

Riboprobe® in vitro Transcription Systems

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

Riboprobe® in vitro Transcription Systems

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Technical Manual. Please contact Promega Technical Services if you have questions on use
of this system. E-mail techserv@promega.com.

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1. 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.

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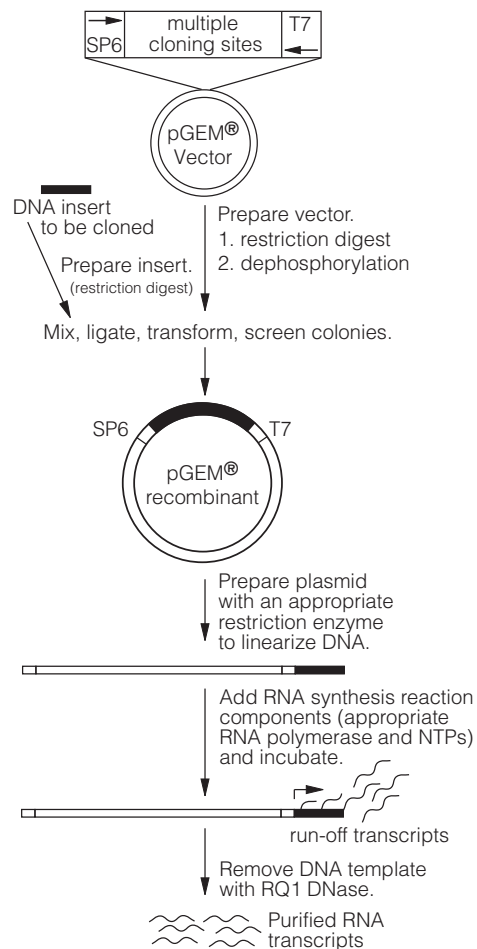


Figure 1. Schematic of the Riboprobe® in vitro Transcription Systems.

2. 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
- 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

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 RNA 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

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 MgCl₂, 10mM DTT, 2mM spermidine, 0.05% Tween®-20, 0.5mM each of rATP, rGTP, rCTP and rUTP, 0.5µCi [³H]rCTP and 2µg of supercoiled pGEM®-5Zf(+) Vector. The reaction conditions for T3 RNA Polymerase are the same except that 2µg of supercoiled pSP6/T3 vector DNA (Invitrogen) is used as the template.

3. General Considerations

3.A. Properties of Promega Vectors Suitable for *in vitro* Transcription

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

The pSP64 Poly(A) and the pSP72–pSP73 Vectors 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 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.# E5750) 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 contain all of the features of the pGEM®-5Zf(+) Vector but are specifically engineered to facilitate the cloning of PCR^(d) products.

The pALTER®-MAX Vector allows expression of the mutated gene products *in vivo* or *in vitro*.

The pSI, pCI and pCI-neo Mammalian Expression Vectors and the pTARGET™ Mammalian Expression Vector System 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 and for expression in mammalian cells. 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 *lacZ* 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, T3, T7	Y	Y	Y	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	N	Y	Y	T3, T7 EEV

S = SP6; M = M13

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

3.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 the first Promega SP6/T7 vectors to incorporate blue/white color screening for the identification of recombinants. These vectors contain a sequence coding for the *lacZ* α -peptide, interrupted by a multiple cloning region. Nonrecombinant plasmids produce a functional α -peptide that, by complementing the product of the host cell *lacZ* Δ M15 gene, leads to production of functional β -galactosidase. Bacterial colonies harboring the *lacZ* Δ M15 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 *lacZ* 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.

3.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 3.A and Table 1, or consult the appropriate Technical Bulletin or Technical Manual at: www.promega.com/tbs

4. RNA Transcription in vitro

4.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.

<i>Aat</i> II	<i>Cfo</i> I	<i>Pvu</i> I
<i>Apa</i> I	<i>Hae</i> II	<i>Sac</i> I
<i>Ban</i> II	<i>Hgi</i> A I	<i>Sac</i> II
<i>Bgl</i> I	<i>Hha</i> I	<i>Sfi</i> I
<i>Bsp</i> 1286 I	<i>Kpn</i> I	<i>Sph</i> I
<i>Bst</i> X I	<i>Pst</i> 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 4.B, below) containing 33 μ M of each dNTP.
2. Add DNA Polymerase I Large (Klenow) Fragment at a concentration of 5u/ μ g and incubate the reaction for 15 minutes at 22°C.
Note: Elevated temperatures, excessive amounts of enzyme, failure to add dNTPs or long reaction times may result in recessed ends due to the 3'→5' exonuclease activity of the enzyme.
3. Proceed with the transcription reaction by adding the nucleotide mix and RNA polymerase.

4.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 ³²P-, ³³P-, ³⁵S- or ³H-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.

Table 3 lists the recommended specific activities of labeled nucleoside triphosphates to be used for transcription reactions *in vitro*. Using an [α-³²P]rCTP label and the conditions described below, RNA transcribed *in vitro* will typically have a specific activity of 2–2.5 × 10⁸cpm/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 4.D and 4.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 7.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

Notes:

1. Synthesize non-radioactive transcripts in a reaction containing a final concentration of 0.5mM each of rATP, rGTP, rUTP and rCTP (Section 4.F).
 2. The mixture should be kept at room temperature while each successive component is added, since DNA can precipitate in the presence of spermidine if kept at 4°C.
 3. 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).
2. Incubate for 1 hour at 37–40°C.
 3. Remove 1μl from the reaction to determine the percent incorporation and specific activity of the probe (Section 4.C). A small aliquot can be analyzed by gel electrophoresis (Positive Control Protocol, Step 3).

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

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

4.B. Synthesis of High-Specific-Activity Radiolabeled RNA Probes (continued)

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 nondenaturing gels loaded with RNA denatured in a formaldehyde/formamide RNA sample buffer.

Add 5µl of the control 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. If the radioactive transcript is also analyzed by gel electrophoresis, mix a small aliquot with 15µl RNA Sample Buffer and 2–5µl RNA Loading Buffer, heat, run on the gel, and analyze by autoradiography or on a phosphorimaging instrument.

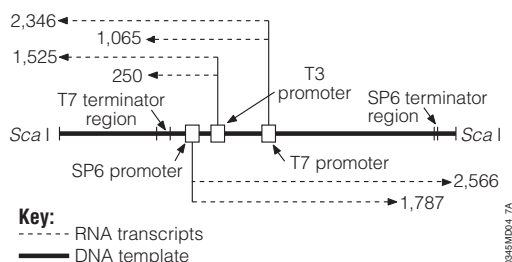


Figure 2. RNA transcripts generated using the appropriate RNA polymerase and the pGEM® Express Positive Control Template.

4.C. Determining 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 ³²P-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:

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

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

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.):

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

$$\text{nmol limiting cold rNTP} = \mu\text{l limiting cold rNTP} \times 100\mu\text{M rNTP} \times \frac{10^3\text{nmol} \times 1\text{L}}{1\mu\text{mol} \times 10^6\mu\text{l}}$$

$$\text{total nmol of limiting rNTP} = \text{nmol of labeled rNTP} + \text{nmol of limiting cold rNTP}$$

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

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

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

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

4. Example:

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

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

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

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

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

The total amount of RNA synthesized is calculated as follows:

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

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

$$\text{total nmol of limiting rNTP} = 0.125 \text{nmol} + 0.24 \text{nmol} = 0.365 \text{nmol}$$

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

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

The specific activity of the RNA probe is:

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

4.D. Removal of the DNA Template Following Transcription

The DNA template should be removed by digestion with DNase I following the transcription reaction. Promega 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 7.A.)

- citrate-saturated phenol (pH 4.7):chloroform:isoamyl alcohol (125:24:1) (available from Sigma-Aldrich [Cat.# P1944, phenol:chloroform 5:1 for molecular biology])

- 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:

- Add RQ1 RNase-Free DNase to a concentration of 1u/μg of template DNA.
- Incubate for 15 minutes at 37°C.
- Extract with 1 volume of citrate-saturated (pH 4.7) phenol:chloroform:isoamyl alcohol (125:24:1). Vortex for 1 minute and centrifuge in a microcentrifuge (12,000 × g) for 2 minutes.
- 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 (2–5 μl) from the aqueous phase obtained above to 15 μl of RNA sample buffer. Add 2–5 μl 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 0.5 μg/ml ethidium bromide. Run the gel under standard conditions for analysis of RNA samples.

- 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.



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.

- 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 4.E. If no further purification is desired, suspend the RNA sample in 10–20 μl of TE buffer or water and store at –70°C.

4.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 Biosciences.

4.E. Removal of Unincorporated Nucleotides (continued)

Once the RNA has been separated from the unincorporated nucleotides, the sample should be ethanol precipitated as described in Section 4.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 10–20 μ l 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.

4.F. Synthesis of Large Amounts of RNA

The standard transcription protocol (Section 4.B) typically yields less than 1 μ g RNA/ μ g DNA. Using the conditions described below, yields of 5–10 μ g RNA/ μ g plasmid DNA can be obtained. Alternatively, the Promega RiboMAX™ Large Scale RNA Production Systems 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	20 μ l
100mM DTT	10 μ l
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)	20 μ l
Linearized template DNA (1.0–2.5mg/ml in water or TE buffer)	2 μ l
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 4.D.

4.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 (Cat.# L4960). However, translation of some transcripts shows a direct dependence on the presence of the m⁷G(5')ppp(5')G cap at the 5'-end. 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).

The protocol for capping synthesized transcripts prior to translation is described below. With this method, the Ribo m⁷G Cap Analog (Cat.# P1711) is directly incorporated into the RNA during the transcription reaction to yield capped RNA substrate.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 7.A.)

- Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511)
- Ribo m⁷G Cap Analog (Cat.# P1711)
- 1mM rGTP

Synthesis of a Capped RNA Transcript in vitro

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

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

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

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.
Note: For small transcripts, the amounts of template can be increased as well.
3. Proceed with DNase I treatment to remove the template, followed by purification of the RNA, as described in Section 4.D.

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	Causes and Comments
Low amounts of RNA synthesized using standard transcription protocol	<p>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.</p> <p>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.</p> <p>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 7.A). Individual transcription components may be purchased directly from Promega.</p> <p>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.</p>
Presence of incomplete transcripts	<p>Premature termination of RNA synthesis. Increase the concentration of the transcripts 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.</p> <p>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).</p>

Symptoms	Causes and Comments
Presence of incomplete transcripts (continued)	<p>Polymerase terminator sequences present. 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.</p> <p>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.</p>
Presence of transcripts larger than expected	<p>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 4.A).</p> <p>Nonlinearized plasmid is present in the sample. Analyze the sample by gel electrophoresis. If undigested vector is noted, redigest with the appropriate restriction enzyme.</p> <p>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).</p> <p>Decrease the concentration of rUTP used in the reaction (10).</p>

6. References

6.A. Cited References

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6.B. Additional References

Generation of Short Oligoribonucleotides Using T7 RNA Polymerase

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Kain, K.C., Orlandi, P.A. and Lanar, D.E. (1991) Universal promoter for gene expression without cloning: Expression-PCR. *BioTechniques* **10**, 366-74.

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**, 1-4.

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Rosemeyer, V., Laubrock, A. and Seib, R. (1995) Nonradioactive 3'-end-labeling of RNA molecules of different lengths by terminal deoxynucleotidyltransferase. *Anal. Biochem.* **224**, 446-9.

Non-Radioactive Labeling of RNA Transcripts Using the Riboprobe® Systems

Holzenberger, M. *et al.* (1997) Selective expression of insulin-like growth factor II in the songbird brain. *J. Neurosci.* **17**, 6974-87.

Lee, W.Y. *et al.* (2000) Nonradioactive 3'-end-labeling of RNA molecules of different lengths by terminal deoxynucleotidyltransferase. *J. Biol. Chem.* **275**, 33998-34008.

7. Appendix

7.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: DEPC is irritating to the respiratory system, skin and eyes. Use a fume hood for all steps prior to autoclaving.

5X 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 MgCl₂
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 of glycerol. Dispense into single-use aliquots and store at -20°C.

citrate-saturated phenol (pH 4.7): chloroform:isoamyl alcohol (125:24:1)

Available from Sigma-Aldrich (Cat.# P1944, phenol:chloroform 5:1 for molecular biology [125:24:1 mixture of phenol, chloroform and isoamyl alcohol])

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

7.B. Sources for Vector Sequence and Restriction Site Information

The 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 sequences are available from GeneBank® and online at: www.promega.com/vectors/

Vector	Literature Number	Vector	Literature Number
pALTER®-MAX Vector	TM041	pGEM®-9Zf(-) Vector	TB070
pCI Vector	TB206	pGEM®-11Zf(-) Vector	TB074
pCI-neo Vector	TB215	pGEM®-11Zf(+) Vector	TB075
pGEM®-3Z Vector	TB033	pGEM®-13Zf(+) Vector	TB073
pGEM®-4Z Vector	TB036	pGEM®-T and pGEM®-T Easy Vectors	TM042
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
pGEM®-7Zf(-) Vector	TB069		

7.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	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*	2,500u	N2511
	10,000u	N2515
Ribo m ⁷ G 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	50µl	G3191
RiboMAX™ Large Scale RNA Production System – SP6	50 × 20µl reactions	P1280
RiboMAX™ Large Scale RNA Production System – T7	50 × 20µl reactions	P1300
Herring Sperm DNA	10mg	D1811

For Laboratory Use.

7.C. Related Products (continued)

Vectors

Product	Size	Cat.#
pALTER [®] -MAX Vector	20µg	Q5761
pGEM [®] -3Z Vector	20µg	P2151
pGEM [®] -4Z Vector	20µg	P2161
pGEM [®] -3Zf(+) Vector	20µg	P2271
pGEM [®] -3Zf(-) Vector	20µg	P2261
pGEM [®] -5Zf(+) Vector	20µg	P2241
pGEM [®] -5Zf(-) Vector	20µg	P2351
pGEM [®] -7Zf(+) Vector	20µg	P2251
pGEM [®] -7Zf(-) Vector	20µg	P2371
pGEM [®] -9Zf(-) Vector	20µg	P2391
pGEM [®] -11Zf(+) Vector	20µg	P2411
pGEM [®] -11Zf(-) Vector	20µg	P2421
pGEM [®] -13Zf(+) Vector	20µg	P2541
pGEM [®] -T Vector System I*	20 reactions	A3600
pGEM [®] -T Vector System II*	20 reactions	A3610
pGEM [®] -T Easy Vector System I*	20 reactions	A1360
pGEM [®] -T Easy Vector System II*	20 reactions	A1380
pSP64 Poly(A) Vector	20µg	P1241
pSP72 Vector	20µg	P2191
pSP73 Vector	20µg	P2221

*For Laboratory Use

Mammalian Expression Vectors

Product	Size	Cat.#
pSI Mammalian Expression Vector	20µg	E1721
pCI Mammalian Expression Vector	20µg	E1731
pCI-neo Mammalian Expression Vector	20µg	E1841
pTARGET [™] Mammalian Expression Vector System	20 reactions	A1410
pF4A CMV Flexi [®] Vector	20µg	C8481
pF4K CMV Flexi [®] Vector	20µg	C8491

Plasmid DNA Purification

Product	Size	Cat.#
PureYield [™] Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

[®]U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803 and Japanese Pat. Nos. 3009458 and 3366596 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor.

[®]U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[®]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.

*In Europe, effective March 28, 2006, European Pat. Nos. 201,184 and 200,362, will expire. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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