

Production of milligram amounts of highly translatable RNA using the RiboMAX™ System

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This article describes the development of an improved method for synthesizing large amounts of RNA (up to 6mg/ml) and describes the unexpected finding that RNA generated with this protocol is of higher quality for rabbit reticulocyte lysate in vitro translation reactions than RNA produced from a standard transcription protocol.

Introduction

In vitro transcription reactions are widely used to synthesize microgram amounts of RNA probes from recombinant DNA templates. Standard RNA probe synthesis reactions typically are optimized to maximize incorporation of radiolabeled ribonucleotides rather than RNA production.

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; for synthesis of tRNA, rRNA, SRP RNA, RNA virus genomes and ribozymes; and for production of substrates for studies of RNA splicing, antisense RNA and RNA-protein interactions.

During the development of Promega's Flexi™ Rabbit Reticulocyte Lysate System* (an optimizable *in vitro* translation system), we quickly recognized the need to produce *in vitro* milligram amounts of highly translatable run-off RNAs. As part of this project, we tested luciferase gene constructs under a variety of translation conditions (1).

Initially, a procedure adapted from Melton (2) was used to produce uncapped run-off RNA transcripts (referred to below as the Standard Protocol). With this method, frequent RNA preparations were necessary because the usual yield of RNA was only 250µg/ml. Unfortunately, inconsistency between RNA batches can affect translational efficiency, complicating comparisons between experiments.

Previous reports of increased RNA production

Gurevich *et al.* (3) reported a preparative RNA transcription protocol which utilized a HEPES, pH 7.5 buffer; increased nucleotide, Mg²⁺ and RNA polymerase concentrations; and as much as 3-fold less DNA template than the Melton procedure. Cunningham and Ofengand (4) also reported that the addition of inorganic pyrophosphatase (PPase) increased the yield of RNA produced in *in vitro* transcription reactions.

The RiboMAX Large Scale RNA Production Systems

A further enhancement of RNA production may be obtained using the RiboMAX protocol described in

this article. Promega's RiboMAX Large Scale Production Systems provide a convenient and cost-effective way to transcribe milligram amounts of RNA. This enhanced protocol for RNA production is described in more detail in Promega Technical Bulletins 127 and 166. [Table 1](#) lists the differences between the Standard Protocol and the RiboMAX Protocol.

Methods

After all transcription reactions, the DNA template was removed by adding RQ1 RNase-Free DNase to a concentration of 1u/μg DNA. This reaction was incubated at 37°C for 15 minutes, and the RNA was purified by two phenol:chloroform:isoamyl alcohol extractions, a chloroform:isoamyl alcohol extraction, and an ethanol precipitation containing 0.3M sodium acetate, pH 5.2, followed by a 70% ethanol wash. The samples were briefly dried under vacuum, resuspended in nuclease-free water and stored at -20°C.

Table 1. Comparison of RNA Production Protocols.

| Protocol | Reaction Components | Polymerase & Template | Incubation Conditions |
|----------|--|---|---|
| Standard | 40mM Tris-HCl, pH 7.5 6mM MgCl ₂ 2mM spermidine 10mM NaCl 10mM DTT 0.5mM each rNTP 1,000u/ml RNasin® Ribonuclease Inhibitor | 400u/ml RNA polymerase 50-100μg/ml DNA template | 2 hours at 37°C |
| RiboMAX | 80mM HEPES-KOH, pH 7.5 16mM MgCl ₂ (for SP6)* 12mM MgCl ₂ (for T3 or T7)* 2mM spermidine 40mM DTT 3mM each rNTP 1,000u/ml RNasin® Ribonuclease Inhibitor 5u/ml yeast inorganic PPase (Sigma Chemical Co.) | 1,800u/ml RNA polymerase 30μg/ml SP6 DNA template* 100μg/ml T3 or T7 DNA template* | 2 hours at 37°C; then add 1,800u/ml more RNA polymerase for an additional 2 hours at 37°C |

*The RiboMAX Protocol reaction components are slightly different for SP6, T3 and T7 RNA polymerase.

Results

Increased RNA yield with HEPES plus pyrophosphatase

[Table 2](#) shows the effect on RNA yield of PPase added to the HEPES buffer reported by Gurevich *et al.* (3, see RiboMAX Protocol buffer composition in [Table 1](#)). Transcripts were generated from two luciferase gene constructs (1), one of which contained a 30-base poly(A) tail. Both of the transcripts contained about 2,000 bases. Using the HEPES buffer without added PPase, about 2-fold more RNA was produced than is usually obtained from the Standard Protocol. The addition of PPase further increased the RNA yield 1.8- to 2.4-fold.

Table 2. RNA Yield of Transcripts Synthesized using the HEPES Buffer With or Without Added PPase.

| Construct | HEPES | HEPES + PPase |
|---------------------|-----------|---------------|
| pPOLY(A) <i>luc</i> | 0.38mg/ml | 0.90mg/ml |
| pGEM- <i>luc</i> | 0.40mg/ml | 0.70mg/ml |

The 100µl reactions were performed at 37°C for 2 hours.

RNA yield enhancement by second addition of RNA polymerase

Encouraged by this increased yield, the HEPES + PPase reaction conditions were used to synthesize three different transcripts (C-JUN, containing the transcription factor C-JUN gene; and B-13 and B-38, both containing the chloramphenicol acetyltransferase gene). A 1µl aliquot of each reaction was removed after 1 hour and analyzed by gel electrophoresis. Since the B-13 and B-38 transcripts were faint (approximately 0.5µg/band), more RNA polymerase was added and the reactions were incubated an additional 2-hours. For all three transcripts, the RNA yields were well above any we had previously obtained.

This result led to the development of the RiboMAX Protocol (see [Table 1](#)), in which two successive 2-hour incubations with RNA polymerase are used in combination with HEPES/PPase reaction buffer to obtain very high levels of RNA synthesis.

[Figure 1](#), panel A compares the amount of each luciferase transcript synthesized with the Standard and the RiboMAX Protocol. For both transcripts, several times more RNA was made with the RiboMAX Protocol. [Figure 1](#), panel B compares the homogeneity of the pGEM^â-*luc* transcripts made with the two protocols. When equal amounts of the two transcripts were loaded, no differences were visible in the banding patterns.

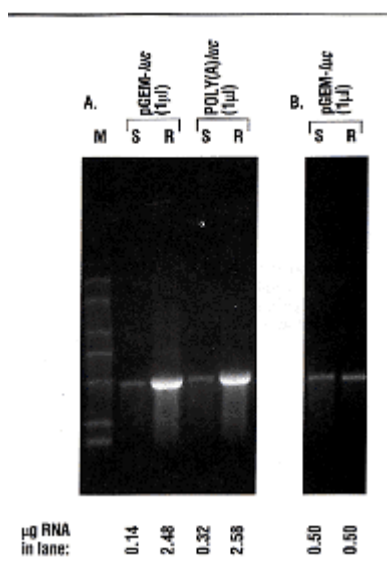


Figure 1. Comparison of Standard and RiboMAX Protocol production of pGEM-*luc* and pPOLY(A)*luc* transcripts. The transcripts indicated were synthesized in 100µl reactions using either the Standard (S) or RiboMAX (R) Protocols described in [Table 1](#). In Panel A, 2µl aliquots of these reactions were added to 20µl of RNA sample buffer (64% formamide; 23% formalin; 26mM MOPS, pH 7.0; 6.45mM sodium acetate; 0.6mM EDTA, pH 8.0) and 3µl of RNA loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 1µg/ml ethidium bromide) and incubated at 65°C for 15 minutes. The samples were quick-chilled on ice and run on a 1% TAE agarose gel. In Panel B, an equal amount of RNA (0.5µg) from each of the pGEM-*luc* reactions was run side by side to compare purity. Lane M, RNA Markers (Promega).

Reduction of translation inhibition at high RNA concentrations

In our early experiments (using RNA synthesized with the Standard Protocol), we observed an interesting phenomenon which had been previously noted by other groups (5,6): *in vitro* translation efficiency actually *decreased* when more than the optimum amount of RNA was added.

By contrast, RNA transcripts produced by the RiboMAX Protocol did not lead to this inhibition. Instead, they could be added at levels 2-3 times beyond the optimum for translation without leading to a substantial drop in translation efficiency (1). This is illustrated in [Table 3](#), which compares the amount of functional luciferase synthesized in *in vitro* translation reactions primed with 1µg (the optimum level) or 3µg of pPOLY(A)*luc* RNA generated with either the Standard or the RiboMAX Protocol.

Table 3. Effect of Greater than Optimal Amounts of RNA on Luciferase Translation Efficiency.

| Protocol | 1µg RNA | 3µg RNA |
|----------|-------------|-------------|
| Standard | 273,000 TLU | 88,000 TLU |
| RiboMAX | 247,000 | 242,000 TLU |

Translation reactions were performed and assayed as described in [Figure 2](#).

Improved translation efficiency

We next compared three sets of transcription conditions for both RNA production levels and for the translation efficiency of the RNA produced. [Figure 2](#), panel A compares the amount of RNA generated

using the Standard Protocol, the HEPES transcription buffer without PPase, and the RiboMAX Protocol (including both PPase and a second addition of RNA polymerase). As observed previously, the use of the HEPES reaction buffer produced greater than 2-fold more RNA than the Standard Protocol. The RiboMAX Protocol gave an additional 4-fold increase in RNA production, to 6mg/ml.

Panel B of [Figure 2](#) compares the amounts of functional luciferase synthesized in translation reactions programmed with equal amounts of RNA generated by each of the three protocols. RNA produced in HEPES buffer gave a 16% increase in translation efficiency, but an even larger increase (65%) in translation efficiency was observed using RNA synthesized with the RiboMAX Protocol.

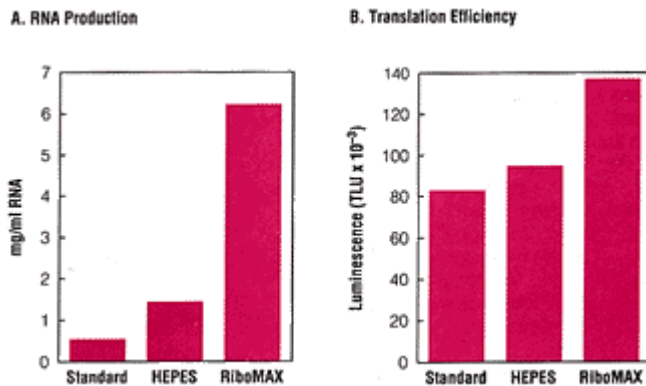


Figure 2. Comparison of RNA production and translation efficiency with three RNA synthesis protocols. Panel A: pGEM-*luc* run-off transcripts were synthesized in 20 μ l transcription reactions using the protocols indicated. The Standard and RiboMAX Protocols were performed as described in [Table 1](#). The HEPES protocol used the conditions described for the RiboMAX Protocol, but without added PPase or the second incubation with RNA polymerase. Panel B: An equal amount (80ng) of RNA produced in each of the three transcription protocols was used to program 50 μ l *in vitro* translation reactions using the FlexiTM Rabbit Reticulocyte Lysate System (Promega). 2.5 μ l of each reaction was mixed with an equal volume of Luciferase Assay Reagent and luminescence was measured for 10 seconds in a luminometer. The synthesis of functional luciferase is expressed as Turner Light Units (TLU).

To verify the increased translation efficiency of RNA from the RiboMAX Protocol, the pGEM-*luc* and pPOLY(A)*luc* luciferase RNA transcripts were generated in duplicate using either the Standard or RiboMAX Protocol. Varying amounts of these RNAs then were used to program rabbit reticulocyte lysate *in vitro* translation reactions ([Figure 3](#) and [Figure 4](#)). In both cases, the RNA produced by the RiboMAX Protocol gave both a higher translation efficiency and, at high concentrations of RNA, less inhibition of translation.

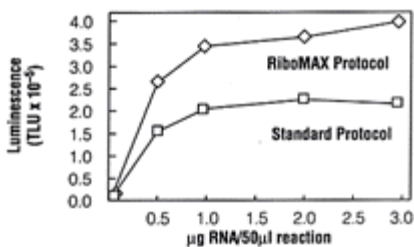


Figure 3. Effect of pGEM-*luc* RNA concentration on *in vitro* translation. The indicated amounts of pGEM-*luc* RNA transcripts were used to program 50 μ l Rabbit Reticulocyte Lysate *in vitro* translation reactions. Expression of functional luciferase was measured as described in [Figure 2](#).

Interestingly, high levels of pPOLY(A)*luc* RNA inhibited translation more than pGEM-*luc* RNA did. The only difference between these two transcripts is the addition of a 30-base poly(A) tail. One possible explanation for this difference might be that high concentrations of the poly(A) tail might reduce the concentration of poly(A) binding protein available for translation.

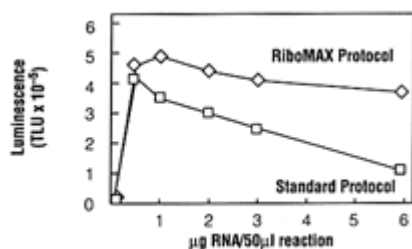


Figure 4. Effect of pPOLY(A)*luc* RNA concentration on *in vitro* translation. The indicated amounts of pPOLY(A)*luc* RNA transcripts were used to program 50µl Rabbit Reticulocyte Lysate *in vitro* translation reactions. Expression of functional luciferase was measured as described in [Figure 2](#).

Effect of RNA clean-up method on translation efficiency

The RNA generated by each of the three protocols described above was cleaned up after transcription using either DNase incubation followed by phenol extractions (see Methods) or by precipitation in 2.5M LiCl. Both methods were followed by precipitation in sodium acetate and ethanol. While there was no observable difference between these RNAs by gel electrophoresis analysis, RNA purified by phenol extraction consistently gave 1.5- to 2-fold better translation efficiencies than LiCl-precipitated RNA (data not shown).

Discussion

Scale up of RNA production

The enhanced *in vitro* RNA synthesis protocol reported here combines key features of two other protocols with a second addition of RNA polymerase to consistently produce 2-6mg/ml of RNA in a 20-100µl reaction, about 10- to 20-fold more than is produced with a standard transcription reaction. We also have successfully scaled up this protocol to 10ml, resulting in a yield of 30mg of RNA from a single reaction.

Effect of HEPES buffer

Additional experiments (data not shown) have suggested that a factor other than PPase may be responsible for the improved translation efficiency of RNA produced with the RiboMAX Protocol. It is unclear, however, how the use of the HEPES-based transcription buffer could lead to such a change. Little salt or other inhibitors should be present after the phenol extractions, ethanol precipitation and washes. The RNAs appear identical upon gel electrophoresis ([Figure 1](#), panel B). Gurevich *et al.* (3) suggest that the HEPES pH 7.5 high-capacity buffer increases the RNA yield by reduced acidification of the reaction mix caused by free protons released during the polymerization reaction.

Perhaps the HEPES reaction conditions also prevent transcription conditions that produce aberrant RNA products. Aberrant double-stranded RNAs have been reported with standard *in vitro* reaction conditions (7,8). These include the "X" RNA, which is produced in a non-template-dependent manner (9), and anti-

sense RNAs produced by RNA polymerase doubling back at the end of a linear DNA template (7).

Effect of inorganic PPase

The inclusion of yeast inorganic pyrophosphatase in the HEPES-based transcription reaction led to a further increase in production of RNA. Inorganic PPase is essential for DNA replication *in vivo*, catalyzing the hydrolysis of pyrophosphate to two molecules of orthophosphate. This drives the DNA polymerization reaction forward by removal of a product (pyrophosphate) which can be utilized in the reverse pyrophosphorolysis reaction (10). Similarly, the removal of orthophosphate or its magnesium complex from RNA polymerization reactions may help drive the RNA polymerization reaction forward by removal of an inhibitory product (4).

While the reasons for the observed improvements in RNA production and translation efficiency are not entirely clear, the RiboMAX Protocol described in this article should be useful to researchers wishing to produce large amounts of RNA for *in vitro* translation. The reduction of components inhibitory to translation also may be advantageous for other applications requiring biologically active RNA.

References

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Ordering Information

Ordering information for the RiboMAX Large Scale RNA Production Systems is provided following the next article, [Stabilized TMB Substrate for Horseradish Peroxidase vs. 4-chloro-1-naphthol: A comparison on Western blots](#). Other Promega products mentioned in this article are listed below.

| Product | Cat# |
|----------------------------|-------|
| Flexi™ Rabbit Reticulocyte | L4540 |

This system contains sufficient reagents to perform 30x50µl reactions. Flexi Lysate is provided in 200µl aliquots.

| Product | Cat.# |
|-------------------------|-------|
| Luciferase Assay System | E1500 |

This system contains sufficient reagents for 100

| Product | Size | Cat.# |
|----------------|------|-------|
| pGEM® -luc DNA | 20µg | E1541 |

The pGEM[®]-luc Vector was constructed by cloning the luciferase in the center of the multiple cloning region of the pGEM[®]-11Zf(-) Vector

| Product | Size | Cat.# |
|---|--------------------------|--------------|
| RQ1 RNase- Free DNase | 1000u | M6101 |
| | 5000u (5 x 1000u) | M6102 |
| SP6 RNA Polymerase | 1000u | P1085 |
| | 5,000u (5 x 1,000u) | P2076 |
| T7 RNA Polymerase | 1,000u | P2075 |
| | 5,000u (5 x 1,000u) | P2076 |
| RNasin [®] Ribonuclease Inhibitor | 2,500u | N2111 |
| | 10,000u (4 x 2,500u) | N2112 |
| | 20,000 (2 x 10,000u) | N2113 |
| | 60,000u (6 x 10,000u) | N2114 |
| Recombinant RNasin [®] Ribonuclease Inhibitor | 2,500u | N2511 |
| | 10,000u (4 x 2,500u) | N2512 |
| | 20,000u (2 x 10,000u) | N2513 |
| | 60,000u (6 x 10,000u) | N2514 |

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