mMESSAGE mMACHINE™

High Yield Capped RNA Transcription Kit

SP6, T7, and T3 Kits
Catalog #s 1340, 1344, 1348

Ambion®

THE RNA COMPANY

mMESSAGE mMACHINE™

Catalog#'s 1340, 1344, 1348

Updated: 1/2/00
Version: 0004E2

Notice: This notice addresses both a product improvement and a printing error.

mMESSAGE mMACHINE™ Kits now contain SUPERase•In™ in the Enzyme Mix

SUPERase•In (patents pending), the ultimate RNase Inhibitor, is now included in the RNA Polymerase Enzyme Mix of all mMESSAGE mMACHINE kits.

An RNase inhibitor is added to the mMESSAGE mMACHINE Enzyme Mix to protect newly transcribed RNA from degradation by RNases that could be inadvertently introduced from one or more of several different sources. Typical sources of RNase contamination in mMESSAGE mMACHINE reactions include plasmid templates, ungloved fingers, contaminated microcentrifuge rotors, and even airborne dust. Previously, Ribonuclease Inhibitor Protein (RIP) was used to inhibit RNase contaminants in mMESSAGE mMACHINE kits, but as part of Ambion's effort to continually improve our products, we have replaced RIP with SUPERase•In.

SUPERase•In, like RIP, is a protein-based RNase inhibitor that works by non-covalently binding RNases. Unlike RIP, SUPERase•In is of nonhuman origin, and it won't release active RNase in the absence of DTT. SUPERase•In protects against a wide range of RNases including A, T1, and I, whereas RIP will only inhibit the RNase A family enzymes. In addition to inhibiting a wider variety of RNases, SUPERase•In also outperforms RIP in the presence of higher levels of RNase. With the addition of SUPERase•In, Ambion's mMESSAGE mMACHINE Kit is even more robust than before.

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Plasmid Templates

~~After linearization~~
~~Terminate the restriction digest by adding a 1-20 volume of 0.5 M EDTA, 1-10 volume of 3 M sodium acetate or 5 M ammonium acetate, and 2 volumes of ethanol. Chill at -20°C for at least 15 minutes and pellet the DNA for 15 minutes in a microcentrifuge at top speed. Remove the supernatant, re-spin the tube for a few seconds, and then 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.~~

After linearization

Terminate the restriction digest by adding the following:

1/20th volume 0.5 M EDTA
1/10th volume of either 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 minutes. Then pellet the DNA for 15 minutes 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.

mMESSAGE mMACHINE™
Instruction Manual

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Manual Version 0004

Literature Citation

We would appreciate if when you are describing a procedure utilizing this product in a Materials and Methods Section for publication that you refer to it as the mMESSAGE mMACHINE™ Kit.

Warranty and Liability

Ambion is committed to providing the highest quality reagents at competitive prices. Ambion warrants that the products meet or exceed the performance standards described in the product specification sheets. If you are not completely satisfied with any product, our policy is to replace the product or credit the full purchase price and delivery charge. No other warranties of any kind, expressed or implied are provided by Ambion. Ambion's liability shall not exceed the purchase price of the product. Ambion shall have no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products. This product is intended for research use only. This product is not intended for diagnostic or drug purposes.

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This product is covered by U.S patent 5,256,555 and patents pending

I. Introduction

A. Background

Ambion's 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 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 ratioof this solution is 4:1, which is optimal for maximizing both RNA yield and the proportion of capped transcripts. Ambion's 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 (U.S. patents issued and pending). The mMESSAGE mMACHINE Kit contains all the buffers and reagents necessary for 25 transcription reactions. Using the control template (Xenopus elongation factor 1α, pTRI Xef) supplied with the kit, each mMESSAGE mMACHINE reaction will yield approximately 20-30 µg RNA using T3 or T7 RNA polymerase, or about 15-25 µg RNA using SP6 RNA polymerase.

B. Storage and Stability

The kit should be stored at -20°C in a non frost-free freezer. Properly stored kits are guaranteed for 6 months from the date received.

C. Reagents Provided With The Kit

Amount	Component
50 µl	Enzyme Mix (SP6, T7, or T3)*: Buffered 50% glycerol containing RNA polymerase, RNase inhibitor, and other components.
50 µl	10X Reaction Buffer (SP6, T7, or T3)* salts, buffer, dithiothreitol, and other ingredients
250 µl	2X NTP/CAP (SP6, T7, or T3)* - a neutralized buffered solution containing:

	SP6 Kits	T7 and T3 Kits
ATP	10 mM	15 mM
CTP	10 mM	15 mM
UTP	10 mM	15 mM
GTP	2 mM	3 mM
cap analog	8 mM	12 mM

Amount	Component
100 µl	GTP* 20 mM in SP6 kits; 30 mM in T3 and T7 kits
45 µl	DNase 1, 2 U/µl (RNase-free)
10 µl	pTRI-Xef, 0.5 mg/ml
1 ml	Ammonium Acetate Stop Solution 5 M ammonium acetate, 100 mM EDTA
1.4 ml	Lithium Chloride Precipitation Solution 7.5 M lithium chloride, 75 mM EDTA
1.75 ml	Nuclease-free Water
1.4 ml	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)

* Starred 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.

NOTE:

The RNA polymerase included in the kit corresponds to the specific type of MEGAscript™ Kit purchased.

D. 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 dH_2O or TE (10 mM Tris-HCl (pH 7-8), 1 mM EDTA).
- Nuclease-free water for dilution following transcription.
- (optional) Labeled nucleotide, for example α-^{32}P]UTP or α-^{32}P]CTP, as a tracer to facilitate quantitation of the RNA synthesized. Any specific activity is acceptable.
- (optional) For purification of RNA following transcription:
  Buffer- or water-saturated phenol/chloroform
  Isopropanol

E. Related Products Available from Ambion

NucAway™ Spin Columns
cat. # 10070

Guaranteed RNase- and DNase-free, Ambion's NucAway Spin Columns provide a fast, efficient way to remove unicorporated nucleotides, and to effect buffer exchange after probe synthesis and other reactions.

Retic Lysate IVT™
cat. # 1200

Ambion's rabbit reticulocyte lysate can be used to translate capped, or uncapped RNA. The template RNA can be from in vitro synthesis reactions, or from complex RNA mixtures such as total or poly(A) cellular RNA. The kit comes with control RNA, and buffers for translating capped or uncapped RNA, using either of two different radiolabled amino acids.

Wheat Germ IVT™
cat. # 1250

This system is an alternative in vitro translation kit when reticulocyte lysate is not appropriate. Buffers for labeling with two different amino acids, and a control RNA are provided with the kit.

Proteinase K
cat. # 2542-2548

Proteinase K is a non-specific serine protease commonly used in molecular biology to remove protein contaminants from nucleis acids. Ambion supplies Proteinase K in lyophilized powder form, and as a 50% glycerol solution.

Cap Analog
cat. # 8048-8052

$m^7G(5')ppp(5')G$, commonly known as cap analog, can be added to in vitro transcription reactions to produce capped RNA transcripts.

DNA-*free*™
cat. # 1906

DNase treatment and removal reagents. This product contains Ambion's ultra-high quality RNase-free DNase 1 and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included.

Electrophoresis Reagents
cat. # see catalog

Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog for a complete listing as this product line is always growing.

II. mMESSAGE mMACHINE™ Protocol

A. 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 mMESSAGE mMACHINE. In general, any DNA with a promoter site, that is suffiently pure to be easily digested with restriction enzymes can be used for in vitro transcription.

Figure 1. Phage polymerase promoters - minimal sequence requirements

T7
+1
TAATACGACTCACTATA**G**GGAGA

SP6
+1
ATTTAGGTGACACTATA**G**AAGNG

T3
+1
AATTAACCCTCACTAAA**G**GGAGA
-17 +6

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.

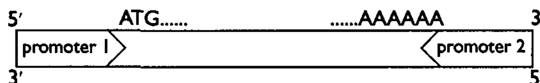
Template size

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 sections IV.C on page 20, and IV.B on page 19.

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).



Transcribing using the RNA polymerase corresponding to promoter 1 will make sense RNA (homologous to what is found in vivo). 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. A DNA miniprep procedure that generally yields high quality template is presented in section IV.G on page 25.

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 a 1 20 volume of 0.5 M EDTA, 1 10 volume of 3 M sodium acetate or 5 M ammonium acteate, and 2 volumes of ethanol. Chill at -20°C for at least 15 minutes and pellet the DNA for 15 minutes in a microcentrifuge at top speed. Remove the supernatant, re-spin the tube for a few seconds, and then 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.

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 minutes at 50°C, follow this with phenol/chloroform extraction and ethanol precipitation.

PCR templates

DNA generated by PCR can be transcribed directly from the PCR reaction provided it contains an RNA Polymerase promoter upstream of the sequence to be transcribed. PCR products should be examined on an agarose gel prior to in vitro transcription to estimate concentration and to verify that the products are unique and of the expected size. A strategy for using PCR to generate transcription templates is descibed in section IV.E on page 23.

B. 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 they are thawed, store the ribonucleotides (2X NTP/CAP) on ice, but ***keep the 10X Reaction Buffer at room temperature.***

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 temperature

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

Add the 10X Reaction Buffer after the water and template DNA are already in the tube.

The following amounts are for a single 20 µl reaction. Reactions may be scaled up or down depending on specific requirements.

IMPORTANT!

The following reaction setup is recommended when the RNA produced will be 300 bases to 5 kb in length. For transcripts shorter or longer than this, see sections IV.C on page 20 and IV.B on page 19, respectively, for modified reaction setup suggestions.

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

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 hour

Typically, 80% yield is achieved after a 1 hour incubation. For maximum yield, we recommend a 2 hour 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 also recommended for templates coding transcripts less than 300 bases, and for templates which are inefficiently transcribed.

IMPORTANT!

At the end of the reaction (before or after DNase treatment), you may want to remove an aliquot of trace-radiolabeled reactions to assess yield by TCA precipitation (see section II.D.3.a on page 11).

5. (optional) Remove template DNA

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

a. Add 1 µl DNase 1, and mix well.

b. Incubate at 37°C for 15 minutes

C. Recovery of the RNA

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

1. 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 in step a below.

a. Stop the reaction and precipitate the RNA by adding 30 µl Nuclease-free Water and 25 µl LiCl Precipitation Solution.

b. Mix thoroughly. Chill the reaction for at least 30 mins. at -20°C.

c. Centrifuge at 4°C for 15 mins. at maximum speed to pellet the RNA.

d. Carefully remove the supernatant. Wash the pellet once with ~1 ml 70% EtOH, and re-centrifuge to maximize removal of unincorporated nucleotides.

e. Carefully remove the wash solution, and resuspend the RNA in a solution or buffer* appropriate for your application. Determine concentration and store frozen at -20°C or -70°C.

2. Spin column chromatography

Spin columns can be used to remove unincorporated nucleotides, including unincorporated cap analog that may inhibit in vitro transcription.

Prepared spin columns such as Ambion's NucAway™ Spin Columns can be used by following the manufacturer's instructions. Alternatively, instructions for preparing spin columns are given in section IV.D on page 22.

3. 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 reactions. Since the RNA is precipitated, it can also be used for buffer exchange.

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* Ambion offers several products for RNA storage, these include:

Nuclease-free Water (not DEPC-treated) - cat. #s 9930-9934
THE RNA Storage Solution - cat.#s 7000, 7001
TE Buffer - cat.#s 9860, 9861
0.1mM EDTA - cat.#s 9911, 9912
RNA*secure*™ - cat.#s 7005, 7006, 7010

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.
(Optional: back-extract the organic phase with 50 µl dH_2O.)

c. Precipitate the RNA by adding 1 volume of isopropanol and mixing well.

d. Chill the reaction for at least 15 minutes at -20°C. Centrifuge at 4°C for 15 minutes 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.

D. Quantitation of Reaction Products

1. Quantitation by UV light absorbance

Simply 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:

$$A_{260} \times \text{dilution factor} \times 40 = \mu g/ml \text{ RNA}$$

2. Quantitation by ethidium bromide fluorescence

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

a. **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 an error of about two-fold.

b. 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 IV.A on page 18 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.

3. Quantitation by trace radiolabeling

a. 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.)

i. To a 12 x 75 mm tube containing 198 µl of H_2O, add 100 µg of carrier DNA or RNA.

ii. Add 2 µl of the mMESSAGE mMACHINE reaction and mix thoroughly.

iii. Transfer 100 µl of the diluted mMESSAGE mMACHINE reaction 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).

iv. Add 2 ml of cold 10% TCA (trichloroacetic acid) to the remaining 100 µl of the dilution in the 12 x 75 mm tube, mix thoroughly and place on ice for 10 minutes. This will precipitate nucleic acids, but not free nucleotides.

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

vi. 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.

vii. Place the filter in a scintillation vial, add aqueous scintillation cocktail, and count in a scintillation counter. (The number will reflect radiolabel that was incorporated into RNA.)

viii. Divide the cpm in Step vii by the cpm in Step iii to determine the fraction of label incorporated into RNA (multiply by 100 for percent incorporation).

b. Calculation of yield

Once the percent incorporation of radiolabel is known, this value can be used to calculate the mass yield of RNA from 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- ^{32}P] 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.

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

III. Troubleshooting

A. Use of the Control Template

The 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 control template in a standard mMESSAGE mMACHINE reaction as described in section II.B on page 7.

2. Expected yield from the control reaction

The yield of RNA from the control reaction for T7 and T3 kits should be 20-30 µg of transcript, and 15-25 µg with the SP6 kits. This corresponds to approximately 15% incorporation of a ^{32}P-NTP added as a tracer.

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

If the yield of RNA from the control reaction is low, probably something is wrong either with the procedure, or with 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 Transcription 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 protocol carefully. If things still don't go well, contact Ambion's Technical Services for more ideas.

B. 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 Ambion 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

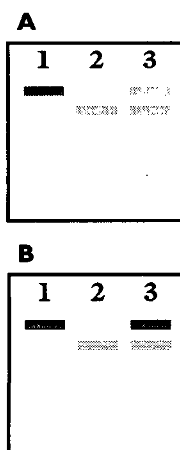


Figure 2. Possible outcomes of mixing experiment

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

Assess the results of the mixing experiment by running 0.5-1 µl of the transcription reaction on a denaturing gel as described in section IV.A on page 18.

a. **Transcription of the control template is inhibited by the presence of your template.** (See figure 2.A)
   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 minutes at 50°C, followed by phenol/chloroform extraction and ethanol precipita-

tion. 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% ethnaol before resuspension.

b. **Addition of your template to the transcription reaction containing the control template does not inhibit synthesis of the control RNA.** (See figure 2.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 not degraded and is of the expected size.

iii. **Extend the reaction time**
Another parameter that can be adjusted is reaction time. Extending the standard 1 hour incubation to 4-6 hours or even overnight may improve yield. One concern about extending the incubation time beyond 4-6 hours is that any ribonuclease held in check by RNase inhibitor present in the Enzyme Mix, may be slowly released during an overnight incubation, degrading some of the RNA.

C. 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 minutes 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 translation 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, one band being smaller than anticipated, but when the same RNA is run on a denaturing polyacrylamide 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 seconday structure

mMESSAGE mMACHINE products occasionally run as two bands; one larger than the expected size, and one 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 extrememly processive, even a small amount of circular template can produce a large amount of RNA.

IV. Additional Procedures

A. 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 IV.F.2 on page 24.

2. Sample preparation

To get good resolution of the RNA, load ~1 µg per gel lane (see section *II.D. Quantitation of Reaction Products* on page 10). Gel Loading Buffer II can be used for both denaturing agarose and polyacrylamide gels. Add an equal volume of Gel Loading Buffer II to each sample, and heat for 3-5 minutes at 80-90°C. To stain with ethidium bromide 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 III.C on page 15 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 minutes 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 IV.F.2 on page 24.

B. 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.

Table 1. Effect of cap analog/GTP ratio on the yield of globin RNA and its translational efficiency

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 $m^7G(5')ppp(5')G$ to GTP was varied as indicated. Reactions were incubated at 37°C using 1 µg of T7 globin template DNA for 1 hour. The globin RNA (6 µg/ml) was then translated in a 25 µl Ambion Retic Lysate IVT™ in vitro translation reactions for 60 minutes at 30°C with 12.5 µCi of [^{35}S]methionine (1200 Ci/mmol). The incorporation of TCA-precipitable cpm was measured.

C. Optimizing Yield of Short Transcripts

The mMESSAGE mMACHINE kit has been 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 results in maximal yields. Increasing the incubation time, template or polymerase concentration does not generally increase the yield of the reaction. However, with smaller templates, these parameters may require adjustment to maximize reaction yields.

Several types of small-transcript templates (<0.3 kb) can be used in mMESSAGE mMACHINE transcription reactions. These include standard plasmid vectors containing small inserts, PCR products, and synthetic oligonucleotides which can either be entirely double-stranded or mostly single-stranded with a double-starnded promoter sequence (Milligan, *et al.*, 1987). In the case of synthetic oligonucleotide, and PCR-derived templates, almost all of the DNA is template sequence, compared to plasmid templates which have non-transcribed vector DNA present.

1. Increase the reaction time

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

2. Increase the template concentration

Increasing the template concentration is the next variable that should be tested. This can be helpful because, with short templates, the initiation step of the transcription reaction is rate-limiting. For a 60 nt transcript generated from an 85 bp PCR-derived DNA template, 50 ng of template was found to be saturating. 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. For instance, the 50 ng of template in the above example provided 0.9 pmoles of template (and 0.9 pmoles of promoters) in the reaction, compared to the approximately 0.3 pmoles provided by 1 µg of the plasmid control template. In general, for optimum yield of short transcripts, use about 0.5-2 pmoles of template. For very short templates (in the 20-30 base range), use the upper end of this range.

If the short template is contained in a plasmid, it may not be possible to add the optimum molar amount. For example, 2 pmoles of template consisting of a 30 bp insert in a 2.8 kb vector would require 4 µg of plasmid DNA. Such large mass amounts of DNA may be detrimental. Thus, it is better to either remove the template from the vector, or to do the transcription reaction under conditions of sub-optimal template concentration.

3. Increase the RNA polymerase concentration

Although the concentration of polymerase in the mMESSAGE mMACHINE kit is optimal for transcription of templates larger than 300 nucleotides, templates coding much smaller transcripts may benefit from adding additional RNA polymerase. The addition of 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 pure high concentration polymerase (e.g. Ambion cat. #s 2075, 2085, and 2063), ***not***

the 10X Enzyme Mix from the kit. Increasing the enzyme should be the last variable tested after increasing incubation time and optimizing template concentration.

D. 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 protocol 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 3 ml spin column thoroughly with distilled water (e.g., Isolabs part. # QS-NP); 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 minutes using a table-top or clinical 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 0.1 ml or less of the nucleic acid sample onto the center of the resin bed, and spin at 2,000 rpm for 10 minutes. 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.

NOTE:

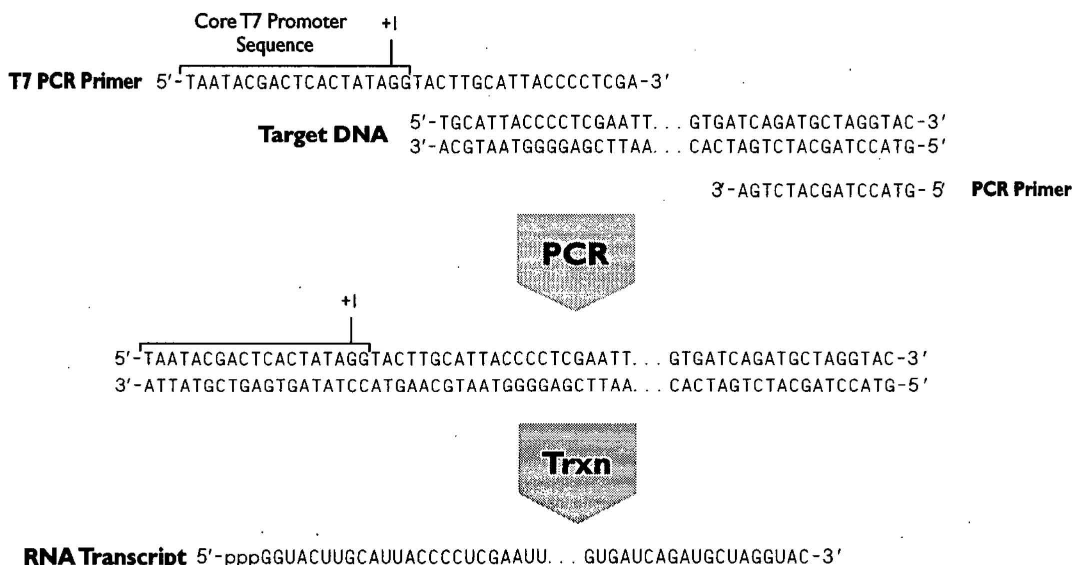
It is important that the centrifugation conditions for column packing and sample purification be identical; varying them could lead to either incomplete recovery or dilution of the sample. The spin column can be tested by loading 100 µl of TE onto it and centrifuging: 100 µl of eluate should be recovered.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

E. PCR Strategy to add a Phage Promotor to DNA

Phage promoter sequences can be added to one or both of the PCR primers to be incorporated into PCR products (Mullis and Faloona, 1987; Stoflet et al., 1988). An example of this strategy is shown below in figure 3 on page 23. One of the PCR primers has the 19 base T7 promoter sequence at the 5' end. Amplification of the target DNA yields PCR product which contains the T7 promoter upstream of the sequence of interest.

The 19 base, core T7 promoter region includes 2 bases which will form the first 2 bases of the transcribed RNA. Yields of transcription product are greatly reduced if the +1 or +2 G are changed. Milligan et al., (1987) has an excellent analysis of the sequence requirements for efficient transcription of T7 promoters.

Figure 3. Strategy for Adding a T7 Promoter by PCR



NOTE:
Using the strategy shown, sense RNA would be transcribed.

We generally add amplified DNA directly to transcription reactions with no further purification after the PCR. Typically, 5 µl of a 100 µl PCR, corresponding to about 0.1-0.2 µg of double-stranded DNA, is used as a template in a standard transcrip-

tion reaction. With shorter templates, however, this amount of template may be suboptional. If the PCR-generated DNA is not concentrated enough to be added directly to the transcription reaction, it can be precipitated (from one or more pooled PCRs) with 2 volumes of ethanol in the presence of 0.5 M ammonium acetate after first extracting with phenol/chloroform.

F. Recipes

1. 10X TBE

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

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

Alternatively, 10X TBE is available from Ambion as a ready-to-resuspend mixture of ultrapure molecular biology grade reagents (Ambion cat.# 9863). Each packet contains the powdered components for 1L of 10X TBE.

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

for 15ml	Component
7.2 g	Urea (high quality)
1.5 ml	10X TBE
1.9 ml	40% Acrylamide (acryl: bis-acryl = 19:1)
to 15 ml	dH_2O

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

120 µl	10% ammonium persulfate in dH_2O
16 µl	TEMED

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

1. Add 0.1% DEPC to double-distilled, deionized H_2O (i.e. add 1 ml per liter of H_2O).
2. Stir well, incubate several hours to overnight at 37°C or 42°C.
3. Autoclave 2 L or smaller volumes for at least 45 minutes. The scent of DEPC should be either not detectable or only very slightly detectable.

G. Miniprep for Isolating Transcription-quality Plasmid DNA

Generally, the cleaner the template DNA, the greater the yield of the transcription reaction. The following miniprep protocol yields high quality transcription template. This protocol differs from the published procedure (Molecular Cloning, A Laboratory Manual) 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 introducing 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 seconds; pour off supernatant, respin briefly (about 5 seconds), and remove residual supernatant via aspiration.

2. Resuspend pellet in 110 µl Solution I

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

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

4. Add 165 µl Solution III

Vortex medium-fast for 10 seconds, incubate 5 min on ice.

Centrifuge 5 minutes 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

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

Centrifuge 5 minutes 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

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 Ambion's RNase Cocktail. Vortex vigorously, incubate about 5 min at 37°C to 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, SDS

Add SDS to a final concentration of 0.5% (usually a 10 to 20% SDS stock solution is used). Add 50 to 100 µg ml Proteinase K. 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 ~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 EtOH, incubate at least 15 min at -20°C.

10. Pellet DNA

Pellet the DNA by microfuging at top speed for 15 minutes. 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 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. Use ~0.5-1 µg of template DNA per 20 µl transcription reaction (typically this will require about 1-3 µl of the 10-20 µl prep).

V. Appendix

A. References

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B. mMESSAGE mMACHINE™ Specification Sheet

Kit Contents:

25 Reactions

Amount	Component
50 µl	Enzyme Mix (SP6, T7, or T3)
50 µl	10X Reaction Buffer (SP6, T7, or T3)
250 µl	2X NTP/CAP (SP6, T7, or T3)
100 µl	GTP (20 mM in SP6 kits; 30 mM in T3 and T7 kits)
45 µl	DNase I (2 U/µl, RNase-free)
10 µl	pTRI-Xef (0.5 mg/ml)
1 ml	Ammonium Acetate Stop Solution
1.4 ml	Lithium Chloride Precipitation Solution
1.75 ml	Nuclease-free Water
1.4 ml	Gel Loading Buffer II

Storage Conditions:

Store at -20°C. Do not store in a frost-free freezer.

Quality Control:

Functional testing

All components are tested in a functional mMESSAGE mMACHINE assay as described in the manual. A 20 µl reaction containing 1 µg of the control template DNA which codes for a 1.92 kb transcript synthesized at least 15-25 µg of capped RNA after a 2 hour incubation.

Nuclease testing

Each component is tested in Ambion's rigorous nuclease assays.

Non-specific endonuclease/nickase activity

None detected after incubation of kit components with supercoiled plasmid DNA, analyzed on 1% agarose.

Exonuclease activity

None detected after incubation of kit components with ^{32}P-labeled *Sau*3A fragments of pUC19, analyzed by PAGE.

RNase activity

None detected after incubation of kit components with ^{32}P-labeled RNA, analyzed by PAGE.

Protease testing

None detected in protein-containing components after incubation with protease substrate, and analysis by spectrophotometry.

Safety:

This kit contains dilute aqueous solutions none of which are thought to present any health hazards. The kits also contain enzymes which use 50% glycerol as a stabilizer, an MSDS for these components is supplied on the following pages.

C. MSDS for Glycerol-Containing Components

Physical Data

Appearance and Odor	Viscous colorless liquid
Boiling Point	182°C
Specific Gravity	1.26
Vapor Density	3.1
Vapor Pressure (mm Hg)	3 mm @ 20°C
Solubility in H_2O	Soluble

Fire and Explosion Hazard Data

Extinguishing Media	Water, CO_2, Foam, Dry Chemical.
Special Fire Fighting	Wear self-contained breathing apparatus and protective clothing.
Unusual Fire/Explosion Hazard	Contact with strong oxidizers may cause fire or explosion.

Health Hazard Data

Effects of Overexposure	Irritant. May be harmful by inhalation or ingestion.
Emergency First Aid	Wash affected area with water. Irrigate eyes for at least 15 min. See physician immediately. If ingested, drink several glasses of water, induce vomiting.

Reactivity Data

Stability	Stable
Incompatibility	Strong oxidizing agents
Hazardous Decomp. Products	Carbon monoxide, carbon dioxide
Hazardous Polymerization	Does not occur

Spill or Leak Procedures

If released or spilled	Absorb on sand and place in a closed container for disposal.
Waste Disposal Method	Dispose according to federal, state and local regulations.

Special Protection and Precaution Information

Respiratory Protection	Not expected to require personal respirator usage. (Use NIOSH approved respirator if necessary)
Ventilation	Not expected to require any special ventilation
Precautionary Labeling	None
Handling and Storage	Use laboratory aprons and gloves. Do not store in aluminum or copper containers. Keep tightly closed in a cool, dry place.

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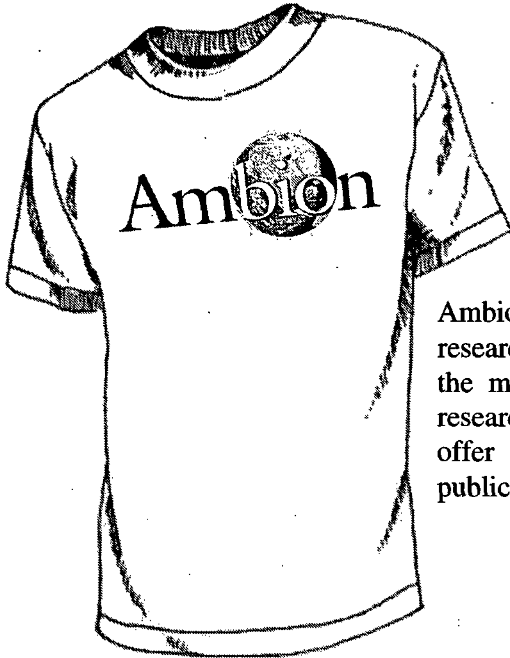

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