Riboprobe® in vitro Transcription Systems

Technical Manual No. 016

INSTRUCTIONS FOR USE OF PRODUCTS P1420, P1430, P1440, P1450 AND P1460.
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I. Description

The Riboprobe® Systems[a,b] are designed for in vitro preparation of high specific activity single-stranded RNA probes or microgram quantities of defined RNA transcripts from cloned DNA inserts (Figure 1). The complete Riboprobe® Systems include the RNA polymerases, all of the required reagents for performing transcription reactions in vitro (excluding radioisotope) and RQ1 RNase-Free DNase (Cat.# M6101) for removal of the template following transcription.

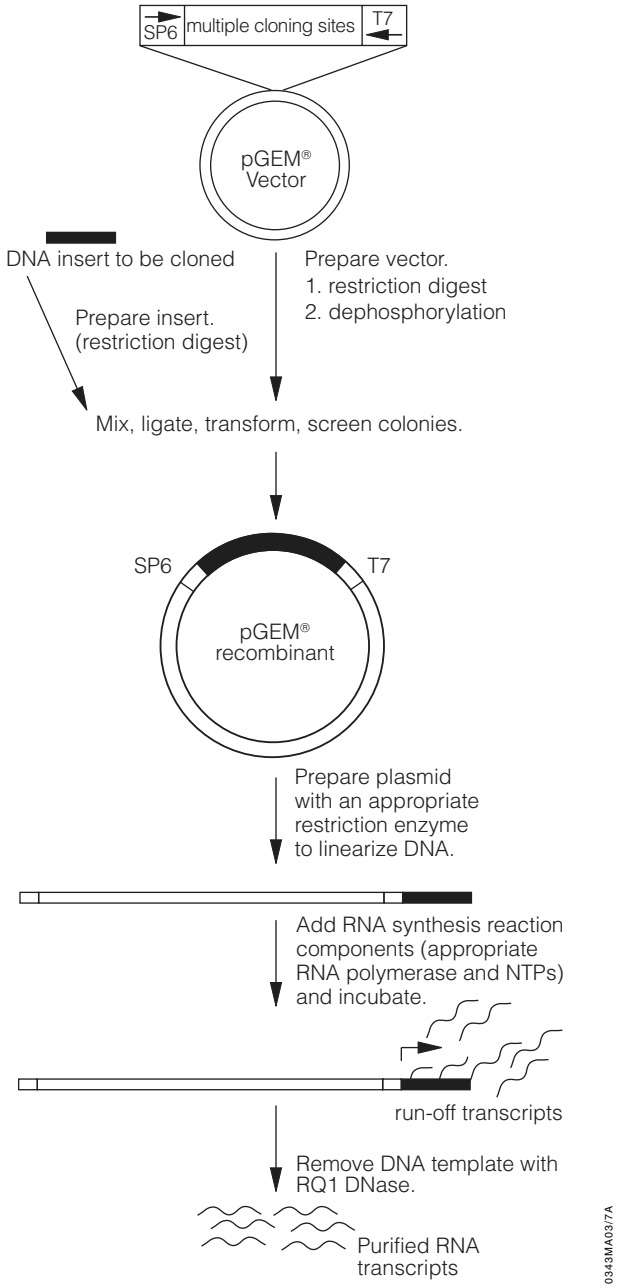

Figure 1. Schematic of the Riboprobe® in vitro Transcription Systems.

II. Product Components

Product	Cat.#
Riboprobe® System – SP6	P1420
Riboprobe® System – T3	P1430
Riboprobe® System – T7	P1440

For Laboratory Use. Each system contains sufficient reagents for 25 standard (20µl) transcription reactions. Includes:

- 500u RNA Polymerase (SP6, T3 or T7)
- 5µg pGEM® Express Positive Control Template(c)
- 500µl Transcription Optimized 5X Buffer
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor(a,b)
- 100µl DTT, 100mM
- 1.25ml Nuclease-Free Water
- 110u RQ1 RNase-Free DNase
- 50µl Each of 4 Unlabeled rNTPs, 10mM
- 1 Protocol

Product	Cat.#
Riboprobe® System - T3/T7	P1450
Riboprobe® System - SP6/T7	P1460

For Laboratory Use. Each system contains sufficient reagents for 25 standard (20µl) transcription reactions with each RNA Polymerase. Includes:

- 500u SP6 RNA Polymerase or T3 polymerase
- 500u T7 RNA Polymerase
- 5µg pGEM® Express Positive Control Template
- 500µl Transcription Optimized 5X Buffer
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor
- 200µl DTT, 100mM (2 × 100µl)
- 2.5ml Nuclease-Free Water (2 × 1.25ml)
- 110u RQ1 RNase-Free DNase
- 2 × 50µl Each of 4 Unlabeled rNTPs, 10mM
- 1 Protocol

Phage RNA Polymerase Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of rCTP into acid-insoluble product in one hour at 37°C in a total volume of 100µl. The reaction conditions for **SP6 and T7 RNA Polymerases** are: 40mM Tris-HCl (pH 7.9 at 25°C), 10mM NaCl, 6mM $MgCl_2$, 10mM DTT, 2mM spermidine, 0.05% Tween®-20, 0.5mM each of rATP, rGTP, rCTP and rUTP, 0.5µCi [^{3}H]rCTP and 2µg of supercoiled pGEM®-5Zf(+) Vector(c). The reaction conditions for **T3 RNA Polymerase** are the same except that 2µg of supercoiled pSP6/T3 vector DNA (Life Technologies, Inc.) is used as the template.

III. General Considerations

A. Properties of Promega Vectors Suitable for in vitro Transcription

The features and applications of Promega's cloning/transcription, expression and mutagenesis vectors are summarized in Table 1.

The pSP64 Poly(A) and the pSP72–pSP73 Vectors[c] are general-purpose cloning vectors that allow the in vitro synthesis of RNA transcripts. The pSP64 Poly(A) Vector allows RNA to be transcribed from one strand of the cloned DNA using the SP6 promoter and also contains a stretch of dA:dT residues at one end of the multiple cloning region. This allows the in vitro synthesis of RNA containing a synthetic 30-base poly(A) tail. The pSP72–pSP73 Vectors and all of the pGEM® Vectors[c] contain opposed SP6 and T7 promoters flanking the multiple cloning region, allowing RNA to be transcribed from either strand of the insert.

The pGEM®-3Z and pGEM®-4Z Vectors incorporate blue/white screening for recombinants. These are general-purpose cloning vectors containing versatile multiple cloning regions and allowing synthesis of RNA transcripts from either strand using the SP6 and T7 RNA polymerase promoters. These vectors are differentiated by the orientation of their multiple cloning region. The pGEM®-3Zf(+/–) Vectors also include the origin of replication of the filamentous bacteriophage f1 for production of single-stranded DNA (ssDNA). The orientation of the f1 origin (either + or –) determines which of the plasmid DNA strands will be secreted as ssDNA in the presence of helper phage.

Other pGEM®-Zf Vectors include the features described above plus unique multiple cloning regions designed for specific applications. The pGEM®-5Zf(+/–) and pGEM®-7Zf(+/–) Vectors are distinct in that their multiple cloning regions contain a central cluster of restriction sites that generate 5´ overhangs flanked by sites that generate 3´ overhangs. This configuration is designed for use with the Erase-a-Base® System (Cat.# E5850) in which a nested set of unidirectional deletions is generated for sequence analysis of large DNA fragments.

The pGEM®-T and pGEM®-T Easy Vectors[c,d] contain all of the features of the pGEM®-5Zf(+) Vector but are specifically engineered to facilitate the cloning of PCR[e] products.

The pGEMEX® Vectors[c,f] are optimized for expression of large quantities of T7 gene 10 fusion proteins from the T7 promoter. RNA transcripts can also be synthesized from either strand of the insert DNA using the SP6 and T3 promoters flanking the multiple cloning region.

The pALTER®[c,g] family of vectors is designed for site-directed mutagenesis of the cloned DNA insert. In addition, the pALTER®-*Ex*1, pALTER®-*Ex*2 and pALTER®-MAX[c,g,h] Vectors allow expression of the mutated gene products in vivo or in vitro.

The pSI, pCI[h] and pCI-neo[c,h] Mammalian Expression Vectors and the pTARGET™ Mammalian Expression Vector System[d,h] are designed to promote constitutive expression of cloned DNA inserts in mammalian cells. Inclusion of the neomycin phosphotransferase marker in the pCI-neo and pTARGET™ Vectors allows the selection of stably transfected mammalian cells in the presence of the antibiotic G-418. Vector design features such as intron and polyadenylation regions provide enhanced RNA stability and subsequent translation.

The pTARGET™ Vector is convenient for cloning PCR products. The pTARGET™ Vector also contains a T7 RNA polymerase promoter sequence and may be used to synthesize RNA transcripts in vitro. The multiple cloning region of the pTARGET™ Vector is within the *lac*Z α–peptide region, allowing identification of recombinant clones by color screening on indicator plates.

Table 1. Features and Applications of Selected Promega Vectors.

Vector	Size (bp)	Ampicillin Selection	Promoters	Blue/White Screening for Recombinants	Single-Stranded DNA Production	Direct dsDNA Sequencing Possible	Sequencing Primers Applicable
pSP64 Poly(A)	3,033	Y	S	N	N	Y	S
pSP72 & pSP73	2,642, 2,464	Y	S, T7	N	N	Y	S, T7
pGEM®-3Z & pGEM®-4Z	2,743 2,746	Y	S, T7	Y	N	Y	S, T7
pGEM®-3Zf(+) & pGEM®-3Zf(–)	3,197	Y	S, T7	Y	Y	Y	S, T7, M
pGEM®-5Zf(+) & pGEM®-5Zf(–)	3,000	Y	S, T7	Y	Y	Y	S, T7, M
pGEM®-7Zf(+) & pGEM®-7Zf(–)	3,000	Y	S, T7	Y	Y	Y	S, T7, M
pGEM®-9Zf(–)	2,925	Y	S, T7	Y	Y	Y	S, T7, M
pGEM®-11Zf(+) & pGEM®-11Zf(–)	3,221	Y	S, T7	Y	Y	Y	S, T7, M
pGEM®-13Zf(+)	3,179	Y	S, T7	Y	Y	Y	S, T7, M
pGEM®-T & pGEM®-T Easy	3,000 3,015	Y	S, T7	Y	Y	Y	T7, M
pGEMEX®-1 & pGEMEX®-2	3,995 3,997	Y	S, T3, T7	N	Y	Y	S, T7, T3
pALTER®-1	5,680	N	S, T7	Y	Y	Y	S, T7, M
pALTER®-*Ex*1	5,858	N	S, T7	Y	Y	Y	S, T7, M
pALTER®-*Ex*2	5,838	N	S, T7	N	Y	Y	S, T7, M
pALTER®-MAX*	5,533	N	T3, T7	Y	Y	Y	T3,T7 EEV
pTARGET™	5,670	Y	T7	Y	Y	Y	T7 EEV
pSI	3,632	Y	T7	N	Y	Y	T7 EEV
pCI	4,006	Y	T7	N	Y	Y	T7 EEV
pCI-neo	5,472	Y	T7, T3	N	Y	Y	T3, T7 EEV

Key: S - SP6
M - M13

***Note:** The T7 EEV (Eukaryotic Expression Vector) Promoter Primer (Cat.# Q6700) is designed specifically for use with Promega's mammalian expression vectors.

B. Applications of Promega Vectors

RNA Transcripts

Transcription of RNA is performed with the appropriate RNA polymerase (T3, T7 or SP6), depending on the RNA polymerase promoter sites present in the vector chosen. Because these polymerases are extremely promoter-specific (i.e., there is almost no transcriptional cross-talk), virtually homogeneous RNA can be obtained using plasmid DNA as the template in a transcription reaction (1). When it is desirable to copy only insert DNA sequences, the plasmid is linearized at an appropriate restriction site prior to the transcription reaction and only discrete "run-off" transcripts are obtained, virtually free of vector sequences (see Figure 1). RNA transcripts may be used to generate probes for hybridization to Northern and Southern blots, plaque and colony lifts, tissue sections and chromosome spreads. RNA transcripts are also useful for S1 nuclease mapping, mRNA synthesis for translation in vitro, and generation of antisense RNAs to block translation.

Blue/White Color Screening

The pGEM®-Z Vectors were Promega's first SP6/T7 vectors to incorporate blue/white color screening for the identification of recombinants. These vectors contain a sequence coding for the *lac*Z α-peptide, interrupted by a multiple cloning region. Nonrecombinant plasmids produce a functional α-peptide that, by complementing the product of the host cell *lac*ZΔM15 gene, leads to production of functional β-galactosidase. Bacterial colonies harboring the *lac*ZΔM15 gene on an F´, and also containing a pGEM®-Z Vector, are blue in color when plated on indicator plates containing IPTG (Cat.# V3955) and X-Gal (Cat.# V3941). However, when the *lac*Z α-peptide is disrupted by cloning into the pGEM®-Z Vector multiple cloning region, complementation does not occur and no β-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM®-Z Vector constructs are white.

C. General Cloning Techniques

For information on general cloning techniques (e.g., ligation, transformation, isolation of recombinant plasmid DNA, Southern and Northern hybridizations), see references 2, 3 and 4. Please contact Promega to request specific information on any of the vectors described in Section III.A and Table 1, or consult the appropriate Technical Bulletin or Technical Manual at www.promega.com/tbs.

IV. RNA Transcription in vitro

Note: An *Experienced User's Protocol* can be found at the end of this Technical Manual.

A. DNA Template Preparation

When the presence of vector sequences on the probe will not interfere with subsequent applications, transcripts can be synthesized using an intact plasmid as the template. Alternatively, the plasmid can be linearized to produce "run-off" transcripts derived from the insert sequence only.

To prepare a plasmid for the production of "run-off" transcripts, linearize the vector with a suitable restriction endonuclease. After the restriction digestion, extract the linearized plasmid with phenol:chloroform:isoamyl alcohol, ethanol precipitate and suspend in TE or water before using the DNA for in vitro transcription reactions.

It is important that the restriction digestion be performed to completion. A small amount of undigested plasmid DNA can give rise to very long transcripts, which may incorporate a substantial fraction of the radiolabeled rNTP.

Extraneous transcripts have been reported to appear in addition to the expected transcript when templates contain 3´ overhangs (5). The extraneous transcripts can contain sequences complementary to the expected transcript as well as sequences corresponding to vector DNA. Therefore, we recommend that plasmids should not be linearized with any enzyme that leaves a 3´ overhang (see Table 2).

Table 2. Commonly Used Restriction Enzymes that Generate 3´ Overhangs.

Aat II	*Cfo* I	*Pvu* I
Apa I	*Hae* II	*Sac* I
Ban II	*Hgi*A I	*Sac* II
Bgl I	*Hha* I	*Sfi* I
*Bsp*1286 I	*Kpn* I	*Sph* I
*Bst*X I	*Pst* I	

Conversion of a 3´ Overhang to a Blunt End

If there is no alternative restriction site, the 3´ overhang should be converted to a blunt end using the 3´→5´ exonuclease activity of DNA Polymerase I Large (Klenow) Fragment (Cat.# M2201) as described below:

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

B. Synthesis of High Specific Activity Radiolabeled RNA Probes

The protocols described below are a modification of the procedure described by Melton (1) for RNA synthesis in vitro. RNA transcripts may be radiolabeled with ^{32}P-, ^{33}P-, ^{35}S- or ^{3}H-labeled ribonucleotides, depending upon the specific application. Throughout these procedures, precautions should be taken to protect against ribonuclease contamination.

Certain applications, such as Southern and Northern blotting procedures, do not require the synthesis of full-length transcripts. Transcription mapping studies, on the other hand, require the synthesis of full-length labeled probes. The yield of full-length transcripts is reduced somewhat as the concentration of the limiting nucleotide falls below 12µM. Therefore, if you wish to omit unlabeled ribonucleotides from the reaction, increase the amount of label used in the reaction to maintain a final concentration of labeled nucleotide of 12–24µM. To keep the final reaction volume from exceeding 20µl, it may be necessary to add the labeled nucleotide to the reaction tube and dry it down before adding the remaining components.

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Table 3 lists the recommended specific activities of labeled nucleoside triphosphates to be used for transcription reactions in vitro. Using an [α-^{32}P]rCTP label and the conditions described below, RNA transcribed in vitro will typically have a specific activity of 2–2.5 x 10^8cpm/µg.

If RNA transcripts are to be used for probe hybridization, unincorporated nucleotides and template DNA should be removed to give lower backgrounds and optimum sensitivity (Sections IV.D and IV.E).

Table 3. Specific Activities of rNTPs Recommended for Transcription in vitro.

Nucleotide	Recommended Microcuries per Reaction	Specific Activity	Final Concentration of Labeled Nucleotide
5´[α-^{32}P]rCTP(10mCi/ml)	50µCi	400Ci/mmol	6.25µM
5´[α-^{33}P]rUTP(10mCi/ml)	150µCi	3,000Ci/mmol	2.5µM
5´[α-^{35}S]rUTP(10mCi/ml)	~240µCi	1,000Ci/mmol	12µM
5,6 [^{3}H]rUTP(1mCi/ml)	25µCi	40Ci/mmol	31µM

Note: We do not recommend using radiolabeled rATP for generating RNA probes since less label is generally incorporated using this nucleotide.

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

- TE buffer
- radiolabeled rNTP
- RNA sample buffer
- RNA loading buffer

Standard Transcription Protocol

1. Add the following components at room temperature in the order listed.

Transcription Optimized 5X Buffer	4µl
DTT, 100mM	2µl
Recombinant RNasin® Ribonuclease Inhibitor	20–40u
rATP, rGTP and rUTP (2.5mM each) (prepared by mixing 1 volume deionized water with 1 volume of each of the 10mM rATP, rGTP and rUTP stocks supplied)	4µl
100µM rCTP (diluted from stock)	2.4µl
Linearized template DNA (0.2–1.0mg/ml in water or TE buffer)	1µl
[α-^{32}P]rCTP (50µCi at 10µCi/µl)	5µl
SP6, T3 or T7 RNA Polymerase	15–20u
Final volume	20µl

Note: Non-radioactive transcripts can be synthesized in a reaction containing a final concentration of 0.5mM each of rATP, rGTP, rUTP and rCTP. See Section IV.F

Note: The mixture should be kept at room temperature during the addition of each successive component, since DNA can precipitate in the presence of spermidine if kept at 4°C.

2. Incubate for 1 hour at 37–40°C.

Note: If full-length transcripts are not obtained and are critical to the success of your experiment the incubation temperature can be lowered to 30°C (6).

3. Remove 1µl from the reaction to determine the percent incorporation and specific activity of the probe (Section IV.C).

Positive Control Protocol

1. Add the following components at room temperature in the order listed:

Transcription Optimized 5X Buffer	4µl
DTT, 100mM	2µl
Recombinant RNasin® Ribonuclease Inhibitor	20–40u
rATP, rCTP, rGTP and rUTP (2.5mM each) (prepared by mixing 1 volume of each of the 10mM rNTP stocks supplied)	4µl
pGEM® Express Positive Control Template	1µg
SP6, T3 or T7 RNA Polymerase	15-20u
Nuclease-Free Water to a final volume of	20µl

2. Incubate for 1 hour at 37–40°C.

3. 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 non denaturing gels loaded with RNA denatured in a formaldehyde/formamide RNA sample buffer.

   Add 5µl of the transcription reaction to 15µl 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. Include RNA markers and run the gel under standard conditions for analysis of DNA samples. The expected sizes of the RNA transcripts are given in Figure 2.

Note: The DNA template can be removed prior to visualization of the transcripts following the procedure in Section IV.D. If the DNA template is not removed, a high-molecular-weight band (approximately 4kb for the pGEM® Express Positive Control Template) will be present when visualizing the transcription products on an agarose gel.

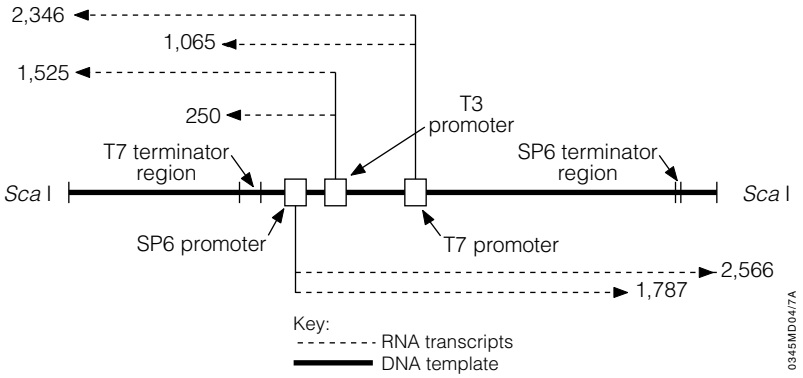

Figure 2. RNA transcripts generated using the appropriate RNA polymerase and the pGEM® Express Positive Control Template.

C. Determination of Percent Incorporation and Probe Specific Activity

Materials to Be Supplied by the User

- Whatman® GF/A filters or equivalent
- 5% TCA (ice cold)
- 1µg/µl carrier nucleic acid (tRNA or Herring Sperm DNA; Cat.# D1811)
- acetone
- scintillation fluid

TCA Precipitation

1. Prepare a 1:10 dilution of the labeled probe in water. Spot 1µl of this 1:10 dilution onto duplicate glass fiber filters (e.g., Whatman® GF/A filters) and let them air-dry. Count the filters directly to determine the total cpm.
2. In duplicate tubes, add 1µl of the 1:10 dilution of labeled probe to 100µl of carrier nucleic acid (tRNA or Herring Sperm DNA at 1µg/µl), mix, and then add 0.5ml of ice-cold 5% TCA and mix again. Leave on ice for at least 5 minutes.
3. Apply the samples to GF/A filters that have been prewet with 5% TCA, under vacuum. Wash twice with 5ml of ice-cold 5% TCA. Rinse the filters with 2ml of acetone and let them air-dry.
4. Insert the dry filters into scintillation vials, add scintillation fluid and count the samples.

   Note: It is not necessary to use scintillation fluid for counting ^{32}P-labeled samples. The Cerenkov radiation emitted from samples without scintillation fluid can be detected by a scintillation counter set to monitor the tritium window. Although the absolute number of counts is not the same between the two methods (because Cerenkov counting is less than half as efficient), they will be proportional from sample to sample.

Calculations

The specific activity of the probe may be expressed as the total incorporated cpm/total micrograms of RNA synthesized. The following equations and example illustrate how to estimate the total incorporated cpm, the total amount of RNA synthesized and the final calculation of probe specific activity.

1. Calculate the percent incorporation and total cpm incorporated as follows:

$$\textbf{\% incorporation} = \frac{\text{incorporated cpm (i.e., TCA precipitated cpm)}}{\text{total cpm}} \times 100$$

$$\textbf{total cpm incorporated} = \text{incorporated cpm x dilution factor} \times \frac{\text{reaction volume}}{\text{volume counted}}$$

2. Next, calculate the total amount of RNA synthesized. (This is determined by the amount of limiting rNTP present in the reaction, the maximum theoretical yield and the percent incorporation):

$$\textbf{nmol of labeled rNTP} = \frac{\text{µCi rNTP in reaction}}{\text{isotope concentration (µCi/nmol)}}$$

$$\textbf{nmol limiting cold rNTP} = \text{µl limiting cold rNTP} \times 100\text{µM rNTP} \times \frac{10^3\text{nmol}}{1\text{µmol}} \times \frac{1\text{L}}{10^6\text{µl}}$$

total nmol of limiting rNTP = nmol of labeled rNTP + nmol of limiting cold rNTP

maximum theoretical RNA yield = nmol of limiting rNTP × 4 rNTPs × $\frac{330ng\ rNTP}{nmol}$

total ng of RNA synthesized = % incorporation × maximum theoretical RNA yield

3. Finally, calculate the specific activity of the RNA probe:

   specific activity = $\frac{\text{total incorporated cpm}}{\text{total µg of RNA synthesized}}$

4. Example:

   In a standard reaction, the limiting rNTP is rCTP; 2.4µl of 100µM rCTP and 50µCi of 400µCi/nmol (=Ci/mmol) rCTP was used. One microliter of a 1:10 dilution was used for TCA analysis. The counts obtained were:

   incorporated: 2.2×10^5cpm
   total: 5.5×10^5cpm

   The percent incorporation and total cpm incorporated are calculated as follows:

   % incorporation = $\frac{2.2 \times 10^5 cpm}{5.5 \times 10^5 cpm} \times 100 = 40\%$

   total cpm incorporated = $2.2 \times 10^5 cpm \times 10 \times \frac{20\mu l}{1\mu l} = 4.4 \times 10^7 cpm$

   The total amount of RNA synthesized is calculated as follows:

   nmol of labeled rNTP = $\frac{50\mu Ci}{400\mu Ci/nmol} = 0.125nmol$

   nmol of limiting cold rNTP = $2.4\mu l \times 100\mu M \times \frac{10^3 nmol}{1mmol} \times \frac{1L}{10^6 \mu l} = 0.24nmol$

   total nmol of limiting rNTP = 0.125nmol + 0.24nmol = 0.365nmol

   maximum theoretical RNA yield = $0.365nmol \times 4 \times \frac{330ng}{nmol} = 482ng = 0.482\mu g$

   total µg of RNA synthesized = $\frac{40}{100} \times 0.482\mu g = 0.193\mu g$

   The specific activity of the RNA probe is:

   $\frac{4.4 \times 10^7 cpm}{0.193\mu g} = 2.28 \times 10^8 cpm/\mu g$

D. Removal of the DNA Template Following Transcription

The DNA template should be removed by digestion with DNase I following the transcription reaction. Promega's RQ1 RNase-Free DNase (Cat.# M6101) has been tested for its ability to degrade DNA while maintaining the integrity of RNA.

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

- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1 [pH 4.5])
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)
- RNA sample buffer
- RNA loading buffer

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 TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1 [pH 4.5]). Vortex for 1 minute and centrifuge in a microcentrifuge (12,000 × *g*) 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 in a microcentrifuge (12,000 × *g*) for 2 minutes.

   Note: At this point the transcripts can be visualized. Add an aliquot (2–5µl) from the aqueous phase obtained above to 15µl of RNA sample buffer. Add 2–5µl of RNA loading buffer and heat the samples 5–10 minutes at 65–70°C prior to loading on an agarose gel containing 1X TAE and 0.5µg/ml ethidium bromide. Run the gel under standard conditions for analysis of RNA samples.
5. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of 100% ethanol. Mix and place at –70°C for 30 minutes. Centrifuge in a microcentrifuge for 20 minutes.
6. Carefully remove the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum. If removal of unincorporated nucleotides is desired, proceed directly to Section IV.E. If no further purification is desired, suspend the RNA sample in 10–20µl of TE buffer or water and store at –70°C.

(!) ***Avoid Storing RNA*** frozen in Transcription Optimized Buffer, as it will precipitate at low temperatures in the presence of spermidine. RNA stored in this way will not run to its true size upon electrophoresis.

E. Removal of Unincorporated Nucleotides

Size Exclusion Chromatography

The newly synthesized RNA may be effectively separated from unincorporated nucleotides by size exclusion chromatography through a small Sephadex® G-100 or G-50 column in 10mM Tris-HCl (pH 7.5), and 0.1% SDS (2). Prepacked columns, which can be equilibrated in SDS-containing buffer for use with RNA, are available from Amersham Pharmacia Biotech.

Once the RNA has been separated from the unincorporated nucleotides, the sample should be ethanol precipitated as described in Section IV.D, Steps 5–6. To ensure that the RNA is recovered successfully, carrier tRNA may be added to the reaction.

Dry the pellet under a vacuum, then suspend in 10–20µl of TE buffer and store at –70°C. Alternatively, the RNA pellet may be stored at –70°C in 100% ethanol prior to the resuspension step.

F. Synthesis of Large Amounts of RNA

The standard transcription protocol (Section IV.B) typically yields less than 1µg RNA/µg DNA. Using the conditions described below, yields of 5–10µg RNA/µg plasmid DNA can be obtained. Alternatively, Promega's RiboMAX™ Large Scale RNA Production Systems[a,b,i,j] can be used. The RiboMAX™ Systems provide high yields of RNA that is particularly suitable for in vitro translation.

1. Add the following components at room temperature in the order listed:

Transcription Optimized 5X Buffer	20µl
100mM DTT	10µl
Recombinant RNasin® Ribonuclease Inhibitor	100u
rATP, rGTP, rCTP and rUTP (2.5mM each) (prepared by mixing equal volumes of each of the four 10mM rNTP stocks supplied)	20µl
Linearized template DNA (1.0–2.5mg/ml in water or TE buffer)	2µl
SP6, T3 or T7 RNA Polymerase	40u
Nuclease-Free Water to a final volume of	100µl

2. Incubate for 1–2 hours at 37–40°C.
3. Clean up the reaction as described in Section IV.D.

G. Capping RNA for in vitro Translation

Capped RNA molecules synthesized in vitro are effective templates for translation. Krieg and Melton (7) have shown that SP6-derived in vitro transcripts are translated as efficiently as native mRNAs in injected oocytes and in wheat germ extracts. These synthetic RNAs have also been successfully translated using the Rabbit Reticulocyte Lysate System[b,j,k] (Cat.# L4960).

Two protocols for capping synthesized transcripts prior to translation are described below. The first protocol utilizes guanylyltransferase to transfer 5´ terminal cap structures to transcripts in vitro. Using the second protocol, the Ribo m^7G Cap Analog (Cat.# P1711) is directly incorporated into the RNA during the transcription reaction to yield capped RNA substrate.

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Many RNA transcripts do not require a cap structure to be translated either in a rabbit reticulocyte lysate system or in a wheat germ extract system. Translation of other transcripts shows a direct dependence on the presence of the $m^7G(5')ppp(5')G$ cap at the 5´-end. It should also be noted that a low level of endogenous capping activity has been detected in wheat germ extract systems using in vitro transcribed RNA as the template.

A cap has been reported to be particularly important for synthesis of biologically active proteins in *Xenopus* oocytes (8). Methylated capped RNA transcripts are also spliced more efficiently than uncapped or unmethylated capped RNAs in an in vitro splicing reaction (9).

Materials to Be Supplied by the User

(Solution compositions are provided in Section VII.A.)

- 1M Tris-HCl (pH 7.9)
- 10mM $MgCl_2$
- 100mM KCl
- 1mM *S*-adenosyl-L-methionine
- guanylyltransferase
- 1mM rGTP
- acetylated BSA (1mg/ml)
- Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511)
- TE-saturated phenol:chloroform: isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)
- Ribo m^7G Cap Analog (Cat.# P1711)

Addition of an $m^7G(5')ppp(5')G$ Cap to RNA Synthesized in vitro

1. Combine in vitro synthesized RNAs with guanylyltransferase as shown below:

RNA transcript (in up to 13µl deionized water)	0.1–1.0µg
1M Tris-HCl (pH 7.9)	1.5µl
$MgCl_2$, 10mM	3.75µl
KCl, 100mM	1.8µl
DTT, 100mM	0.75µl
acetylated BSA, 1mg/ml	3µl
Recombinant RNasin® Ribonuclease Inhibitor (20–40u/µl)	1µl
S-adenosyl-L-methionine, 1mM	3µl
rGTP, 1mM	1.2µl
guanylyltransferase (at 1–5u/µl)	1u/µg RNA
Nuclease-Free Water to a final volume of	30µl

2. Incubate for 45 minutes at 37°C.
3. Extract with phenol:chloroform:isoamyl alcohol and then with chloroform:isoamyl alcohol alone. Precipitate with ethanol (see Section IV.D, Steps 3–6).

Synthesis of a Capped RNA Transcript in vitro

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

Transcription Optimized 5X Buffer	10µl
DTT, 100mM	5µl
Recombinant RNasin® Ribonuclease Inhibitor	50u
nucleotides (2.5µl each of 10mM rATP, rCTP, rUTP plus 2.5µl 1mM rGTP)	10µl
Ribo m^7G Cap Analog, 5mM	5µl
linearized template DNA (1mg/ml in water or TE buffer)	5µl
SP6, T7 or T3 RNA Polymerase	40u
Nuclease-Free Water to a final volume of	50µl

2. Incubate at 37°C for 1 hour. To increase the RNA yield, add an additional 40 units of RNA polymerase and incubate for an additional hour.
3. Proceed with DNase I treatment to remove the template, followed by purification of the RNA, as described in Section IV.D.

Note: Higher yields of longer capped transcripts may be obtained by increasing the concentration of rGTP.

Note: To increase the yield of RNA, add an additional 40u of RNA polymerase and incubate for an additional hour. For small transcripts, the amount of template can be increased as well.

V. Troubleshooting

Symptoms	Possible Causes	Comments
Low amounts of RNA synthesized using standard transcription protocol	Insufficient amount of template in solution	The DNA template may be precipitated by spermidine in the Transcription Optimized 5X Buffer. Make sure the components of the reaction are assembled at room temperature and in the stated order.
	NaCl concentration 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, reprecipitating the template in the presence of another salt, and washing the resulting pellet 1–2 times with 70% ethanol.
	RNase contamination	The use of Recombinant RNasin® Ribonuclease Inhibitor is recommended for all in vitro transcription reactions. User-supplied transcription 5X buffer should be autoclaved. The other solutions used in the reaction (e.g., DTT and rNTPs) should be prepared with water that has been treated with 0.2% DEPC (Section V.A). Individual transcription components may be purchased directly from Promega.

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**

V. Troubleshooting (continued)

Symptoms	Possible Causes	Comments
Low amounts of RNA synthesized using standard transcription protocol (continued)	Inactive RNA polymerase	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 transcripts	Premature termination of RNA synthesis	Increase the concentration of the limiting rNTP (probe synthesis only). Additional "cold" rNTP can be added to the reaction to increase the proportion of full-length transcripts. The improvement in yield of full-length product is gained at the expense of reducing the specific activity of the probe.
		Lower the temperature of incubation from 37°C to 30°C. This has been shown to increase the proportion of full-length transcripts in some cases (6).
	Presence of polymerase terminator sequences	Subclone the 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.
	RNA sample buffer is degraded	If the RNA sample buffer is old or has undergone multiple freeze-thaw cycles, the RNA will not run at its true size.
Presence of transcripts larger than expected	Transcription from the wrong strand of DNA	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 (5). If it is impossible to avoid using a restriction enzyme of this type, the linear DNA should be "blunt-ended" with DNA Polymerase I Large (Klenow) Fragment before use in a transcription reaction (Section IV.A).

V. Troubleshooting (continued)

Symptoms	Possible Causes	Comments
Presence of transcripts larger than expected (continued)	Nonlinearized plasmid is present in the sample	Analyze the sample by gel electrophoresis. If undigested vector is noted, redigest with the appropriate restriction enzyme.
	Product cannot form stable secondary structures at the 3´-end	Decrease the incubation time of the reaction from 60 minutes to 15–30 minutes (10).
		Decrease the concentration of rUTP used in the reaction (10).

VI. References

A. Cited References

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3. *Protocols and Applications Guide,* Third Edition (1996) Promega Corporation.
4. Ausubel, F.M. *et al*. (1988) *Current Protocols in Molecular Biology,* John Wiley and Sons, NY.
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B. Additional References

Generation of Short Oligoribonucleotides Using T7 RNA Polymerase

Milligan, J.F. *et al*. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucl. Acids Res*. **15,** 8783.

Milligan, J.F. and Uhlenbeck, O.C. (1989) Synthesis of small RNAs using T7 RNA polymerase. *Meth. Enzymol*. **180**, 51.

VI. References (continued)

PCR Amplification Using Primers Containing the T7 Promoter Sequence

Kain, K.C., Orlandi, P.A. and Lanar, D.E. (1991) Universal promoter for gene expression without cloning: expression-PCR. *BioTechniques* **10,** 366.

van der Luijt, R. *et al.* (1994) Rapid detection of translation-terminating mutations at the adenomatous polyposis coli (APC) gene by direct protein truncation test. *Genomics* **20,** 1.

Nor-Radioactive Labeling of RNA Transcripts

Richardson, R.W. and Gumport, R.I. (1983) Biotin and fluorescent labeling of RNA using T4 RNA ligase. *Nucl. Acids Res.* **11,** 6167.

Rosemeyer, V., Laubrock, A. and Seib, R. (1995) Nonradioactive 3´-end-labeling of RNA molecules of different lengths by terminal deoxynucleotidyltransferase. *Anal. Biochem.* **224,** 446.

VII. Appendix

A. Composition of Buffers and Solutions

DEPC-treated water

0.2ml	DEPC
100ml	deionized water

Add DEPC to water; shake vigorously to mix. Autoclave to inactivate the DEPC. Store at room temperature.

Caution: Use a fume hood for all steps prior to autoclaving.

MOPS buffer

0.2M	MOPS (pH 7.0)
50mM	sodium acetate
5mM	EDTA (pH 8.0)

Transcription Optimized 5X Buffer (provided)

200mM	Tris-HCl (pH 7.9)
30mM	$MgCl_2$
10mM	spermidine
50mM	NaCl

RNA loading buffer

50%	glycerol
1mM	EDTA
0.4%	bromophenol blue
1mg/ml	ethidium bromide

Prepare using a very high grade glycerol. Dispense into single-use aliquots and store at –20°C.

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

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1). **Note:** For removal of the DNA template following transcription (Section IV.D), use TE buffer at pH 4.5 rather than TE buffer at pH 8.0.

RNA sample buffer

10.0ml	deionized formamide
3.5ml	37% formaldehyde
2.0ml	MOPS buffer

Dispense into single use aliquots and store at –20°C in tightly sealed screw-cap tubes. These can be stored for 3–6 months. Do not freeze-thaw.

TE buffer

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

1X TAE buffer

40mM	Tris acetate (with respect to Tris)
1mM	EDTA

B. Sources for Vector Sequence and Restriction Site Information

The sequence and location of restriction sites for each vector are provided in the sources listed below, available upon request from Promega and on the Internet at www.promega.com/vectors.

Vector	Literature Number	Vector	Literature Number
pALTER®-1 Vector	TM001	pGEM®-7Zf(–) Vector	TB069
pALTER®-*Ex*1 Vector	TM001	pGEM®-9Zf(–) Vector	TB070
pALTER®-*Ex*2 Vector	TM001	pGEM®-11Zf(–) Vector	TB074
pALTER®-MAX Vector	TM041	pGEM®-11Zf(+) Vector	TB075
pCI Vector	TB206	pGEM®-13Zf(+) Vector	TB073
pCI-neo Vector	TB215	pGEM®-T and pGEM®-T	
pGEM®-3Z Vector	TB033	Easy Vectors	TM042
pGEM®-4Z Vector	TB036	pGEMEX® Vectors	TB253
pGEM®-3Zf(+) Vector	TB086	pSI Vector	TB206
pGEM®-3Zf(–) Vector	TB045	pSP64 Poly(A) Vector	TB052
pGEM®-5Zf(+) Vector	TB047	pSP72 Vector	TB040
pGEM®-5Zf(–) Vector	TB068	pSP73 Vector	TB041
pGEM®-7Zf(+) Vector	TB048	pTARGET™ Vector	TM044

C. Related Products

System Components and Buffers

Product	Size	Cat.#
rATP*	0.5ml	P1132
rCTP*	0.5ml	P1142
rGTP*	0.5ml	P1152
rUTP*	0.5ml	P1162
rATP, rCTP, rGTP, rUTP*	0.5ml each	P1221
pGEM® Express Positive Control Template(c)	10µg (2 × 5µg)	P2561
DTT (100mM)*	100µl	P1171
Transcription Optimized 5X Buffer*	200µl	P1181
Nuclease-Free Water*	50ml (2× 25ml)	P1193
RQ1 RNase-Free DNase*	1,000u	M6101
Recombinant RNasin® Ribonuclease Inhibitor(a,b)*	2,500u	N2511
	10,000u	N2515
Ribo m7G Cap Analog	10 A_{254} units	P1711
	25 A_{254} units	P1712

*For Laboratory Use

RNA Polymerase Promoter Sequencing Primers

Product	Size	Cat.#
SP6 Promoter Primer	2µg	Q5011
T7 Promoter Primer	2µg	Q5021
T3 Promoter Primer	2µg	Q5741
T7 EEV Promoter Primer	2µg	Q6700

RNA Polymerases

Product	Conc. (u/µl)	Size	Cat.#
SP6 RNA Polymerase	10–20	1,000u	P1085
	10–20	5,000u	P1081
SP6 RNA Polymerase (High Conc.)	80	2,500u	P4084
T3 RNA Polymerase	10–20	1,000u	P2083
T3 RNA Polymerase (High Conc.)	80	2,500u	P4024
T7 RNA Polymerase	10–20	1,000u	P2075
	10–20	5,000u	P2077
T7 RNA Polymerase (High Conc.)	80	10,000u	P4074

Each includes Transcription Optimized 5X Buffer and 100mM DTT.
For Laboratory Use.

Other Products for Transcription and RNA Analysis

Product	Size	Cat.#
RNA Markers, 0.28–6.58kb[b]	50µl	G3191
RiboMAX™ Large Scale RNA Production System – SP6[a,b,j]	50 × 20µl reactions	P1280
RiboMAX™ Large Scale RNA Production System – T7[a,b,i,j]	50 × 20µl reactions	P1300
RiboMark® Labeling System[a,b]	10 reactions	P1550
Herring Sperm DNA	10mg	D1811

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Vectors

Product	Size	Cat.#
pALTER®-1 Vector[c,g]	20µg	Q6301
pALTER®-*Ex*1 Vector[c,g]	20µg	Q6121
pALTER®-*Ex*2 Vector[c,g]	20µg	Q6111
pALTER®-MAX[c,g,h]	20µg	Q5761
pGEM®-3Z Vector[c]	20µg	P2151
pGEM®-4Z Vector[c]	20µg	P2161
pGEM®-3Zf(+) Vector[c]	20µg	P2271
pGEM®-3Zf(–) Vector[c]	20µg	P2261
pGEM®-5Zf(+) Vector[c]	20µg	P2241
pGEM®-5Zf(–) Vector[c]	20µg	P2351
pGEM®-7Zf(+) Vector[c]	20µg	P2251
pGEM®-7Zf(–) Vector[c]	20µg	P2371
pGEM®-9Zf(–) Vector[c]	20µg	P2391
pGEM®-11Zf(+) Vector[c]	20µg	P2411
pGEM®-11Zf(–) Vector[c]	20µg	P2421
pGEM®-13Zf(+) Vector[c]	20µg	P2541
pGEMEX®-1 Vector[c,f]	20µg	P2211
pGEMEX®-2 Vector[c,f]	20µg	P2551
pGEM®-T Vector System I*[c,d]	20 reactions	A3600
pGEM®-T Vector System II*[c,d]	20 reactions	A3610
pGEM®-T Easy Vector System I*[c,d]	20 reactions	A1360
pGEM®-T Easy Vector System II*[c,d]	20 reactions	A1380
pSP64 Poly(A) Vector	20µg	P1241
pSP72 Vector[c]	20µg	P2191
pSP73 Vector[c]	20µg	P2221

*For Laboratory Use

Mammalian Expression Vectors

Product	Size	Cat.#
pSI Vector	20µg	E1721
pCI Vector[h]	20µg	E1731
pCI-neo Vector[c,h]	20µg	E1841
pTARGET™ Mammalian Expression Vector System[d,h]	20 reactions	A1410

Usage Restrictions for the T7 Expression System
The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patents and patent applications assigned to Brookhaven Science Associates, LLC (BSA). This technology, including bacteria, phage and plasmids that carry the gene for T7 RNA polymerase, is to be used for academic or nonprofit laboratory or licensed commercial research purposes only. By accepting or using the T7 expression technology you agree to be bound by the following conditions set forth by BSA. The initial purchaser may refuse to accept the conditions of this notice by returning this product and the enclosed materials to Promega unused.

Academic and NonProfit Laboratories
No materials that contain the cloned gene for T7 RNA polymerase may be distributed further to third parties outside of your laboratory unless the recipient receives a copy of this assurance notice and agrees to be bound by its terms. This limitation applies to Bacterial Strains JM109(DE3) and BL21(DE3)pLysS, to the following Promega products that include these bacterial strains (pGEMEX®-1 and pGEMEX®-2 Vectors, and to any derivatives thereof.

Commercial Laboratories
A license is required for any commercial use of the T7 expression system, including use of the T7 system for research purposes or for production purposes by any commercial entity. Information about commercial licenses may be obtained from the Licensing Office, Brookhaven National Laboratory, Upton, NY 11973, Telephone: 631-344-7134, FAX: 631-344-3729.

[a]U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803, and Japanese Pat. No. 3009458 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI).

[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]U.S. Pat. No. 4,766,072.

[d]Licensed under one or both of U.S. Pat. No. 5,487,993 and European Pat. No. 0 550 693.

[e]The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

[f]Usage restrictions apply to Bacterial Strains JM109(DE3) and BL21(DE3)pLysS, to the Promega products that include these bacterial strains (pGEMEX®-1 and pGEMEX®-2 Vectors) and to any derivatives thereof. Please read the statement directly above this box describing these restrictions before purchasing any of these products.

[g]U.S. Pat. No. 5,955,363 has been issued to Promega Corporation for a vector for in vitro mutagenesis and use thereof.

[h]The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

[i]The RiboMAX™ Large Scale RNA Production Systems—T7 and T3 (Cat.# P1290 and P1300) are covered by U.S. Pat. No. 5,256,555 and are sold under a license from Ambion, Inc. They are intended for research use only. Parties wishing to use these products for other applications should contact Ambion, Inc.

[j]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.

[k]U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, 5,814,471 and Australian Pat. No. 649289 and European Pat. No. 0 553 234 have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay. Other patents are pending.

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Riboprobe® in vitro Transcription Systems: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Sections IV.A through IV.G) the first time you use the Riboprobe® in vitro Transcription System.

Template Preparation (Section IV.A)

1. Prepare the DNA template. Intact plasmid can be used if the presence of vector sequences on the probe will not interfere with subsequent applications. To produce "run-off" transcripts derived from the insert sequence only, linearize the vector with a suitable restriction endonuclease. Plasmids should not be linearized with restriction enzymes that leave a 3´ overhang (see Table 2).
2. After restriction digestion, extract the DNA with phenol:chloroform: isoamyl alcohol.

Transcription Protocol (Section IV.B)

1. Add the following components at room temperature in the order listed:

Transcription Optimized 5X Buffer	4µl
DTT, 100mM	2µl
rRNasin® Ribonuclease Inhibitor	20u–40u
rATP, rGTP and rUTP (2.5mM each) (prepared by mixing 1 volume deionized water with 1 volume of each of the 10mM rATP, rGTP and rUTP stocks supplied)	4µl
100µM rCTP (diluted from stock)	2.4µl
linearized template DNA (0.2–1.0mg/ml in water or TE)	1µl
[α-^{32}P]rCTP (50µCi at 10µCi/µl)	5µl
SP6, T3 or T7 RNA Polymerase	15–20u
Nuclease-free water to a final volume	20µl

2. Incubate for 1 hour at 37–40°C.
3. Remove 1µl from this reaction to determine the percent incorporation and specific activity of the probe (Section IV.C).

Positive Control Protocol (Section IV.B)

1. Add the following components at room temperature in the order listed:

Transcription Optimized 5X Buffer	4µl
DTT, 100mM	2µl
rRNasin® Ribonuclease Inhibitor	20u–40u
rATP, rCTP, rGTP and rUTP (2.5mM each) (prepared by mixing 1 volume of each of the 10mM rNTP stocks supplied)	4µl
pGEM® Express Positive Control Template	1µg
SP6, T3 or T7 RNA Polymerase	15–20u
Nuclease-Free Water to a final volume of	20µl

2. Incubate for 1 hour at 37–40°C.

Analysis of Transcripts (Section IV.B)

1. 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).
2. Add 5µl of transcription reaction to 15µl of sample buffer. Add 2–5µl of RNA loading buffer and heat the sample for 5–10 minutes at 65–70°C prior to loading. Include RNA markers and run the gel under standard conditions for analysis of RNA samples.

Removal of Template DNA (Section IV.D)

1. Add RQ1 RNase-Free DNase (1u/µg DNA). Incubate 15 minutes at 37°C.
2. Extract with 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1[pH 4.5]). Vortex for 1 minute and centrifuge in a microcentrifuge (12,000 × *g*) for 2 minutes.
3. 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 in a microcentrifuge (12,000 x *g*) for 2 minutes.
4. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of 100% ethanol. Mix and place at –70°C for 30 minutes. Centrifuge in a microcentrifuge for 20 minutes.
5. Carefully remove the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum. If removal of unincorporated nucleotides is desired, see Section IV.E. If no further purification is desired, suspend the RNA in 10–20µl of TE buffer or water and store at –70°C.

Capping RNA for in vitro Translation (Section IV.G)

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

Transcription Optimized 5X Buffer	10µl
DTT, 100mM	5µl
rRNasin® Ribonuclease Inhibitor	50u
nucleotides (2.5µl of each of 10mM rATP rCTP, rUTP plus 2.5µl 1mM rGTP)	10µl
Ribo m^7G Cap Analog, 5mM	5µl
linearized template DNA (1mg/ml in water or TE buffer)	5µl
SP6, T3 or T7 RNA Polymerase	40u
Nuclease-Free Water to a final volume of	50µl

2. Incubate for 1 hour at 37°C. To increase the RNA yield, add an additional 40 units of RNA polymerase and incubate for 1 hour.
3. Proceed with DNase I treatment to remove the template, followed by purification of the RNA as described above and in Section IV.D.

Alternative procedure for Capped RNA Transcripts in vitro

4. Combine in vitro synthesized RNAs with guanylyltransferase in the following reaction mix:

RNA transcript (in up to 13µl deionized water)	0.1–1.0µg
1M Tris-HCl (pH 7.9)	1.5µl
$MgCl_2$, 10mM	3.75µl
KCl, 100mM	1.8µl
DTT, 100mM	0.75µl
acetylated BSA, 1mg/ml	3µl
rRNasin® Ribonuclease Inhibitor (20–40u/µl)	1µl
S-adenosyl-L-methionine, 1mM	3µl
rGTP, 1mM	1.2µl
guanylyltransferase (at 1–5u/µl)	1u/µg RNA
Nuclease-Free Water to a final volume of	30µl

5. Incubate for 45 minutes at 37°C.
6. Extract with phenol:chloroform:isoamyl alcohol (25:24:1), then with chloroform:isoamyl alcohol (24:1) alone. Precipitate with ethanol. Suspend the RNA in 10–20µl of TE or water and store at –70°C.