 Reaction Buffer may cause precipitation of the template DNA if it is not diluted into a mixture of template, water, and 2X NTP Mix. For the same reason it is important to assemble the transcription reaction at room temperature.
   - If you are using a water bath or heat block for the 37°C transcription reaction incubation, then use a cabinet incubator to eliminate condensation that may form during the reaction.
   - Check the temperature of the transcription reaction incubation to confirm that it is 37°C ±2°C.

e. If the digested siRNA band is less than 50% as intense as the undigested dsRNA, then try the following things to attenuate the nuclease digestion reaction.
   - Reduce the amount of nuclease in the reaction by adding only 25–50% the volume of RNase and DNase to the digestion reaction (step 1. on page 13).
   - Reduce the stringency of the digestion reaction (step 1. on page 13) by incubating for 2 hr at room temp rather than at 37°C.

f. If the digested siRNA band is the expected intensity but the digested and purified siRNA band is less than 50% as intense as the undigested dsRNA band, then try the following things to improve the purification step.
   - Confirm that the siRNA Binding and Wash Buffers were prepared by adding 5.3 mL and 11 mL 100% ethanol respectively before using them the first time (step 2. on page 14). It is also important to mix the buffers well by vortexing or shaking after adding the ethanol.
   - Incubate the dsRNA in the siRNA Binding Buffer for at least 2 min before applying it to the Filter Cartridge (step 3. on page 14).
   - Elute the siRNA from the Filter Cartridge using 2, 50 µL elutions with Nuclease-free Water instead of a single 100 µL elution (step 7. on page 14).
   - During the siRNA elution (step 7. on page 14), apply the Nuclease-free Water to the Filter Cartridge, and incubate the Filter Cartridge for 5 min at 37–50°C before centrifuging.

g. If none of these suggestions improve the yield of the positive control, then call Life Technologies's Technical Services Staff for more advice.

Low yield

Silencer® siRNA Construction Kit reactions should routinely yield at least 10 µg of siRNA. Lower yields could result from inefficient transcription of 1 or both of the siRNA templates, overdigestion of the dsRNA preparation, or poor binding or elution from the Filter Cartridge during siRNA purification.

1. Do the positive control reaction

   Perform the positive control reaction to confirm that the components in the kit are functioning properly (section "Using the Control DNA supplied with the kit" on page 16). If the positive control reaction yields less than 10 µg of RNA, try the troubleshooting experiment described in section "Troubleshooting low yield from the positive control reaction" on page 16.

2. If the positive control reaction works, but your templates give low siRNA yield, try the transcription troubleshooting experiment

   If the positive control provides the expected yield but your templates do not, then confirm that both your sense and antisense templates are functioning properly by doing the experiment described below.

   a. Transcription troubleshooting experiment

      i. Using your sense and antisense template oligonucleotides, follow the standard procedure through step 3. on page 13.
      Reaction products from this experiment will be compared to the product of the positive control experiment (described in section "Using the Control DNA supplied with the kit" on page 16). If you do not have at least 10 µL left from the positive control reaction to run on the gel, do the positive control reaction while doing this experiment.

      ii. In step 4. on page 13, mix only 10 µL of the sense transcription reaction with 10 µL of the antisense transcription reaction for the overnight incubation. There will be 3 tubes:

10 µL sense strand reaction
10 µL antisense strand reaction
20 µL reaction for dsRNA

      iii. After the overnight incubation, digest half (10 µL) of the reaction containing both the sense and antisense templates with RNase and DNase as follows:

Amount	Component
10 µL	dsRNA transcription reaction (containing both sense and antisense template)
1.5 µL	Digestion Buffer
12 µL	Nuclease-free Water
0.75 µL	RNase
0.6 µL	DNase

      iv. Run 4 µL of each of the 3 transcription reactions and 10 µL of the digested siRNA on a 2% agarose gel. Also include a 10 µL sample of the product from the positive control reaction on the gel. (Gel instructions are in section "Gel analysis of siRNA" on page 25.)

   b. Expected result

      The sense and antisense reactions should generate distinct, 31 nt products with roughly equivalent intensities.

      The undigested transcription reaction containing a mixture of the sense and antisense templates (undigested dsRNA) should generate a primary product that migrates differently than the single-stranded RNAs. The dsRNA product typically migrates more slowly than the single-stranded products, but some sequences migrate faster when double-stranded.

      The digested transcription reaction containing both templates should generate a product that migrates faster than the undigested dsRNA. Its intensity should be ≥50% the intensity of the undigested dsRNA.

c. If none of the transcription reactions have good yield:

If neither of the single template reactions, nor the reaction with both templates generates a robust product (when compared to the positive control reaction), then consider the following:

- Check the sense and antisense templates used for transcription (i.e. the reaction products at step 7. on page 12) using the polyacrylamide gel electrophoresis procedure (see section "Gel analysis of siRNA" on page 25).
  Both templates should migrate as distinct ~50 bp bands. If they do not, then consider gel purifying the template oligonucleotides (see section "Gel purification of nucleic acids" on page 26) before using them in the *Silencer*® siRNA Construction Kit.
- Confirm that the sequence of the oligonucleotides includes the 8 nt sequence required to hybridize to the T7 Promoter Primer provided with the *Silencer*® siRNA Construction Kit (see section 4. on page 9 for instructions on template oligonucleotide design).
- The presence of significant amounts of EDTA or salt can inhibit transcription reactions. If the template oligonucleotides might be contaminated with either, then ethanol precipitate the oligonucleotides and wash the pellets with 70% ethanol before starting the procedure.

d. If one template generates much less RNA than the other (or none), or if the dsRNA contains a significant amount of ssRNA:

If one of the templates generates significantly less product than the other in the transcription reactions with a single template, or if the dsRNA product has a significant amount of single-stranded product, then one of the transcription reactions is probably not working properly. This will reduce the yield of dsRNA and subsequently, siRNA. Consider the following suggestions:

- Recheck the concentrations of the template oligonucleotides by measuring their A_{260} and doing the calculations to determine the molar concentration (see step 2. on page 10). The *Silencer*® siRNA Construction Kit procedure requires that the template oligonucleotide concentration is 100 µM so that equimolar amounts of template oligonucleotide and T7 Promoter Primer are present in the hybridization step (step 5. on page 12).
- Gel purify the oligonucleotide that generates the less effective template. This treatment could increase transcription yield by increasing the concentration of full-length transcription template, and/or by eliminating inhibitors of transcription from the template.
- If one template doesn't generate any product, confirm the sequence of its template oligonucleotide to make sure it includes the 8 nt sequence required to hybridize to the T7 Promoter Primer (the T7 promoter sequence is required for transcription).
- The presence of significant amounts of EDTA or salt can inhibit transcription reactions. If the template oligonucleotide might be contaminated with either, then ethanol precipitate it and wash the pellet with 70% ethanol before starting the procedure.

- Increase the duration of the transcription reaction in separate tubes (step 3. on page 13) to overnight at 37°C, then combine the sense and antisense transcription reactions and incubate the mixture 1 hr at 42°C instead of at 37°C. Elevating the temperature of the hybridization increases the liklihood that RNA:DNA template hybrids will come apart so that RNA:RNA hybrids can form.

e. All transcription reactions generate a robust product, but the digested dsRNA is not 21 bp, or it is <50% as intense as the undigested dsRNA:

If the sense and antisense transcription reactions as well as the combined transcription reaction generate robust products, but the digested dsRNA is not 21 bp, or it generates a band that is less than 50% as intense as the signal seen with the undigested dsRNA, then consider the following:

- Confirm that the sequences for the sense and antisense templates encode fully complementary RNAs.
- Reduce the amount of nuclease in the reaction by adding only 25–50% the volume of RNase and DNase to the digestion reaction (step 1. on page 13).
- Reduce the stringency of the nuclease digestion reaction (step 1. on page 13) by incubating for 2 hr at room temperature rather than at 37°C.

Transfected siRNA does not reduce gene expression

Many researchers who are using siRNA have observed that some siRNAs simply do not reduce gene expression. Currently it is not clear why some siRNAs cause gene silencing and others do not. To troubleshoot siRNA that does not reduce gene expression, first check the siRNA on a gel to make sure that it was synthesized properly, then troubleshoot transfection. If the siRNA looks as expected on a gel and options for optimizing transfection are exhausted, but the siRNA still does not reduce gene expression, redesign the siRNA.

1. Run the siRNA on an agarose gel to see if it is the expected size

   See section "Gel analysis of siRNA" on page 25 for instructions and expected results.

   If the siRNA does not have the expected appearance on a gel, see troubleshooting tips in section "Gel analysis shows multiple bands or incorrect siRNA size" on page 22.

2. Troubleshoot transfection

   Although this is not intended to be an exhaustive list of troubleshooting tips for transfection, it does provide solutions for some of the common problems associated with siRNA experiments in mammalian cells. For more thorough information, please refer to the provider of your transfection agent.

   a. Cell toxicity or nonspecific reduction in RNA or protein levels

   - Use less siRNA in the transfection.
   - Dilute the cultured cells 2 fold with fresh medium 24 hr after transfection.
   - Try a different transfection agent or procedure.
   - Design an siRNA to a different target sequence.
   - Gel purify the siRNA (see section "Gel purification of nucleic acids" on page 26).

b. No reduction in the target RNA or protein levels
   - Increase the amount of siRNA in the transfection.
   - Try a different transfection agent or procedure.
   - Try an siRNA to a different target sequence.

Gel analysis shows multiple bands or incorrect siRNA size

1. Expected appearance of siRNA on a 12% nondenaturing polyacrylamide gel

   Using the *Silencer*® siRNA Construction Kit, we routinely detect a primary siRNA product that is a 21–22 bp dsRNA and a secondary product that contains a 27 nt RNA in the siRNA duplex. The secondary product is typically less than 30% as intense as the primary product. The secondary product appears to have no detrimental effects on siRNA experiments and is likely to be active in targeting mRNA degradation. dsRNA products that are not present in 1 of these 2 bands could potentially limit the effectiveness of an siRNA experiment.

2. Troubleshooting multiple bands

   If gel analysis shows multiple bands in addition to the primary and secondary products described above, then consider the following:

   a. Check the templates.

      Using polyacrylamide gel electrophoresis (see section "Gel analysis of DNA oligonucleotides" on page 25), check the sense and antisense siRNA transcription templates, i.e. the reaction products at step 7. on page 12, to confirm that there is only a single distinct template band. If it turns out that there are multiple bands in the template, then consider gel purifying the template oligonucleotides used to prepare the transcription templates (see section "Gel purification of nucleic acids" on page 26).

   b. Multiple bands can result from ineffective nuclease digestion.
      - If the product bands are primarily larger than the expected 21–22-mer dsRNA, then increase the time of the nuclease digestion reaction (step 1. on page 13) to 4–16 hr or increase the amount of RNase in the reaction from 3 µL to ≤6 µL.
      - If the product bands are primarily smaller than the expected 21–22-mer dsRNA, then check the sequences of the sense and antisense template oligonucleotides to confirm that they are complementary. You can also consider incubating the nuclease digestion reaction (step 1. on page 13) at room temperature instead of 37°C, or decrease the amount of RNase in the reaction from 3 µL to ≥1 µL.

   c. If enough of the dsRNA is the correct length, you can gel purify it to generate a pure sample of siRNA.

      See instructions in section "Gel purification of nucleic acids" on page 26.

3. Troubleshooting siRNA that looks too large on a gel

   If gel analysis reveals a single band that migrates as though it is larger than a 21 bp dsRNA, consider the following:

   a. There is a problem with the nuclease digestion

      If there is a single band, and it is the same size as the undigested dsRNA, then the nuclease digestion is not functioning. Confirm that the nuclease digestion reaction (step 1. on page 13) is being incubated at 37°C.

b. Gel mobility comparison is not always an accurate way to estimate siRNA size

The gel mobility of siRNA is highly dependent on its nucleotide composition. Because of this, the gel migration of siRNAs with different sequences cannot reliably be compared to determine size. A better standard to estimate the size of an siRNA is the undigested dsRNA from which it is derived.

4. Troubleshooting siRNA that looks too small on a gel

If gel analysis reveals a single band that migrates as though it is smaller than a 21 bp dsRNA, consider the following:

a. Gel mobility comparison is not always an accurate way to estimate siRNA size

The gel mobility of siRNA is highly dependent on its nucleotide composition. Because of this, the gel migration of siRNAs with different sequences cannot reliably be compared to determine size. A better standard to estimate the size of an siRNA is the undigested dsRNA from which it is derived.

b. Check the size of the template oligonucleotides and siRNA transcripts

Confirm that the sense and antisense template oligonucleotides are 29 nt, and that the individual siRNA transcripts are approximately 31 nt by running them on a polyacrylamide gel (you would need to reserve an aliquot of the sense and antisense transcripts from step 3. on page 13 before combining them). A shorter than expected template oligonucleotide or a sequence in the template that disrupts T7 RNA polymerase could create smaller than expected siRNAs. If the template oligonucleotide is smaller than expected, then have it resynthesized. If the template is the correct size but either or both of the transcripts is smaller than expected, then design oligonucleotides to a different target sequence.

c. Confirm that the sense and antisense oligonucleotides are entirely complementary.

A single base mismatch could create a cleavage site for the RNase used in the digestion reaction, resulting in a smaller than expected siRNA.

A Supplemental Information

Additional procedures

Gel analysis of siRNA

siRNA should be assessed by gel electrophoreisis on 2% agarose in TBE (see section "Gel analysis of siRNA" on page 25 for instructions on pouring a 2% agarose gel).

Instructions for gel electrophoresis

1. Mix up to 10 µL of siRNA sample with 2 µL of a native gel loading buffer (see a typical recipe in section on page 28).
2. Load the sample on a 2% agarose gel and electrophorese at about 5–10 mAmps/cm.
3. Stop electrophoresis when the bromophenol blue dye front has migrated two-thirds of the way down the gel.
4. Stain the gel for ~10 min in a 1 µg/mL solution of ethidium bromide.
5. Visualize the siRNA using a UV transilluminator.

Expected appearance of the siRNA on the gel

The siRNA should migrate as a 21–22 bp band that runs slightly behind the bromophenol blue dye front. A second, less intense band may be apparent running behind the primary siRNA band. This band represents a dsRNA where the leader sequence of 1 of the strands of siRNA was only partially digested. The underdigested RNA strand is 27 nt and does not create any nonspecific effects when transfected into cells.

Gel analysis of DNA oligonucleotides

DNA oligonucleotides can be assessed by gel electrophoresis using a nondenaturing 12% polyacrylamide gel (see section on page 28) for a 12% polyacrylamide gel recipe).

Instructions for gel electrophoresis

1. Mix up to 5 µL of oligonucleotide with 2 µL of a native gel loading buffer (see a typical recipe in section on page 28).
2. Load the sample on a nondenaturing 12% polyacrylamide gel and electrophorese at 200–250 V.
3. Stop electrophoresis when the bromophenol blue dye front has migrated two-thirds of the way down the gel.
4. Stain the gel for 2–5 min in a 1 µg/mL solution of ethidium bromide.
5. Soak the gel for 2–5 min in water.

6. Visualize the siRNA using a UV transilluminator.

Gel purification of nucleic acids

Gel purification can be used to prepare oligonucleotides or siRNA of a single, defined length. Both siRNA and DNA oligonucleotides should be fractionated using a 12% nondenaturing acrylamide gel (see section on page 28 for a recipe). For this application, it is useful to have a "preparative scale" comb with teeth about 1–2 cm wide that will form large capacity wells.

1. Fractionate the nucleic acids using a polyacrylamide gel

   To prepare siRNA for electrophoresis, add 20 µL of native gel loading buffer (e.g. see a typical recipe in step on page 28) to the siRNA following the nuclease digestion step (step 4. on page 13).

   For oligonucleotides, mix 1 volume native gel loading buffer (e.g. as in section on page 28) with 2–4 volumes of oligonucleotide.

   Load the entire sample(s) into the freshly-rinsed well(s) of the 12% polyacrylamide gel and run for about 20 min–1 hr at 100–300 volts until the bromophenol blue is approximately 2/3rd of the way to the bottom of the gel.

2. Isolate the gel fragment containing the siRNA or DNA oligonucleotide

   Nucleic acids can be visualized by UV shadowing. UV shadowing works best if the gel is removed from both glass plates and enclosed in thin plastic wrap. The wrapped gel should then be laid on a Fluor-coated TLC plate (Cat #AM10110). It may alternatively be possible to use an intensifying screen for visualizing the band, but it will be less sensitive than a Fluor-coated TLC plate.

   To see the nucleic acid, direct short wave UV light onto the gel surface in the dark. There must be at least ~400 ng of siRNA or oligonucleotide present in the band to use UV shadowing. Nucleic acids will appear as a dark purple or black band. The xylene cyanol and bromophenol blue bands will also be visible when the gel is illuminated with UV light; if the band of interest comigrates with 1 of these bands, it may be difficult to distinguish between nucleic acid and dye (consider running a lane of loading buffer alone in order to differentiate the dyes from the band of interest).

   The siRNA or correct oligonucleotide is usually the most intense band on the gel; it should be excised with a clean scalpel and transferred to about 350 µL of an RNase-free solution in an RNase-free microfuge tube. DEPC-treated water can be used for the elution, however, we recommend using 0.5 M ammonium acetate/ 1 mM EDTA/ 0.2% SDS (Probe Elution Buffer that is included in Life Technologies's family of nuclease protection assay kits). The EDTA and SDS will inactivate low levels of nuclease and the salt will precipitate the siRNA or oligonucleotide when 3 volumes of 100% ethanol is added.

3. Elution of siRNA or DNA oligonucleotides from acrylamide gel slices

   To elute the nucleic acids, incubate the gel slice in solution at 37°C overnight. Remove the gel slice from the elution solution and precipitate the siRNA or DNA oligonucleotide with 3 volumes of 100% ethanol. If the elution solution contained less than 300 mM Na^+ or NH_4^+ ions, then add 1/10th volume of 5 M ammonium acetate or sodium acetate to the elution solution before adding the ethanol. Incubate the precipitation reaction at –20°C for 15 min. Pellet the nucleic acids by centrifuging at 13,200 rpm for 15 min. Aspirate the ethanol and wash the pellet with 70% ethanol. Both siRNAs and DNA oligonucleotides can be dissolved in nuclease-free water and stored at –20°C until they are needed.

Assessing mammalian cells following siRNA transfection

Cells transfected with effective siRNAs exhibit a reduction in the amount of the targeted mRNA and the protein that it encodes. To assess whether siRNA-mediated gene silencing is occurring, levels of the target RNA or the target protein can be monitored. Life Technologies offers a comprehensive line of products for both RNA isolation and analysis. Please see section Appendix A, "Supplemental Information" on page 25 for a partial listing of Life Technologies products that can be used to assess gene silencing in cells transfected with siRNA made with this kit.

Additional recipes

10X TBE

TBE is generally used at 1X final concentration for preparing gels and/or for gel running buffer.

IMPORTANT! Do not treat TBE with diethylpyrocarbonate (DEPC).

Concentration	Component	for 1 L
0.9 M	Tris base	109 g
0.9 M	Boric Acid	55 g
20 mM	0.5 M EDTA solution	40 mL

Dissolve with stirring in about 850 mL nuclease-free water. Adjust the final volume to 1 L.

Alternatively, Life Technologies offers nuclease-free solutions of 10X TBE (Cat. nos. AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (Cat. no. AM9864). Both are made from of ultrapure molecular biology grade reagents.

2% agarose gel

- Melt 2 g of agarose in 90 mL of water to make 100 mL of gel solution. Typically this is done by putting the mixture in a flask and heating in a microwave. Swirl the mixture in the flask well as the agarose melts to obtain a uniform solution.
- Allow the gel mixture to cool to ~60°C, add 10 mL 10X TBE (for a final concentration of 1X), and swirl to mix well.
- Pour the gel solution into the mold, place the comb, and allow the gel to solidify.
- Place the gel in the gel tank and cover with 1X TBE, then remove the comb carefully.

12% acrylamide gel

For 15 mL, enough for a 13 cm x 15 cm x 0.75 mm thick gel:

Amount	Component
1.5 mL	10X TBE
4.5 mL	40% acrylamide (acrylamide: bis acrylamide = 19:1)
9 mL	water (double distilled, deionized)
Stir to mix, then add:	
150 µL	10% ammonium persulfate
15 µL	TEMED
Mix briefly after adding the last 2 ingredients, and pour gel immediately.	

Nondenaturing gel loading buffer

Amount	Component
50 %	sucrose
0.25 %	bromophenol blue
0.25 %	xylene cyanol

RNase-free water

a. Add DEPC to 0.05% to double-distilled, deionized water (i.e. add 0.5 mL per liter of water).

b. Stir well, incubate several hours to overnight at 37°C or 42°C.

c. Autoclave 2 L or smaller volumes for at least 45 min. The scent of DEPC should be either not detectable or only very slightly detectable.

Related products available from Life Technologies

RNase*Zap*® Cat. nos. AM9780, AM9782, AM9784	RNase Decontamination Solution. RNase*Zap*® is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*®.
Silencer® siRNAs	Ambion® *Silencer*® Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer*® siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer*® siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer*® siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects.
Silencer® siRNA Transfection II Kit Cat. no. AM1631	The *Silencer*® siRNA Transfection II Kit contains both siPORT™ *NeoFX*™ and siPORT™ *Lipid* Transfection Agents in addition to a well-characterized siRNA targeting human, mouse, and rat GAPDH. This kit is ideal for developing an optimal transfection protocol for your cells. Also included are a highly validated non-targeting negative control siRNA and a detailed Protocol.
Negative Control siRNA and Templates	Universal scrambled siRNA control sequences are available separately as either prepared and tested siRNA or templates for use in the *Silencer* siRNA Construction Kit. The scrambled controls have no significant homology to mouse, rat, or human gene sequences and are ideal for use as negative controls in any siRNA experiment.
Antibodies for siRNA Research	For select *Silencer*® Control and Validated siRNAs, Life Technologies offers corresponding antibodies for protein detection. These antibodies are ideal for confirming mRNA knockdown results by analyzing concomitant protein levels.
NorthernMax® Kits Cat. nos. AM1940, AM1946	Ambion® NorthernMax® Kits: NorthernMax®, and NorthernMax-Gly®, combine ultrasensitive, reliable Northern blot protocols with unsurpassed quality control to ensure optimal results in less time.
RPA III™ Cat. nos. AM1414, AM1415	Ribonuclease Protection Assay Kit for the detection and quantitation of mRNA. This kit incorporates Life Technologies exclusive one-tube format, for fast, sensitive detection of RNA with no proteinase K or phenol extraction steps.

B

Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:
Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.).
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/ 29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**

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