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T7 RNA Polymerase

Technical Bulletin 8033-1

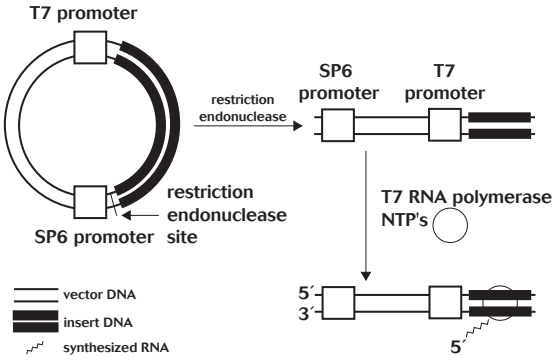
T7 RNA Polymerase is a DNA-dependent RNA polymerase present in *E. coli* infected with bacteriophage T7 (1). The enzyme has extremely high specificity for promoter sequences found in T7 bacteriophage DNA and in various cloning vectors containing the T7 promoter (*eg.* pCR®II-TOPO®, pCR®-Blunt II-TOPO®, pET-DEST42). Many vectors used for *in vitro* transcription contain two different phage promoter sequences flanking the DNA insert, so both strands of the cloned DNA can be transcribed from a single plasmid using two different polymerases. T7 RNA Polymerase is used to generate specific RNA transcripts *in vitro* from DNA containing the T7 promoter sequence. The DNA sequence of interest is cloned into the polylinker region adjacent to the T7 promoter sequence of the vector. To prepare the DNA template for transcription, the recombinant plasmid is linearized at a restriction endonuclease site downstream of the DNA insert to be transcribed. T7 RNA Polymerase initiates synthesis at the T7 promoter sequence and produces an RNA transcript of the DNA (figure 1).

The RNA transcripts are used as hybridization probes for DNA and RNA blots and *in situ* studies (2), in ribonuclease protection assays where the transcript is hybridized with target mRNA sequences (3), to study post-transcriptional modifications, including RNA splicing (4-6) and polyadenylation (7), and for *in vitro* translation (8,9).

As hybridization probes, RNA transcripts offer advantages over DNA probes. Sensitivity is increased because RNA probes are single-stranded; therefore, there are no complementary labeled strands than can compete with each other during hybridization to target sequences. In addition, unhybridized RNA probes can be removed from hybridized filters by RNase A digestion, which substantially reduces nonspecific background (10).

T7 RNA Polymerase is the product of the T7 gene 1 (11) and has been cloned in an expression vector under the control of an inducible *lac* promoter (12). The enzyme is supplied with 1 ml of 5X T7 RNA Polymerase Reaction Buffer [0.2 M Tris-HCl (pH 8.0), 0.125 M NaCl, 40 mM $MgCl_2$, 10 mM spermidine-$(HCl)_3$]. It is stored in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1 mM Na_2EDTA, 1 mM dithiothreitol (DTT), 50% (v/v) glycerol, and 0.01% (v/v) Triton X-100. One unit of T7 RNA Polymerase incorporates 1 nmol of ribonucleotide into acid-precipitable material in 1 h at 37°C. No detectable contaminating activity is observed in endodeoxyribonuclease, 3´ and 5´ exodeoxyribonuclease, ribonuclease, and DNA nicking assays. Yield of high specific activity RNA and percentage of full-length copy are determined by transcription of a 9.5-kb template. This bulletin describes conditions that use T7 RNA Polymerase to prepare large amounts of unlabeled or low specific activity RNA for RNA processing studies or protein translation.

Figure 1 - Summary of *in vitro* transcription



Materials

In addition to the enzyme and buffer, the following materials are required to prepare RNA transcripts from DNA and subsequently to remove the DNA template:

- Linearized DNA template with a T7 promoter sequence
- 10 mM UTP
- 0.1 M Na_2EDTA (pH 8.0)
- 10 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA (TE)
- Autoclaved distilled water
- DNase I (RNase-free)
- 10% (w/v) trichloroacetic acid, 1% (w/v) sodium pyrophosphate (TCA-1% NaPPi)
- Absolute ethanol
- Glass fiber filters (1 x 2 cm), Whatman GF/C or equivalent
- Autoclaved 1.5-ml microcentrifuge tubes
- Microcentrifuge
- NTP stock (10 mM each ATP, GTP, and CTP)
- [α-^{32}P]UTP (400 Ci/mmol)
- 50 mM dithiothreitol (DTT)
- 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$
- 1 mg/ml yeast tRNA (RNase-free)
- 5% (w/v) trichlroacetic acid (TCA)
- Buffer-saturated phenol
- Buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)]
- 7.5 M ammonium acetate [DEPC (diethylpyrocarbonate)-treated and filtered through a sterile 0.45-µm nitrocellulose filter]
- 37°C water bath

NOTE: Always wear gloves when performing the following assays. For best results, solutions should be sterile and microcentrifuge tubes should be autoclaved and baked at 115°C. DEPC-treatment of tubes and solutions may be helpful if RNase contamination is a problem (13).

Protocol For preparing high specific activity RNA transcripts

The following procedure is optimized to prepare high specific activity (~ 5 x 10^8 dpm/µg) RNA (500-750 ng) for hybridization probes, especially from long DNA templates (5 to 10 kb). Linearized templates should be phenol extracted (14), ethanol precipitated (15), and dissolved in autoclaved, distilled water prior to transcription reaction. After transcription, the DNA template is removed by DNase I digestion.

1. Lyophilize 700 pmol (280 µCi) of [α-^{32}P]UTP (400 Ci/mmol) to a final volume of 1 to 2 µl in an autoclaved, 1.5-ml microcentrifuge tube. (The final concentration of [α-^{32}P]UTP is 70 µM in a 10 µl reaction volume.)
2. At room temperature, add the following components to the tube containing the radiolabeled nucleotide:

Component	Amount	Final Concentration
5X T7 RNA Polymerase Reaction Buffer	2 µl	1X
NTP stock of 10 mM each ATP, CTP, GTP	1 µl	1 mM
50 mM DTT	1 µl	5 mM
Linearized DNA template	0.1-0.5 µg	10-50 µg/ml
Autoclaved distilled water	up to 9 µl (total volume)	

NOTE: Due to the potential precipitation of DNA in the presence of spermidine, do not prepare the reaction on ice. Always add the components in the order indicated.

3. Equilibrate the reaction mixture at 37°C for 5 min.
4. Add 1 µl (50 units) T7 RNA Polymerase. Mix by gentle pipetting. Do not vortex.
5. Incubate at 37°C for 10 min.
6. Add 90 µl of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$.
7. Dilute 1 µl of DNase I (5 to 15 µl/µl) to 0.5 µg/µl with 50 mM Tris (pH 7.5), 10 mM $MgCl_2$.
8. Add 1 µl of the diluted DNase I to the reaction mixture. Incubate for 10 min. at 37°C.
   NOTE: Do not exceed 10 min (16).
9. Add 10 µl of 0.1 M Na_2EDTA (pH 8.0) to terminate the DNase I reaction.
10. Add 110 µl of buffer-saturated phenol, vortex thoroughly, and centrifuge 5 min at 15,000 X *g* at room temperature to separate the phases. Transfer the upper aqueous phase to a new tube.
    Warning: The phenol solution contains radiolabeled material and should be disposed of properly.
11. Extract with 110 µl of buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] as described in step 10.
12. To the aqueous phase, add 0.5 volume of 7.5 M ammonium acetate followed by 2.5 volumes of absolute ethanol. Centrifuge at 15,000 X *g* at room temperature for 30 min (15). Carefully remove the supernate.
13. Wash the pellet in 70% (v/v) ethanol and centrifuge briefly. Remove the supernate.
14. Evaporate residual ethanol. Dissolve the RNA in TE. The RNA may be stored at -20°C for up to two weeks.

Protocol for preparing large amounts of RNA transcripts

For RNA processing studies or *in vitro* translation, high specific activity RNA is not necessary. The following procedure is used to produce large quantities of unlabeled or low specific activity RNA by raising the concentration of the NTPs to 2mM and by increasing the T7 RNA Polymerase to 5000 units/ml. A trace amount of [α-^{32}P]UTP is included in the reaction so that the exact amount of RNA synthesis can be determined. Under these conditions, it is possible to synthesize 50 µg of a 2 to 3 kb RNA transcript from 1 µg of DNA template.

1. At room temperature, add the following into an autoclaved 1.5-ml microcentrifuge tube:

Component	Amount	Final Concentration
5X T7 RNA Polymerase Reaction Buffer	10 µl	1X
NTP stock of 10mM each ATP, CTP, GTP	10 µl	2 µM
400 Ci/mmol [α-^{32}P] UTP	1 µl	--
10 mM UTP	10 µl	2 µM
50 mM DTT	5 µl	5 mM
Linearized DNA template	1 µg	20 µg/ml
Autoclaved distilled water	up to 45 µl (total volume)	

2. Equilibrate the reaction mixture at 37°C for 1 h.
3. Add 5 µl (250 units) of T7 RNA Polymerase. Mix by gentle pipetting.
4. Incubate at 37°C for 1 h.
5. Add 50 µl of 0.1 M Na_2EDTA to dissolve any precipitate that may have formed.
6. Add 100 µl of buffer-saturated phenol, vortex thoroughly, and centrifuge 5 min at 15,000 X *g* at room temperature to separate the phases. Transfer the upper aqueous phase to a new tube.
7. Extract with 100 µl of buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] as described in step 6.
8. To the aqueous phase, add 0.5 volume of 7.5 M ammonium acetate followed by 2.5 volumes of absolute ethanol. Centrifuge at 15,000 X *g* at room temperature for 30 min (15). Carefully remove the supernate.
9. Wash the pellet in 70% ethanol and centrifuge briefly. Remove the supernate.
10. Evaporate residual ethanol. Dissolve the RNA in TE and store at –70°C.

Troubleshooting

Some possible causes of synthesizing incomplete RNA transcripts with T7 RNA Polymerase are described below and accompanied by suggested solutions.

Possible Causes	Suggested Solutions
Nucleotide concentrations limiting	Use nucleotide concentrations as stated in the protocols. For high specific activity hybridization probes, be sure to use the recommended specific activity isotope. Addition of a higher than recommended specific activity nucleotide will result in decreased molar concentration of the labeled nucleotide and will cause premature termination. For high specific activity hybridization probes to ensure representation of the entire sequence of a large probe, subclone smaller restriction fragments of the insert DNA into the vector. A mixture of templates from the subclones, representing the entire insert sequence, should then be used for synthesis of the RNA probe (16).
DNA template linearized incorrectly	Be sure to linearize the DNA template at a restriction endonuclease site downstream of the DNA sequence to be transcribed. If the restriction enzyme cleaves within the DNA sequence to be transcribed, T7 RNA Polymerase stops transcribing at the end of the linear DNA.
Sample contaminated with RNases	Be sure solutions and microcentrifuge tubes are sterile. DEPC-treat tubes and solutions. Add RNaseOUT™ Recombinant Ribonuclease Inhibitor.
T7 RNA Polymerase handled incorrectly	Store T7 RNA Polymerase at –20°C. Do not vortex solutions containing T7 RNA Polymerase. Do not dilute the enzyme.

Additional information

Storage and stability

T7 RNA Polymerase is susceptible to denaturation when exposed to air. It is shipped on wet ice and must be stored at –20°C. Handle the enzyme very gently. Shaking or vortexing the vial results in denaturation and aggregation. T7 RNA Polymerase is very sensitive to dilution. Therefore, do not dilute the enzyme.

Dual promoter vectors

The pCR®II-TOPO® and pCR®-Blunt II-TOPO® vectors have been constructed with the T7 promoter flanking one side of the polylinker region and the SP6 RNA Polymerase promoter on the other side. Such dual promoter vectors are useful for transcription of both strands of the cloned DNA from a single plasmid construct by linearizing at different sites within the polylinker.

Nonspecific transcription

Although the specificity of T7 RNA Polymerase for its promoter is very high, initiation of transcription from other sequences can occur. The ends of DNA restriction fragments can serve as initiation sites, especially if they contain 3´-protruding ends. The level of spurious transcription is ~3% to 5% of that observed from the promoter sequence. This can be suppressed ~eight-fold by raising the NaCl concentration to 100 mM, although total transcription is also decreased by 50% (17,18). Preferably, restriction enzymes that generate blunt or 5´-protruding ends should be used to linearize plasmids for transcription assays.

Translation of RNA transcripts

Synthetic RNA prepared with bacteriophage RNA polymerases can be used in eukaryotic *in vitro* translation systems more efficiently if a 5-methylguanisine cap is incorporated during transcription (19-21).

RNase inhibitors

RNaseOUT™ Recombinant Ribonuclease Inhibitor can be used if RNase contamination is a problem.

Ordering information

Description	Concentration	Quantity	Cat. No.
T7 RNA Polymerase	50 units/µl	2500 units	18033-019
	50 units/µl	2 x 2500 units	18033-100
RNaseOUT™ Recombinant Ribonuclease Inhibitor	40 units/µl	5000 units	10777-019
Dithiothreitol (DTT)		5 g	15508-013
Phenol		500 g	15509-037
TOPO TA Cloning® Kit Dual Promoter (with PCR®II-TOPO® vector)			
with One Shot® TOP10 Chemically Competent *E.coli*		20 rxns	K4600-01
		40 rxns	K4600-40
with One Shot® TOP10F´ Chemically Competent *E.coli*		20 rxns	K4650-01
		40 rxns	K4650-40
with One Shot® TOP10F Electrocomp™ *E.coli*		20 rxns	K4660-01
		40 rxns	K4660-40
Zero Blunt® TOPO® PCR Cloning Kit			
with One Shot® TOP10 Chemically Competent *E.coli*		20 rxns	K2800-20
		40 rxns	K2800-40
with One Shot® TOP10 Electrocomp™ *E.coli*		20 rxns	K2860-20
		40 rxns	K2860-40
pET-DEST42 Gateway™ destination vector		6 µg	12276-010

For research use only. Not for diagnostic or therapeutic use in humans or animals.

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