MEGAscript® RNAi Kit

Part Number AM1626

Instruction Manual

Manual 1626M Revision B Revision Date: October 2009

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I. Introduction

A. Background

The MEGAscript® RNAi Kit is a system for the preparation of double-stranded RNA (dsRNA), free of protein and other contaminating molecules, for use in RNA interference (RNAi) or other experiments. This kit is designed for the preparation of dsRNAs larger than ~200 bp. For preparation of small interfering dsRNA, known as siRNA (~20 bp long), we recommend the Ambion *Silencer*® siRNA Construction Kit, or Custom siRNA Synthesis Service.

The procedure begins with a high yield transcription reaction based on the Ambion MEGAscript technology, to synthesize two complementary RNA transcripts from template(s) supplied by the user. The RNA strands are hybridized either during or after the transcription reaction to form dsRNA. Next, DNA and any single-stranded RNA (ssRNA) are removed with a nuclease digestion. Finally, the dsRNA is purified with a a solid-phase adsorption system to remove protein as well as mono- and oligonucleotides. The dsRNA produced can be introduced into the organism of interest by a variety of means, including injection, electroporation, or chemically-mediated transfection, depending on the organism being studied.

RNAi, the phenomenon by which long dsRNAs specifically suppress expression of a target gene, was originally discovered in worms (Fire 1998), but this phenomenon has now been found in a large number of organisms, including flies (Misquitta and Paterson 1999), trypanosomes (Ngo 1998), planaria (Sánchez-Alvarado and Newmark 1999), hydra (Lohmann 1999), and zebrafish (Wargelius 1999). The RNAi mechanism is currently being investigated, but it appears to work through smaller dsRNA intermediates. The parent dsRNA is broken down into these smaller fragments in vivo, and this siRNA directs a post-transcriptional breakdown of the targeted mRNA (Zamore 2000). An unusual feature of this process is that it works non-stoichiometrically and can spread between cells (Fire 1998). RNAi is a powerful method to investigate gene function through suppression of gene expression.

B. Reagents Provided with the Kit and Storage Conditions

The kit contains the components listed in the following tables to synthesize 20 dsRNAs.

Amount	Component	Storage
10 mL	Nuclease-free Water	any temp*
20	Filter Cartridges	room temp
40	Collection Tubes	room temp
40 µL	T7 Enzyme Mix T7 RNA polymerase, RNase Inhibitor Protein, and other components in buffered 50% glycerol	–20°C
40 µL	10X T7 Reaction Buffer	–20°C
40 µL	ATP Solution (75 mM)	–20°C
40 µL	CTP Solution (75 mM)	–20°C
40 µL	GTP Solution (75 mM)	–20°C
40 µL	UTP Solution (75 mM)	–20°C
1 mL	10X Binding Buffer	–20°C
4 mL	Elution Solution	–20°C
40 µL	RNase	–20°C
45 µL	DNase I	–20°C
100 µL	10X Digestion Buffer	–20°C
12 mL	2X Wash Solution Add 12 mL 100% ethanol before use	–20°C
20 µL	Control Template (500 ng/µL)	–20°C

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

C. Materials Not Provided with the Kit

Gene-specific template(s)

A transcription template(s) is needed with T7 RNA polymerase promoters positioned to transcribe sense and antisense RNA corresponding to the target RNA. See section II starting on page 4 for a detailed discussion of template requirements and preparation.

For dsRNA purification

- 100% ethanol: ACS grade or better
- Equipment to draw solutions through the Filter Cartridges: Use either a ***microcentrifuge*** capable of at least 8,000 X g, or a ***vacuum manifold with sterile 5 mL syringe barrels*** mounted to support the Filter Cartridges.

To assess the reaction products

Reagents and equipment for agarose gel electrophoresis

Spectrophotometer

D. Related Products Available from Applied Biosystems

RNase*Zap*® Solution P/N AM9780 and AM9784	RNase Decontamination Solution. RNase*Zap* solution 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* solution.
High concentration SP6 and T7 RNA Polymerases P/N AM2075 and AM2085	Cloned, high purity RNA polymerases. These RNA polymerases are rigorously tested for superior performance, and for contaminants.
Electrophoresis Reagents See web or print catalog for P/Ns	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing; this product line is always growing. A list of Invitrogen products, including novel electrophoresis reagents and E-Gels, is also available at www.invitrogen.com.
Antibodies for siRNA Research See web or print catalog for P/Ns	For select *Silencer* Control and Validated siRNAs, Ambion offers corresponding antibodies for protein detection. These antibodies are ideal for confirming mRNA knockdown results by analyzing concomitant protein levels. A list of antibody products is also available at Invitrogen (www.invitrogen.com).

II. Preparation of Template DNA

A. Choosing the dsRNA Sequence

RNAi experiments are typically done with dsRNA 400 bp and larger; 200 bp is the minimum size of dsRNA recommended for RNAi. Typically templates for transcription of dsRNA for use in RNAi correspond to most or all of the target message sequence.

B. Strategies for Transcription of dsRNA

RNAi experiments requiredouble-stranded RNA (dsRNA). Since the T7 RNA polymerase used in this kit synthesizes single-stranded RNA (ssRNA), use one of the following strategies to produce dsRNA:

- Prepare one DNA template with opposing T7 promoters at the 5' ends of each strand, and use it in a single transcription reaction to synthesize dsRNA without a separate annealing step.
- Use two DNA templates that are identical except that a single T7 promoter sits at opposite ends of the region to be transcribed. With this strategy, the templates can both be added to a single reaction. Although both templates should be transcribed at the same rate, if they are not, the final dsRNA yield will be dictated by the template with the lower transcription efficiency.

  Alternatively, the two templates can be transcribed in separate reactions to make complementary RNA molecules, which are then mixed and annealed. For the annealing step, the two transcripts can be mixed in precisely equimolar amounts. Note, however, that if the templates are transcribed in separate transcription reactions, this kit contains enough reagents to produce only 10 different dsRNAs.

Figure 1. T7 Polymerase Promoter: Minimal Sequence Requirement

The +1 base (in bold) is the first base incorporated into RNA. The underline shows the minimum promoter sequence needed for efficient transcription.

C. PCR Templates

Amplification strategies to add T7 promoter sequences to DNA

T7 promoter sequences can be added to DNA using PCR to generate templates that can be directly added to the MEGAscript RNAi Kit transcription reaction. Begin by synthesizing PCR primers with the T7 promoter sequence appended to the 5' end of the primer. The T7 promoter-containing PCR primers (sense and antisense) can either be used in separate PCRs, or in a single PCR to generate transcription template for both strands of the dsRNA. The two strategies for adding T7 promoter to DNA are shown below:

Two separate PCRs with a single T7 promoter-containing PCR primer in each	A single PCR with the T7 promoter appended to both PCR primers
	
• Typically the yield of PCR product is higher with this strategy than if both primers include a T7 promoter. • This strategy requires 4 PCR primers and 2 PCRs. • After transcription, the RNA products from each reaction will require a separate annealing step to make dsRNA.	• Yield may be lower than when only one primer includes a T7 promoter. • Only 2 PCR primers and a single PCR are needed to make template for the dsRNA. • If the RNA products are ≤800 nt, an annealing step will not be needed after the transcription reaction; dsRNA will form during the transcription reaction.

PCR amplification profile suggestions

- Calculate the annealing temperatures of the entire PCR primer (with the T7 promoter site) and the gene specific portion of the PCR primer separately.
- Since the first cycles of PCR use only the 3' half of the PCR primer(s), the gene-specific part, the annealing temperature for the first 5 PCR cycles should be ~5°C higher than the calculated T_m for the gene-specific region of the primer. We have found that using the calculated annealing temperature for the initial cycles often results in synthesis of spurious PCR products.
- Once some PCR product is made, subsequent primer annealing events (cycle 6 and thereafter) use the entire primer site; therefore use the calculated T_m for the ***entire*** PCR primer plus ~5°C for subsequent cycles.
- We recommend using primers at 100 nM in the PCR mix. Higher concentrations may result in synthesis of primer dimers.

Check PCR products on a gel before using them in this procedure

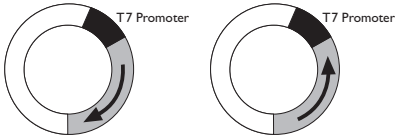
PCR products should be examined on an agarose gel prior to in vitro transcription to estimate concentration and to verify that the products are unique and of the expected size.

D. Plasmid Templates

Cloning strategy

When using plasmid templates in the MEGAscript RNAi Kit, it is best to make two separate clones with the same target region in both orientations.

Figure 2. Cloning in plasmids



NOTE

Although some dsRNA templates have been cloned in plasmids containing opposing T7 promoters (on either side of the linker region Kamath 2001, Timmons and Fire 1998, Morris 2001), these constructs will result in synthesis of dsRNA with significant stretches of RNA corresponding to the polylinker region of the plasmid.

PCR products can be cloned into plasmid vectors using any of the following strategies:

- Amplify the target by PCR and ligate the product into a PCR vector with a T7 promoter. Identify plasmids with the insert in both orientations with regard to the T7 promoter.
- Or, include the T7 promoter sequence at the 5' end of one or both of the PCR primers, then perform PCR to incorporate them into the fragment. Finally, ligate the PCR product into a PCR cloning vector (one that does not include a T7 promoter).
- Or, include both a T7 promoter and a restriction site at the 5' end of one or both PCR primers to incorporate them into the fragment during PCR. Ligate the PCR product into a cloning vector using the added restriction sites.

Plasmid linearization

Plasmid templates must be linearized downstream of the insert to create a transcription termination site—the RNA polymerase will literally fall off the end of the DNA molecule. Linearize each template, then examine the DNA on a gel to confirm that cleavage is complete. Since initiation of transcription is the rate limiting step of in vitro transcription reactions, even a small amount of circular plasmid in a template prep will generate a large proportion of transcript, wasting much of the synthetic capacity of the reaction.

NOTE

We routinely use all types of restriction enzymes. However, there has been one report of low level transcription from the inappropriate template strand in plasmids cut with restriction enzymes leaving 3' overhanging ends (produced by Kpn I, Pst I, etc.; Schenborn and Mierindorf, 1985).

Figure 3. Linearized plasmids

After linearization

Terminate the restriction digest by adding each of the following:

- 1/20 volume 0.5 M EDTA
- 1/10 volume of either 3 M NaOAc ***or*** 5 M NH_4OAc
- 2 volumes of ethanol

Mix well and chill at –20°C for at least 15 min. Then pellet the DNA for 15 min in a microcentrifuge at top speed. Remove the supernatant, respin the tube for a few seconds, and remove the residual fluid with a very fine-tipped pipet. Resuspend in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) at a concentration of 0.5–1 µg/µL.

Plasmid DNA purity

DNA should be relatively free of contaminating proteins and RNA. The greatest yields of dsRNA will be obtained with very clean template preparations. Most commercially available plasmid preparation systems yield DNA that works well in the MEGAscript RNAi Kit. Alternatively, a DNA miniprep procedure that generally yields high quality template is presented in section V.D on page 22.

Note that DNA from some miniprep procedures may be contaminated with residual RNase A. Also, restriction enzymes occasionally introduce RNase or other inhibitors of transcription. When transcription from a template is suboptimal, it is often helpful to treat the template DNA with proteinase K before performing the transcription reaction (section *IV. Proteinase K treatment* on page 16).

III. MEGAscript RNAi Kit Procedure

A. Before Using the Kit for the First Time

Prepare the Wash Solution

1. Add 12 mL ACS grade 100% ethanol to the bottle labeled 2X Wash Solution.
2. Mix well, and store at room temperature.

We suggest crossing out the 2X from the bottle label after adding the ethanol. In these instructions this reagent will be called ***Wash Solution*** once the ethanol is added.

B. Transcription Reaction Assembly

1. Thaw the frozen reagents at room temp then place them in ice

Remove the T7 Enzyme Mix from the freezer and place it directly in ice; it is stored in glycerol and will not freeze at –20°C.

Vortex the 10X T7 Reaction Buffer and the 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP) until they are completely in solution. Once they are thawed, store the ribonucleotides (ATP, CTP, GTP, and UTP) on ice, but ***keep the 10X Reaction Buffer at room temperature***.

Microcentrifuge all reagents briefly before opening to prevent loss and/or contamination of any material on the rim of the tube.

2. Assemble transcription reaction at room temperature

Assemble the transcription reaction at room temperature in the order shown below. The following amounts are for a single 20 µL transcription reaction. Reactions may be scaled up or down if desired.

IMPORTANT

The following reaction setup is recommended when the RNA produced will be ≥400 nt in length. For transcripts shorter than this, see section V.C. Optimizing Yield of Short Transcripts on page 21 for modified reaction setup suggestions.

Amount	Component
to 20 µL	Nuclease-free Water
1–2 µg	Linear template DNA Use either 1 µg of a template with opposing T7 promoters flanking the transcription region, or use a mixture of 1 µg of each template when the T7 promoter template is on separate molecules.

Amount	Component
2 µL	10X T7 Reaction Buffer
2 µL	ATP Solution
2 µL	CTP Solution
2 µL	GTP Solution
2 µL	UTP Solution
2 µL	T7 Enzyme Mix

3. Mix thoroughly

Gently flick the tube or pipette the mixture up and down, then briefly microcentrifuge to collect the reaction mixture at the bottom of the tube.

4. Incubate at 37°C for 2–4 hr

The first time a new template is transcribed, the recommended incubation time is 2–4 hr. The optimal incubation time for a given template varies depending on its size and transcriptional efficiency.

For transcripts <400 nt, a longer incubation time (up to ~16 hr) may be advantageous, since more transcription initiation events are required to synthesize a given mass amount of short RNA, compared to transcription of longer templates. Instead of doing overnight incubations, we suggest freezing the reaction overnight and then thawing and resuming the reaction the next day. Reactions are stable for several days when frozen at –20°C; however, storage will result in gradual loss of enzyme activity.

To determine the optimum incubation time for maximum yield with a given template, a time-course experiment can be done. To do this, set up a transcription reaction, and remove aliquots of the reaction at various intervals (for example after 2 hr, 4 hr, 6 hr, and overnight incubation). Assess results by looking for a decrease in absorbance at 260 nm as the free nucleotides are incorporated into RNA, or by running 0.5 µL samples of the RNA on a gel stained with ethidium bromide (see instructions in section V.B on page 19).

C. Annealing RNA to Maximize Duplex Yield

Include this annealing step for the following types of reactions:

- All >800 nt dsRNA synthesis reactions
- ≤800 nt dsRNA synthesis reactions when the two strands were synthesized from separate transcription templates (in the same or in separate transcription reactions).

Annealing the complementary RNA is often unnecessary for transcripts ≤800 nt made from a single template with opposing T7 promoters because RNA products in this size range will typically hybridize during the transcription reaction. With transcripts >800 nt, however, at least a portion of the transcripts form aggregates (presumably branched structures) rather than the dsRNA.

1. Mix the transcription reactions containing complementary RNA

- If sense and antisense RNA were synthesized in separate transcription reactions, add the entire contents of one of the reactions to the other.
  If desired, reserve a 0.5 µL aliquot of each template before mixing for gel analysis.
- If sense and antisense RNA was synthesized in a single transcription reaction, both strands of RNA will already be in a single tube; proceed to step 2.

2. Incubate at 75°C for 5 min, then cool to room temperature

Incubate at 75°C for 5 min then leave the mixture on the bench to cool to room temperature. The RNA will anneal as it cools, forming dsRNA. ***Do not put the reaction on ice to cool.***

3. Check 1/400th of the dsRNA on an agarose gel

Run 1/400th of the dsRNA on a 1% agarose gel (nondenaturing) to examine the integrity and efficiency of duplex formation.

- 1/400th of a 20 µL dsRNA solution is 5 µL of a 1:100 dilution.
- Dilute the gel samples in TE (10 mM Tris, 1 mM EDTA) or in gel loading buffer.

(Instructions for running a gel are in section V.B on page 19.) The dsRNA will migrate slightly slower than DNA markers of the same length. See Figure 4 on page 15 for an example of how the dsRNA reaction products will look on a gel.

D. Nuclease Digestion to Remove DNA and ssRNA

This DNase/RNase treatment digests template DNA and any ssRNA that did not anneal. RNase will not degrade dsRNA when using the reaction conditions specified below.

1. Assemble RNase digestion reaction on ice

The amounts shown are for a 20 µL transcription reaction; scale up if your transcription reaction was larger.

Amount	Component
20 µL	dsRNA (from step B.4 or step C.2)
21 µL	Nuclease-free Water
5 µL	10X Digestion Buffer
2 µL	DNase I
2 µL	RNase

2. Incubate at 37°C for 1 hr

The ssRNA will be digested after 15 min but allow the incubation to proceed for 1 hr to completely digest the DNA template.

Do ***not*** continue this incubation longer than 2 hr.

E. Purification of dsRNA

This purification removes proteins, free nucleotides, and nucleic acid degradation products from the dsRNA.


NOTE

For the quickest dsRNA purification, preheat the Elution Solution to ~95°C before starting the purification procedure.

1. Assemble the dsRNA binding mix

Assemble the dsRNA binding mix by adding 10X Binding Buffer, water, and 100% ethanol to the dsRNA according to the table below.

Amount	Component
50 µL	dsRNA (from step D.2 above)
50 µL	10X Binding Buffer
150 µL	Nuclease-free Water
250 µL	100% Ethanol

Gently mix the reaction by pipetting up and down.

2. Apply binding mix to the Filter Cartridge, and draw it through

Pipet the entire 500 µL dsRNA binding mix onto the filter in the Filter Cartridge, and draw it through by centrifugation or with a vacuum manifold.

Centrifuge users:

a. For each dsRNA sample, place a Filter Cartridge in a Collection Tube. Use the Collection Tubes supplied with the kit.

b. Pipet the entire 500 µL dsRNA mixture onto the filter in the Filter Cartridge. Centrifuge at maximum speed for 2 min.

c. Discard the flow-through and replace the Filter Cartridge in the Collection Tube.

Vacuum manifold users:

a. For each dsRNA sample, place a 5 mL syringe barrel on the vacuum manifold, load it with a Filter Cartridge, and turn on the vacuum.

b. Pipet the entire 500 µL dsRNA mixture onto the filter in the Filter Cartridge. The vacuum will draw the lysate through the filter.

3. Wash the Filter Cartridge with 2 X 500 µL Wash Solution

IMPORTANT

Verify that 12 mL of 100% ethanol was added to the 2X Wash Solution.

a. Pipet 500 µL of Wash Solution onto the filter in the Filter Cartridge. Draw the wash solution through the filter as in the previous step.

b. Repeat with a second 500 µL of Wash Solution.

c. After discarding the Wash Solution, continue centrifugation, or leave on the vacuum manifold for ~10–30 sec to remove the last traces of liquid.

4. Recover the dsRNA 2 X 50–100 µL Elution Solution

a. *The Elution Solution provided with the kit is 10 mM Tris-HCl pH 7, 1 mM EDTA. It is compatible with dsRNA injection, or 2X Injection Buffer can be added to the purified dsRNA for a final concentration of 1X Injection Buffer. Alternatively, the dsRNA can be eluted into any sterile low salt solution (≤30 mM), e.g. 5 mM KCl, 0.1 mM sodium phosphate buffer as used by Rubin and Spradling (1982).*Transfer the Filter Cartridge to a fresh Collection Tube.

b. Apply 50–100 µL (hot) Elution Solution to the filter in the Filter Cartridge.
   - Apply preheated (≥95°C) Elution Solution to the filter, ***or***
   - Apply room temperature Elution Solution, close the tube lid over the Filter Cartridge, and incubate in a heat block set to 65°C or warmer for 2 min.

c. Centrifuge for 2 min at maximum speed.

d. Repeat steps b–c with a second 50–100 µL aliquot of Elution Solution collecting the RNA into the same Collection Tube.
Most of the RNA will be eluted in the first elution. The second elution is included to recover any remaining RNA.

5. Quantitate and store the dsRNA

Quantitate the reaction product by measuring its absorbance at 260 nm and calculating the concentration (see section *V.A. Quantitation of RNA by Spectrophotometry* on page 19).

The dsRNA is stable when stored at –20°C in Elution Solution.

6. Check 1/400th of the purified dsRNA on an agarose gel

Run 1/400th of the dsRNA on a 1% agarose gel (nondenaturing) to examine the integrity and efficiency of duplex formation.

- 1/400th of 100 µL elution volume is 2.5 µL of a 1:10 dilution
- 1/400th of 200 µL elution volume is 5 µL of a 1:10 dilution
- Dilute the gel samples in TE (10 mM Tris, 1 mM EDTA) or in gel loading buffer

(Instructions for running the gel are in section V.B on page 19). The dsRNA will migrate slightly slower than DNA markers of the same length. See Figure 4 on page 15 for an example of how the dsRNA reaction products will look on a gel.

IV. Troubleshooting

A. Use of the Control Template

The Control Template is a linear dsDNA fragment with opposing T7 promoters. It yields a 500 bp dsRNA product.

Positive control reaction instructions

1. Use 2 µL (1 µg) of Control Template in standard MEGAscript RNAi Kit reactions as described in section III.B starting on page 8.
2. Incubate the transcription reaction (step III.B.4 on page 9) for 2 hr.
3. Skip section *III.C. Annealing RNA to Maximize Duplex Yield* on page 10 as the sense and antisense RNA strands will hybridize during the transcription reaction.
4. Follow the procedure as described in sections III.D and III.E starting on page 11 to purify the dsRNA.
5. (Optional) Run 1–2 µg of the positive control reaction product on a 1% agarose gel to verify that the dsRNA is 500 bp.

Expected yield from the control reaction

The yield of RNA from the positive control reaction should be 50–80 µg of a 500 bp dsRNA.

What to do if the positive control reaction doesn't work as expected

If the yield of RNA from the control reaction is low, something is probably wrong with the procedure, the kit, or the quantitation.

1. Double check the RNA quantitation
   - When assessing yield by UV spectrophotometry, be sure to use TE (10 mM Tris-HCl, 1 mM EDTA) to blank the spectrophotometer and dilute the RNA.
   - To confirm that the quantitation is correct, verify the yield by an independent method. For example if UV spectrophotometry was used to assess yield, try also running an aliquot of the reaction on an agarose gel and comparing its intensity to a sample of known concentration.
2. Consider repeating the positive control reaction
   If the yield is indeed low by two different measurements, there may be a technical problem with the way the kit is being used. You may want to consider repeating the positive control reaction. If you are certain that the procedure was carried out correctly, and the control reaction does not give the expected results, contact Ambion's Technical Services.

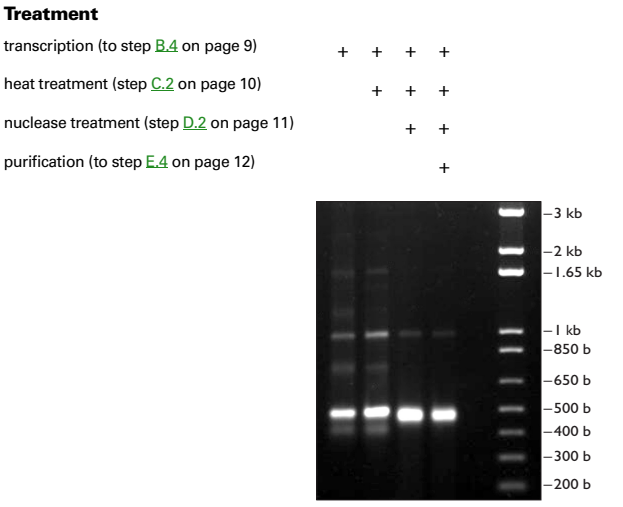


Figure 4. Positive Control Reaction

Samples (1/400th of the eluted dsRNA) were taken from the positive control reaction as indicated and run on a 1% agarose gel stained with ethidium bromide. The ~1 kb band visible in the picture is seen occasionally, and doesn't indicate problems with RNAi synthesis. Since it is precisely two-fold the size of the expected product, it probably represents persistent secondary structure or reinitiation of transcription without transcript release.

B. Low Yield

The amount of RNA synthesized in a standard 20 µL transcription reaction should be 50 µg or more; however, there is a great deal of variation in yield among different templates. If the yield is low, the first step in troubleshooting the reaction is to use the Control Template in a standard MEGAscript RNAi Kit transcription reaction (section IV.A on page 14) to determine if the problem is with the template, the reagents, or procedure.

Neither my template nor the control reaction works

If the positive control does not work, it could be an indication that something is wrong with the kit; call Ambion's Technical Support group for more troubleshooting help.

The control reaction works, but my template gives low yield

If the transcription reaction with your template generates full-length, intact RNA, but the reaction yield is significantly lower than the amount of RNA obtained with the Control Template, it is possible that contaminants in the DNA are inhibiting the RNA polymerase. A mixing experiment can help to differentiate between problems caused by

inhibitors of transcription and problems caused by the sequence of a template. Include three reactions in the mixing experiment using the following DNA templates:

1. 2 µL Control Template
2. 1–2 µg experimental DNA template
3. a mixture of 1 and 2

Assess the results of the mixing experiment by running 0.5 µL of the transcription reaction on an agarose gel as described in section V.B on page 19.

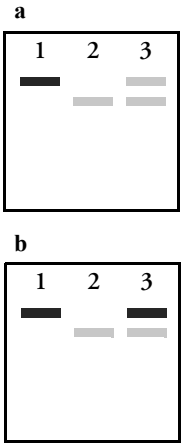


Figure 5. Possible outcomes of mixing experiment

1 – Control Template
2 – experimental DNA template
3 – mixture of 1 and 2

a. Transcription of the Control Template is inhibited by your template. (See Figure 6.a)

This implies that inhibitors are present in your DNA template. Typical inhibitors include residual SDS, salts, EDTA, and RNases. Proteinase K treatment followed by phenol:chloroform extraction frequently improves template quality.

Proteinase K treatment

Treat template DNA with Proteinase K (100–200 µg/mL) and SDS (0.5%) for 30 min at 50°C, followed by phenol/chloroform extraction and ethanol precipitation. Carry-over of SDS can be minimized by diluting the nucleic acid several fold before ethanol precipitation, and excess salts and EDTA can be removed by vigorously rinsing nucleic acid pellets with 70% ethanol before resuspension.

b. Adding your template to the reaction with the Control Template does not inhibit synthesis of the control RNA.

(See Figure 6.b.) This indicates that the problem may be inherent to your template.

- Check the amount and quality of template

  Template quantitation may be inaccurate. If quantitation was based on UV absorbance and the DNA prep had substantial amounts of RNA or chromosomal DNA, the amount of template DNA may be substantially less than the calculated value.

  Also, check an aliquot of the template DNA on an agarose gel to make sure it is intact and that it is the expected size. If there is even a small amount of circular template in the transcription reaction it will reduce the yield of dsRNA (see section *Plasmid linearization* on page 6).

- Extend the reaction time

  Another parameter that can be adjusted is reaction time. Extending the standard 2–4 hr incubation to 6–10 hr, or even overnight, may improve yield.

- To transcribe <400 nt RNA, it may be helpful to increase the RNA polymerase concentration

See section V.C.3 on page 22 for more information.

- Change your priming region

  Some sequences are simply inefficient transcription templates. If you get low RNA yields, even after checking the template and trying overnight incubation of the transcription reaction, it may be necessary to prepare a transcription template from a different region of the gene. Often simply moving the transcription start point can overcome problems with inefficient transcription; typically there are several regions of the gene that will transcribe with equal efficiency. Also, transcription efficiency may be higher when the transcription template contains 2–3 bases of purines immediately following the GGG sequence at positions +1 to +3 in the T7 promoter sequence (T7 promoter sequence is shown in Figure on page 4).

- Use 2 separate templates

  Sometimes the template will simply not transcribe well with opposing promoters. In this case, the two strands of RNA need to be made from separate templates and annealed after synthesis (see section II.B on page 4).

C. Multiple Reaction Products, Transcripts of the Wrong Size

Reaction products produce a smear when run on a gel

This problem is usually seen with single-strand transcriptions. If the RNA appears degraded (e.g. smeared), remove residual RNase from the DNA template preparation before in vitro transcription. Do this by digesting the DNA prep with proteinase K (section *IV. Proteinase K treatment* on page 16). The RNase Inhibitor in the transcription reaction can only inactivate moderate RNase contamination. Large amounts of RNase in the DNA template will compromise the size and amount of transcription products.

Reaction products run as more than one band, or as a single band smaller than expected

Premature termination of transcription

If gel analysis shows multiple discrete bands or a single band smaller than the expected size, there may be problems with premature termination by the polymerase.

Even if transcription of only one of the strands was prematurely terminated, the single-stranded portion of the duplex will be digested during the nuclease treatment, resulting in a shorter than expected dsRNA.

- Possible causes of premature termination are sequences which resemble the phage polymerase termination signals, stretches of a single nucleotides, and GC-rich templates.

- Termination at single polynucleotide stretches can sometimes be alleviated by decreasing the transcription reaction temperature (Krieg 1990). We suggest testing incubations at 30°C, 20°C and 10°C, but be sure to increase the reaction time to offset the decrease in yield caused by incubation at suboptimal temperatures.
- There is a report that single-stranded binding (SSB) protein increased the transcription efficiency of a GC rich template (Aziz and Soreq, 1990).

Reaction products are larger than expected

Products occasionally run as two bands after the nuclease digestion and dsRNA purification; one at the expected size, and one that is double the expected size. If this occurs, check the size of the transcription template on a gel to verify that it is pure and sized correctly. dsRNA that contains a double-sized band can be used for RNAi with no problems, in fact double-sized bands are sometimes seen from the Control Template (see Figure 4 on page 15).

Multi-strand aggregates are present in the mixture

Larger than expected bands or ethidium bromide staining in the wells could be seen as a result of aggregates of multiple RNA strands. These can be denatured by heating the solution to 75–100°C for ~3 min, then allowing it to cool to room temperature. Be sure that RNA is in a solution containing at least 1 mM EDTA (such as the Elution Solution supplied with the kit) for the heat treatment.

V. Additional Procedures

A. Quantitation of RNA by Spectrophotometry

The concentration of dsRNA can be determined by diluting an aliquot of the preparation (usually a 1:10 to 1:25 dilution) in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and reading the absorbance in a spectrophotometer at 260 nm. The concentration of RNA in µg/mL can be calculated as follows:

1 A_{260} = 40 µg RNA/mL

so, A_{260} x dilution factor x 40 = µg/mL RNA

B. Analysis of dsRNA by Agarose Gel Electrophoresis

Ideally there will be a single, tight band at the expected molecular weight. See section IV.C on page 18 for troubleshooting suggestions if this is not what appears on your gel.

Solutions for Agarose Gel Electrophoresis

10X TBE

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

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, Ambion offers 10X TBE as a ready-to-resuspend mixture of ultrapure molecular biology grade reagents (Ambion Cat #9863). Each packet makes 1 L of 10X TBE.

6X non-denaturing gel loading buffer

Concentration	Component	for 10 mL
37 %	glycerol (100%)	3.7 mL
0.025 %	bromophenol blue	2.5 mg
0.025 %	xylene cyanol	2.5 mg
20 mM	1 M Tris-HCl, pH 8	200 µL
5 mM	500 mM EDTA	100 µL
	nuclease-free water	to 10 mL

Alternatively, Ambion offers an all-purpose Gel Loading Solution for native agarose gels: Cat #8556. This 10X solution is rigorously tested for nuclease contamination and functionality.

Pouring and running agarose gels

Staining with ethidium bromide

There are several different ways to stain agarose gels. They are all equivalent in terms of efficacy, but no two researchers can agree which is the most convenient.

- Add 0.5 µg/mL ethidium bromide to the gel mix, the running buffer, or both.
- Add 10 µg/mL ethidium bromide to the gel loading buffer. All the samples should have the same amount of ethidium bromide, because it will affect the electrophoretic mobility of nucleic acids.

Gel mix

i. Weigh 1 g agarose per 100 mL of gel needed.

ii. Pour 1X TBE into a flask, using the volume of gel desired, in a flask with a capacity 5–10 fold the gel volume.

iii. Add the measured agarose to the flask with the TBE and heat with intermittent swirling until the agarose is completely melted.

iv. Leave at room temp to cool to ~65°C.

Pouring the gel

i. While the gel is cooling, prepare the gel mold. (Gel running and pouring devices are all quite different, so follow the manufacturer's instructions for set-up.)

ii. When the agarose has cooled so that touching the flask is tolerable, the agarose can be slowly poured into the mold.

iii. Place the comb, and pop any bubbles that have formed with a clean pipet tip, or a heated needle.

Preparing samples and running the gel

i. Put 0.5 µL of each sample into a fresh tube, and add gel loading buffer to samples for a final concentration of 1X. Flick or vortex the tubes to mix.

ii. Place the gel in the electrophoresis chamber and fill it with running buffer (1X TBE) to cover the gel completely. Remove the comb from the gel carefully.

iii. Check that the wells are intact and free of debris, and carefully load the samples into separate wells. It is convenient to load a molecular weight marker to identify bands after the electrophoresis.

iv. Attach the electric leads, and turn on the power. Typically, native agarose gels are run at 3.5–5.5 volts/cm (measured between the electrodes). Stop the electrophoresis when the bromophenol blue (the faster-migrating dye) has moved 1/2 to 2/3 of the length of the gel.

v. View the gel on a UV transilluminator.

C. Optimizing Yield of Short Transcripts

The MEGAscript RNAi Kit has been designed to function best with transcription templates larger than about 400 bases. Under these conditions, 1 µg of plasmid DNA template per 20 µL reaction results in maximal yields. Increasing the incubation time, template or polymerase concentration does not generally increase the yield of the reaction. However, with smaller templates, these parameters may require adjustment to maximize reaction yields. Several types of small-transcript templates (<400 bases) can be used in MEGAscript RNAi Kit transcription reactions. These include standard plasmid vectors containing small inserts, PCR products, and synthetic oligonucleotides which can either be entirely double- stranded or mostly single-stranded with a double-stranded promoter sequence (Milligan 1987). In the case of synthetic oligonucleotide, and PCR-derived templates, almost all of the DNA is template sequence, compared to plasmid templates which have non-transcribed vector DNA present.

1. Increase the reaction time

Increasing the incubation time is the easiest variable to change and should be tried first. Try increasing the incubation time to 4 or 6 hr. This allows each RNA polymerase molecule to engage in a greater number of initiation events.

2. Increase the template concentration

Increasing the template concentration is the next variable that should be tested. This can be helpful because, with short templates, the initiation step of the transcription reaction is rate-limiting. Remember that 1 µg of a short template contains a much larger molar amount of DNA than 1 µg of a longer template. For instance, 50 ng of an 85 bp template provides 0.9 pmoles of template (and 0.9 pmoles of promoters) in the reaction, compared to approximately 0.3 pmoles provided by 1 µg of the control template. In general, for optimum yield of short transcripts, use about 0.5–2 pmoles of template. For very short templates (in the 20–30 base range), use the upper end of this range. If the short template is contained in a plasmid, it may not be possible to add the optimum molar amount. For example, 2 pmoles of template consisting of a 30 bp insert in a 2.8 kb vector would require 4 µg of plasmid DNA. Such large mass amounts of DNA may be detrimental. Thus, it is better to either remove the template from the vector, or to do the transcription reaction under conditions of sub-optimal template concentration.

3. Increase the RNA polymerase concentration

The concentration of RNA polymerase in the kit is optimal for transcription of templates ≥400 nt, templates coding much smaller transcripts may benefit from adding additional RNA polymerase. Adding 200 units more polymerase may increase yields with very short templates by allowing more initiation events to occur in a given amount of time. We suggest adding high concentration polymerase (e.g., Ambion P/N AM2075 and AM2085), not the 10X Enzyme Mix from the MEGAscript RNAi Kit. Increasing the enzyme should be the last variable tested after increasing incubation time and optimizing template concentration.

D. Miniprep for Isolating Transcription-quality Plasmid DNA

Generally, the cleaner the template DNA, the greater the yield of the transcription reaction. The following miniprep protocol yields high quality transcription template. This protocol is derived from a published procedure (Molecular Cloning, A Laboratory Manual), but differs in that the phenol/chloroform extraction is done after linearization of the plasmid with restriction enzyme(s), and proteinase K treatment (Step 9). In this way, any possible ribonuclease contamination from the restriction enzyme is eliminated without an additional proteinase K or phenol/chloroform extraction step. If you have difficulty getting good restriction digestion of your plasmid prep, it may be necessary to include a phenol/chloroform extraction before the ethanol precipitation at Step 5.

Solution I

Amount	Component
50 mM	glucose
10 mM	EDTA, pH 8
25 mM	Tris-HCl, pH 8

Autoclave for 15 min. Store at 4°C in small aliquots.

Solution II (make fresh)

Amount	Component
0.2 N	NaOH
1 %	SDS

Solution III

for 100 mL	Component
60 mL	5 M potassium acetate
11.5 mL	glacial acetic acid
28.5 mL	water (distilled deionized)

Store at room temperature.

1. Pellet cells

Centrifuge a 1.5 mL bacterial culture (grown overnight) for about 30 sec; pour off supernatant, respin briefly (about 5 sec), and remove residual supernatant via aspiration.

2. Resuspend pellet in 110 µL Solution I, vortex

Add 110 µL of Solution 1 and vortex vigorously to resuspend the pellet. Check for complete resuspension of pellet by inverting the tube and confirming that the solution is homogenous.

3. Add 220 µL Solution II, incubate 1 min on ice

Add 220 µL of Solution II and invert the tube several times to mix. Incubate the tube on ice for at least 1 min.

4. Add 165 µL Solution III, incubate 5 min on ice, centrifuge 5 min

Add 165 µL of Solution III and vortex medium-fast for 10 sec. Incubate the tube on ice for 5 min.

Centrifuge for 5 min at maximum speed; this spin should be done at 4°C if possible. Most of the proteins, genomic DNA, and other cellular components will pellet during this spin.

5. Add supernatant to a fresh tube with 1 mL ethanol, incubate 5 min on ice, centrifuge 5 min

Add the supernatant to a fresh tube containing 1 mL of 100% ethanol, and invert several times to mix. Incubate the mixture for 5 min on ice to precipitate the plasmid DNA and some of the RNA.

Centrifuge for 5 min at maximum speed at 4°C if possible. This will pellet the plasmid DNA. Pour off the supernatant, centrifuge briefly, and aspirate off any residual supernatant.

6. Resuspend in ~50 µL TE containing RNase, incubate 5 min at 37°C

Resuspend the DNA pellet in ~50 µL TE (10 mM Tris HCl, pH 8 and 1 mM EDTA).

Add 0.5 U or 1 µg RNase A or use 1 µL of Ambion's RNase Cocktail. Vortex vigorously, incubate about 5 min at 37–42°C, and vortex again to thoroughly solubilize the pellet.

7. Digest with appropriate restriction enzyme

Digest with an enzyme that will linearize the plasmid so that the promoter site will be upstream of the sequence you want to transcribe. The volume of the restriction digest should be about 2–3 times the volume of plasmid DNA used. Follow the recommendations of the restriction enzyme supplier for buffer composition, units of enzyme to use, and incubation conditions.

8. Treat with Proteinase K and SDS

Add SDS to a final concentration of 0.5% (usually a 10 to 20% SDS stock solution is used). Add 50–100 µg/mL Proteinase K (final concentration). Mix well by inversion, and incubate at 50°C for at least 30 min.

9. Phenol/chloroform extract and ethanol precipitate

Add an equal volume of phenol/chloroform or phenol/chloroform/IAA, vortex vigorously, centrifuge ~1 min at room temp.

Remove the aqueous (top) phase to fresh tube, add 1/10 volume of 5 M ammonium acetate (RNase-free), add 2 volumes ethanol, and incubate at least 15 min at –20°C.

10. Pellet DNA

Pellet the DNA by centrifuging at top speed for 15 min. After the spin, discard the supernatant, re-spin briefly and remove any residual supernatant.

Resuspend the DNA in 10–20 µL nuclease-free water per 1.5 mL culture. Vortex until the pellet has completely dissolved.

11. Gel analysis

Assess the DNA by running an aliquot on an agarose gel in the presence of ethidium bromide. Estimate the concentration of the DNA by comparison to a known quantity of similar-sized DNA run on the same gel.

VI. Appendix

A. References

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B. Quality Control

Functional Testing

The Control Template (1 µg) is used in a MEGAscript RNAi Kit reaction as described in section IV.A on page 14, and is shown to produce ≥50 µg of ~500 bp dsRNA.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chem-

ical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com), telephone (650-554-2756; USA), or fax (650-554-2252; USA) your request, specifying the catalog or part number(s) and the name of the product(s). The associated MSDSs will be e-mailed unless you request fax or postal delivery. Requests for postal delivery require 1 to 2 weeks for processing.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.