MEGAshortscript™ Kit

(Part Number AM1354)

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I. Introduction

A. Background

The MEGAshortscript™ High Yield Transcription Kit is designed for high yields of in vitro transcribed RNA products, in the 20–500 nucleotide range. Using conventional methods, transcription of small templates often results in low mass yields of RNA; short transcripts require many more transcription initiation events to synthesize a given mass of product; since the initiation step is the rate-limiting step in transcription reactions, the yield of product from short templates is often low. The MEGAshortscript Kit has been designed to overcome the drawbacks involved in conventional in vitro transcription to maximize the yield of short RNA transcripts.

Each kit contains reagents for 25 transcription reactions (20 µL each). each reaction will yield at least 100 µg of RNA using the pTRI-RNA 18S control template supplied with the kit in a 4 hr incubation. The MEGAshortscript Kit is compatible with user supplied DNA templates made from linearized plasmid, synthetic oligonucleotides, or PCR products containing a T7 promoter.

T7 RNA polymerase will effectively incorporate many modified nucleotides, and can be used to synthesize fluorescein-, biotin-, and digoxigenin-labeled RNA. Because of the high nucleotide concentrations in MEGAshortscript reactions, this kit is not recommended for synthesis of high specific-activity radiolabeled probes. MEGAshortscript Kit can be used to synthesize larger transcripts, but the Ambion MEGAscript® Kit is more cost effective for this purpose.

B. Reagents Provided with the Kit and Storage

This kit provides reagents for 25 in vitro transcription reactions. The kit should be stored in a non-frost-free freezer.

Amount	Component	Storage
50 µL	T7 Enzyme Mix	–20°C
50 µL	T7 10X Reaction Buffer	–20°C
50 µL	T7 ATP Solution (75 mM)	–20°C
50 µL	T7 CTP Solution (75 mM)	–20°C
50 µL	T7 GTP Solution (75 mM)	–20°C
50 µL	T7 UTP Solution (75 mM)	–20°C
10 µL	pTRI-RNA 18S	–20°C
100 µL	TURBO DNase (2 U/µL)	–20°C
1.4 mL	Gel Loading Buffer II	–20°C
1 mL	Ammonium Acetate Stop Solution*	–20°C
1.75 mL	Nuclease-free Water	any temp†

* 5 M ammonium acetate, 100 mM EDTA

† Nuclease-free Water may be stored at –20°C, 4°C or room temp

The life of the kit can be prolonged by storing the ATP, CTP, GTP, and UTP solutions at –80°C.

C. Materials Not Supplied with the Kit

- DNA template containing a T7 promoter—effective template can be made from synthetic oligonucleotides, PCR products, or linearized plasmids. See section II on page 4 for template guidelines.
- (optional) Reagents for purification of RNA transcripts synthesized with the kit:
  – MEGAclear™ (P/N AM1908), or
  – Phenol/chloroform, isopropanol or ethanol, and RNase-free water
- (optional) Spectrophotometer—we recommend the Nanodrop® ND-1000A UV-Vis Spectrophotometer. With the NanoDrop Spectrophotometer only 1.5 µL of sample is needed and no dilutions or cuvettes are necessary.

D. Related Products Available from Applied Biosystems

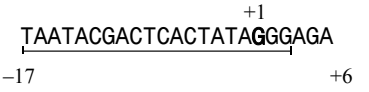
RNase*Zap*® P/N AM9780, AM9782, AM9784	RNase Decontamination Solution. RNase*Zap* is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*.
RNase-free Tubes & Tips See web or print catalog for P/Ns	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog for specific information.
TURBO DNA-*free*™ P/N AM1907	The TURBO DNA-*free* Kit employs Ambion's exclusive TURBO DNase (patent pending); a specifically engineered hyperactive DNase that exhibits up to 350% greater catalytic efficiency than wild type DNase I. The kit also includes a novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation—and without heat inactivation, which can cause RNA degradation. TURBO DNA-*free* is ideal for removing contaminating DNA from RNA preparations.
MEGAclear™ P/N AM1908	MEGAclear purifies RNA from transcription, and other enzymatic reactions yielding high quality RNA free of unincorporated nucleotides, enzymes and buffer components with close to 100% recovery of input RNA.
Proteinase K P/N AM2542–AM2548	Proteinase K is a non-specific serine protease commonly used in molecular biology to remove protein contaminants from nucleic acids. Ambion supplies Proteinase K in lyophilized powder form, and as a 50% glycerol solution.
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 as this product line is always growing.
Decade™ Markers P/N AM7778	The Decade Marker System is a set of reagents to prepare radiolabeled low molecular weight RNA markers: from 10–100 nt in 10 nt increments. The user supplies only [γ-^{32}P]ATP to end label a single, gel purified RNA transcript which is then cleaved into the 10 molecular weight markers in a simple 5 minute reaction.
RNA Century™, and Century™-Plus Markers P/N AM7140, AM7145	RNA Century Markers: 100, 200, 300, 400 and 500 bases. RNA Century-Plus Markers: 100, 200, 300, 400, 500, 750 and 1000 bases. Both are available unlabeled for staining with ethidium bromide, or biotinylated for subsequent nonisotopic detection.

II. Template Requirements

A. Important Template Parameters

Linearized plasmid DNA, synthetic oligonucleotides, and PCR products that contain a T7 RNA polymerase promoter site can be used as templates for in vitro transcription with MEGAshortscript. In general, any DNA with a T7 promoter site (Figure 1), that is pure enough to be easily digested with restriction enzymes can be used for in vitro transcription.

Figure 1. T7 polymerase promoter: minimal sequence requirements.



The +1 base (in bold) is the first base incorporated into RNA during transcription. The underline shows the minimum promoter sequence needed for transcription. The two G's following G+1 are recommended for highly efficient transcription.

Template size

The MEGAshortscript Kit is designed to function best with templates that code for RNA transcripts ***in the 20 to 500 nt range***. The kit can be used to produce longer RNA but the Ambion MEGAscript Kit is more economical for this purpose.

Template input

The amount of template needed will depend on the amount of product desired and the length of the transcript. The rate-limiting step of a transcription reaction is the initiation step. Therefore, the shorter the transcript being synthesized, the greater the number of transcription initiation events that will be required to synthesize a given mass amount of RNA (see Figure 2). For example, the difference in the template amount needed with a 200 nt and a 20 nt template should be about 10 fold; transcription of 25 nmoles of a 200 nt template will produce a similar mass amount of product RNA as 250 nmoles of a 20 nt template.

Optimal template input is dependent on template size

For maximum yield of transcripts in the 100–300 base range, the optimum template concentration usually falls in the 25–125 nM range. For shorter transcripts, maximum yields are obtained with higher template concentrations, up to several micromolar for transcripts around 20 bases in length. The optimal concentration of a particular template must be determined empirically for that template.

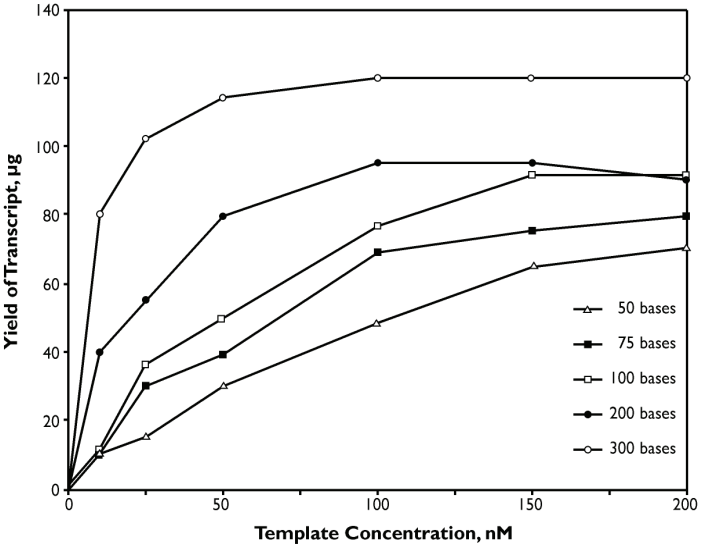


Figure 2. Template Concentration and Yield of Transcripts of Various Sizes.

MEGAshortscript reactions were performed according to protocol using five different template sizes at six different concentrations. All reactions were incubated 2 hr, then quantitated in micrograms. While molar yields are expected to be similar among transcripts of different sizes with the same input concentration, the mass yield increases proportionally to the length of the transcript. Note that longer transcripts will saturate the reaction at a lower concentration.

Calculating moles of template from mass

To calculate moles of DNA from the mass, use the equation below. To simplify the calculation, the average molecular weight of all four nucleotides (330 g/mol) can be used in place of the exact molecular weight of the sequence (assuming that all four nucleotides are in roughly equal proportions).

MW of template = Ave MW per bases x # of base (x 2 for double-stranded)

$$\text{Moles of template} = \frac{\text{Mass of template}}{\text{MW of template}}$$

For example, to calculate the moles in 2.5 µg of a 120 bp PCR product (double stranded):

MW of template = 330 g/mol x 120 x 2 = 7.92×10^4 g/mol

$$\text{Moles of template} = \frac{2.5\ \mu g}{7.92 \times 10^4\ g/mol} = 31\ \text{pmol of template}$$

Standard 20 µL transcription reactions can accommodate a maximum of about 8 µg of template DNA. Thus, for some plasmid templates it may not be possible to use the optimum concentration of template, because too great a mass amount of DNA would be required. Often a lower molar concentration of template can be offset by increasing the incubation time of the reaction (see "Optimize incubation time" on page 15).

Template purity

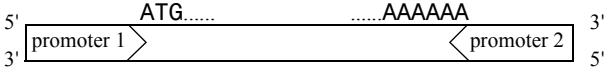
The template DNA should be free of contaminating proteins and RNA. Most commercially available plasmid preparation systems yield DNA that works well in the MEGAshortscript Kit.

If your template preparation has inhibitors of RNA synthesis (such as salts, detergents, proteases, or ribonucleases) adding more template to the reaction will increase the problems caused by these contaminants and may lead to a lower RNA yield. (see section IV.B.1.a on page 14 for suggestions on improving template quality.)

Orientation

If ***sense RNA*** is needed, it is important to transcribe using the RNA polymerase corresponding to the phage promoter at the 5', or amino-terminal side of the coding region of the protein (using promoter 1 in the diagram below). If the template consists of a plasmid, it should be linearized at the opposite (3' or carboxy-terminal side) of the protein-coding region.

Antisense (mRNA-complementary) transcripts will be synthesized if the RNA polymerase corresponding to the RNA phage promoter at the 3', or carboxy-terminal side of the coding region of the protein is used (using promoter 2 in the diagram below).



Transcription using the RNA polymerase corresponding to promoter 1 will make sense RNA (the same sequence as the mRNA). If the RNA polymerase for promoter 2 is used, antisense RNA will be transcribed.

B. Different Template Types

Several different types of template can be used with the MEGAshortscript Kit. The best transcription efficiency is usually obtained with linearized plasmid, followed by PCR templates; templates made from annealed oligos are easy to construct, but are sometimes inefficiently transcribed due to the inherent instability of a short base-paired sequence.

PCR templates

A template can be transcribed directly from a PCR provided it contains a T7 RNA polymerase promoter upstream of the sequence to be transcribed. PCR products should be examined on an agarose gel before use as a template in MEGAshortscript to estimate their concentration, and to verify that the products are unique, and of the expected size.

Plasmid templates

Most commercial plasmid purification kits will produce plasmid compatible with this protocol. For a plasmid purification protocol see:
www.ambion.com/techlib/append/supp/plasmidDNA.html

Linearization

Plasmid DNA must be linearized with a restriction enzyme downstream of the insert so transcription will be terminated by run-off. Circular plasmid templates will generate extremely long, heterogeneous RNA transcripts because RNA polymerases are very processive. We recommend examining the linearized template DNA on a gel to confirm that cleavage is complete, since even a small amount of circular plasmid in a template will generate a large proportion of longer transcripts.

Although we routinely use all types of restriction enzymes, 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.; Schendorn and Mierindorf, 1985).

After linearization

Terminate the restriction digest by adding the following:

- 1/20th volume 0.5 M EDTA
- 1/10th volume of 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 water or TE buffer at a concentration of 0.5–1 µg/µL.

Proteinase K treatment

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 as described in section "Proteinase K Digestion" on page 18.

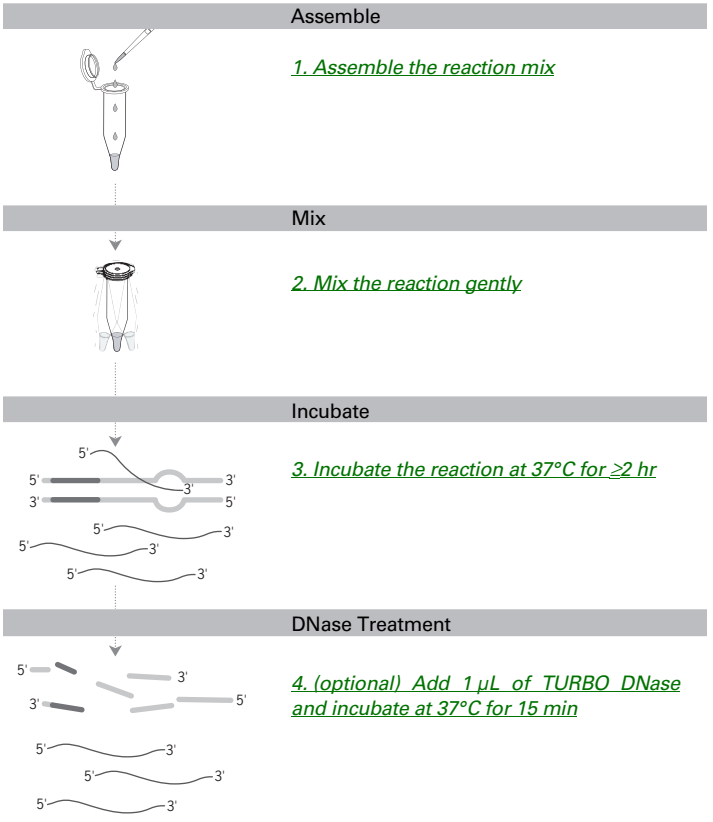
Synthetic oligonucleotide templates

Very short transcription templates can be prepared from synthetic oligonucleotides. Complementary oligonucleotides of the same length, containing phage promoter sequences, can be annealed to make a completely double-stranded template. Alternatively, a partially single-stranded template can be prepared by annealing a synthetic oligonucleotide which is complementary only to the promoter region of the longer bottom strand of the template (Figure 6 on page 20). See section V.F on page 19 for a protocol on annealing synthetic oligonucleotides to make a transcription template. The oligonucleotides should be PAGE or HPLC purified to remove partial sequences.

III. MEGAshortscript™ Kit Procedure

A. Procedure Overview

Figure 3. Transcription Reaction Assembly and Incubation



B. Transcription Reaction Assembly and Incubation

Thaw the T7 10X Reaction Buffer, four ribonucleotide solutions, and Water at room temperature. Briefly vortex the T7 10X Reaction Buffer and ribonucleotide solutions. Microfuge all reagents briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube. Keep the T7 Enzyme Mix on ice during assembly of the reaction.

1. Assemble the reaction mix

Assemble the reaction in an RNase-free microfuge tube at ***room temperature*** in the order shown. For convenience, all four nucleotides can be premixed; add 8 µL of the mixture to a standard 20 µL reaction instead of adding the ribonucleotides separately.

The following amounts are for a single 20 µL reaction. Reactions may be scaled as needed.

NOTE

Components in the transcription buffer can lead to precipitation of the template DNA if the reaction is assembled on ice.

Amount	Component
Water (Nuclease-free) to 20 µL final volume.	
2 µL	T7 10X Reaction Buffer
2 µL	T7 ATP Solution (75 mM)
2 µL	T7 CTP Solution (75 mM)
2 µL	T7 GTP Solution (75 mM)
2 µL	T7 UTP Solution (75 mM)
~1 µL	(optional) Labeled ribonucleotide
<8 µL	Template DNA
2 µL	T7 Enzyme Mix

2. Mix the reaction gently

Mix contents thoroughly by gently flicking the tube, then microfuge the tube briefly to collect the reaction mixture at the bottom of the tube.

3. Incubate the reaction at 37°C for ≥2 hr

Incubate the reaction at 37°C for at least 2 hr. For most applications a 2–4 hr incubation is sufficient; however, the optimal incubation time will be template-dependent. To determine the optimum incubation time for maximum yield with a given template, a time-course experiment should be done (see *Optimize incubation time* on page 15).

4. (optional) Add 1 µL of TURBO DNase and incubate at 37°C for 15 min

To remove the DNA template, add 1 µL of TURBO DNase to the reaction, mix well, and continue the incubation at 37°C for 15 min. If very large mass amounts of DNA template were used, more TURBO DNase may be required.

NOTE

For many applications it may not be necessary to degrade the template DNA, since it will be present at a very low concentration relative to the RNA.

C. Termination of the Reaction and RNA Recovery

The degree of purification required after the transcription reaction depends on what will be done with the transcript RNA. Choose the method according to your application and resources.

MEGAclear™ Kit

The MEGAclear™ Kit (Ambion P/N AM1908) is a glass filter-based system for purification RNA transcripts (>100 nt) from salts, free nucleotides, and enzymes in an easy 15 minute procedure that requires no organic solvents.

Phenol:chloroform extraction and alcohol precipitation

Phenol:chloroform extraction followed by alcohol precipitation will remove all enzymes and most of the free nucleotides from MEGAshortscript reactions. Since the RNA is precipitated, this method can also be used for buffer exchange. The following procedure is for a standard 20 µL reaction.


NOTE

Extraction with acid phenol will cause most of the DNA to partition into the organic phase. This procedure is an alternative to TURBO DNase for removing the DNA template (Kedzierski and Porter, 1991).

a. Add 115 µL nuclease-free water and 15 µL Ammonium Acetate Stop Solution or 3 M sodium acetate, then mix thoroughly.
   Ammonium ions can inhibit some downstream applications, such as end-labeling with polynucleotide kinase. If this is the case, substitute 15 µL of 3 M sodium acetate for the Ammonium Acetate Stop Solution.

b. Extract with an equal volume of phenol/chloroform (water-saturated, buffer-saturated, or acidic), and then with an equal volume of chloroform. Recover the aqueous phase and transfer to a new tube.

c. Precipitate the RNA by adding 2 volumes of ethanol and mixing well. Chill the mixture for at least 15 minutes at –20°C.

d. Centrifuge at 4°C for 15 minutes at maximum speed (≥10,000 x g) to pellet the RNA. Carefully remove the supernatant solution and resuspend the RNA in a solution or buffer appropriate for your application.

e. Store the RNA at –20°C or –70°C.

Alcohol precipitation

Precipitation with ethanol or isopropanol is simpler and faster, but not as rigorous as phenol:chloroform extraction. However, it is sufficient for most purposes. RNA transcripts will be purified away from most protein, salts and free nucleotides.

a. Add 115 µL nuclease-free water and 15 µL Ammonium Acetate Stop Solution or 3 M sodium acetate, then mix thoroughly.
   Ammonium ions can inhibit some downstream applications, such as end-labeling with polynucleotide kinase. If this is the case, substitute 15 µL of 3 M sodium acetate for the Ammonium Acetate Stop Solution.

b. Precipitate the RNA by adding 2 volumes of ethanol and mixing well. Chill the mixture for at least 15 minutes at –20°C.

c. Centrifuge at 4°C for 15 minutes at maximum speed (≥10,000 x g) to pellet the RNA. Carefully remove the supernatant and resuspend the RNA in a solution or buffer appropriate for your application.

d. Store the RNA at –20°C or –70°C.

Gel purification

If the transcript will be gel purified, stop the reaction by adding an equal volume of Gel Loading Buffer II. Once in the Gel Loading Buffer II, the RNA can be safely stored at –20°C. See section V.D on page 18 for instructions on gel purification.

IV. Troubleshooting

A. Positive Control Reaction

Control template construct

The pTRI-RNA 18S control template contains a highly conserved 80 bp cDNA fragment from the human 18S rRNA, inserted downstream from tandem SP6, T7 and T3 promoters in the antisense orientation. The construct has been linearized with *Eco*R I and *Bam*H I, and it will generate a 128 base sense-strand transcript from the T7 promoter (sequence shown in Figure 4).

Transcribe 2 µL of pTRI-RNA 18S in a 20 µL MEGAshortscript reaction

Use 2 µL (1 µg) of the pTRI-RNA 18S control template in a 20 µL reaction following the instructions starting at step III.B.1 on page 10. Incubate the MEGAshortscript reaction for 4 hr.

NOTE

Alternatively, you can incubate the transcription reaction for the same amount of time as your experimental samples are incubated, but remove a 2 µL sample of the reaction after 4 hr of incubation to check yield.

Clean up the reaction and assess yield

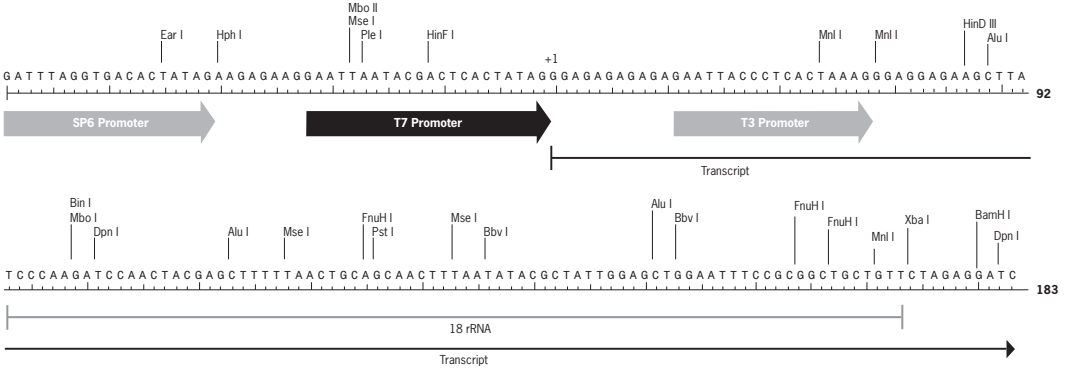
Purify the reaction products by one of the methods described in section III.C on page 11, and assess the yield by UV absorbance at 260 nm.

Expected result of the positive control reaction

Approximately 90 µg of transcript is expected from a 4 hr incubation of the positive control reaction.

If the positive control reaction yields <80 µg of RNA, it is an indication that something may be wrong with the kit; contact Ambion's Technical Services Department for further assistance.

Figure 4. Sequence of the Control Template.



B. Low Yield

Do the positive control reaction

The amount of RNA synthesized in a standard MEGAshortscript reaction is dependent on the template concentration and on the size (and to a lesser extent the sequence) of the transcript. A typical reaction containing the optimum template concentration for a transcript of 75 bases or longer should yield about 80 µg of RNA in 4 hr. If the yield is low, the first step for troubleshooting the reaction is to perform the control MEGAshortscript reaction (section IV.A).

1. The control reaction works, but my template gives low yield

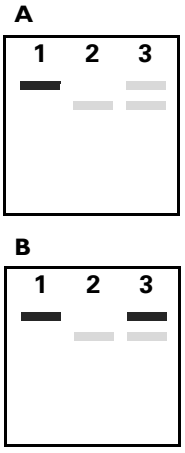


Figure 5. Possible outcomes of mixing experiment

1 – control template
2 – experimental template
3 – mixture of 1 and 2

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 pTRI-RNA 18S 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 pTRI-RNA 18S control template
2. experimental DNA template
3. a mixture of 1 and 2

Assess the results of the mixing experiment by running 1–2% of each transcription reaction on a denaturing gel as described in section V.G.3.

a. Transcription of the control template is inhibited by the presence of your template (see Figure 5.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 and ethanol precipitation frequently improves template quality (see section V.B on page 18 for procedure).

b. Adding your template to the reaction with the control template does not inhibit synthesis of the control RNA (see figure 5.B)
This result indicates that the problem may be inherent to your template.

i. Quantitate yield by an alternative method
To confirm that the quantitation is correct, try to verify the yield by an independent method. For example, if UV absorption was used to assess yield, try also running an aliquot of the reaction on a polyacrylamide gel.

ii. Check that the template DNA is intact

Run a sample of the template DNA on a gel to determine if it is intact. Intact DNA should appear as a sharp band, whereas degraded DNA will appear as multiple bands or as a smear.

iii. Optimize incubation time

The optimal incubation time depends on the size and concentration of the template, the amount of transcript needed, and the intrinsic transcriptional efficiency of the template.

To determine the optimum incubation time for a given template, perform a time-course experiment by removing aliquots of the reaction at various time intervals (for example after 2, 4, 6 hr, and overnight incubation), then assess the yield (see section V.A. for a procedure on assessing yields).

In general, it is safer and more effective to improve yields by increasing the template concentration than by increasing the incubation time, except when using synthetic oligonucleotide templates, in which case overnight incubations may substantially improve yields of very short transcripts.

C. Multiple Reaction Products, Transcripts of the Wrong Size

Reaction products produce a smear when run on a denaturing gel

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 as described in section V.B on page 18. Although the RNase inhibitor that is present in the transcription reaction will inactivate normal levels of RNase contamination, large amounts of RNase contamination 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

a. Sample is not adequately denatured in the gel

If the size of the MEGAshortscript reaction product is unexpected, consider that the RNA may be running aberrantly due to secondary structure. To ensure that the RNA is completely denatured, use the Gel Loading Buffer II supplied with the kit, and heat the RNA in loading buffer at 95°C for the full 3 min before loading it onto the gel.

b. Premature termination of transcription

If denaturing gel analysis shows the presence of multiple bands or of a single band smaller than the expected size, there may be problems with premature termination by the T7 RNA polymerase. This can be caused by sequences which resemble the polymerase termination signals, stretches of a single nucleotides, and GC-rich templates.

- Termination at single polynucleotide stretches can sometimes be alleviated by decreasing the reaction temperature. We suggest testing the reaction at 30, 20 and 10°C. However, decreasing the reaction temperature will also significantly decrease the yield of the reaction.
- 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

Circular template

Longer-than-expected transcription products will be seen if plasmid template preps are incompletely digested, leaving circular template molecules in the reaction. Since the RNA polymerases are extremely processive, even a small amount of circular template can produce a large amount of RNA. If this is suspected, gel purify the linear plasmid.

V. Additional Procedures

A. Assessing Yield

The appropriate method for assessing MEGAshortscript reaction yield depends partly on the size of the transcript and the extent to which unincorporated nucleotides were removed; several suggested methods are discussed below.

Assessing RNA by UV Absorbance

We recommend the Nanodrop® ND-1000A UV-Vis Spectrophotometer (www.nanoambion.com). With the NanoDrop Spectrophotometer only 1.5 µL of sample is needed, and no dilutions or cuvettes are necessary.

Dilute the sample 1:50 to 1:500 in water to bring the concentration into the linear range of the spectrophotometer (if using the NanoDrop, no dilution is necessary). Blank the spectrophotometer with water. Read the absorbance at 260 nm and 280 nm. Quantitate as discussed below or assess the RNA purity by comparing A_{260}/A_{280}.

Approximate quantitation

After determining the A_{260} value by multiplying the spectrophotometer reading by the dilution factor, the RNA concentration can be calculated with either of the following equations:

$$C\ (\text{mol/L}) = A_{260}/(\varepsilon \times l)$$

$$C\ (\mu\text{g/mL}) = \frac{A_{260}}{\varepsilon \times l} \times M \times 1000 = \frac{1000 \times M}{\varepsilon \times l} \times A_{260}$$

Where:

- ε = extinction coefficient (L x mol^{-1} x cm^{-1})
- l = path length (cm): all modern spectrophotometers have a path length of 1 cm.
- M = molecular weight (g/mol)
- C = concentration

For short nucleic acids (<200 nt), 1000 x M/ε x l ≈ 33, the approximate concentration can be determined with the following formula:

Small RNA <200 nt: C (µg/mL) ≈ 33 x A_{260}

Total RNA, or >200 nt: C (µg/mL) ≈ 40 x A_{260}

Assess purity of RNA

The purity of the RNA can be assessed from the ratio of A_{260}/A_{280}. For highly pure RNA a ratio of 1.8–2.1 is expected.

RiboGreen®

If a fluorometer, or a fluorescence microplate reader is available, Molecular Probes' RiboGreen® fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen. It is not necessary to remove free nucleotides to quantitate by this method.

B. Proteinase K Digestion

Treat template DNA with Proteinase K to remove nucleases and other protein contamination.

a. Add Proteinase K (100–200 µg/mL) and SDS (0.5%) to the sample and digest for 30 minutes at 50°C.

b. Follow Proteinase K treatment 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 cold 70% ethanol before resuspension.

C. Assessing the Quality of Transcripts

The best way to analyze the outcome of a transcription reaction is to run an aliquot of the reaction on a denaturing polyacrylamide gel and visualize by staining with ethidium bromide. See section V.G.3 on page 21 for information on preparing and running gels. Alternatively, for transcripts longer than 100 nt, the reaction can be analyzed using the Agilent 2100 bioanalyzer and RNA6000 LabChip® Kit.

D. Gel Purification of Transcripts

1. Separate products on an acrylamide gel

Add an equal volume of Gel Loading Buffer II to the sample and heat for 3 min at 95–100°C. Load the products on a denaturing polyacrylamide gel and electrophorese the gel at ~10–25 mA until the bromophenol blue reaches the bottom of the gel. (see "Additional Recipes" on page 21 for guidelines and gel recipes.)

2. Excise the gel fragment containing the full-length nucleic acid

After electrophoresis, remove one glass plate from the gel, and stain in ethidium bromide or other nucleic acid staining solution. Cut out the area of the gel that contains the full-length transcript with a razor blade or a scalpel, and transfer it to an RNase-free microfuge tube. Remove the smallest possible fragment of gel that contains the full-length probe.

3. Elute nucleic acid from acrylamide gel slices

To elute the full-length probe, add 100–150 µL probe elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS) to the gel slice and incubate at 37°C for 30 min. Transfer the buffer, which contains the eluted probe, to a clean microfuge tube. Repeat with 50–100 µL of probe elution buffer and pool the two elution fractions.

Since longer probes elute more slowly from the gel, we recommend increasing the elution time for probes longer than 40 nt to at least 1 hr. For convenience, or to maximize recovery from the gel, incubation can be carried out overnight with ~200 µL or more of probe elution buffer. The probe elution buffer contains EDTA and SDS which will inactivate low levels of nuclease contamination.

E. Synthesis of Transcripts Containing Modified Nucleotides

The MEGAshortscript Kit can be used to synthesize large amounts of both biotinylated and digoxigenin labeled transcripts. Modified nucleotides may not be incorporated as efficiently as unmodified ones and greater sensitivity is obtained with a mixture of modified and unmodified nucleotides. We recommend using a ratio of modified nucleotide to standard nucleotide of 1:2 or 1:3. For example, to synthesize a biotin-UTP labeled transcript, add 0.66 µL 75 mM biotin-UTP and 1.34 µL 75 mM UTP to a standard 20 µL MEGAshortscript reaction. Because of the difficulty of measuring small volumes, and the expense of modified nucleotides, it may be convenient to use a larger volume of a less concentrated modified nucleotide stock, e.g., 3.3 µL of a 15 mM stock solution of modified nucleotide.

Note that Heer et al. (1994) have found that transcription efficiency with digoxigenin-modified nucleotides is template dependant and may vary widely. In the case of low transcription yields, the group suggests decreasing the digoxigenin to unmodified nucleotide ratio to 1:5.

For most efficient incorporation, the pH of nucleotide solutions should be close to neutral.

For more information on incorporating modified nucleotides see:
www.ambion.com/techlib/tb/tb_173.html

F. Annealing Synthetic Oligonucleotides to Make a Transcription Template

It is only necessary that the promoter sequence of a template be double-stranded; however, completely double-stranded templates may result in better yields in transcription reactions.

There are two options for making a template from synthetic oligonucleotides: the two oligonucleotides can simply be annealed to form a double-stranded promoter and used directly as a template for transcription

(Figure 6). Alternatively, after annealing the oligonucleotides, the single-stranded region can be filled-in to create a completely double-stranded template.

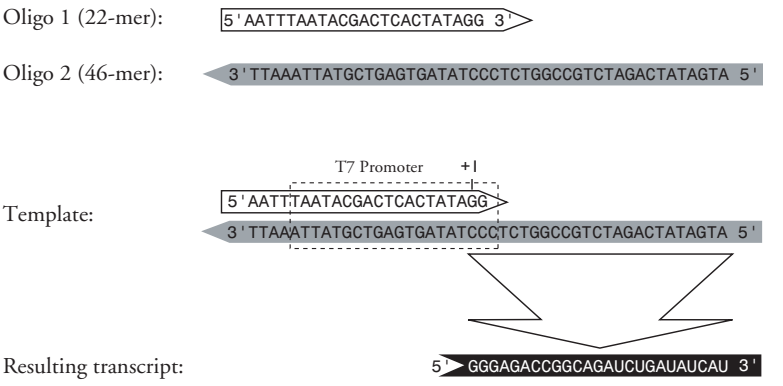
Partially double-stranded templates

1. Suspend the oligonucleotides in TES (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.1 M NaCl), and mix equimolar amounts to a final concentration of ~10–50 µM of each oligonucleotide.
2. Bring the mixture to ~95°C in a heat block or in a beaker of hot water.
3. Allow the solution to cool to room temperature on the bench.

Completely double-stranded templates

To make a completely double-stranded template, use the annealed oligonucleotides from step 3 above and fill-in using the Klenow enzyme according to the suppliers directions.

Figure 6. Oligonucleotides Annealed to Make Partially Single-stranded Templates for 25 nt Runoff Transcript



G. Additional Recipes

1. TE Buffer

10 mM Tris (pH 8), 1 mM EDTA

2. 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, Ambion offers nuclease-free solutions of 10X TBE (P/N AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (P/N AM9864). Both are made from of ultrapure molecular biology grade reagents.

3. Preparing and running denaturing acrylamide gels

Choose the appropriate percent gel

The choice of what percent polyacrylamide gel to use will depend on the size and desired resolution. General guidelines are given below:

- Transcripts >100 bases use 5% polyacrylamide gel
- Transcripts ≥50 bases use 10% polyacrylamide gel
- Transcripts ≤50 bases use 20% polyacrylamide gel

8 M urea polyacrylamide gel recipe

15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel

Amount			
5% gel	10% gel	20% gel	Component
7.2 g	7.2 g	7.2 g	Urea
1.5 mL	1.5 mL	1.5 mL	10X TBE
1.9 mL	3.4 mL	6.8 mL	40% acrylamide (acrylamide:bis acrylamide = 19:1)
to 15 mL	to 15 mL	to 15 mL	Nuclease-free water

a. Stir at room temp until the urea is completely dissolved, then add:

75 µL	10% ammonium persulfate
15 µL	TEMED

b. Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour gel immediately.

Gel set up

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1X TBE as the gel running buffer.
- It is very important to rinse the wells of urea-containing gels immediately before loading the samples.

Electrophoresis conditions

Gels should be run at about 20 V/cm gel length; for 13 cm long gel this will be about 250 V. Alternatively, denaturing acrylamide gels of this size can be run at ~25 mAmp, constant current.

Heat and load sample

Mix the sample with at least one volume of Gel Loading Buffer II. Heat samples for 3 min at 95°C just prior to running the gel.

More detailed information on preparing and running acrylamide gels can be found in *Molecular Cloning, A Laboratory Manual* (2001) or *Current Protocols in Molecular Biology* (2003).

4. 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.

VI. Appendix

A. References

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B. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

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 glasses, 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.

C. Quality Control

Functional Analysis

All components are functionally tested in a MEGAshortscript reaction as described in this protocol.

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.