USER GUIDE

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mMESSAGE mMACHINE® T7 Ultra Kit

Synthesis of Translation Enhanced Capped Transcripts

Catalog Number AM1345

Publication Number 1345M
Revision H

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About this guide

Revision history

Revision	Date	Description
H	February 2014	• Clarified instructions for the TURBO DNase step in the Capped transcription reaction assembly section and the addition of E-PAP in the Poly(A) tailing procedure. • Updated Safety, Documentation and support, and Legal statements to align with current document templates. • Updated company name from Life Technologies Corp. to Thermo Fisher Scientific Inc.
G	November 2012	Baseline for revision history

mMESSAGE mMACHINE® T7 Ultra Kit

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

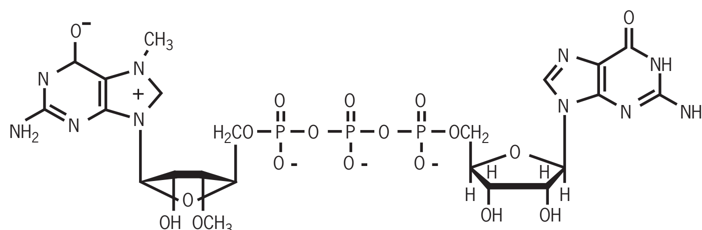
Introduction

Background

Synthesis of capped RNA

mMESSAGE mMACHINE® T7 Ultra Kit is designed for the *in vitro* synthesis of large amounts of efficiently and correctly capped RNA. In addition, this kit includes reagents for *in vitro* poly(A) tailing to make mRNA specifically for microinjections and transfections. mMESSAGE mMACHINE® T7 Ultra reactions include a novel cap analog called Anti-Reverse Cap Analog or ARCA (Stepinski J et al. 2001 and Peng et al. 2002). In ARCA, one of the 3' OH groups (closer to ^{7}MG) is eliminated from the cap analog and is substituted with $-OCH_3$ (see Figure below). This modification allows T7 RNA polymerase to initiate transcription only with the remaining –OH group and thus synthesize RNAs capped exclusively in the correct orientation. Substitution of traditional Cap Analog with ARCA allows for synthesis of capped RNAs that are 100% functional, in contrast to transcription reactions using traditional cap analog where only half of the cap analog is incorporated in the correct orientation. GTP and ARCA are supplied in a single solution with an ARCA:GTP ratio of 4:1, which is optimal for maximizing both RNA yield and the proportion of capped transcripts.

Figure 1 ARCA Structure



The high yields are achieved by optimizing reaction conditions for RNA synthesis in the presence of high nucleotide concentrations. In addition, the RNA synthesized is protected from degradation by any contaminating ribonucleases that may be present with RNase inhibitor—a component of the T7 Enzyme Mix. The mMESSAGE mMACHINE® T7 Ultra Kit contains all the buffers and reagents necessary for ten 20 µL transcription reactions. Using the control template supplied with the kit (Xenopus elongation factor 1α, pTRI Xef), each mMESSAGE mMACHINE® T7 Ultra reaction will yield approximately 20–30 µg of RNA.

Poly(A) tailing of RNA

Poly(A) tailing reagents are also included with the mMESSAGE mMACHINE® T7 Ultra Kit; they can be used to add a ≥50–100 base poly(A) tail to the RNA transcripts. This is accomplished using *E. coli* Poly(A) Polymerase (*E*-PAP) and ATP. The resulting capped and tailed RNA can then be used in transfection or microinjection experiments where enhanced translation over untailed mRNAs may be seen due to increased mRNA stability and translation efficiency (Bernstein and Ross 1989, Gallie 1991, Harland and Misher 1989, Khaleghpour et al. 2001).

Materials provided with the kit

The mMESSAGE mMACHINE® T7 Ultra Kit should be stored in a non-frost-free freezer. Keep all reagents on ice while using the kit; the nucleotides and enzymes (T7 Enzyme Mix and *E*-PAP) are especially labile.

Reagents are included for 10 mMESSAGE mMACHINE® T7 Ultra reactions.

Transcription and capping components:

Amount	Component	Storage
1.75 mL	Nuclease-free Water	any temp†
20 µL	T7 Enzyme Mix Buffered 50% glycerol containing RNA polymerase, RNase Inhibitor, and other components	–20°C
20 µL	10X T7 Reaction Buffer salts, buffer, dithiothreitol, and other ingredients	–20°C
100 µL	T7 2X NTP/ARCA a neutralized buffered solution containing: 15 mM ATP 15 mM CTP 15 mM UTP 3 mM GTP 12 mM ARCA	–20°C
40 µL	T7 GTP (30 mM)	–20°C
100 µL	TURBO DNase 1 (2 U/µL)	–20°C
10 µL	pTRI-Xef, 0.5 mg/mL (Control Template)	–20°C
1 mL	Ammonium Acetate Stop Solution 5 M ammonium acetate, 100 mM EDTA	–20°C
1.4 mL	Lithium Chloride Precipitation Solution 7.5 M lithium chloride, 50 mM EDTA	–20°C
1 mL	Formaldehyde Load Dye	–20°C

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

Poly(A) tailing components:

Amount	Component	Storage
40 µL	*E*-PAP (2 U/µL)	–20°C
240 µL	5X *E*-PAP Buffer	–20°C
100 µL	ATP Solution (10 mM)	–20°C
100 µL	25 mM $MnCl_2$	–20°C

Materials not provided with the kit

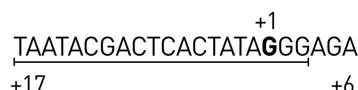
- DNA template: The DNA template must have the T7 RNA polymerase promoter site upstream of the sequence to be transcribed. The suggested template concentration is 0.5 µg/µL in water or TE (10 mM Tris-HCl (pH 7–8), 1 mM EDTA).
- (optional) Labeled nucleotide(s): [α-^{32}P] UTP or [α-^{32}P]CTP can be used as a tracer for transcription and [α-^{32}P]ATP can be used as a tracer to assess the poly(A) tailing reaction. Any specific activity is acceptable.
- (optional) For purification of the synthesized RNA:
  - Buffer- or water-saturated phenol/chloroform
  - Isopropanol
  - Spin Columns

mMESSAGE mMACHINE® T7 Ultra Kit procedure

Preparation of template DNA

Linearized plasmid DNA and PCR products that contain a T7 RNA polymerase promoter site can be used as templates for *in vitro* transcription with mMESSAGE mMACHINE® T7 Ultra. In general, any DNA with a T7 promoter site, that is pure enough to be easily digested with restriction enzymes can be used for *in vitro* transcription.

Figure 2 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 efficient transcription.

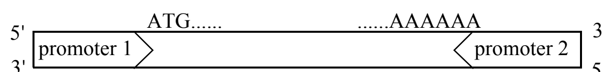
Template size

The mMESSAGE mMACHINE® T7 Ultra kit is designed to function best with templates that code for RNA transcripts ***in the 0.3 to 5 kb range***. The kit can be used to produce shorter RNA, but modify the reaction as described in section "Optimizing yield of short transcripts" on page 25.

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 in the polylinker 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.

PCR templates

DNA generated by PCR can be transcribed directly from the 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 mMESSAGE mMACHINE® T7 Ultra to estimate concentration, and to verify that the products are unique, and the expected size.

Plasmid templates

DNA should be relatively free of contaminating proteins and RNA. We observe the greatest yields with very clean template preparations. Most commercially available plasmid preparation systems yield DNA that works well in the mMESSAGE mMACHINE® T7 Ultra Kit. Otherwise, a DNA miniprep procedure that generally yields high quality template is presented in section “ Miniprep for isolating transcription-quality plasmid DNA” on page 26.

Linearization

Plasmid DNA must be linearized with a restriction enzyme downstream of the insert to be transcribed. Circular plasmid templates will generate extremely long, heterogeneous RNA transcripts because RNA polymerases are very processive. It is generally worthwhile to examine the linearized template DNA on a gel to confirm that cleavage is complete. Since initiation of transcription is one of the limiting steps of *in vitro* transcription reactions, even a small amount of circular plasmid in a template prep will generate a large proportion of transcript.

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

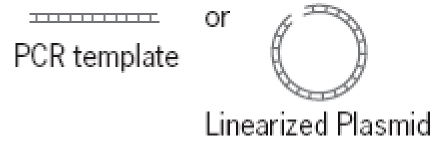
Mix well and chill at –20°C for at least 15 min. Then pellet the DNA for 15 min in a microcentrifuge at top speed. Remove the supernatant, re-spin the tube for a few seconds, and remove the residual fluid with a very fine-tipped pipet. Resuspend in dH2O or TE buffer at a concentration of 0.5–1 µg/µL.

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 (100–200 µg/mL) and 0.5% SDS for 30 min at 50°C, follow this with phenol/chloroform extraction (using an equal volume) and ethanol precipitation.

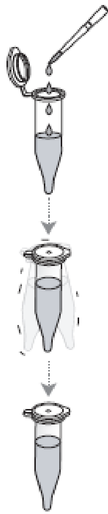
Procedure overview

Figure 3 Transcription reaction assembly and incubation

Preparation of template DNA

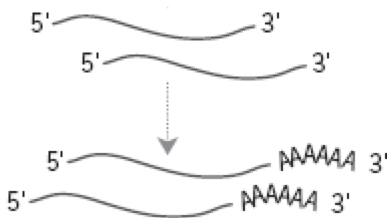


Capped transcription reaction assembly



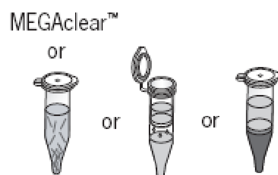
1. “Thaw the frozen reagents” on page 13
2. “Assemble transcription reaction at room temp” on page 13
3. “Mix thoroughly” on page 13
4. “Incubate at 37°C, 1 hr” on page 13
5. “Add 1 µL TURBO DNase, mix well and incubate 15 min at 37°C” on page 14

Poly(A) tailing procedure



1. “Add tailing reagents” on page 14
2. “Reserve 2.5 µL of the reaction mixture” on page 14
3. “Add 4 µL of E-PAP, and mix gently.” on page 14
4. “Incubate at 37°C for 30–45 min” on page 14

Recovery of the RNA



1. “MEGAclear™ Kit” on page 14
2. “Lithium chloride precipitation” on page 15
3. “Spin column chromatography” on page 15
4. “Phenol:chloroform extraction and isopropanol precipitation” on page 15

Capped transcription reaction assembly

1. Thaw the frozen reagents

   Place the RNA Polymerase Enzyme Mix on ice, it is stored in glycerol and will not be frozen at –20°C.

   Vortex the 10X T7 Reaction Buffer and the T7 2X NTP/ARCA until they are completely in solution. Once thawed, store the ribonucleotides T7 (2X NTP/ARCA) on ice, but ***keep the 10X T7 Reaction Buffer at room temperature while assembling the reaction***.

   All reagents should be microfuged briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube.

2. Assemble transcription reaction at room temp

   The spermidine in the 10X T7 Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.

   Add the 10X T7 Reaction Buffer after the water and the ribonucleotides are already in the tube.

   The following amounts are for a single 20 µL reaction. Reactions may be scaled up or down if desired.

   IMPORTANT! The following reaction setup is recommended when the RNA produced will be 300 bases to 5 kb in length. For transcripts longer or shorter than this, consider the suggestions in sections “Optimizing yield of long transcripts” on page 24 and “Optimizing yield of short transcripts” on page 25, respectively.

Amount	Component
to 20 µL	Nuclease-free Water
10 µL	T7 2X NTP/ARCA
2 µL	10X T7 Reaction Buffer
(1 µL)	(optional) [α-^{32}P]UTP as a tracer
0.1–1 µg	linear template DNA†
2 µL	T7 Enzyme Mix

† Use 0.1–0.2 µg PCR-product template or ~1 µg linearized plasmid template.

3. Mix thoroughly

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

4. Incubate at 37°C, 1 hr

   Typically, 80% yield is achieved after a 1 hr incubation. For maximum yield, we recommend a 2 hr incubation.

   A second hour of incubation is recommended for synthesis of <300 base transcripts and for inefficiently transcribed templates. (See sections “Optimizing yield of long transcripts” on page 24 and “Optimizing yield of short transcripts” on page 25 for optimizing yield from templates coding RNA outside the 0.3–5 kb range.

If the transcription reaction is trace-labeled:

After the incubation (before or after TURBO DNase treatment), remove an aliquot of trace-radiolabeled reactions to assess yield by TCA precipitation (see step 5., “Quantitation by trace radiolabeling” on page 16).

5. Add 1 µL TURBO DNase, mix well and incubate 15 min at 37°C

   This DNase treatment removes the template DNA. For many applications it may not be necessary because the template DNA will be present at a very low concentration relative to the RNA.

Poly(A) tailing procedure

1. Add tailing reagents

   Add the tailing reagents to the 20-µL mMESSAGE mMACHINE® T7 Ultra reaction in the following order:

Amount	Component
(20 µL)	mMESSAGE mMACHINE® T7 Ultra reaction
36 µL	Nuclease-free Water
20 µL	5X *E*-PAP Buffer
10 µL	25 mM $MnCl_2$
10 µL	ATP Solution

2. Reserve 2.5 µL of the reaction mixture

   Remove 2.5 µL of the reaction mixture before adding the *E*-PAP enzyme; this minus-enzyme control will be run on a gel next to the tailed RNA at the end of the experiment.

3. Add 4 µL of *E*-PAP, and mix gently.

   The final reaction volume is 100 µL.

4. Incubate at 37°C for 30–45 min

   After the 30–45 min incubation at 37°C, place the reaction on ice.

   If the poly(A) tailing reaction is trace-labeled:

   After the incubation (before RNA recovery), remove an aliquot of trace-radiolabeled reactions to assess yield by TCA precipitation (see section “TCA precipitation” on page 17.)

Recovery of the RNA

The degree of purification required after the transcription reaction depends on what will be done with the RNA. Four different methods follow, choose one or more according to your application and resources.

1. MEGAclear™ Kit

   The MEGAclear Kit was developed specifically for purifying RNA from high yield *in vitro* transcription reactions. The quick and simple procedure removes nucleotides, short oligonucleotides, proteins, and salts from RNA. The RNA recovered can be used for any application that requires high purity RNA.

2. Lithium chloride precipitation

   Lithium Chloride (LiCl) precipitation is a convenient and effective way to remove unincorporated nucleotides and most proteins. Lithium chloride precipitation, however, does not precipitate transfer RNA and may not efficiently precipitate RNAs smaller than 300 nucleotides. Also, the concentration of RNA should be at least 0.1 µg/µL to assure efficient precipitation. To precipitate from mMESSAGE mMACHINE® T7 Ultra reactions that are thought to have relatively low yields of RNA, do not dilute the transcription reaction with water prior to adding the LiCl Precipitation Solution the first substep below.

   a. Stop the reaction and precipitate the RNA by adding 50 µL LiCl Precipitation Solution.
   b. Mix thoroughly. Chill for ≥30 min at –20°C.
   c. Centrifuge at 4°C for 15 min at maximum speed to pellet the RNA.
   d. Carefully remove the supernatant. Wash the pellet once with ~1 mL 70% ethanol, and re-centrifuge to maximize removal of unincorporated nucleotides.
   e. Carefully remove the 70% ethanol, and resuspend the RNA in a solution or buffer† appropriate for your application. Determine the RNA concentration and store frozen at –20°C or –70°C.

3. Spin column chromatography

   Spin columns will remove unincorporated nucleotides, including unincorporated ARCA that may inhibit *in vitro* translation.

   Prepared spin columns such as NucAway™ Spin Columns can be used by following the manufacturer's instructions. Alternatively, instructions for preparing spin columns are given in section " Spin column preparation and use" on page 26.

4. Phenol:chloroform extraction and isopropanol precipitation

   This is the most rigorous method for purifying transcripts. It will remove all enzyme and most of the free nucleotides from mMESSAGE mMACHINE® T7 Ultra Kit reactions. Since the RNA is precipitated, this method can also be used for buffer exchange.

   a. Add 10 µL Ammonium Acetate Stop Solution, and mix thoroughly.
   b. Extract with an equal volume of phenol/chloroform (it can be water-saturated, buffer-saturated, or acidic), and then with an equal volume of chloroform. Recover aqueous phase and transfer to new tube.
   c. Precipitate the RNA by adding 1 volume of isopropanol and mixing well.
   d. Chill the mixture for at least 15 min at –20°C. Centrifuge at 4°C for 15 min at maximum speed to pellet the RNA. Carefully remove the supernatant solution and resuspend the RNA in a solution or buffer† appropriate for your application.
   e. Store frozen at –20°C or –70°C.

† We offer several products for RNA storage, these include:
Nuclease-free Water (not DEPC-treated): Cat. nos. AM9930–AM9939
THE RNA Storage Solution: Cat. nos. AM7000, AM7001
TE Buffer: Cat. nos. AM9860, AM9861
0.1 mM EDTA: Cat. no. AM9912

Quantitation of reaction products

1. Quantitation by UV light absorbance

   Reading the A_{260} of a diluted aliquot of the reaction is clearly the simplest way to determine yield, but any unincorporated nucleotides and/or template DNA in the mixture will contribute to the reading. Typically, a 1:100 dilution of an aliquot of a mMESSAGE mMACHINE® T7 Ultra reaction will give an absorbance reading in the linear range of a spectrophotometer.

   For single-stranded RNA, 1 A_{260} unit corresponds to 40 µg/mL, so the RNA yield can be calculated as follows:

   $$A_{260} \text{ x dilution factor x } 40 = \mu\text{g/mL RNA}$$

2. Assessing RNA yield with RiboGreen® assay

   If you have a fluorometer, or a fluorescence microplate reader, 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® assay.

3. Quantitation by ethidium bromide fluorescence

   The intensity of ethidium bromide staining can be used to get a rough estimation of the RNA yield.

   Ethidium bromide spot assay

   If unincorporated nucleotides have been removed, an ethidium bromide spot assay can be used to quantitate RNA concentration. Make a standard curve with several 2-fold dilutions of an RNA solution of known concentration. Start at about 80 ng/µL, and go down to about 1.25 ng/µL. Make a few dilutions of the unknown RNA, and add ethidium bromide to 1 ng/µL to each dilution of both RNAs. Spot 2 µL of the standard curve RNA samples and the unknown RNA dilutions onto plastic wrap placed on a UV transilluminator. Compare the fluorescence of the RNAs to estimate the concentration of the unknown RNA sample. Make sure that the sample dilutions are in the linear range of ethidium bromide fluorescence. This assay will detect as little as 5 ng of RNA with about a 2-fold error.

   Denaturing gel electrophoresis

   If unincorporated nucleotides have not been removed from the reaction, an aliquot of the mMESSAGE mMACHINE® T7 Ultra reaction should be run on a denaturing agarose gel alongside an aliquot of an RNA of known concentration. See section "Denaturing agarose gel electrophoresis" on page 23 for instructions on running gels. Stain the samples with ethidium bromide, and simply compare the intensity of the unknown sample to the known RNA to estimate its concentration.

4. Agilent® Bioanalyzer® system and RNA LabChip® Kits

   RNA can be evaluated on an Agilent® 2100 Bioanalyzer® system using one of their RNA LabChip® Kits to get an idea of what percentage of the transcription products are full-length. Follow the manufacturer's instructions for using the bioanalyzer and the RNA LabChip® Kit.

5. Quantitation by trace radiolabeling

   mMESSAGE mMACHINE® T7 Ultra reactions can be trace radiolabeled during either the transcription reaction or the poly(A) tailing reaction. Trace labeling the transcription reaction makes it possible to accurately determine the transcript yield; trace labeling the poly(A) tailing reaction enables determination of the amount of ATP incorporated into the poly(A) tail.

- To determine RNA yield from a trace labeled transcription reaction, TCA precipitate an aliquot of the reaction as described in section below, and use the results to calculate RNA yield as described in step 5., "Quantitation by trace radiolabeling" on page 16.
- Trace labeling the poly(A) tailing reaction will provide an indication of how well the tailing reaction worked. To trace label poly(A) tailing reactions, simply add 1 µL of [α-^{32}P]ATP (any specific activity can be used) to the tailing reaction at step . After the reaction is complete, TCA precipitate an aliquot of the reaction as described in section below.
- Successful reactions incorporate 50% or more radiolabel.

TCA precipitation

a. Dilute 5 µL of the completed mMESSAGE mMACHINE® T7 Ultra reaction with 5 µL TE Buffer in a nuclease-free 1.5-mL microfuge tube, and vortex thoroughly to ensure that the newly synthesized RNA is solubilized.

b. Add 150 µL of carrier DNA or RNA (1 mg/mL) (Sheared Salmon Sperm DNA Cat. no. AM9680 can be used for this) to the diluted reaction products, and mix thoroughly.

c. Transfer 50 µL of the RNA + carrier nucleic acid mixture to aqueous scintillation cocktail and count in a scintillation counter. This will measure the total amount of radiolabel present in the reaction mixture (unincorporated and incorporated counts).

d. Transfer another 50 µL of the RNA + carrier nucleic acid mixture to a 12 x 75 mm glass tube, and add 2 mL of cold 10% TCA (trichloroacetic acid). Mix thoroughly and place on ice for 10 min. This will precipitate nucleic acids, but not free nucleotides.

e. Collect the precipitate via vacuum filtration through a Whatman GF/C glass fiber filter (or its equivalent).

f. Rinse the tube twice with 1 mL of 10% TCA and then rinse once with 3–5 mL of 95% ethanol. Pass each of the rinses through the GF/C filter.

g. Place the filter in a scintillation vial, add aqueous scintillation cocktail, and count in a scintillation counter. This will give the TCA precipitated counts (radiolabel that was incorporated into RNA).

h. Divide the cpm in Step g. by the cpm in Step c. to determine the fraction of label incorporated into RNA (multiply by 100 for percent incorporation).

$$\frac{\text{TCA ppt cpm}}{\text{Total cpm}} \times 100 = \%\text{ incorporation}$$

Calculation of transcription reaction yield

Once the percent incorporation of radiolabel is known, it can be used to calculate the mass yield of RNA transcribed in the mMESSAGE mMACHINE® T7 Ultra reaction. The concentration of GTP limits the amount of RNA that can be synthesized. For any tracer other than labeled GTP (e.g. [α-^{32}P] UTP), each 1% incorporation corresponds to about 2 µg of RNA synthesized.

1% incorporation = 2 µg/RNA
For example: if % incorp. = 10%
10 x 2 µg = 20 µg RNA

In a mMESSAGE mMACHINE® T7 Ultra reaction, if all four nucleotides are incorporated equally, 39.6 μg of RNA will be produced if all of the 1.5 mM of GTP is incorporated into RNA (the sum of the molecular masses of the 4 nucleotides in RNA is about 1320). Since the ratio of cap analog to GTP is 4:1, this represents a maximal theoretical incorporation of 20% of the label.

$$\frac{1.5\ \text{mM}}{10^6\ \mu\text{L}} \times \frac{1320\ \text{g}}{1000\ \text{mM}} \times 20\ \mu\text{L} = \frac{39.6 \times 10^3\ \text{g}}{10^9} = 39.6 \times 10^{-6}\ \text{g} = 39.6\ \mu\text{g}$$

Troubleshooting

Use of the Control Template

The pTRI-Xef control template is a linearized TRIPLEscript™ plasmid containing the 1.85 kb Xenopus elongation factor 1α gene under the transcriptional control of tandem SP6, T7, and T3 promoters (pTRI-Xef 1). When transcribed with the mMESSAGE mMACHINE® T7 Ultra Kit, a ***1.89 kb transcript*** will be produced (before capping). This transcript codes for a 50.2 kDa protein.

1. Reaction setup

   Use 2 μL (1 μg) of pTRI-Xef in a standard mMESSAGE mMACHINE® T7 Ultra reaction as described in sections “Capped transcription reaction assembly” on page 13 and “Poly(A) tailing procedure” on page 14. Don’t neglect to remove an aliquot of the reaction at step just before adding the *E*-PAP so that the transcription reaction can be assessed separately from the tailing reaction.

   Analysis of the positive control reaction

   Run 2.5 μL of the tailed transcript and 2.5 μL of the minus-*E*-PAP control on a denaturing agarose gel as described in section .

   - The pTRI-Xef template should produce an 1890 base RNA.
   - The poly(A) tailing reaction should extend the Xef transcript ≥50 additional bases.

   The yield from the control reaction for T7 should be 20–30 μg of RNA. If a [^{32}P]NTP was added to the transcription reaction as a tracer, approximately 15% of the radiolabel should be incorporated during transcription.

2. What to do if the control reaction doesn’t work as expected

   If the yield of RNA from the control reaction is low or if the poly(A) tail extends less than ~50 bases, something may be wrong either with the procedure or the kit.

   a. Double check the RNA quantitation

      To confirm that the quantitation is correct, verify the yield by an independent method. For example if TCA precipitation was used to assess yield, try also running an aliquot of the reaction on an agarose gel.

   b. Try the positive control reaction again

      If the yield is indeed low by two different measurements, there may be a technical problem with the way the kit is being used. For example, the spermidine in the 10X T7 Reaction Buffer may cause precipitation of the template DNA if it is not diluted by the other ingredients prior to adding the DNA. (This is the reason that the water is added first.) Repeat the reaction, following the procedure carefully. If things still don’t go well, contact Technical Services for more ideas.

Troubleshooting low yield from the transcription reaction

The amount of RNA synthesized in a standard 20 µL mMESSAGE mMACHINE® T7 Ultra reaction should be 15–20 µg and may exceed 30 µg; however, there is a great deal of variation in yield from different templates. If the yield is low, the first step in troubleshooting the reaction is to use the pTRI-Xef control template in a standard mMESSAGE mMACHINE® T7 Ultra reaction.

1. Neither my template nor the control reaction works

   Double check that you have followed the procedure accurately, and consider trying the control reaction a second time. If the kit control still doesn't work, it is an indication that something may be wrong with the kit, call our Technical Support group for more ideas.

2. 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 pTRI-Xef 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.	1 µL pTRI-Xef control template
2.	experimental DNA template (0.5 µg plasmid or 2–6 µL PCR product)
3.	a mixture of 1 and 2

   Assess the results of the mixing experiment by running 1 µL of each transcription reaction on a denaturing gel as described in section "Denaturing agarose gel electrophoresis" on page 23.

   a. Transcription of the control template is inhibited by the presence of your template. (See Figure 4.A)

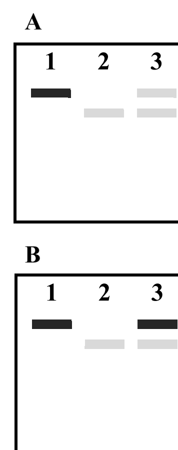


Figure 4 Possible outcomes of mixing experiment
1 – control template
2 – experimental template
3 – mixture of 1 and 2

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

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 cold 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 4.B)

This result indicates that the problem may be inherent to your template.

i. Check the amount and quality of template

Inaccurate template quantitation could cause low yield of transcript. 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.

ii. Extend the reaction time

Another parameter that can be adjusted is transcription reaction time (step 4. on page 13). Extending the standard 1 hr incubation to 4–6 hr or even overnight may improve yield.

Multiple transcription reaction products, transcripts of the wrong size

1. Transcription reaction products produce a smear when run on a denaturing gel

If the RNA appears degraded (e.g. smeared) before the poly(A) tailing reaction, remove residual RNase from the DNA template preparation before *in vitro* transcription. Do this by digesting the DNA prep with proteinase K (100–200 μg/mL) in the presence of 0.5% SDS for 30 min at 50°C, follow this with phenol/chloroform extraction. The RNase Inhibitor that is present in the transcription reaction, can only inactivate trace RNase contamination. Large amounts of RNase contamination will compromise the size and amount of transcription products.

2. Transcription 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 amount of RNA produced is acceptable, but the size of the 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 Formaldehyde Load Dye supplied with the kit, heat the RNA in loading dye at 75°C for the full 10 min and be sure to use fresh reagents to prepare the gel mix.

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 before the poly(A) tailing reaction, there may be problems with premature termination by the polymerase. Possible causes of this 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 reaction temperature (Krieg, P.A. 1990). We suggest testing 30°C, 20°C, 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).

3. Transcription reaction products are larger than expected

   a. Persistent secondary structure

   mMESSAGE mMACHINE® T7 Ultra products occasionally run as 2 bands; 1 larger than the expected size, and 1 at the expected size. This may occur with transcripts from the pTRI-Xef control template, even when the RNA is denatured during the electrophoresis. This phenomenon occurs because of persistent secondary structure. To verify this, the band that migrates at the expected size can be excised from the gel and run in a second denaturing gel. If the RNA runs as a doublet in the second gel also, it is a good indication that the larger band is simply an artifact of electrophoresis.

   b. Circular template

   Longer-than-expected transcription products will be seen if any of the template molecules are circular. This is typically caused by incomplete digestion of a plasmid template. Since the RNA polymerases are extremely processive, even a small amount of circular template can produce a large amount of RNA

No visible size shift seen after the poly(A) tailing reaction

Check the kit components by doing the positive control reaction

(See section “Use of the Control Template” on page 18 for instructions.)

Verify that the positive control reaction adds a poly(A) tail that is longer than ~50 bases to the Xef transcript.

The positive control works, but the experimental transcript does not

- Inadequate gel resolution:

  If the experimental transcript is several kb, then the resolution of an agarose gel may not be adequate to resolve a size change. Check the reaction by trace labeling instead (see next section).

- Trace label the tailing reaction

  If the positive control reaction product produces a band >50 bases larger than the minus-enzyme control, but the experimental transcript does not, then add a trace label to the poly(A) tailing reaction at step 1. on page 14. Do not radiolabel transcription reactions when the poly(A) tailing reaction will be radiolabeled, and remember to remove an aliquot of the reaction mix before adding the *E*-PAP as a minus-enzyme control (step 2. on page 14). Trace radiolabeling makes it relatively easy to determine how much of the ATP was incorporated into poly(A) tail at the end of the reaction.

  Determine the fraction of label incorporated into RNA by TCA precipitation as described in step 5. on page 16.

  Successful reactions incorporate up to 50% or more radiolabel.

  If less than 50% of the label is incorporated, but radiolabel incorporation is greater than background, increasing either the amount of enzyme or the concentration of ATP may help to generate a poly(A) tail of the desired length.

Supplemental information

Additional procedures

Denaturing agarose gel electrophoresis

NorthernMax® reagents for Northern Blotting include everything needed for denaturing agarose gel electrophoresis. These products are optimized for ease of use, safety, and low background, and they include detailed instructions for use.

An alternative to using the NorthernMax® reagents is to use the procedure described below for electrophoresis in a formaldehyde denaturing agarose gel. This procedure is modified from "Current Protocols in Molecular Biology", Section 4.9 (Ausubel et al. editors). It is more difficult and time-consuming than the NorthernMax® method, but it gives similar results.

1. Prepare the gel

   a. Pour a denaturing agarose-formaldehyde gel of the appropriate percentage for the size of the untailed transcript. For 100 mL of gel, dissolve 1–2.5 g of agarose in 72 mL of water and cool to 60°C.

Transcript size	Recommended % agarose
≤ 500 bases	2.5% agarose
> 500 bases	1% agarose

CAUTION! Formaldehyde is toxic through skin contact and inhalation of vapors. Manipulations involving formaldehyde should be done in a chemical fume hood.

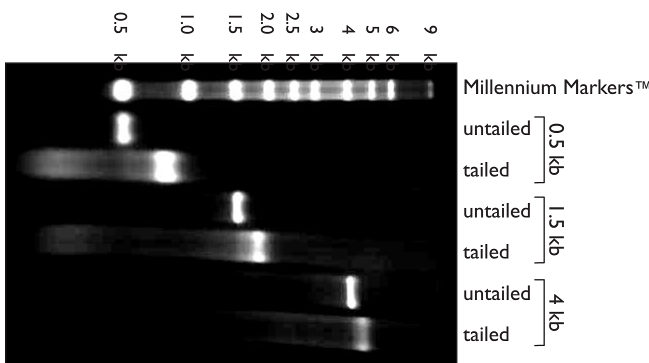
   b. Add 10 mL of 10X MOPS running buffer, and 18 mL of 37% formaldehyde (12.3 M).

10X MOPS running buffer	
Concentration	**Component**
400 mM	MOPS, pH 7.0
100 mM	sodium acetate
10 mM	EDTA

   c. Pour the gel and allow it to set. The wells should be large enough to accommodate at least 60 µL. Remove the comb, and place the gel in the gel tank. Cover with a few millimeters of 1X MOPS running buffer.

2. Prepare the RNA samples
   a. Prepare a 2.5-µL aliquot of each tailing reaction, and the corresponding minus-enzyme control. Also plan to run an RNA size marker (e.g. RNA Century™-Plus Markers Cat. no. AM7145 or Millennium™ Markers Cat. no. AM7150).
   b. Mix 7.5 µL of Formaldehyde Load Dye containing 50 µg/mL of ethidium bromide, and 2.5 µL of RNA sample.
   c. Heat samples at 75°C for 10 minutes
3. Electrophoresis
   a. Load the samples, and run the gel in 1X MOPS buffer at ~5 volts/cm until the bromophenol blue dye is near the bottom of the gel. (If ethidium bromide was not added to the Formaldehyde Load Dye, post-stain the gel for ~20 min in 1X MOPS running buffer with 0.5 µg/mL of ethidium bromide, and destain with two 10 min incubations in water.)
   b. Examine the gel on a UV light box. The tailed RNA should be ≥50 bases longer than the corresponding RNA that was not tailed (minus-*E*-PAP control).

Figure 5 Tailing transcripts of different sizes. Transcripts of 0.5 kb, 1.5 kb, and 4 kb were generated from mMESSAGE mMACHINE® T7 Ultra reactions and each RNA was tailed using the mMESSAGE mMACHINE® T7 Ultra Kit according to the protocol. The RNA was run on 1% denaturing agarose stained with ethidium bromide.



Optimizing yield of long transcripts

When synthesizing transcripts that are larger than 5 or 6 kb, GTP will become rate limiting and may result in low yield, premature termination of transcription, or both. To circumvent this, it may be desirable to supplement the reaction with extra GTP. Shown below is the effect of adding the indicated volumes of the GTP supplied with the kit to an otherwise ordinary mMESSAGE mMACHINE® T7 Ultra reaction.

Added GTP	Final ARCA:GTP Ratio	Fraction of Transcripts Capped
0 µL	4.0:1	80%
1 µL	2.0:1	67%
2 µL	1.3:1	57%
3 µL	1.0:1	50%

For templates in the 5–8 kb range we suggest initially testing the addition of 1 µL of GTP. For larger templates you should try titrating additional GTP to determine the minimum amount needed. Adding GTP will decrease the fraction of transcripts synthesized with a cap, but it will result in higher yields of full length product. The fraction of capped transcripts is proportional to the ratio of ARCA to GTP in the transcription reaction.

Optimizing yield of short transcripts

The mMESSAGE mMACHINE® T7 Ultra Kit is designed to function best with transcription templates in the 0.3–5 kb range. Under these conditions, 1 µg of plasmid DNA template per 20-µL reaction gives maximal RNA yield. 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 (<0.3 kb) can be used in mMESSAGE mMACHINE® T7 Ultra reactions. These include 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 et al. 1987). Using oligonucleotides, and PCR-derived templates, almost all of the DNA is template sequence, compared to plasmid templates which include non-transcribed vector DNA.

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 more 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. It is important to remember that 1 µg of a short template contains a much larger molar amount of DNA than 1 µg of a longer template. 50 ng of an 85-bp, PCR-derived template provides 0.9 pmoles of template (and 0.9 pmoles of promoters), compared to the approximately 0.3 pmoles template in 1 µg of the pTRI-Xef control template. In general, for optimum yield of short transcripts, use about 0.5–2 pmoles of template. For very short templates (that is, ~20–30 nt), 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 larger than 300 nucleotides, 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 (for example, Cat. nos. AM2075, AM2085, and AM2063), ***not*** the 10X T7 Enzyme Mix from the mMESSAGE mMACHINE® T7 Ultra Kit. Increasing the enzyme should be the last variable tested after increasing incubation time and optimizing template concentration.

Spin column preparation and use

Unincorporated labeled nucleotides can be removed by size exclusion chromatography on RNase-free Sephadex G-25 or G-50 spin columns. The following is a procedure for the preparation and use of spin columns:

1. Resuspend and equilibrate Sephadex G-25 or G-50 with 2 volumes of TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA), then wash with several volumes of TE.
2. Place the resuspended and washed resin in 1.5 volumes of TE in a glass bottle and autoclave. Store at 4°C until use.
3. Rinse a 1–3 mL spin column thoroughly with distilled water; frits may be pre-installed, or made by plugging the bottom of a 1 mL syringe with a support such as siliconized glass beads.
4. Pipet 1–3 mL of the prepared, well mixed resin into the washed spin column. Place the column in a 15 mL plastic centrifuge tube and spin at 2,000 rpm for 10 min in a centrifuge with a swinging-bucket rotor.
5. Place the end of the spin column containing the spun resin into an appropriate microfuge tube (typically, 0.5 mL) and insert the assembly into a new 15 mL centrifuge tube.
6. Load 20–100 µL of the sample onto the center of the resin bed (dilute sample with nuclease-free water or TE Buffer if necessary), and spin at 2,000 rpm for 10 min. The eluate collected in the microfuge tube should be approximately the same volume as the sample loaded onto the column, and it will contain about 75% of the nucleic acid applied to the column.

   IMPORTANT! It is important that the centrifugation conditions for column packing and sample purification be identical; varying them could lead to either incomplete recovery or dilution of the sample. The spin column can be tested by loading 100 µL of TE onto it and centrifuging: 100 µL of eluate should be recovered. If recovery is much greater or less than 100 µL, the column is not equilibrated and should be tested again.

Miniprep for isolating transcription-quality plasmid DNA

Generally, the cleaner the template DNA, the greater the yield of the transcription reaction. The following miniprep procedure yields high quality transcription template. This procedure is derived from a published procedure (Molecular Cloning, A Laboratory Manual), but differs in that the phenol/$CHCl_3$ 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/$CHCl_3$ extraction step. If you have difficulty getting good restriction digestion of your plasmid prep, it may be necessary to include a phenol/$CHCl_3$ 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	H_2O

Store at room temperature.

1. Pellet cells

   Centrifuge a 1.5-mL overnight bacterial culture 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

   Vortex vigorously to resuspend the pellet. Check for complete resuspension of pellet by inverting the tube and looking to see that the solution is homogenous.

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

   Invert tube several times to mix, incubate tube on ice for at least 1 min.

4. Add 165 µL of Solution III, incubate for 5 min on ice, and centrifuge for 5 min:

   Vortex medium-fast for 10 sec to mix, incubate 5 min on ice.

   Centrifuge 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 of EtOH, incubate for 5 min on ice, and centrifuge for 5 min

   Invert several times to mix, incubate 5 min on ice. This will precipitate the plasmid DNA and some of the RNA.

   Centrifuge 5 min at maximum speed: this spin should be done at 4°C if possible. This will pellet the plasmid DNA. Pour off the supernatant, respin briefly, and aspirate off any residual supernatant.

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

   TE consists of 10 mM Tris HCl, pH 8 and 1 mM EDTA.

   Use 0.5 U or 1 µg of RNase A or use 1 µL of RNase Cocktail. Vortex vigorously, incubate for about 5 min at 37–42°C and revortex to thoroughly solubilize the pellet.

7. Digest with appropriate restriction enzyme

   Use an enzyme that will linearize the plasmid so that the polymerase promoter site will be upstream of the sequence you want to transcribe. The volume of the restriction digest should be about 2–3 times 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 of Proteinase K (final concentration). Mix well by inversion, and incubate at 50°C for at least 30 min.

9. Phenol/$CHCl_3$ extract and ethanol precipitate

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

   Remove the aqueous (top) phase to new tube, add 1/10th volume of 5 M NH_4OAc (RNase-free), add 2 volumes of EtOH, incubate for at least 15 min at –20°C.

10. Pellet DNA

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

    Resuspend the DNA in 10–20 µL RNase-free dH_2O per 1.5 mL of culture. Vortex until the pellet is 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. For example, if 0.5 µg of a phage lambda/Hind III digest is used for comparison, the 2.2 kb band, which will probably be close to your template DNA in size, will contain about 22 ng of DNA.

Recipes

1. 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 of nuclease-free water. Adjust the final volume to 1 L.

Alternatively, we offer 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. Denaturing acrylamide gel mix

5% acrylamide /8M urea gel

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

a. Mix the following:

for 15mL	Component
7.2 g	Urea (high quality) (Cat. no. AM9902)
1.5 mL	10X TBE
1.9 mL	40% Acrylamide (19 acryl:1 bis-acryl) (Cat. nos. AM9022, AM9024)
to 15 mL	ddH_2O

b. Stir at room temperature until the urea is completely dissolved, then add:

120 µL	10% ammonium persulfate
16 µL	TEMED

c. Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour gel immediately. (It is not necessary to treat the gel mixture with diethylpyrocarbonate)

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.

3. RNase-free water
   a. Add DEPC to 0.05% to double-distilled, deionized water (that is, 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 Thermo Fisher Scientific

Product	Description
MEGAclear™ Kit Cat. no. AM1908	The MEGAclear™ kit 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.
mMESSAGE mMACHINE® Cat. nos. AM1340, AM1344, AM1348	High yield transcription kits for production of large amounts of capped RNA. These kits employ our novel, patented MEGAscript® technology, and include cap analog. Kits are available with T7, SP6, and/or T3 RNA polymerase.
RNase-free Tubes & Tips	RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free.
RNase*Zap*® RNase Decontamination Solution Cat. nos. AM9780, AM9782, AM9784	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.
NucAway™ Spin Columns Cat. no. AM10070	Guaranteed RNase- and DNase-free, NucAway™ Spin Columns provide a fast, efficient way to remove unincorporated nucleotides, and to effect buffer exchange after probe synthesis and other reactions.
RNA Storage Solutions	Three different choices for safe, RNase-free resuspension of RNA pellets. Choose one or more of the following: THE RNA Storage Solution, Cat. nos. AM7000, AM7001 0.1 mM EDTA, Cat. no. AM9912 TE Buffer, Cat. nos. AM9860, AM9861
TURBO DNA-*free*™ Kit Cat. no. AM1907	The TURBO DNA-*free*™ Kit employs our 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*™ kit is ideal for removing contaminating DNA from RNA preparations.
Electrophoresis Reagents	We offer gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis.
Proteinase K Cat. nos. AM2542, AM2548	Proteinase K is a non-specific serine protease commonly used in molecular biology to remove protein contaminants from nucleic acids. We offer Proteinase K in powder form and as a 50% glycerol solution.
Phenols	We offer a full line of prepared phenol solutions for most molecular biology needs. These premixed, quality-tested, saturated phenols are ready-to-use and eliminate the handling concerns associated with preparing phenol for use from solid phenol.

Quality control

Functional testing

All components are tested in a functional mMESSAGE mMACHINE® T7 Ultra assay as described in this procedure. A 20-µL reaction containing 1 µg of the control template DNA which codes for a ~1.9-kb transcript synthesized at least 15–25 µg of capped RNA after a 2-hr incubation. The Poly (A) Tailing reaction is shown to append an >100 base poly(A) tail to the RNA transcripts.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity

A sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

A sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

A sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

A sample is incubated with protease substrate and analyzed by fluorescence.

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

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. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organization, Laboratory Biosafety Manual, 3rd Edition, WHO /CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

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21 February 2014