

INSTRUCTION MANUAL

*Klentfeld
Physics 173*

mMESSAGE
mMACHINE™

*Large Scale in vitro Transcription kit
for Synthesis of Capped RNA*

out of 2 ≈ \$200⁻
SP6, T7 and T3 Kits
Catalog #s 1340, 1344, and 1348

Ambion
The RNA Company™

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Manual Version 0001

Literature Citation

We would appreciate if when you are describing a procedure utilizing this kit in a Materials and Methods Section for publication that you refer to this product 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 a pending patent.

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 (m⁷G(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. 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. patent protected). The mMESSAGE mMACHINE Kit contains all the buffers and reagents necessary for 25 transcription reactions. Using the control template (*Xenopus* elongation factor 1 α , pTRI Xef-1) supplied with the kit, each mMESSAGE mMACHINE reaction will yield approximately 20-30 μ g RNA using T3 or T7 RNA polymerase, or about 15-25 μ g 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 μ l	Enzyme Mix (SP6, T7, or T3)* Buffered 50% glycerol solution containing RNA polymerase, RNase inhibitor, and other components.																		
50 μ l	10X Reaction Buffer (SP6, T7, or T3)* salts, buffer, dithiothreitol, and other ingredients																		
250 μ l	2X NTP/CAP (SP6, T7, or T3)* - a neutralized buffered solution containing:																		
	<table border="1"> <thead> <tr> <th></th> <th>SP6 Kits</th> <th>T7 and T3 Kits</th> </tr> </thead> <tbody> <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> </tbody> </table>		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
	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																	
100 μ l	GTP* 20 mM in SP6 kits; 30 mM in T3 and T7 kits																		
45 μ l	DNase I 2 U/ μ l (RNase-free)																		
10 μ l	pTRI-Xef 1 0.5 μ g/ μ l																		
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																		

*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.

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 μ g/ μ l in dH₂O or TE buffer (10 mM Tris-HCl, (pH 7-8), 1 mM EDTA).
- (optional) Labeled nucleotide, for example α -³²P]UTP or α -³²P]CTP, as a tracer to facilitate quantitation of the RNA synthesized. Any specific activity is acceptable.

- (optional) For purification of RNA following transcription:
Buffer-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 unincorporated 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 radiolabeled 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 nucleic acids. Ambion supplies Proteinase K in lyophilized powder form, and as a 50% glycerol solution.

Cap Analog cat. # 8048-8052

m⁷G(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 I 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

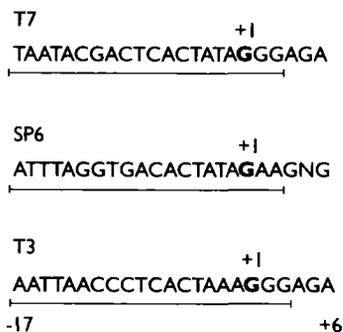
Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog for a complete listing as this product line is always growing.

II. mMESSAGE mMACHINE™ Protocol

A. 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 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.

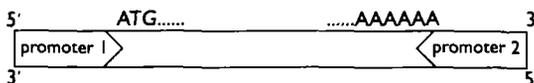
Template size

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 modifications to the reaction as described in sections IV.A on page 15, and IV.B on page 16 should be considered.

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 (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 (homologous to what is found in vivo). If the RNA polymerase for promoter 2 is used, antisense RNA will be transcribed.

Plasmid Templates

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).

Preparation and concentration

Linearized template DNA should be resuspended in water or TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) at a final concentration of 0.5-1.0 mg/ml. 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.H on page 24. After restriction digestion to linearize template, stop the reaction by adding EDTA to 25 mM (chelates magnesium and causes enzyme to fall apart), then EtOH precipitate with 0.3M NaOAc or 0.5M NH₄OAc. Wash the pellet with 70% cold EtOH and resuspend as above.

Note that DNA from some miniprep procedures may be contaminated with residual RNase A which may not be completely eliminated by phenol extraction alone. Template DNA should be digested with proteinase K (100-200 µg/ml for 30 minutes at 50°C) with 0.5% SDS, and then phenol/chloroform extracted

after any RNase A treatment. This Proteinase K/phenol extraction procedure is often unnecessary, it should be considered, however, when transcription from a template is suboptimal.

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 described in section IV.F on page 21.

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.A on page 15 and IV.B on page 16, respectively, for modified reaction setup suggestions.

Amount	Component
to 20 μ l	Nuclease-free Water
2 μ l	10X Reaction Buffer
10 μ l	2X NTP/Cap
(1 μ l)	(optional) [α - 32 P]UTP as a tracer
1 μ g	linear template DNA
2 μ l	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 hr.

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 IV.D on page 19).

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.

- Add 1 μ l DNase 1, and mix well.
- Incubate at 37°C for 15 minutes

C. Recover 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.

I. Lithium chloride precipitation

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 Lithium Chloride Precipitation Solution in step a below.

- a. Stop the reaction and precipitate the RNA by adding 30 µl Nuclease-free Water and 25 µl Lithium Chloride Precipitation Solution.
- b. Mix thoroughly. Chill the reaction for at least 30 minutes at -20°C.
- c. Centrifuge at 4°C for 15 minutes 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.E on page 20.

3. Phenol:chloroform extraction and isopropanol precipitation

This is the most rigorous of the methods for purifying transcripts. This treatment will remove all enzyme and most of the free nucleotides from mMESSAGE mMACHINE reaction. Since the RNA is precipitated, it also can be used for buffer exchange.

- a. Add 115 µl Nuclease-free Water and 15 µl Ammonium Acetate Stop Solution, and mix thoroughly.

* Ambion offers several products for RNA storage, these include:
 Nuclease-free Water (not DEPC-treated) - cat. #s 9930-9934
 TRIE RNA Storage Solution - cat.#s 7000, 7001
 TE Buffer - cat.#s 9860, 9861
 0.1mM EDTA - cat.#s 9911, 9912
 RNAssecure™ - cat.#s 7005, 7006, 7010

- 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.
- c. (Optional: back-extract the organic phase with 50 μ l dH₂O.)
- d. Precipitate the RNA by adding 1 volume of isopropanol and mixing well.
- e. 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.
- f. 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 μ g/ml, so the RNA yield can be calculated as follows:

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

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. 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. The Gel Loading Buffer II supplied with the kit can be used for either type of gel. Stain the samples with ethidium bromide, and simply compare the intensity of the unknown sample to the known RNA to estimate its concentration. To stain with ethidium bromide, either add 0.5 μ g/ml to the gel, or add 10 μ g/ml to the RNA and gel loading buffer before loading the gel.

b. 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/ μ l, and go down to about 1.25 ng/ μ l. Make a few dilutions of the unknown RNA, and add ethidium bromide to 1 ng/ μ l to each dilution of both RNAs. Spot 2 μ l 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.

3. TCA precipitation

If a trace amount of radiolabel was included in the reaction, it can be used to determine yield; this is described in section IV.D on page 19.

III. Troubleshooting

A. Use of the Control Template

The control template is a linearized TRIPL.Escript plasmid containing the 1.85 kb *Xenopus* elongation factor 1 α gene under the transcriptional control of tandem SP6, T7, and T3 promoters (pTRI-Xef1). 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:

SP6: 1.92 kb

T7: 1.89 kb

T3: 1.86 kb

These transcripts will produce a 50.2 kD protein when translated.

Reaction setup

Use 2 μ l of pTRI-Xef1 control template in a standard mMES-SAGE mMACHINE reaction as described in section II.B on page 6.

Expected yield from the control reaction

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

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.

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.

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 μ l mMESSAGE mMACHINE reaction should be at 15-20 μ g and may exceed 30 μ g; 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-XefI control template in a standard mMESSAGE mMACHINE reaction.

The control reaction works, but my template gives low yield

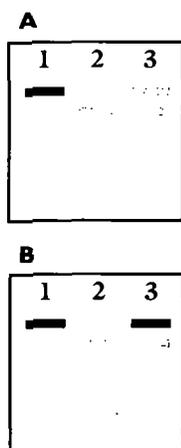


Figure 2. Possible outcomes of mixing experiment

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

If the transcription reaction with your template generates full-length, intact RNA, but the reaction yield is significantly lower than the amount of RNA obtained with the pTRI-XefI 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 μ l pTRI-Xef control template
2. experimental DNA template (0.5 μ g plasmid or 2-6 μ l PCR product)
3. a mixture of 1 and 2

Assess the results of the mixing experiment by running 2 to 4 μ l of the transcription reaction on a denaturing gel as described in section IV.C 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 μ g/ml) and SDS (0.5%) for 30 minutes at 50°C, followed by phenol/chloroform extraction and ethanol precipitation. Additionally, carry-over of SDS can be minimized by diluting the nucleic acid several-fold before ethanol precipitation, and excess salts can be removed by vigorously rinsing nucleic acid pellets with cold 70% ethanol 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.

Use a different polymerase for transcription

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.

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.

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 from 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

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 translation products.

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

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.

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).

IV. Additional Procedures

A. Optimizing Yields with Short Templates

The mMESSAGE mMACHINE kit has been designed to function optimally with transcription templates in the 0.3-5 kb range. Under these conditions, 1 μ g of plasmid DNA template per 20 μ l 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 the mMESSAGE mMACHINE transcription reaction. These include standard plasmid vectors containing small inserts, PCR products, and synthetic oligonucleotides which can either be double stranded or single stranded except for the promoter sequence itself (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. To optimize reaction conditions for maximum yields of small transcripts, it is instructive to consider the yields obtained with the control template for the 489 bp transcript, supplied with the kit. If 20% of the nucleotides are incorporated into RNA in a standard reaction containing 1 μ g of the control template, this corresponds to a yield of 39.6 μ g of product, or 569 copies of RNA per template molecule. If the plasmid template generates a 30 base transcript, however, in order to achieve the same mass yield, each template molecule would have to be copied 9,275 times. Since the template would need to be copied many more times, the initiation step of the transcription reaction becomes the rate-limiting step.

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 increasing the number of substrate molecules favors transcription initiation. 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 for short templates, an equal mass represents a larger molar amount compared to longer templates. 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. Note we suggest adding pure high concentration polymerase (e.g. Ambion cat. #s 2075, 2085, and 2063), *not* than the 10X Enzyme Mix from the kit. We recommend that increasing the enzyme be the last variable tested after increasing incubation time and optimizing template concentration.

B. Optimizing Yields with Long Templates

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 μ l	4.0: 1	80%
1 μ l	2.0: 1	67%
2 μ l	1.3: 1	57%
3 μ l	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.

How much additional GTP should be added?

For templates in the 5-8 kb range we suggest initially testing the addition of 1 μ l 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.

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 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 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 [³⁵S]methionine (1200 Ci/mmol). The incorporation of TCA-precipitable cpm was measured.

C. Analysis of Transcription Products by Gel Electrophoresis

Type of gel

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%) can be better for sizing smaller transcripts. Since secondary structure in the transcript may result in aberrant migration and/or the presence of multiple bands, the gel should be run under denaturing conditions. For agarose gels, this means glyoxal or formaldehyde gels should be 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.G.2 on page 23.

Sample preparation

Gel loading buffer is provided with the kit and is suitable for denaturing agarose or polyacrylamide gels. Remove an aliquot of the reaction, add an equal volume of gel loading buffer, and heat the sample for 3-5 minutes at 80-90°C. The RNA can be visualized with 0.5 µg/ml ethidium bromide in gel running buffer. (Note, because single-stranded nucleic acid does not bind ethidium as efficiently as does double-stranded, the fluorescence of RNA samples on a denaturing agarose gel will be less than for the same amount of DNA.)

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 μ l of 800 Ci/mmol, 10 mCi/ml [α - 32 P] UTP was used in the mMES-SAGE mMACHINE reaction) are about 10-30 minutes with an intensifying screen, or several hours to overnight without a screen, when 1 μ l 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.G.2 on page 23.

D. Determination of Yield using a Trace Radiolabel

I. TCA precipitation

First use TCA precipitation to determine the proportion of radiolabel that was incorporated into RNA.

1. To a 12 x 75 mm tube containing 198 μ l of H₂O, add 100 μ g of carrier DNA or RNA.
2. Add 2 μ l of the mMES-SAGE mMACHINE reaction and mix thoroughly.
3. Transfer 100 μ l of the diluted mMES-SAGE 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).
4. Add 2 ml of cold 10% TCA (trichloroacetic acid) to the remaining 100 μ l 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.
5. Collect the precipitate via vacuum filtration through a Whatman GF/C glass fiber filter (or its equivalent).
6. 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.
7. 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.)
8. Divide the cpm in Step 7 by the cpm in Step 3 to determine the fraction of label incorporated into RNA (multiply by 100 for percent incorporation).

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 1.98 μ g of RNA synthesized in a T7 or T3 reaction. For SP6 kits, each 1% incorporation corresponds to 1.26 μ g of RNA synthesized.

In a T7 or T3 reaction, if all four nucleotides are incorporated equally, 39.6 μ g 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 theoretical yield for SP6 mMESSAGE mMACHINE reactions is 26.4 μ g.

E. 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 μ l of TE onto it and centrifuging: 100 μ l of eluate should be recovered. If recovery is much greater or less than 100 μ l, the column is not equilibrated and should be tested again.

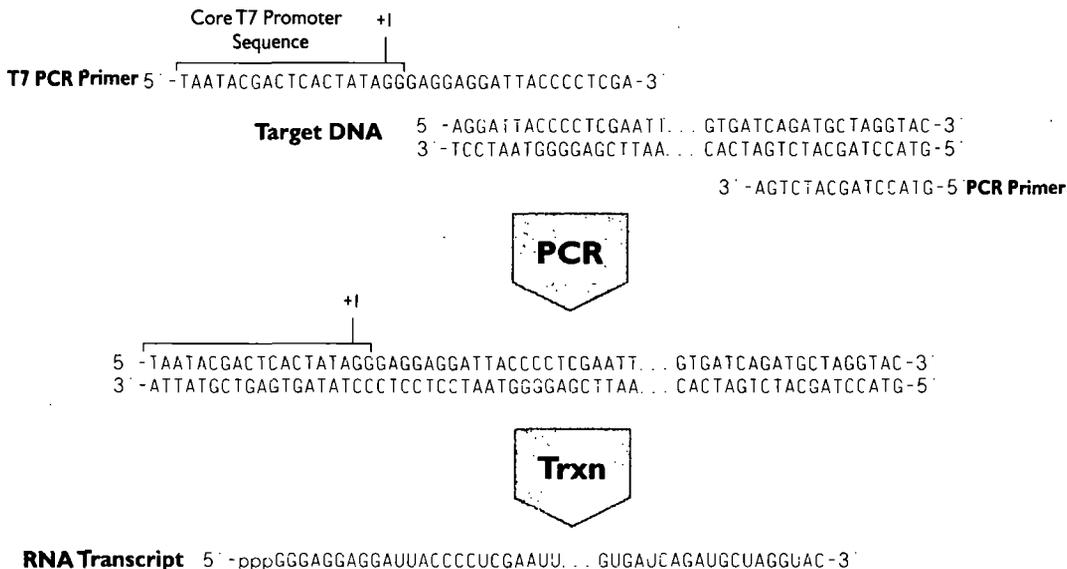
F. PCR Strategy to add a Phage Promotor to DNA

Phage promoter sequences can be added to one or both of the PCR primers to be incorporated into PCR products (Mullis and Faloona, 1987; Stoffel et al., 1988). An example of this strategy is shown below in figure 3 on page 22. 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.

We generally add amplified DNA directly to transcription reactions with no further purification after the PCR. Typically, 5 μ l of a 100 μ l PCR, corresponding to about 0.1-0.2 μ g of double-stranded DNA, is used as a template in a standard transcription reaction. With shorter templates, however, this amount of template may be suboptimal. 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.

Figure 3. Strategy for Adding a T7 Promoter by PCR



NOTE:

Using the strategy shown, sense RNA would be transcribed.

G. Recipes

I. 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)

for 1 L	Final concentration	Component
109 g	0.9 M	Tris base
55 g	0.9 M	Boric Acid
40 ml	20 mM	EDTA- use 0.5 M stock

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 ₂ O

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

120 µl	10% ammonium persulfate in dH ₂ O
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.

RNase-free water

1. Add 0.1% DEPC to double-distilled, deionized H₂O (i.e. add 1 ml per liter of H₂O).
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.

H. 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/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 introducing 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

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)

0.2 N NaOH
1 % SDS

Solution III

for 100 ml

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 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 μ l 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 μ l TE containing RNase**

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

Use 0.5 U or 1 μ g RNase A or use 1 μ l 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 μ g/ml Proteinase K. 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 minutes. After the spin, discard the supernatant, re-spin briefly and remove residual supernatant.

Resuspend the DNA in 10-20 μ l 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 μ g 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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- Schenborn, E.T. and Mierendorf, R.C. (1985) A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. *Nucl. Acids Res.* **13**: 6223-6236.
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B. mMESSAGE mMACHINE™ Specification Sheet

Kit Contents: 25 Reactions

Amount	Description
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
10 µl	pTRJ-Xcf 1
45 µl	DNase 1
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 ³²P-labeled *Sau3A* fragments of pUC19, analyzed by PAGE.

RNase activity

None detected after incubation of kit components with ³²P-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 ₂ O	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

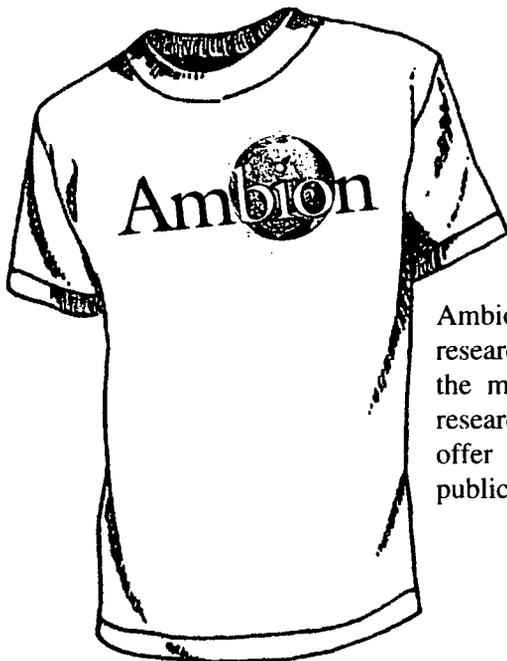
If released or spilled	Absorb on sand and place in a closed container for disposal.
Waste Disposal Method	Dispose according to federal, state and local regulations.

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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