TECHNICAL BULLETIN

RiboMAX™ Large Scale RNA Production Systems—SP6 and T7

Instructions for use of Products
P1280 and P1300

Revised 12/13
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Promega

RiboMAX™ Large Scale RNA Production Systems—SP6 and T7

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1. Description

In vitro transcription reactions are widely used to synthesize microgram amounts of RNA probes from recombinant DNA templates. Most transcription reactions designed to generate RNA probes are optimized to maximize incorporation of radiolabeled ribonucleotides rather than to produce large amounts of RNA. However, in vitro transcription also is used for many other applications that require larger amounts of biologically active RNA. The production of large amounts of RNA is potentially valuable for in vitro translation and for synthesis of tRNA, rRNA, other small functional RNAs, RNA virus genomes and ribozymes. Large-scale RNA preparation is also useful for production of substrates for studies of RNA splicing, RNA secondary structure, antisense RNA and RNA:protein interactions.

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The RiboMAX™ Large Scale RNA Production Systems[a] produce milligram amounts of RNA. Transcripts up to 14kb have been generated using the RiboMAX™ System; however, they are more commonly used to generate transcripts up to 5–6kb in size. The RiboMAX™ Systems consistently produce 2–5mg/ml of RNA in a 1ml reaction, about 10- to 20-fold more RNA than is produced with the standard Riboprobe® System transcription reaction. The RiboMAX™ Systems reactions differ from those of the Riboprobe® Systems in three primary ways: a HEPES (pH 7.5) buffer is used rather than a Tris-HCl (pH 7.9) buffer; rNTP and magnesium concentrations are elevated at levels appropriate for either SP6 or T7 RNA Polymerase (1); and recombinant inorganic pyrophosphatase is included in the reaction (2). Both systems include Recombinant RNasin® Ribonuclease Inhibitor.

An additional advantage of the RiboMAX™ Systems is that the RNA synthesized is of higher quality for in vitro translation in rabbit reticulocyte translation systems than RNA synthesized by standard methods (3). This enhanced "translatability" is especially evident at high RNA concentrations that normally inhibit in vitro translation (4,5). While the reasons for the observed improvements in RNA production and translation efficiency are not entirely clear, the RiboMAX™ Systems are useful to researchers wishing to produce large amounts of RNA for in vitro translation.

Because the RiboMAX™ Systems produce large quantities of RNA, these systems are not recommended for generating high specific-activity RNA probes. The amount of radiolabeled nucleotide required to produce this type of probe would be prohibitively expensive.

Selected Citations Using the RiboMAX™ Large Scale RNA Production Systems

- Pratt, S.D. *et al.* (2004) A strategy for discovery of novel broad-spectrum antibacterials using a high-throughput *Streptococcus pneumoniae* transcription/translation screen. *J. Biomol. Screen.* **9**, 3–11.

  Several different sequences from a *S. pneumoniae* pA promoter region were cloned into the pSP-*luc*+ vector. The RiboMAX™ SP6 Large Scale RNA Production System was used to transcribe luciferase-encoding mRNAs.

- Shim, E.Y. *et al.* (2002) Broad requirement for the mediator subunit RGR-1 for transcription in *Caenorhabditis elegans* embryo. *J. Biol. Chem.* **377**, 30413–6.

  RNA interference (RNAi) was performed by synthesizing sense and antisense rgr-1 cDNA using the RiboMAX™ Large Scale RNA Production System.

For additional peer-reviewed articles that cite use of the RiboMAX™ Large-Scale RNA Production Systems—SP6 and T7, visit: **www.promega.com/citations/**

2. Product Components and Storage Conditions

Product	Cat.#
RiboMAX™ Large Scale RNA Production System—SP6	P1280
RiboMAX™ Large Scale RNA Production System—T7	P1300

Each system contains sufficient reagents for a 1ml reaction or 50 standard 20µl reactions. Includes:

- 120µl Enzyme Mix (RNA Polymerase, Recombinant RNasin® Ribonuclease Inhibitor and Recombinant Inorganic Pyrophosphatase)
- 240µl Transcription 5X Buffer
- 100µl Each of 4 rNTPs, 100mM
- 110u RQ1 RNase-Free DNase, 1u/µl
- 10µl Linear Control DNA, 1mg/ml
- 1ml 3M Sodium Acetate (pH 5.2)
- 1.25ml Nuclease-Free Water

Storage Conditions: Store all components at –20°C.

3. DNA Template Preparation

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.)

- chloroform:isoamyl alcohol (24:1)
- TE-saturated (pH 8.0) phenol:chloroform:isoamyl alcohol (25:24:1)
- ethanol (70% and 95%)

3.A. Linearizing DNA Template

Optimal RNA yields depend on a high-quality DNA template. The Wizard® *Plus* SV Minipreps System (Cat.# A1470) and the PureYield™ Plasmid Midiprep System (Cat.# A2492) yield DNA suitable for transcription reactions. The DNA template must be free of RNase. If the presence of RNase is suspected, treat the DNA with Proteinase K (100µg/ml) and SDS (0.5%) in 50mM Tris-HCl (pH 7.5), 5mM $CaCl_2$ for 30 minutes at 37°C (6). Purify the DNA further by extraction with TE-saturated (pH 8.0) phenol:chloroform: isoamyl alcohol (25:24:1) and ethanol precipitation (Section 4.B, Steps 3–6).

DNA templates are usually linearized prior to in vitro transcription to produce RNA of defined length. Linearize the DNA by digestion with an appropriate restriction endonuclease followed by an appropriate clean-up procedure, such as phenol extraction followed by ethanol precipitation, or the Wizard® DNA Clean-Up System (Cat.# A7280). Start with at least 30% more DNA than is required for the transcription reaction to allow for DNA loss during purification and visualization by gel electrophoresis.

Avoid the use of restriction enzymes that produce 3´ overhangs (see Table 1). Extraneous transcripts have been reported to appear in addition to the expected transcript when such templates are transcribed (7). The extraneous transcripts can contain sequences complementary to the expected transcript as well as

sequences corresponding to the vector DNA. If these enzymes must be used, the linearized template ends can be made blunt using DNA Polymerase I Large (Klenow) Fragment (Cat.# M2201) prior to transcription (7; Section 3.B).

Table 1. Commonly Used Restriction Enzymes That Generate 3´ Overhangs.

AatII	**ApaI**	**BanII**
BglI	**Bsp1286I**	**BstXI**
CfoI	**HaeII**	**HgiAI**
HhaI	**KpnI**	**PstI**
PvuI	**SacI**	**SacII**
SfiI	**SphI**	

PCR-generated DNA containing an appropriate phage promoter can be used in transcription reactions. The phage promoter sequences can be incorporated into the DNA by using primers that flank the phage promoter sequences in the vector or by having the promoter sequence within the 5´ oligomer used in the PCR. The resulting PCR-generated DNA can be purified using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281).

The purified linear DNA should be examined by agarose or polyacrylamide gel electrophoresis prior to transcription to verify complete linearization and ensure the presence of a clean (nondegraded) DNA fragment of the expected size.

3.B. Conversion of a 3´ Overhang to a Blunt End

1. Set up a standard in vitro transcription reaction (Section 4.A) minus the nucleotides and RNA polymerase.
2. Add DNA Polymerase I Large (Klenow) Fragment at a concentration of 5u/µg and incubate the reaction mixture for 15 minutes at 22°C.
3. Proceed with the transcription reaction by adding the nucleotide mix and RNA polymerase.

4. Transcription Protocol

This protocol was developed by combining and modifying two published protocols that use HEPES buffer (1) and yeast inorganic pyrophosphatase (2). The development of this system, a comparison to a standard transcription protocol (8), and data demonstrating the enhanced "translatability" of RNA generated by this system are described in reference 3.

The Linear Control DNA supplied with each system contains a luciferase gene under the control of the appropriate SP6 or T7 RNA polymerase promoter. This DNA produces a transcript approximately 1,800 bases long. Since luciferase must be full-length to show activity, transcription and translation of the Control DNA followed by a luciferase assay is a convenient means to verify that full-length transcripts have been generated.

4.A. Synthesis of up to Milligram Quantities of RNA

1. Set up the appropriate reaction for SP6 or T7 RNA Polymerase at room temperature. Add the reaction components in the order shown, being careful to dissolve the DNA template in water before adding it to the reaction.

   For convenience, mix equal volumes of the 4 individual 100mM rNTPs provided to produce a solution that is 25mM for each nucleotide.

SP6 Reaction Components	Sample Reaction	Control Reaction
SP6 Transcription 5X Buffer	20µl	4µl
rNTPs (25mM ATP, CTP, GTP, UTP)	20µl	4µl
linear DNA template (5–10µg total) plus Nuclease-Free Water	50µl	1µl (control DNA) 9µl (water)
Enzyme Mix (SP6)	10µl	2µl
final volume	**100µl**	**20µl**

T7 Reaction Components	Sample Reaction	Control Reaction
T7 Transcription 5X Buffer	20µl	4µl
rNTPs (25mM ATP, CTP, GTP, UTP)	30µl	6µl
linear DNA template (5–10µg total) plus Nuclease-Free Water	40µl	1µl (control DNA) 7µl (water)
Enzyme Mix (T7)	10µl	2µl
final volume	**100µl**	**20µl**

   Notes:
   DNA can precipitate in the presence of spermidine (a component of the Transcription 5X Buffer) if reactions are set up at colder temperatures.

   These reactions can be scaled up or down to suit your template requirements. A 1ml reaction will typically produce 2–5mg of RNA in 2–4 hours.

2. Gently pipet the reaction to mix and incubate at 37°C for 2–4 hours.

Note: Do not freeze the completed transcription reaction. After the transcription reaction is complete, proceed directly to the DNase step or the removal of unincorporated nucleotides.

4.B. Removal of the DNA Template Following Transcription

The DNA template may be removed by digestion with DNase following the transcription reaction. RQ1 RNase-Free DNase (Cat.# M6101) has been tested for its ability to degrade DNA while maintaining the integrity of RNA. For some uses, it may not be necessary to remove the DNA template. For example, 1–10µl of a 1:10 to 1:50 dilution of the transcription reaction can be added directly to a rabbit reticulocyte in vitro translation reaction. The RNA should be DNase treated if accurate RNA concentration determination is desired or to remove potentially inhibitory or interfering components.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.)

- citrate-saturated phenol:chloroform:isoamyl alcohol (125:24:1; pH 4.7) (e.g., Sigma Cat.# 77619 [Fluka])
- isopropanol
- ethanol (70% and 95%)

Note: If DNase treatment is not being performed, proceed to Step 3.

After performing the in vitro transcription reaction:

1. Add RQ1 RNase-Free DNase to a concentration of 1u/µg of template DNA.
2. Incubate for 15 minutes at 37°C.
3. Extract with 1 volume of citrate-saturated phenol (pH 4.7):chloroform: isoamyl alcohol (125:24:1). Vortex for 1 minute and spin at top speed in a microcentrifuge for 2 minutes.
4. Transfer the upper, aqueous phase to a fresh tube, and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as described in Step 3. At this point, unincorporated nucleotides may be removed (Section 4.C), or the RNA may be precipitated directly (Step 5, below).
5. Transfer the upper, aqueous phase to a fresh tube. Any transferred chloroform can be removed by performing a quick spin (10 seconds) in a microcentrifuge followed by removal of the bottom phase with a micropipet. Add 0.1 volume of 3M Sodium Acetate (pH 5.2), and 1 volume of isopropanol or 2.5 volumes of 95% ethanol. Mix and place on ice for 2–5 minutes. Spin at top speed in a microcentrifuge for 10 minutes.
6. Carefully pour off or aspirate the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and suspend the RNA sample in TE buffer or Nuclease-Free Water to a volume identical to that of the transcription reaction. Store at –70°C.

4.C. Chromatographic Removal of Unincorporated Nucleotides

MicroSpin® G-25 columns (GE Healthcare Cat.# 27-5325-01), which allow purification of 25–50µl of transcription reaction per column, are recommended for purification of RNA from small-scale transcription reactions. For transcription reactions of less than 50µl, add water to bring the load volume up to 50µl. Dilution of reactions 1:2 with water before purification may increase recovery; however, the 50µl loading limit per column should still be observed.

NAP®-5 (GE Healthcare Cat.# 17-0853-01) and NAP®-10 (GE Healthcare Cat.# 17-0854-01) columns are recommended for purification of RNA from large-scale transcription reactions. NAP®-5 columns should be used for transcription volumes of 0.5–1.0ml. These columns should be equilibrated with water before sample application and elution are performed according to manufacturer's instructions.

4.D. Determining RNA Concentration and Visualizing RNA by Electrophoresis

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.)

- RNA loading buffer
- RNA sample buffer

After removing the DNA template and unincorporated nucleotides, the RNA concentration can be quantitated most easily by ultraviolet light absorbance. A 1:100 to 1:300 dilution of the RNA is prepared and the absorbance is read at a wavelength of 260nm. One A_{260} unit equals approximately 40µg/ml of RNA. Alternatively, trace amounts of radiolabeled nucleotide can be added to the reaction ([^{32}P]rUTP, for example), and the percent incorporation can be determined by TCA precipitation. However, this is unnecessary for most applications other than probe synthesis.

The DNase-treated in vitro transcript can be examined by denaturing gel electrophoresis to determine the accuracy of the A_{260} quantitation and the integrity of the full-length transcript. Including RNA Markers (Cat.# G3191) allows determination of the RNA transcript size. The Linear Control DNA produces a transcript approximately 1,800bp long. This RNA can be added to a translation extract (rabbit reticulocyte or wheat germ), and the expression of functional luciferase can be determined in a non-radioactive assay using the Luciferase Assay System (Cat.# E1500).

Prepare either an agarose gel in 1X TAE containing 0.5µg/ml ethidium bromide or an acrylamide minigel, depending upon the length of the transcript involved (0.7–2.0% agarose for transcripts from 200 to several thousand nucleotides; 5% acrylamide for transcripts from 50–1,000 nucleotides). While denaturing gels (containing formaldehyde, glyoxal or 8M urea) provide the greatest resolution of the denatured RNA, we have found that acceptable results usually can be obtained using nondenaturing gels loaded with RNA denatured in a formaldehyde/formamide sample buffer. Add 1–2µl of RNA to 18–20µl of RNA sample buffer. Add 2–5µl of RNA loading buffer, and heat the sample for 5–10 minutes at 65–70°C before loading. Run the gel under standard conditions for the analysis of DNA samples.

4.E. Synthesis of Capped RNA Transcripts

Most eukaryotic mRNAs contain a m^{7}G(5´)ppp(5´)G cap at the 5´-end, which is important for binding translation initiation factors and contributes to mRNA stability. The use of capped RNA is suggested for programming certain translation systems (e.g., *Xenopus* oocytes). In rabbit reticulocyte- and wheat germ-based translation systems, some capped transcripts may demonstrate increased translation efficiency.

Uncapped messages can be used effectively in reticulocyte and wheat germ systems, provided the proper concentration of the appropriate potassium salt is supplied (1). In rabbit reticulocyte lysate, potassium chloride (not potassium acetate) at levels 20mM above the maximal stimulatory level has been shown to provide the optimal conditions for the synthesis of authentic products from

uncapped mRNA (9). Flexi® Rabbit Reticulocyte Lysate System (Cat.# L4540) provides lysate devoid of added salts or DTT and provides potassium chloride, magnesium chloride and DTT for optimization of translation of uncapped or capped messages.

The following protocol incorporates a cap analog into the transcript during the RiboMAX™ transcription reaction. It is the same protocol used in Section 4.A, but the final GTP concentration is reduced to 0.6mM and $m^7G(5')ppp(5')G$, such as the Ribo m^7G Cap Analog (Cat.# P1711), is added to a final concentration of 3mM. Incorporating a cap analog may reduce the yield of RNA to 20–50% of the standard reaction. The ratio of cap analog:GTP is 5:1 in the following protocol but can be varied from 10:1 to 1:1 to balance the percentage of capped products with the efficiency of the transcription reaction. Higher yields of longer capped transcripts may be optimized by increasing the concentration of GTP in the following protocol. Higher yields of smaller capped transcripts may be obtained by increasing the incubation time, the amount of RNA polymerase and the concentration of template DNA.

1. Synthesize RNA in vitro using the following reaction mix:

SP6 Reaction Components	**Sample Reaction**
SP6 Transcription 5X Buffer	20.0µl
rNTPs (25mM ATP, CTP, UTP and 3mM GTP)	20.0µl
linear DNA template (5–10µg total) plus Nuclease-Free Water	42.5µl
Ribo m^7G Cap Analog, 40mM	7.5µl
Enzyme Mix (SP6)	10.0µl
final volume	**100.0µl**

T7 Reaction Components	**Sample Reaction**
T7 Transcription 5X Buffer	20.0µl
rNTPs (25mM ATP, CTP, UTP and 2mM GTP)	30.0µl
linear DNA template (5–10µg total) plus Nuclease-Free Water	32.5µl
Ribo m^7G Cap Analog, 40mM	7.5µl
Enzyme Mix (T7)	10.0µl
final volume	**100.0µl**

Notes:
Free cap analog can inhibit processes such as translation. Unincorporated cap analog should be removed by precipitation (Section 4.B) or chromatography (Section 4.C).

Larger scale reactions may be performed by increasing the volumes proportionally.

2. Gently pipet the reaction to mix and incubate at 37°C for 2–4 hours.

3. Remove DNA template as described in Section 4.B.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Comments
Low amounts of RNA synthesized using standard transcription protocol	DNA template can precipitate in presence of the spermidine in the Transcription 5X Buffer. Make sure the components of the reaction are assembled at room temperature and in the order listed (Section 4.A).
	NaCl concentration is too high (>30mM). Residual NaCl used to precipitate the template DNA may inhibit the RNA polymerase activity by as much as 50%. The template DNA may be desalted by column chromatography and reprecipitated in the presence of another salt. Wash the resulting pellet 1–2 times with 70% ethanol.
	RNase contamination can cause RNA degradation. We recommend using Recombinant RNasin® Ribonuclease Inhibitor for all in vitro transcription reactions. Any solutions not provided should be made up in water that has been treated with 0.1% DEPC. Individual transcriptioncomponents may be purchased from Promega.
	RNA polymerase has lost activity. The activity of the individual RNA polymerase may be evaluated by in vitro transcription of the control template or supercoiled plasmid containing the appropriate RNA polymerase promoter.
Presence of incomplete RNA synthesis	RNA synthesis is terminating prematurely. Subclone the transcript sequence of interest into a different vector in which transcription is initiated by a different RNA polymerase. Some sequences recognized as terminators by one RNA polymerase are not recognized as efficiently by another.
	Lower the incubation temperature from 37°C to 30°C. This can increase the proportion of full-length transcripts in some cases (10).
Presence of transcripts larger than expected	Nonlinearized plasmid is present in the sample. Analyze the sample by gel electrophoresis. If undigested vector is noted, redigest with the appropriate restriction enzyme.

5. Troubleshooting (continued)

Symptoms	Comments
Presence of transcripts larger than expected (continued)	Protruding 3´ termini on the DNA template. If the DNA template was linearized with a restriction enzyme that generates a protruding 3´ terminus, transcription results in the synthesis of significant amounts of long RNA molecules that are initiated at the terminus of the template (7). If it is impossible to avoid using a restriction enzyme of this type, the ends of the linear DNA should be made blunt using DNA Polymerase I Large (Klenow) Fragment before use in a transcription reaction (see Section 3.B).

6. Composition of Buffers and Solutions

5X MOPS buffer

0.2M	MOPS (pH 7.0)
50mM	sodium acetate
5mM	EDTA (pH 8.0; Cat.# V4231)

RNA loading buffer

50%	glycerol
1mM	EDTA (Cat.# V4231)
0.4%	bromophenol blue
1mg/ml	ethidium bromide (Cat.# H5041)

Use a high-grade glycerol. Lower grades of glycerol contain ribonuclease activity. Aliquot RNA loading buffer and store at –20°C.

RNA sample buffer

10.0ml	deionized formamide (Cat.# H5051)
3.5ml	37% formaldehyde
2.0ml	5X MOPS buffer (final concentration 7%)

Dispense into aliquots and store at –20°C for up to 6 months. Do not freeze-thaw more than twice.

TE buffer

10mM	Tris-HCl (pH 8.0; Cat.# H5121)
1mM	EDTA (Cat.# V4231)

TE-saturated phenol:chloroform: isoamyl alcohol (25:24:1) (pH 8.0)

Mix equal parts of the TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower phenol phase with 1 part of the chloroform:isoamyl alcohol (24:1).

Transcription 5X Buffer (provided)

400mM	HEPES-KOH (pH 7.5)
160mM	$MgCl_2$ (for SP6)
120mM	$MgCl_2$ (for T7)
10mM	spermidine
200mM	DTT (Cat.# P1171)

7. Related Products

Related Systems

Product	Size	Cat.#
Riboprobe® System—SP6	1 system	P1420
Riboprobe® System—T3	1 system	P1430
Riboprobe® System—T7	1 system	P1440
Riboprobe® System Buffers	1 system	P1121
Ribo m^{7}G Cap Analog	10 A_{254} units	P1711
	25 A_{254} units	P1712

DNA Purification Products

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
Wizard® *Plus* SV Minipreps DNA Purification System*	50 preps	A1330
+ Vacuum Adaptors	50 preps	A1340
Wizard® DNA Clean-Up System	100 preps	A7280
Wizard® PCR Preps DNA Purification System	50 preps	A7170
Wizard® SV Gel and PCR Clean-Up System	50 preps	A9281
	250 preps	A9282

*Available in additional sizes.

Translation Systems

Product	Size	Cat.#
Flexi® Rabbit Reticulocyte Lysate	5 × 200µl	L4540
Rabbit Reticulocyte Lysate, Nuclease Treated	5 × 200µl	L4960
Wheat Germ Extract	5 × 200µl	L4380
Wheat Germ Extract Plus	40 × 50µl	L3250
	10 × 50µl	L3251
Rabbit Reticulocyte Lysate/Wheat Germ Extract Combination System	12 reactions	L4330

8. References

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8. References (continued)

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[a]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.