USER GUIDE

ambion®
by life technologies™

mMESSAGE mMACHINE® Kit

High Yield Capped RNA Transcription Kit

SP6, T7, and T3 Kits

Catalog Numbers AM1340, AM1344, AM1348

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life technologies™

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mMESSAGE mMACHINE® Kit

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

Introduction

Background

mMESSAGE mMACHINE® Kits are designed for the in vitro synthesis of large amounts of capped RNA. Capped RNA mimics most eukaryotic mRNAs found in vivo, because it has a 7-methyl guanosine cap structure at the 5' end. mMESSAGE mMACHINE® Kit reactions include cap analog [m^7G(5')ppp(5')G] in an ultra high-yield transcription reaction. The cap analog is incorporated only as the first or 5' terminal G of the transcript because its structure precludes its incorporation at any other position in the RNA molecule. mMESSAGE mMACHINE® Kits have a simplified reaction format in which all four ribonucleotides and cap analog are mixed in a single solution. The cap analog:GTP ratio of this solution is 4:1, which is optimal for maximizing both RNA yield and the proportion of capped transcripts. mMESSAGE mMACHINE® Kits are ideal for the routine synthesis of capped RNAs for oocyte microinjection, in vitro translation, transfection and other applications.

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 Enzyme Mix. The mMESSAGE mMACHINE® Kit contains all the buffers and reagents necessary for 25 transcription reactions. Using the control template supplied with the kit (Xenopus elongation factor 1α, pTRI Xef), each mMESSAGE mMACHINE® reaction will yield approximately 20–30 µg of RNA using T3 or T7 RNA polymerase, or about 15–25 µg RNA using SP6 RNA polymerase.

Materials provided with the kit

The mMESSAGE mMACHINE® Kit should be stored in a non-frost-free freezer. Keep all reagents on ice while using the kit; the nucleotides and enzymes are especially labile.

Reagents are included for 25 mMESSAGE mMACHINE® reactions.

<table>
<tr><th>Amount</th><th>Component</th><th>Storage</th></tr>
<tr><td>1.75 mL</td><td>Nuclease-free Water</td><td>any temp‡</td></tr>
<tr><td>50 µL</td><td>Enzyme Mix (SP6, T7, or T3)†:
Buffered 50% glycerol containing RNA polymerase, RNase Inhibitor, and other components</td><td>–20°C</td></tr>
<tr><td>50 µL</td><td>10X Reaction Buffer (SP6, T7, or T3)†
salts, buffer, dithiothreitol, and other ingredients</td><td>–20°C</td></tr>
<tr><td>250 µL</td><td>2X NTP/CAP (SP6, T7, or T3)†: a neutralized buffered solution containing:
<table>
<tr><th></th><th>SP6 Kits</th><th>T7 and T3 Kits</th></tr>
<tr><td>ATP</td><td>10 mM</td><td>15 mM</td></tr>
<tr><td>CTP</td><td>10 mM</td><td>15 mM</td></tr>
<tr><td>UTP</td><td>10 mM</td><td>15 mM</td></tr>
<tr><td>GTP</td><td>2 mM</td><td>3 mM</td></tr>
<tr><td>cap analog</td><td>8 mM</td><td>12 mM</td></tr>
</table></td><td>–20°C</td></tr>
<tr><td>100 µL</td><td>GTP† 20 mM in SP6 Kits; 30 mM in T3 and T7 Kits</td><td>–20°C</td></tr>
<tr><td>100 µL</td><td>TURBO DNase (2 U/µL)</td><td>–20°C</td></tr>
<tr><td>10 µL</td><td>pTRI-Xef, 0.5 mg/mL (Control Template)</td><td>–20°C</td></tr>
<tr><td>1 mL</td><td>Ammonium Acetate Stop Solution
5 M ammonium acetate, 100 mM EDTA</td><td>–20°C</td></tr>
<tr><td>1.4 mL</td><td>Lithium Chloride Precipitation Solution
7.5 M lithium chloride, 50 mM EDTA</td><td>–20°C</td></tr>
<tr><td>1.4 mL</td><td>Gel Loading Buffer II: a 1–2X gel loading solution for TBE polyacrylamide and agarose gels
95% formamide
0.025% xylene cyanol, 0.025% bromophenol blue
18 mM EDTA
0.025% SDS</td><td>–20°C</td></tr>
</table>

† Components are specifically calibrated for each lot and kit type. Mixing components from different lots, or from kits for different enzymes (SP6, T7, T3) will compromise RNA yield.

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

Materials not provided with the kit

- DNA template: The DNA template must have the correct RNA polymerase promoter site (T7, T3, or SP6) 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).

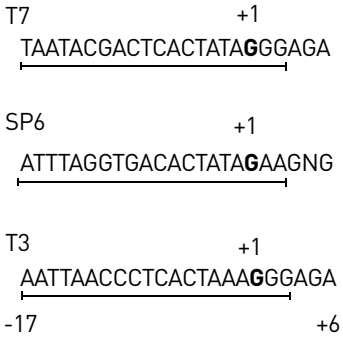
- (optional) Labeled nucleotide(s): [α-^{32}P] UTP or [α-^{32}P]CTP can be added to the reaction as a tracer to facilitate quantitation of the RNA synthesized. Any specific activity is acceptable.
- (optional) For purification of the synthesized RNA:
  - Buffer- or water-saturated phenol/chloroform
  - Isopropanol
  - Spin Columns

mMESSAGE mMACHINE® Kit Procedure

Preparation of template DNA

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

Figure 1 Phage Polymerase Promoters: 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.

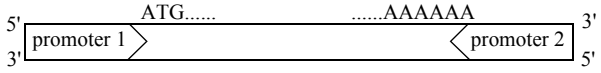
Template size

The mMESSAGE mMACHINE® 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, or longer RNA, but modify the reaction as described in section "Optimizing yield of short transcripts" on page 21.

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.

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® 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 23.

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 Na acetate ***or*** 5 M NH_4 acetate
- 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 dH_2O 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 (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.

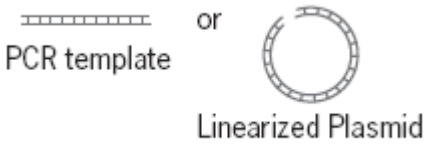
PCR templates

DNA generated by PCR can be transcribed directly from the PCR provided it contains an 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® to estimate concentration, and to verify that the products are unique and the expected size.

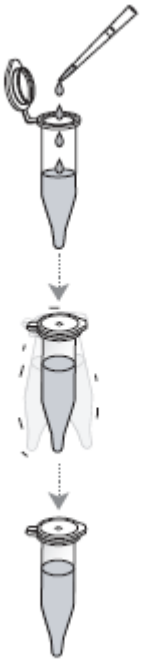
Procedure overview

Figure 2 Transcription reaction assembly and incubation

Preparation of template DNA

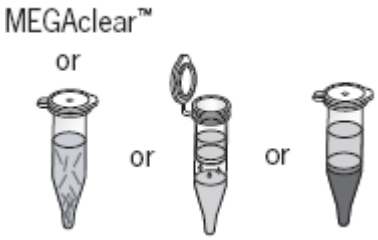


Capped transcription reaction assembly



1. "Thaw the frozen reagents" on page 11
2. "Assemble transcription reaction at room temp" on page 11
3. "Mix thoroughly" on page 11
4. "Incubate at 37°C, 1 hr" on page 11
5. "(optional) Add 1 µL TURBO DNase, mix well and incubate 15 min at 37°C" on page 12

Recovery of the RNA



1. "MEGAclear™ Kit" on page 12
2. "Lithium chloride precipitation" on page 12
3. "Spin column chromatography" on page 12
4. "Phenol:chloroform extraction and isopropanol precipitation" on page 13

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 Reaction Buffer and the 2X NTP/CAP until they are completely in solution. Once thawed, store the ribonucleotides (2X NTP/CAP) on ice, but ***keep the 10X 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 Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.

   Add the 10X 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 20 and "Optimizing yield of short transcripts" on page 21, respectively.

Amount	Component
to 20 µL	Nuclease-free Water
10 µL	2X NTP/CAP
2 µL	10X Reaction Buffer
(1 µL)	(optional) [α-32P]UTP as a tracer
0.1–1 µg	linear template DNA†
2 µL	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. Since SP6 reactions are somewhat slower than T3 and T7 reactions, they especially may benefit from the second hour of 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 20 and "Optimizing yield of short transcripts" on page 21 for optimizing yield from templates coding RNA outside the 0.3–5 kb range.

If the 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 14).

5. (optional) 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.

   a. Add 1 µL TURBO DNase, and mix well.

   b. Incubate at 37°C for 15 min.

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® 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 30 µL Nuclease-free Water and 30 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 cap analog that may inhibit in vitro translation.

† Life Technologies offers 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

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 22.

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® Kit reactions. Since the RNA is precipitated, this method can also be used for buffer exchange.

   a. Add 115 µL Nuclease-free Water and 15 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.

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® 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:

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

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.

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® reaction should be run on a denaturing agarose or acrylamide gel alongside an aliquot of an RNA of known concentration. See section "Additional procedures" on page 19 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® 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

TCA precipitation

If a trace amount of radiolabel was included in the mMESSAGE mMACHINE® reaction, it can be used to determine yield. First precipitate with TCA to determine the proportion of radiolabel that was incorporated into RNA. (TCA will precipitate nucleic acids as small as 18 nt.)

a. Dilute 1 µL of the completed mMESSAGE mMACHINE® reaction with 1 µL TE Buffer in a nuclease-free 1.5 mL microfuge tube, and vortex thoroughly to ensure that the newly synthesized RNA is completely 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 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® reaction. The concentration of GTP limits the amount of RNA that can be synthesized. For any tracer other than labeled GTP (e.g. [α-32P] UTP), each 1% incorporation corresponds to about 2 µg of RNA synthesized in a T7 or T3 reaction. For SP6 Kits, each 1% incorporation corresponds to about 1.3 µg of RNA synthesized.

1% incorporation = 2 µg/RNA

For example: if % incorp. = 10%

10 x 2 µg = 20 µg RNA

In a T7 or T3 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.

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

The standard SP6 mMESSAGE mMACHINE® reaction contains 1.0 mM GTP, so when that value is substituted in the above equation the maximum theoretical yield for SP6 mMESSAGE mMACHINE® reactions is 26.4 µg.

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). Any one of the three RNA polymerases can be used to synthesize the control RNA. When transcribed with the following RNA polymerases, sense transcripts of the indicated length are produced from this template:

Enzyme	Transcript Size
SP6	1.92 kb
T7	1.89 kb
T3	1.86 kb

These transcripts will produce a 50.2 kD protein when translated.

1. Reaction setup

   Use 2 µL (1 µg) of pTRI-Xef in a standard mMESSAGE mMACHINE® reaction as described in section “Capped transcription reaction assembly” on page 11.

2. Expected yield from the control reaction

   The yield from the control reaction for T7 and T3 Kits should be 20–30 µg of RNA, and 15–25 µg with the SP6 Kits. If a [32P]NTP was added to the transcription reaction as a tracer, approximately 15% of the radiolabel should be incorporated.

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

   If the yield of RNA from the control reaction is low, something may be wrong either with the procedure or the kit, or the quantitation is in error.

   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 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

The amount of RNA synthesized in a standard 20 µL mMESSAGE mMACHINE® 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® 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

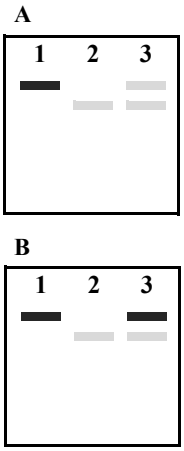
   If the transcription reaction with your template generates full-length, intact RNA, but the reaction yield is significantly lower than the amount of RNA obtained with the pTRI-Xef control template, it is possible that contaminants in the DNA are inhibiting the RNA polymerase. A mixing experiment can 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 0.5–1 µL of each transcription reaction on a denaturing gel as described in section , "Additional procedures" on page 19.

a. Transcription of the control template is inhibited by the presence of your template. (See Figure 3.A)

Figure 3 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 3.B)

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

i. Use a different polymerase for transcription if possible.

Templates differ in transcription efficiency depending on the initiation efficiency of their promoter, the presence of internal termination signals, and their length. If the problem is due to the first or second of these issues, changing the RNA polymerase promoter used to transcribe the fragment may alleviate the problem.

ii. Check the amount and quality of template

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

Also, check an aliquot of the template DNA on an agarose gel to make sure it is intact and that it is the expected size.

iii. Extend the reaction time

Another parameter that can be adjusted is reaction time. Extending the standard 1 hr incubation to 4–6 hr or even overnight may improve yield.

Multiple reaction products, transcripts of the wrong size

1. 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 (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. 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. Sometimes the RNA will run as two distinct bands on a native agarose gel, but when the same RNA is run on a denaturing gel, it will migrate as a single band of the expected size.

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

      - Different phage polymerases recognize different termination signals, so using a different polymerase promoter may help.
      - 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. Reaction products are larger than expected

   a. Persistent secondary structure

      mMESSAGE mMACHINE® 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.

A Supplemental Information

Additional procedures

Analysis of transcription products by gel electrophoresis

1. Agarose or Acrylamide?

   The size of mMESSAGE mMACHINE reaction products can be assessed by running an aliquot of the reaction on an agarose or polyacrylamide gel. Transcripts larger than about 1.5 kb should be run on agarose gels, whereas polyacrylamide gels (4–5%) are better for sizing smaller transcripts. Since secondary structure in the transcript may cause aberrant migration and/or multiple bands, the gel should be run under denaturing conditions. For agarose gels, this means glyoxal or formaldehyde gels, prepared and run according to standard procedures (Molecular Cloning, A Laboratory Manual, 1989). Instructions for preparing and running denaturing acrylamide gels are supplied in section "Denaturing acrylamide gel mix" on page 25.

2. Sample preparation

   To get good resolution of the RNA, load ~1 µg per gel lane. For denaturing polyacrylamide gels add an equal volume of Gel Loading Buffer II to each sample, and heat for 3–5 min at 80–90°C. (Gel Loading Buffer II cannot be used with glyoxal agarose gels and it will not completely denature samples run on formaldehyde agarose gels. Use a loading buffer specifically formulated for the type of agarose gel you plan to run.)

   To stain the RNA with ethidium bromide during electrophoresis do one of the following:

   a. Add 0.5 µg/mL ethidium bromide to the gel mix
   b. Add 0.5 µg/mL ethidium bromide to the running buffer
   c. Add 10 µg/mL ethidium bromide to the RNA samples (and gel loading buffer) before loading the gel.

   (Because single-stranded nucleic acids bind ethidium less efficiently than double-stranded nucleic acids, the fluorescence of RNA samples on a denaturing agarose gel will be less intense than the same amount of DNA.)

3. Visualizing reaction products

   a. Ethidium bromide stained samples

      View ethidium bromide stained gels on a UV transilluminator. Ideally there will be a single, tight band at the expected molecular weight. See section "Multiple reaction products, transcripts of the wrong size" on page 18 for troubleshooting suggestions if this is not what appears on your gel.

   b. Radioactively-labeled transcripts

If the transcription reaction contained a radiolabeled nucleotide tracer (e.g. [α-^{32}P]UTP), the RNA can be visualized by autoradiography. Agarose gels should be dried before exposing to X-ray film, but thin (0.75 mm thick) polyacrylamide gels may be transferred to filter paper, covered with plastic wrap, and exposed directly (when ^{32}P is used). Approximate exposure times for visualizing low specific activity transcripts (e.g. when 1 µL of 800 Ci/mmol, 10 mCi/mL [α-^{32}P] UTP was used in the mMESSAGE mMACHINE reaction) are about 10–30 min with an intensifying screen, or several hours to overnight without a screen, when 1 µL of the undiluted reaction is run on the gel. A recipe for standard denaturing (i.e. 8 M urea) polyacrylamide gels is given in section "Denaturing acrylamide gel mix" on page 25.

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 reaction.

Added GTP*	Final Cap Analog: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%

*The GTP supplied with T7 and T3 mMESSAGE mMACHINE Kits is 30 mM. The GTP supplied with SP6 mMESSAGE mMACHINE Kits is 20 mM

1. How much additional GTP should be added?

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 Cap Analog to GTP in the transcription reaction.

2. There is a trade-off between RNA yield, and good capping efficiency

   We tested the effect of varying the ratio of cap analog to GTP on the yield of RNA from transcription reactions and on the efficiency of translation of the resulting RNA in a reticulocyte lysate in vitro translation system (See Table 1). The translation of globin RNA in a reticulocyte lysate is known to be very cap dependent. As the ratio of cap analog to GTP increases, the yield of RNA decreases. Conversely, the translational efficiency of the RNA synthesized increases with increasing cap analog to GTP ratios. This reflects the increasing fraction of transcripts with a 5' cap. Note that a cap analog to GTP ratio of 4:1 provides a good compromise between RNA yield, and capping efficiency in the case of globin. The presence of uncapped transcripts in Xenopus oocyte microinjection experiments does not usually present a problem. These uncapped transcripts are presumably rapidly degraded by the oocyte.

Cap analog: GTP ratio	RNA yield	Protein yield (cpm)
0:1	49.3 µg	5,466 cpm
1:1	43.5 µg	56,450 cpm
2:1	37.4 µg	65,632 cpm
4:1	25.7 µg	80,636 cpm
8:1	14.8 µg	86,867 cpm
10:1	12.0 µg	93,834 cpm

T7-mMESSAGE mMACHINE reactions were done under standard conditions except that the ratio of m7G(5')ppp(5')G to GTP was varied as indicated. Reactions were incubated at 37°C using 1 µg of T7 globin template DNA for 1 hr. The globin RNA (6 µg/mL) was then translated in a 25 µL Retic Lysate IVT™ in vitro translation reactions for 60 min at 30°C with 12.5 µCi of [35S]methionine (1200 Ci/mmol). The incorporation of TCA-precipitable cpm was measured.

Optimizing yield of short transcripts

The mMESSAGE mMACHINE 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 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 (i.e. ~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 (e.g. Cat. nos. AM2075, AM2085, and AM2063), ***not*** the 10X Enzyme Mix from the mMESSAGE mMACHINE 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/CHCl3 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/CHCl3 extraction step. If you have difficulty getting good restriction digestion of your plasmid prep, it may be necessary to include a phenol/CHCl3 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	H2O

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 Solution II, incubate 1 min on ice

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

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

   Vortex medium-fast for 10 sec, 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 EtOH, incubate 5 min on ice, centrifuge 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 TE containing RNase, incubate 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 RNase A or use 1 µL of RNase Cocktail. Vortex vigorously, incubate 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 Proteinase K (final concentration). Mix well by inversion, and incubate at 50°C for at least 30 min.

9. Phenol/CHCl3 extract and ethanol precipitate

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

   Remove the aqueous (top) phase to new tube, add 1/10th volume of 5 M NH4OAc (RNase-free), add 2 volumes EtOH, incubate 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 dH2O per 1.5 mL 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 nuclease-free water. Adjust the final volume to 1 L.

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

2. 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 (i.e. add 0.5 mL per liter of water).
   b. Stir well, incubate several hours to overnight at 37°C or 42°C.
   c. Autoclave 2 L or smaller volumes for at least 45 min. The scent of DEPC should be either not detectable or only very slightly detectable.

Related products available from Life Technologies

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® T7 Ultra Kit Cat. no. AM1345	The mMESSAGE mMACHINE® T7 Ultra Kit incorporates Anti-Reverse Cap Analog (ARCA) into Life Technologies's patented high yield transcription technology to generate RNA transcripts that yield much higher amounts of protein when translated in vitro or in vivo, than messages with traditional cap analog. The increased translation efficiency provided by ARCA is further enhanced by the addition of a poly(A) tail to the transcripts. Experiments comparing ARCA and ARCA/poly(A) tailed transcripts to cap analog and cap analog/poly(A) tailed transcripts show significantly higher levels of protein synthesis with ARCA capped RNA.
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	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 Life Technologies's exclusive TURBO™ DNase, 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.
Electrophoresis Reagents	Life Technologies offers 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. Life Technologies supplies Proteinase K in powder form, and as a 50% glycerol solution.
Phenols	Life Technologies offers 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.
ARCA Cat. no. AM8045	7-methyl (3'-0-methyl) GpppG, anti-reverse cap analog, can be added to in vitro transcription reactions to produce capped RNA transcripts that incorporate the cap only in the correct orientation.

Quality control

Functional testing

All components are tested in a functional mMESSAGE mMACHINE® 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.

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

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

institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

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

In the EU:

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

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Documentation and Support

Obtaining SDSs

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

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

Obtaining support

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

www.lifetechnologies.com/support

At the website, you can:

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

Limited product warranty

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

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit lifetechnologies.com/support or email techsupport@lifetech.com

lifetechnologies.com
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