TECHNICAL BULLETIN

T7 RiboMAX™ Express Large Scale RNA Production System

Instructions for use of Product
P1320

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T7 RiboMAX™ Express Large Scale RNA Production System

All technical literature is available on the Internet at www.promega.com/protocols Please visit the web site to verify that you are using the most current version of this Technical Bulletin. Please contact Promega Technical Services if you have questions on use of this system. E-mail techserv@promega.com.

1. Description

The T7 RiboMAX™ Express Large Scale RNA Production System is an in vitro transcription system designed for the consistent production of milligram amounts of RNA in a short amount of time. This is about 10- to 20-fold more RNA than produced in a standard Riboprobe® System transcription reaction. The T7 RiboMAX™ Express System reaction differs from those of the Riboprobe® System in three primary ways: a HEPES (pH 7.5) (1) buffer is used rather than a Tris-HCl (pH 7.9) buffer, NTP and magnesium concentrations are elevated and inorganic pyrophosphatase is included in the reaction (2).

The T7 RiboMAX™ Express Large Scale RNA Production System differs from the standard RiboMAX™ System in two primary ways:

Time savings: The T7 RiboMAX™ Express System produces milligram amounts of RNA in as little as 30 minutes rather than the 2–4 hours required with other commercially available systems including the original RiboMAX™ System.

Convenience: The rNTPs and Transcription Buffer are combined, minimizing pipetting errors and setup time.

The production of large amounts of RNA is potentially valuable for in vitro translation; synthesis of tRNA, rRNA and other small, functional RNAs; transcription of RNA virus genomes and ribozymes; and for production of substrates for studying RNA splicing, RNA secondary structure, antisense RNA and RNA:protein interactions. Because the T7 RiboMAX™ Express System produces large quantities of RNA, this system is 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. The T7 RiboMAX™ Express System is not recommended for the production of capped RNA.

Selected Citations Using the T7 RiboMAX™ Express System

- Carlson, B.A. *et al.* (2004) Identification and characterization of phosphoseryl-tRNA$^{[Ser]Sec}$ kinase. *Proc. Natl. Acad. Sci. USA* **101**, 12848–53.

  Synthetic tRNA$^{[Ser]Sec}$ was transcribed from an expression vector containing the mouse Sec tRNA$^{[Ser]Sec}$ gene using the T7 RiboMAX™ Express Large Scale RNA Purification System.

- Islam, M.K. *et al.* (2005) Pyrophosphatase of the roundworm *Ascaris suum* plays an essential role in the worm's molting and development. *Infect. Immun.* **73**, 1995–2004.

  A cDNA insert was amplified by PCR using primers to attach T7 promoter recognition sites to both 5´ and 3´ ends. Complemetary dsRNA was generated from the PCR product using the T7 RiboMAX™ Express Large Scale RNA Purification System.

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

2. Product Components and Storage Conditions

Product	Cat.#
T7 RiboMAX™ Express Large Scale RNA Production System	P1320

Each system contains sufficient reagents for 50 standard 20µl reactions. Includes:

- 100µl Enzyme Mix, T7 Express (T7 RNA Polymerase, Recombinant RNasin® Ribonuclease Inhibitor and Recombinant Inorganic Pyrophosphatase)
- 500µl RiboMAX™ Express T7 2X Buffer
- 110 units RQ1 RNase-Free DNase, 1u/µl
- 2 × 5µg pGEM® Express Positive Control Template, 1mg/ml
- 1ml 3M Sodium Acetate (pH 5.2)
- 1.25ml Nuclease-Free Water

Storage and Stability: 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 starting with a high-quality DNA template. Both cesium chloride purification and the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330) yield DNA suitable for transcription reactions. The DNA template must be free from 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) and 5mM $CaCl_2$ for 30 minutes at 37°C (3). Purify the DNA further by extraction with TE-saturated (pH 8.0) phenol:chloroform: isoamyl alcohol (25:24:1) and ethanol precipitate (Section 4.B, Steps 3–6).

DNA templates are usually linearized prior to in vitro transcription to produce RNA transcripts of defined length. Linearize the DNA by digestion with an appropriate restriction endonuclease, then perform the appropriate clean-up procedure, such as phenol extraction followed by ethanol precipitation. Alternatively, the Wizard® DNA Clean-Up System (Cat.# A7280) can be used. It is useful to 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, in addition to the expected transcript, have been reported when such templates are transcribed (4). These 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 ends of the linearized template can be made blunt prior to transcription using DNA Polymerase I Large (Klenow) Fragment or T4 DNA polymerase.

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® PCR Preps DNA Purification System (Cat.# A7170) or Wizard® SV Gel and PCR Clean-Up System (Cat.# A9282).

3.A. Linearizing DNA Template (continued)

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

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 pGEM® Express Positive Control Template DNA supplied with the system produces transcripts that are 1.1kb and 2.3kb in length. **The transcripts produced from the pGEM® Express Positive Control Template are not suitable for in vitro translation.**

4.A. Synthesizing Large Quantities of RNA

1. Set up the appropriate reaction size at room temperature. Add the reaction components in the order shown; be careful to dissolve the DNA template in water before adding it to the reaction.

T7 Reaction Components	Sample Reaction	Control Reaction
RiboMAX™ Express T7 2X Buffer*	10µl	10µl
linear DNA template (1µg total)	1–8µl	–
pGEM® Express Positive Control Template (1µg)	–	1µl
Nuclease-Free Water	0–7µl	7µl
Enzyme Mix, T7 Express	2µl	2µl
final volume	**20µl**	**20µl**

*Frozen RiboMAX™ Express T7 2X Buffer will contain a precipitate that can be dissolved by warming the buffer at 37°C and mixing well.

2. Mix gently and incubate at 37°C for 30 minutes.

Note: Do NOT freeze transcription reactions. After the transcription reaction is complete, proceed directly to the DNase step or removal of unincorporated rNTPS.

4.B. Removing DNA Template and Unincorporated rNTPs Following Transcription

The DNA template can 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. 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.)

- phenol (pH 4–5):chloroform:isoamyl alcohol [125:24:1; available from Sigma (Fluka Cat.# 77619, phenol, chloroform and isoamyl alcohol mixture BioChemika Ultra, for molecular biology, 125:24:1)]
- 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 1 unit per microgram of template DNA.
2. Incubate for 15 minutes at 37°C.
3. Extract with 1 volume of phenol (pH 4–5):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.
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 micropipette.
6. 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.
7. Carefully pour off or aspirate the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and resuspend 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. Removing Unincorporated Nucleotides by Chromatography

We recommend removing unincorporated nucleotides using chromatography methods. 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. Diluting reactions 1:2 with water before purification may increase RNA recovery; however, the 50µl loading limit per column should be observed.

4.C. Removing Unincorporated Nucleotides by Chromatography (continued)

NAP®-5 (GE Healthcare Cat.# 17-0853-01) and NAP®-10 (GE Healthcare Cat.# 17-0854-01) columns are recommended for purification of mRNA 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 the manufacturer's instructions.

4.D. Determining RNA Concentration and Visualizing 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. Prepare a 1:100 to 1:300 dilution of the RNA and read the absorbance at a wavelength of 260nm. One A_{260} unit equals approximately 40µg/ml of RNA. Alternatively, quantitation can be performed using a product such as RiboGreen® Assay (Molecular Probes).

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) on the gel can help determine the size and concentration of the RNA sample. The pGEM® Express Positive Control Template produces two RNA transcripts approximately 2.3kb and 1.1kb in length.

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). As an alternative to ethidium bromide, staining gels with SYBR® Green II following electrophoresis will provide greater sensitivity. While denaturing gels (containing formaldehyde, glyoxal or 8M urea) provide the greatest resolution of the denatured RNA, we have found that perfectly acceptable results can usually be obtained using nondenaturing gels loaded with RNA that is denatured in sample buffer prior to being loaded on the gel. 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 prior to loading. Perform electrophoresis under standard conditions used for the analysis of DNA samples.

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 and Possible Causes
Low amounts of RNA synthesized using standard transcription protocol	Spermidine in the Transcription 2X Buffer is causing the DNA to precipitate. Make sure the components of the reaction are assembled at room temperature and in the order listed.
	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 precipitated in the presence of another salt. Wash the resulting pellet 1–2 times with 70% ethanol.
	RNase contamination is resulting in degradation of the RNA. The use of Recombinant RNasin® Ribonuclease Inhibitor is recommended for all in vitro transcription reactions. Any solutions not provided should be made up in water that has been treated with 0.1% DEPC.
	RNA polymerase has become inactive. The activity of the RNA polymerase may be evaluated by in vitro transcription of the control template or supercoiled plasmid containing the T7 RNA polymerase promoter.
Presence of incomplete transcripts	RNA synthesis is terminated prematurely. Lower the temperature of incubation from 37°C to 30°C. This can increase the proportion of full-length transcripts in some cases (5).
Presence of larger transcripts than expected	Protruding 3´ termini on the DNA template. If the DNA template has been 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 (4). If it is impossible to avoid using a restriction enzyme of this type, the ends of linear DNA should be made blunt using DNA Polymerase I Large (Klenow) Fragment before use in a transcription reaction.
	Nonlinearized plasmid is present in the sample. Analyze the sample by gel electrophoresis. If undigested vector is noted, redigest with the appropriate restriction enzyme.

6. Composition of Buffers and Solutions

phenol (pH 4–5): chloroform:isoamyl alcohol (125:24:1)

Available from Sigma (Fluka Cat.# 77619, phenol, chloroform and isoamyl alcohol mixture BioChemika Ultra, for molecular biology, 125:24:1)

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. Dispense RNA loading buffer into aliquots 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. The mix 1 part of the lower phenol phase with 1 part of the chloroform:isoamyl alchol (24:1).

7. Related Products

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
RiboMAX™ Large Scale RNA Production System—SP6	1 system	P1280
RiboMAX™ Large Scale RNA Production System—T7	1 system	P1300

DNA Purification Products

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps**	A2492
PureYield™ Plasmid Maxiprep System	10 preps**	A2392
Wizard® *Plus* SV Minipreps DNA Purification System	50 preps**	A1330
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

**Larger sizes available.

Single-Stranded RNA Markers

Product	Size	Cat.#
RNA Markers	50µl	G3191

Translation Systems

Product	Size	Cat.#
Flexi® Rabbit Reticulocyte Lysate	1ml	L4540
Rabbit Reticulocyte Lysate, Nuclease Treated	1ml	L4960
Wheat Germ Extract	1ml	L4380
Wheat Germ Extract Plus	40 × 50µl	L3250
	10 × 50µl	L3251
Rabbit Reticulocyte Lysate/Wheat Germ Extract Combination System	12 reactions	L4330

8. References

1. Gurevich, V.V. *et al*. (1991) Preparative in vitro mRNA synthesis using SP6 and T7 RNA polymerases. *Anal. Biochem*. **195**, 207–13.
2. Cunningham, P.R. and Ofengand, J. (1990) Use of inorganic pyrophosphatase to improve the yield of in vitro transcription reactions catalyzed by T7 RNA polymerase. *BioTechniques* **9**, 713–4.
3. Herrmann, B.G. and Frischauf, A.M. (1987) Isolation of genomic DNA. *Methods Enzymol*. **152**, 180–3.
4. Schenborn, E.T. and Mierendorf, R.C. (1985) A novel transcription property of SP6 and T7 RNA polymerases: Dependence on template structure. *Nucleic Acids Res.* **13**, 6223–36.
5. Krieg, P.A. (1990) Improved synthesis of full-length RNA probe at reduced incubation temperatures. *Nucleic Acids Res*. **18**, 6463.