RNAMaxx High Yield Transcription Kit

Instruction Manual

Catalog #200339

Revision C.0

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RNAMaxx High Yield Transcription Kit

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RNAMaxx High Yield Transcription Kit

MATERIALS PROVIDED

Materials Provided[a]	Volume
T7 RNA polymerase, 200 U/µl	50 µl
5× RNAMaxx transcription buffer	250 µl
100 mM rATP	50 µl
100 mM rGTP	50 µl
100 mM rCTP	50 µl
100 mM rUTP	50 µl
0.75 M dithiothreitol (DTT)	250 µl
0.75 U/µl yeast inorganic pyrophosphatase	25 µl
Diethylpyrocarbonate (DEPC)-treated water	1 ml
RNase block	50 µl

[a] Kit contains enough reagents for 50 transcription reactions.

STORAGE CONDITIONS

All Components: –20°C

ADDITIONAL MATERIALS REQUIRED

Proteinase K
RNase-free DNase (optional)

Revision C.0

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INTRODUCTION

Traditional transcription methods often produce RNA yields that are insufficient for many downstream applications. The RNAMaxx High Yield Transcription Kit has been designed to produce large quantities of RNA for many gene expression analysis applications including RNA interference, RNase protection assays, antisense or microinjection studies, and the study of RNA binding proteins. Modified ribonucleotides are easily incorporated into the transcripts for use in constructing non-isotopically labeled RNA probes for hybridization studies or microarrays. The RNAMaxx transcription kit produces 80 to 100 μg or more of RNA from 1 μg of DNA template in just 2 hours. In addition to producing large quantities of RNA, the RNAMaxx transcription kit easily produces transcripts from a wide range of DNA template sizes, up to 9 kb.

The RNAMaxx transcription kit uses T7 RNA polymerase to synthesize RNA from a double-stranded DNA template containing the T7 promoter. To generate high RNA yields, high concentrations of ribonucleotides are added to the reaction mixture. When a ribonucleotide is incorporated into RNA, a molecule of pyrophosphate is released. High concentrations of pyrophosphate have been shown to inhibit further polymerization. To remove this inhibition, yeast inorganic pyrophosphatase has been added to the reaction.[1] The use of a higher concentration of ribonucleotides and the addition of yeast inorganic pyrophosphatase in the reaction mixture increases the RNA yield 10 to 30 times the yield produced by traditional in vitro transcription methods.

PREVENTING SAMPLE CONTAMINATION

Preventing RNase Contamination

Ribonucleases are very stable enzymes that hydrolyze RNA. RNase A can be temporarily denatured under extreme conditions, but it readily renatures. RNase A can therefore survive autoclaving and other standard methods of protein inactivation. The following precautions can prevent RNase contamination:

- Wear gloves at all times during the procedures and while handling materials and equipment, as RNases are present in the oils of the skin.

- Exercise care to ensure that all equipment is as free as possible from contaminating RNases. Avoid using equipment or areas that have been exposed to RNases. Use sterile tubes and micropipet tips only.

- Micropipettor bores can be a source of RNase contamination, since material accidentally drawn into the pipet or produced by gasket abrasion can fall into RNA solutions during pipetting. Clean micropipettors according to the manufacturer's recommendations. We recommend rinsing both the interior and exterior of the micropipet shaft with ethanol or methanol.

- Linearized DNA templates for transcription must be RNase free. CsCl preps are advisable, but minipreps may be used if care is taken to remove contaminating RNases. Linearized plasmid DNA or PCR products that are used as template may be purified using Agilent's StrataPrep PCR purification kit (Catalog #400771 or 400773).

- Working with RNA is simplified by using a ribonuclease inhibitor in transcription reactions. The RNase block included in the transcription reaction mixture has been shown to effectively inhibit RNases from degrading transcripts.

Sterilizing Labware

Disposable Plasticware

Disposable sterile plasticware is generally free of RNases. If disposable sterile plasticware is unavailable, components such as 1.5-ml microcentrifuge tubes can be sterilized and treated with diethylpyrocarbonate (DEPC), which chemically modifies and inactivates enzymes (refer to Sambrook, *et al.*).[2]

Caution *DEPC is toxic and extremely reactive. Always use DEPC in a fume hood.* ***Read and follow the manufacturer's safety instructions.***

Electrophoresis Gel Boxes

To inactivate RNases on electrophoresis gel boxes, treat the gel boxes with 3% (v/v) hydrogen peroxide for 10–15 minutes and then rinse them with RNase-free water.

Glassware or Metal

To inactivate RNases on glassware or metal, bake the glassware or metal for a minimum of 8 hours at 180°C.

Treating Solutions with DEPC

Add DEPC to water and solutions (except those containing Tris base) to a final concentration of 0.1% (v/v) DEPC. During preparation, mix the DEPC-treated solution thoroughly, allow it to incubate overnight at room temperature, and then autoclave it prior to use. If a solution contains Tris base, prepare the solution with autoclaved DEPC-treated water.

PREPROTOCOL CONSIDERATIONS

Nonspecific Initiation

T7 RNA polymerase is highly specific; however, nonspecific initiation of RNA transcripts may occur at the ends of the DNA template. This is most prevalent with a 3´-protruding terminus. Use restriction enzymes and DNA polymerases that leave blunt or 5´-protruding ends when possible.

If the T7 RNA polymerase enzyme is used in molar excess of the DNA template, there is a risk of polymerization from a T3 RNA promoter. T7 RNA polymerase can synthesize RNA inefficiently from a plasmid containing only a T3 promoter. Synthesis is extremely promoter specific when both promoters are present, provided that the enzyme is not in molar excess of the specific promoter.

DNase Treatment after Transcription (optional)

The DNA template will be present after the transcription reaction and can be removed with RNase-free DNase. After the transcription reaction, add 10 U of RNase-free DNase/μg of DNA template and incubate at 37°C for 30 minutes. The RNA transcript must be purified following the DNase treatment after transcription. We recommend using Agilent's Absolutely RNA RT-PCR miniprep kit (Catalog #400800) to purify the RNA after DNase digestion.

PREPARING THE DNA TEMPLATE

T7 RNA Polymerase Promoter

The in vitro transcription reaction uses T7 RNA polymerase to produce RNA from a double-stranded DNA template containing a T7 promoter. The T7 promoter is minimally a 19 base pair sequence (5´ TAATACGACTCACTATAGG 3´) that directs T7 RNA polymerase transcription initiation with high specificity.

DNA Template Size

Large quantities of RNA can be produced using a wide range of DNA template sizes, from as small as 500 bp to larger than 9 kb.

Orientation

The synthesis of the sense or antisense RNA transcript depends on the orientation of the T7 promoter sequence to the target sequence. The target sequence must be cloned downstream of the T7 promoter for sense RNA to be transcribed. For antisense RNA transcription, the target sequence must be inverted.

DNA Template

Plasmid Templates

The plasmid template is linearized with an enzyme that cleaves the DNA downstream of the T7 promoter and the insert in the multiple cloning site. The end of the RNA transcript during the transcription reaction depends upon the position of the restriction site selected to linearize the template. Use a restriction enzyme that leaves blunt or 5´-protruding ends when possible to avoid nonspecific initiation (see *Preprotocol Considerations*). Following restriction digest, analyze a sample of the linearized DNA on an agarose gel to ensure complete digestion.

Proteinase K Treatment: It is strongly advised to treat the digested DNA with proteinase K to remove the restriction enzyme and RNases prior to transcription. We have found that proteinase K treatment greatly increases RNA transcript yields. Add proteinase K to the reaction mixture following digestion to a final proteinase K concentration of 0.25 μg/μl. Incubate the reaction for 30 minutes at 37°C. Purify the DNA template prior to transcription using an RNase-free DNA purification kit or by phenol–chloroform extraction and ethanol precipitation. Resuspend the purified DNA with RNase-free water.

PCR-Generated Templates

A PCR product containing the T7 promoter sequence upstream of the target sequence may be used as template in the transcription reaction. Use a DNA polymerase that leaves blunt ends when possible to avoid nonspecific initiation (see *Preprotocol Considerations*). PCR products for transcription must be RNase free, and may be purified using the StrataPrep PCR purification kit. Following purification, analyze an aliquot of the sample on an agarose gel to ensure a single PCR product of expected size.

TRANSCRIPTION PROTOCOL

1. The following amounts are for a typical 25-µl transcription reaction. Keep the ribonucleotides, T7 RNA polymerase, yeast inorganic pyrophosphatase and RNase block on ice and the remaining components at room temperature while preparing the reaction mixture. Add the components *in order* while mixing gently.

   Note *RNA is commonly labeled by incorporating modified ribonucleotides, such as amino allyl UTP or biotin CTP, when used as a hybridization probe. Modified ribonucleotides are incorporated into the RNA transcript by including the ribonucleotides in a one-to-one ratio of normal to modified ribonucleotides. When generating modified RNA, maintain the original final ribonucleotide concentration by decreasing the amount of normal ribonucleotide used.*

Component	Amount per reaction
5× RNAMaxx transcription buffer	5 µl
DEPC-treated water	*X* µl
100 mM rUTP	1 µl
100 mM rATP	1 µl
100 mM rGTP	1 µl
100 mM rCTP	1 µl
Linearized DNA template (1 µg)	*X* µl
0.75 M DTT	1 µl
0.75 U/µl of yeast inorganic pyrophosphatase	0.5 µl
RNase block	1 µl
200 U/µl T7 RNA polymerase	1 µl
Total reaction volume:	25 µl

2. Incubate the reaction at 37°C for 2 hours.

3. **Optional:** To stop the reaction following the 2-hour incubation, add 1 µl of 10 U/µl RNase-free DNase. Incubate at 37°C for 30 minutes.

4. Purify and analyze the reaction products immediately or store the reaction at –20°C until analysis.

RNA PURIFICATION

The RNA purification method chosen is dependent on the type of experiment that the RNA is needed for. Protocols for RNA purification can be found in Sambrook, *et al.* (1989).[2] Alternatively, Agilent's Absolutely RNA RT-PCR miniprep kit purifies high-quality, RNase-free RNA following the in vitro transcription reaction.

RNA ANALYSIS

Quantitating RNA Yield Using Spectrophotometry

Note *Accurate spectrophotometric measurement requires an $OD_{260} \geq 0.05$.*

1. Blank the spectrophotometer at 260 nm with an appropriate buffer (e.g., 10 mM Tris, pH 7.5) near neutral pH.

2. Prepare an appropriate dilution of the RNA (1:50–1:100) in the same buffer used to blank the spectrophotometer. Place a piece of laboratory film (e.g., Parafilm® laboratory film) over the top of the cuvette and mix the sample well. The conversion factor for RNA is 0.040 µg/µl per OD_{260} unit. Take the spectrophotometric reading. For a reading of 0.20, calculate the concentration as follows:

   Concentration = A_{260} × dilution factor × conversion factor

   Example $0.20 \times 500/5 \times 0.040\ \mu g/\mu l = 0.8\ \mu g/\mu l$

3. Calculate the yield of RNA by multiplying the volume in microliters by the concentration. The sample volume in this example is 100 µl, therefore, the RNA yield is 80 µg.

Quantitating RNA Yield Using RiboGreen®

The use of a fluorescence-based system provides a more sensitive RNA quantitation method. Using this system, a fluorescence microplate reader or fluorometer and an RNA-specific intercalating fluorescent dye (i.e. RiboGreen® RNA Quantitation Kit, Molecular Probes, Inc.) are required. The RNA concentration of a sample is determined by comparing the sample absorbance to a standard curve of known sample concentrations. Refer to the manufacturer's instructions for fluorescence-based RNA quantitation.

Determining RNA Quality Using Formaldehyde Gel Electrophoresis

Caution *Formaldehyde is a suspected carcinogen and must be used and disposed of in accordance with federal, state and local regulations. Always use formaldehyde in a fume hood.*

The secondary structure of the RNA must be denatured if the molecules are to migrate at their true molecular weight. The percentage of agarose used affects resolution; agarose concentrations of 0.8–1.2% are recommended. The RNA can be visualized using either ethidium bromide or SYBR® Green II RNA gel stain.

1. Dilute the purified RNA sample to 0.5–1 µg/µl in RNase-free water.

2. For 100 ml of a 1% agarose gel, melt 1 g of agarose in 88 ml of RNase-free water.

3. Add 10 ml of 10× MOPS buffer[§] to the agarose solution. Allow the gel solution in the flask to cool to approximately 60°C while preparing an electrophoresis gel apparatus. Place the gel apparatus on a level space inside a fume hood. Add 2.7 ml of 37% formaldehyde to the cooled agarose. Swirl to mix and quickly pour the agarose into the gel apparatus. If the RNA on the gel will be transferred to a membrane, the gel should only be thick enough to handle easily (0.5–0.75 cm). Allow the gel to solidify in the fume hood.

4. While the gel is solidifying, prepare 10 µl of sample loading buffer for each sample. Prepare the loading buffer by mixing the components listed below (no more than 12 hours before use):

   For 100 µl total volume of loading buffer use:

   10 µl of 10× MOPS buffer
   11.5 µl of RNase-free water
   50 µl of deionized formamide
   17.5 µl of 37% formaldehyde solution
   10 µl of 10× loading dye[§]
   1 µl of 10 mg/ml ethidium bromide (optional)

   Note *This solution is not stable.* ***Do not*** *use after 12 hours.*

5. Cover the solidified gel with 1× MOPS buffer. Carefully pull the comb out and connect the electrophoresis apparatus to a power supply.

6. Add the RNA to 5–10 µl of loading buffer. Heat the sample at 65°C for 10–15 minutes, chill on ice for 1–2 minutes, centrifuge to collect condensation and immediately load onto the gel.

7. Electrophorese the gel until the bromphenol blue has run one-half to three-quarters the length of the gel (depending on the resolution desired). Ethidium bromide (if used) in the loading buffer will migrate to the negative electrode, and the bromphenol blue and xylene cyanol will travel to the positive electrode with the RNA sample.

   Note *Formaldehyde gels are more fragile than other agarose gels. Use caution when moving the gel.*

8. **Visualization using ethidium bromide:** Examine the gel with UV illumination.

   Note *Wear UV-protective safety glasses or a full safety mask to prevent UV damage to the face and skin.*

   Visualization using SYBR Green II: Incubate the gel in 50 ml of TE buffer[§] containing 3 µl of SYBR Green II gel stain at room temperature for 40 minutes with gentle rocking. The stained RNA can be visualized on an imaging instrument using SYBR Green filters.

[§] See *Preparation of Reagents*.

TROUBLESHOOTING

Observation	Suggestion(s)
Low RNA yield	Precipitation of the DNA template by components in the transcription buffer may be occurring. Water must be added to the transcription reaction first to dilute buffer components to the proper concentrations
	The DNA template must be pure. DNA should be purified with multiple phenol–chloroform extractions until the interface is clear. Cesium chloride banding is the preferred method of purifying DNA. Check the $OD_{260/280}$ ratio (expect 1.8–2.0)
	The DNA template may be contaminated with RNase. If RNase is used during the minipreparation of DNA, perform either multiple phenol–chloroform extractions or proteinase K treatment
	The T7 RNA polymerase may be inhibited by excessive NaCl from the input DNA template. Make sure all NaCl is removed from the DNA preparation. A 70% (v/v) ethanol wash of the DNA pellet is recommended after precipitation

PREPARATION OF REAGENTS

10× Loading Dye

50% sterile glycerol
1 mM ethylenediaminetetraacetic acid (EDTA)
0.25% bromophenol blue
0.25% xylene cyanol FF

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

10× MOPS Buffer

0.2 M MOPS (3-[N-morpholino] propanesulfonic acid)
0.05 M sodium acetate
0.01 M ethylenediaminetetraacetic acid (EDTA)
Bring to a final pH of 7.0 with NaOH
Do not autoclave

REFERENCES

1. Cunningham, P. and Ofengand, J. (1990) *Biotechniques* 9(6):713–714.
2. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

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QUICK-REFERENCE PROTOCOL

- Prepare the 25-μl transcription reaction as indicated below:

  5 μl of 5× RNAMaxx transcription buffer
  X μl of DEPC-treated water
  1 μl of 100 mM rUTP
  1 μl of 100 mM rATP
  1 μl of 100 mM rGTP
  1 μl of 100 mM rCTP
  X μl of linearized DNA template (1 μg)
  1 μl of 0.75 M DTT
  0.5 μl of 0.75 U/μl of yeast inorganic pyrophosphatase
  1 μl of RNase block
  1 μl of 200 U/μl T7 RNA polymerase

- Incubate the reaction at 37°C for 2 hours.

- **Optional:** Stop the reaction by adding 1 μl of 10 U/μl RNase-free DNase. Incubate at 37°C for 30 minutes.

- Purify and analyze the reaction products immediately or store the reaction at 20°C until analysis.