

Take the “Express Way” to RNA

Fast & Efficient Production of RNA with the T7 RiboMAX™ Express System

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Abstract

The T7 RiboMAX™ Express Large Scale RNA Production System is a new in vitro transcription system designed to rapidly produce very high concentrations of RNA. The new system offers improvements over the standard T7 RiboMAX™ Large Scale Production System in terms of both time savings and convenience by including the ribonucleotide triphosphates as part of the RiboMAX™ Express T7 2X Buffer. In this article we illustrate the benefits of the new T7 RiboMAX™ Express System.

The new RiboMAX™ Express System is intended for the rapid production of large quantities of RNA in a significantly reduced amount of time.

Introduction

In vitro transcription has become a universal technique for generating large quantities of biologically active RNA. The procedure can be used to synthesize mRNA, rRNA, tRNA, other small, functional RNAs, antisense RNA, dsRNA for making siRNA, ribozymes and RNA viral genomes, as well as substrates for RNA splicing, RNA:protein interaction and secondary structure studies. Coupled transcription/translation reactions are common, but RNA generated first by in vitro transcription allows modifications to be incorporated into the transcript. For example, RNAs used for ribosome display technology are modified at the 3'-end to allow linkage of the RNA to the translated protein (1). Shorter transcription reaction times are very important to reduce cycle time in applications such as ribosomal display. However, the transcription reaction times are the rate limiting step. If transcription times can be reduced to 30 minutes, a complete screen can be performed in only one day.

Butler, Chamberlin and their colleagues first described in vitro transcription using phage RNA polymerases in 1982 (2,3). The techniques were further developed by several researchers including Krieg and Melton in 1984 (4,5), Cunningham and Ofengand in 1990 (6) and Gurevich *et al.* in 1991 (7). Promega developed the Riboprobe® Systems^(a,b) for labeling RNA in 1983 and expanded the product line by introducing the RiboMAX™ Large Scale RNA Production Systems^(a,b,c,d) in 1992 (8). The new T7 RiboMAX™ Express Large Scale RNA Production System^(a,b) continues this tradition of advancement. The new T7 RiboMAX™ Express System is intended for the rapid production of large quantities of RNA in a significantly reduced amount of time. However,

for synthesis of transcripts that are capped on the 5'-end using m⁷G[5']ppp[5']G analogs such as the Ribo m⁷G Cap Analog (Cat.# P1711) we recommend continuing to use the original RiboMAX™ Large Scale RNA Production System. This process requires optimizing the GTP concentration, which can not be done with the RiboMAX™ Express System because the rNTPs are all included in the RiboMAX™ Express T7 2X Buffer.

We investigated the effectiveness of the T7 RiboMAX™ Express System by performing the following experiments. First, we demonstrated the improved transcription rate and its relationship to DNA template concentration. Next, we analyzed the Enzyme Mix and RiboMAX™ Express T7 2X Buffer stability after incubation at 37°C for up to 24 hours, as well as the buffer's stability through 30 freeze-thaw cycles. Finally, we showed that the system is capable of generating large amounts of short transcripts.

Transcription Rate

To demonstrate the rate at which the T7 RiboMAX™ Express System can transcribe RNA, we prepared reactions using the pGEM® Express Positive Control Template^(e) (Cat.# P2561), which produces transcripts 1.1kb and 2.3kb in length. We incubated the reactions at 37°C, quenched them at 10-minute intervals from 10–60 minutes, and determined the concentration of RNA generated as described in Methods. As shown in Figure 1, the reaction was greater than 50% complete at 10 minutes and nearly complete after 20 minutes, generating just under 8mg/ml of RNA. Agarose gel analysis of the 30-minute reaction shows the 1.1 and 2.3kb transcripts (Figure 1, Panel B). Each T7 RiboMAX™ Express reaction contains sufficient nucleotides to generate a theoretical maximum of 10mg/ml RNA. However, the actual amount of RNA produced is template-specific and depends upon the G:C/A:T ratio and template complexity.

The T7 RiboMAX™ Express System is designed to generate the maximum amount of transcript when using 50µg/ml of template DNA in only 30 minutes. However, higher concentrations of RNA can be produced from lower amounts of template by increasing incubation times. We demonstrated this by incubating reactions using 1, 5 or 10µg/ml of pGEM® Express Positive Control Template at 37°C for just over 8.5 hours. Figure 2, Panel A, shows that even 1µg/ml of the control template produced 3mg/ml RNA in ~8 hours. Increasing the template concentrations reduces the time needed for maximum RNA production. Agarose gel analysis (Figure 2, Panel B) shows that only the 1.1 and 2.3kb transcripts are generated.

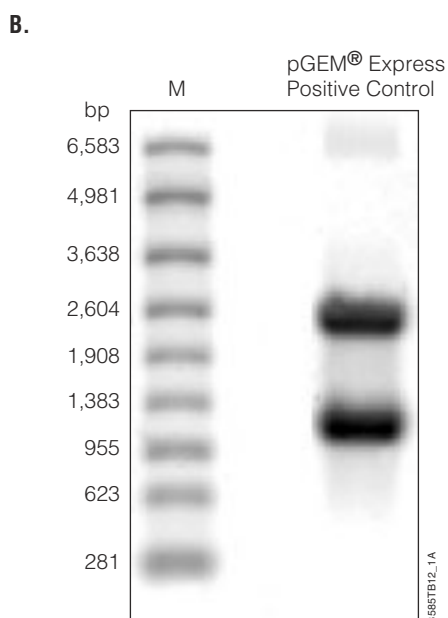
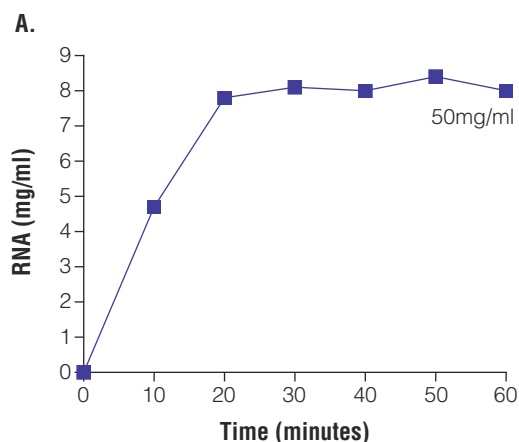


Figure 1. Reaction time-course. A 250 μ l transcription reaction was prepared according to the description in Methods, adding 1 μ g of pGEM[®] Express Positive Control Template per 20 μ l of reaction mix (50 μ g/ml final concentration), and adding the Enzyme Mix last. Twenty-microliter aliquots of the reaction were immediately dispensed and placed at 37°C. Duplicate reactions were removed at the times indicated, placed on ice and quenched with 180 μ l of TE buffer. **Panel A:** A portion of each reaction was diluted 1,000-fold and the RNA concentration was determined as described in Methods. **Panel B:** A 15 μ l aliquot of the reaction incubated for 30 minutes was analyzed by agarose gel electrophoresis as described in Methods. Lane M, 10 μ l of RNA Markers^(b) (Cat.# G3191).

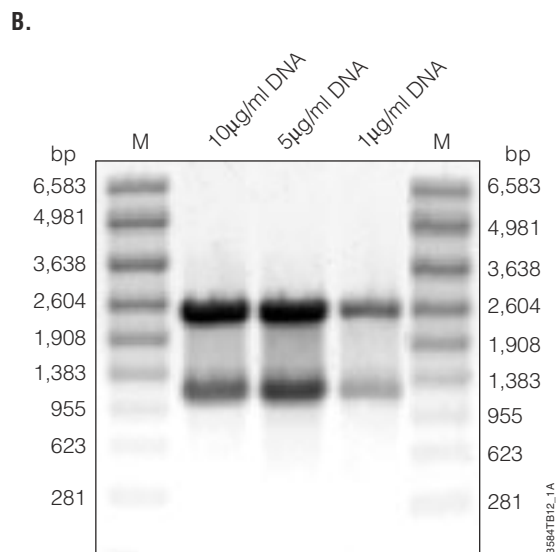
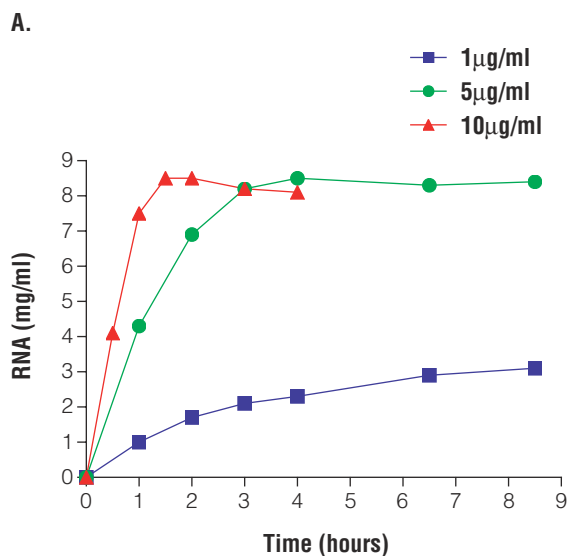


Figure 2. Template DNA concentrations versus time. Transcription reactions were prepared as in Figure 1, containing 1, 5 or 10 μ g/ml of pGEM[®] Express Positive Control Template (final concentration) per 20 μ l reaction volume and adding the Enzyme Mix last. The reactions were immediately dispensed and placed at 37°C. At the times indicated, duplicate reactions were removed and quenched as described in Figure 1. **Panel A:** A portion of each reaction was diluted 1,000-fold and assayed for the RNA concentration. **Panel B:** A 15 μ l aliquot of each reaction was incubated for 4 hours and analyzed by agarose gel. Lanes M, 10 μ l of RNA Markers.

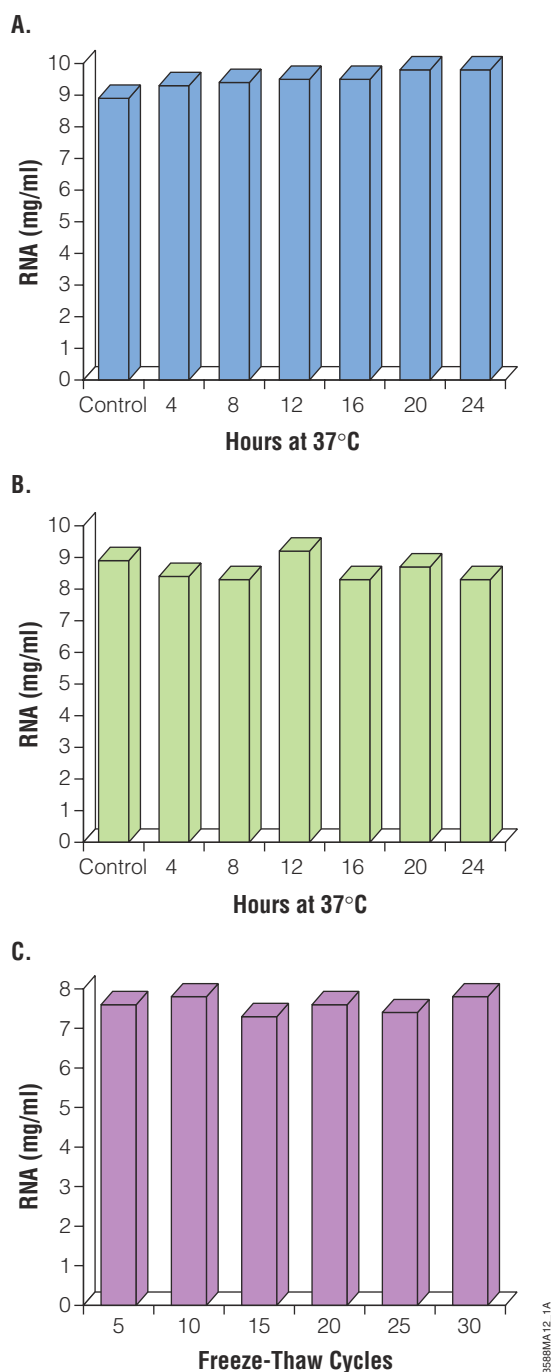


Figure 3. Reagent stability. **Panel A:** A sample (250 μ l) of the Enzyme Mix was incubated at 37°C, 25 μ l aliquots were removed at the indicated times and stored at -20°C. Duplicate transcription reactions were prepared, incubated for 30 minutes at 37°C and quenched as described in Figure 1. The reactions were diluted 1,000-fold and assayed for RNA concentration. **Panel B:** A sample (400 μ l) of RiboMAX™ Express T7 2X Buffer was incubated at 37°C, and 50 μ l aliquots were removed and reactions prepared and assayed as in Panel A. **Panel C:** A sample (1ml) of freshly prepared Transcription 2X Buffer was frozen and thawed (~5 minutes at 37°C) for a total of 30 cycles. Fifty-microliter aliquots were removed after cycles 4, 9, 14, 19, 24 and 29 and frozen. All of the samples were thawed a final time and reactions prepared and assayed as in Panel A.

Reagent Stability

The long-term stability of molecular biology reagents is a concern for many scientists. Figure 3, Panels A and B, demonstrates the stability of the Enzyme Mix and RiboMAX™ Express T7 2X Buffer in the T7 RiboMAX™ Express System after incubation at 37°C for various times (we used fresh Enzyme Mix when testing the RiboMAX™ Express T7 2X Buffer and vice versa). Incubating the Enzyme Mix for 24 hours at 37°C did not decrease the amount of RNA produced. The buffer stability test was especially important, since it is commonly thought that nucleotide triphosphates may be less stable in the presence of magnesium ions. However, after 24 hours at 37°C the RiboMAX™ Express T7 2X Buffer showed only a slight decrease in RNA production and still produced >8mg/ml of RNA.

At the recommended storage temperature, the RiboMAX™ Express T7 2X Buffer is frozen. When the buffer is thawed, a precipitate forms consisting primarily of the magnesium salt of nucleotide triphosphates. This precipitate can be dissolved by heating at 37°C for roughly 5 minutes, mixing periodically. The precipitate will re-form if the buffer is placed on ice; therefore, we recommend keeping the buffer at room temperature while preparing transcription reactions. To demonstrate that freezing and thawing the RiboMAX™ Express T7 2X Buffer does not affect its stability, we subjected a sample to 30 freeze-thaw cycles, removing an aliquot after every 5 cycles. The results show no significant change in the maximum amount of RNA generated and demonstrate that the RiboMAX™ Express T7 2X Buffer is stable through 30 freeze-thaw cycles (Figure 3, Panel C).

Short Transcripts

In vitro transcription of equal molar levels of short RNA transcripts (e.g., tRNAs, which are 80–100 bases in length compared to the average transcript length of 1–3kb) is thought to be more difficult because of the greater number of polymerase initiation events required. The T7 RiboMAX™ Express System can effectively generate such short transcripts. We prepared a reaction using the Century™ marker templates (Ambion), which produce transcripts in 100-base increments from 100–500 bases. In addition we prepared a reaction containing the control template DNA from the MEGAshortscript™ pT7-RNA-18S (Ambion), which is expected to generate a 116-base transcript. A 2-hour reaction generated 8mg/ml RNA from the Century™ marker templates and 5mg/ml of transcript from the pT7-RNA-18S control template. The

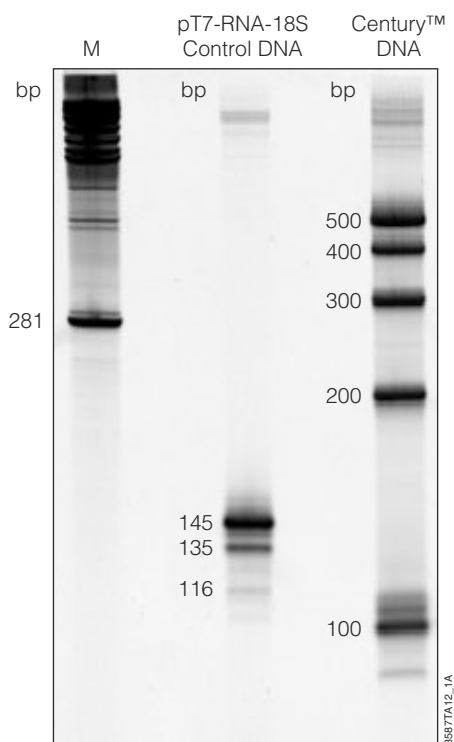


Figure 4. Production of short transcripts. Duplicate reactions were prepared using 1µg (2µl of 0.5µg/µl) of the Century™ marker templates or 1µg (2µl of 0.5µg/µl) of the pT7-RNA-18S control DNA from the MEGAshortscript™ kit (Ambion) as a template. The Enzyme Mix was added last. The reactions were performed as described in Methods. After quenching, they were diluted 500-fold and the RNA concentrations were determined. A half a microliter of the Century™ marker reaction and 0.25µl of the pT7-RNA-18S reaction were analyzed by denaturing PAGE gel (Invitrogen); Lane M, 10µl of RNA Markers.

TBE-urea PAGE gel results (Figure 4) demonstrate that the distribution among the 5 bands for the Century™ marker templates is comparatively equal. We determined the size of the bands in the pT7-RNA-18S Control DNA lane using the designated transcript sizes for the Century™ markers. It is not clear why the pT7-RNA-18S template generated a transcript larger than the expected 116 bases.

Discussion

The T7 RiboMAX™ Express Large Scale RNA Production System was developed for researchers who need to rapidly produce large amounts of RNA. It is especially valuable in techniques such as ribosome display, because it reduces the rate limiting step in the overall cycle time of transcription, translation, ligand capture, reverse transcription and amplification (9). The T7 RiboMAX™ Express Large Scale RNA Production System is a stable, robust and versatile method for rapidly producing large quantities of RNA.

The time-course experiments demonstrate that the T7 RiboMAX™ Express System generates close to the maximum amount of RNA transcript possible in less

than 30 minutes. When template concentration is limiting, maximum RNA production can be achieved with longer incubation times. However, the rate of transcription is template-specific; therefore, not all templates will generate their maximum in the same amount of time.

The reaction components of the T7 RiboMAX™ Express Large Scale RNA Production System are stable, even after 24 hours at 37°C. The RiboMAX™ Express T7 2X Buffer has no change in activity after freeze-thaw cycles. A precipitate, which forms upon cooling below room temperature, dissolved completely with each cycle and does not adversely affect the performance.

The T7 RiboMAX™ Express Large Scale RNA Production System was shown to be very versatile, producing transcripts between 100 bases and 2.3kb. Although not shown here, transcripts generated by in vitro transcription have been shown to have significant biological activity, especially for translation of proteins, as demonstrated by Beckler using the T7 RiboMAX™ Large Scale RNA Production System (8).

Methods

All transcription reactions were performed according to the procedure outlined in Technical Bulletin #TB298. Each 20µl reaction consists of 10µl of RiboMAX™ Express T7 2X Buffer, 2µl of Enzyme Mix, and 8µl of Nuclease-Free Water containing the indicated amount of template DNA (usually 1µg).

The RNA concentration was determined using the RiboGreen® RNA Quantitation Reagent Kit (Molecular Probes). The reagent was diluted 200-fold into TE buffer containing 0.1mg/ml BSA (to reduce nonspecific binding in the microtiter plates), and 100µl of this solution was mixed with 100µl of diluted RNA reactions or standards in a black Cliniplate microtiter plate (Labsystems). The RNA standards were prepared from the stock solution found in the RiboGreen® kit (a mixture of 16S and 23S rRNA from *E. coli*), diluting the 100µg/ml solution to 1µg/ml in TE buffer (prepared from the 20X solution in the kit) and a 0.75, 0.5, and 0.25µg/ml solution was prepared.

Transcript lengths were analyzed by electrophoresis using 1% TAE agarose gels containing SYBR® Green II RNA gel stain (1:10,000 dilution of stock solution), or using TBE urea PAGE gels soaked in SYBR® green II stain (1:10,000 dilution). Gels were visualized with a Storm® PhosphorImager® /FluorImager® (Molecular Dynamics). For TAE gels, 6µl of each diluted reaction was added to 20µl of RNA sample buffer and 4µl RNA loading buffer and heated at 65°C for 10 minutes. For TBE-urea PAGE analysis, 1µl of the pT7-RNA-18S Control DNA reactions or 2µl of the Century™ reactions were combined with 19 or 18µl of gel loading buffer II (Ambion), heated at 95°C, and 5µl was loaded on the gel.

References

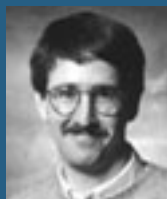
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Protocols

- ◆ *T7 RiboMAX™ Express Large Scale RNA Production System Technical Bulletin #TB298*, Promega Corporation.
(www.promega.com/tbs/tb298/tb298.html)



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Ordering Information

Product	Size	Cat.#
T7 RiboMAX™ Express Large Scale RNA Production System	1 system	P1320

For Laboratory Use.

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		P1121
RiboMAX™ Large Scale RNA Production System – SP6	1 system	P1280
RiboMAX™ Large Scale RNA Production System – T7	1 system	P1300
RNA Markers	50µl	G3191

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^(a) U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

^(b) U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

^(c) 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.

^(d) The RiboMAX™ Large Scale RNA Production System—T7 (Cat.# P1300) is covered by U.S. Pat. No. 5,256,555 and is sold under a license from Ambion, Inc. It is intended for research use only. Parties wishing to use this product for other applications should contact Ambion, Inc.

^(e) U.S. Pat. No. 4,766,072.