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SP6 RNA Polymerase

Technical Bulletin 8018-1

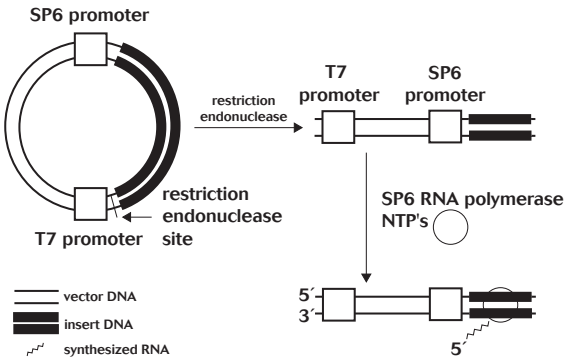
SP6 RNA Polymerase is a DNA-dependent RNA polymerase present in *Salmonella typhimurium* infected with bacteriophage SP6. The enzyme has an extremely high specificity for promoter sequences found in SP6 bacteriophage DNA and in various cloning vectors containing the SP6 promoter (*eg.* pCR®II-TOPO®, pCR®-Blunt II-TOPO®)(1,2). Many DNA vectors used for *in vitro* transcription contain 2 different phage promoter sequences flanking the DNA insert, so both strands of the cloned DNA can be transcribed from a single plasmid by using two different polymerases. SP6 RNA Polymerase is used to generate specific RNA transcripts *in vitro* from DNA containing the SP6 promoter sequence (3). The DNA sequence of interest is cloned into the polylinker region adjacent to the SP6 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. SP6 RNA Polymerase initiates synthesis at the SP6 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 (4), in ribonuclease protection assays where the transcript is hybridized with target mRNA sequences (5), to study post-transcriptional modifications, including RNA splicing (6-8) and polyadenylation (9), and for *in vitro* translation (10, 11).

As hybridization probes, RNA transcripts offer advantages over double-stranded 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 (12).

SP6 RNA Polymerase has been cloned in an expression vector under the control of the inducible *tac* promoter and is purified from *E. coli* containing this plasmid (13). The enzyme is supplied with 1 ml of 5X SP6 RNA Polymerase Reaction Buffer [0.2 M Tris-HCl (pH 7.9), 30 mM $MgCl_2$, 10 mM spermidine-$(HCl)_3$]. It is stored in 50 mM Tris-HCl (pH 7.9), 0.1 M NaCl, 0.1 mM Na_2EDTA, 14 mM 2-mercaptoethanol, 50% (v/v) glycerol, and 0.1% (v/v) Triton X-100. One unit of SP6 RNA Polymerase incorporates 1 nmol of ribonucleotide into acid-precipitable material in 1 h at 37°C. No detectable contaminating activity is observed in endodeoxynuclease, 3´ and 5´ exodeoxyribonuclease, ribonuclease, and DNA nicking assays. The yield of high specific activity RNA and percentage of full-length copy are determined by transcription of a 1.1-kb template. This bulletin describes conditions that use SP6 RNA Polymerase to prepare high specific activity RNA for hybridization probes, and 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 SP6 promoter sequence
- NTP stock (10 mM each ATP, GTP, and CTP)
- 10 mM UTP
- [α-^{32}P]UTP (3000 Ci/mmol)
- 0.1 M Na_2EDTA (pH 8.0)
- 10 mM dithiothreitol (DTT)
- 10 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA (TE)
- 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$
- Autoclaved distilled water
- 1 mg/ml yeast tRNA (RNase-free)
- DNase I (RNase-free)
- 5% (w/v) trichloroacetic acid (TCA)
- 10% (w/v) trichloracetic acid, 1% (w/v) sodium pyrophosphate (TCA-1% NaPPi)
- 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]
- Absolute ethanol
- 70% ethanol
- Glass fiber filters (1 x 2 cm), Whatman GF/C or equivalent
- Autoclaved 1.5-ml microcentrifuge tubes
- Microcentrifuge
- 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 (14).

Protocol for preparing high specific activity RNA transcripts

The following procedure is optimized to prepare high specific activity (1 x 10^8 to 1 x 10^9 dpm/µg) RNA (~0.1 µg) for use as hybridization probes. In order to achieve these specific activities, it is necessary to lower the concentration of UTP to 10 µM to avoid excessive dilution of the ^{32}P-label. The concentration of the other nucleotides is 400 µM. The limiting concentration of the UTP causes premature termination of transcripts. Therefore, only templates of <2 kb should be used to ensure that the majority of the transcripts are full-length (15). Linearized templates should be phenol extracted (16), ethanol precipitated (17), and dissolved in autoclaved, distilled water prior to the transcription reaction.

1. Dilute 1 µl of the 10-mM stock of ATP, GTP, and CTP to 10 µl with autoclaved, distilled water to yield a final concentration of 1 mM. Add 4 µl of this dilution to an autoclaved 1.5-ml microcentrifuge tube.
2. Dilute 1 µl of 10 mM UTP to 1000 µl with autoclaved, distilled water to yield a final concentration of 0.01 mM. Add 6.7 µl (67 pmol) of this dilution to the microcentrifuge tube containing the other nucleotides.
3. Add 100 µCi of 3000 Ci/mmol (33 pmol) [α-^{32}P] UTP.
   NOTE: Total amount of UTP added is 100 pmol.
4. Dry all four NTPs together with the radioactive label by evaporation or lyophilization. Do not over dry.
5. At room temperature, to the microcentrifuge tube containing the dried NPTs add:

Component	Amount	Final Concentration
5X SP6 RNA Polymerase Reaction Buffer	2 µl	1X
10 mM DTT	1 µl	1 mM
Linearized DNA template	0.2 µg	20 µg/ml
Autoclaved distilled water	up to 9.5 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.

6. Equilibrate the reaction mixture at 37°C for 5 min.
7. Add 0.5 µl of SP6 RNA Polymerase (5 to 10 units). Mix by gentle pipetting. Do not vortex.
8. Incubate 1 h at 37°C.
9. Add 90 µl of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$.
10. Dilute 1 µl of DNase I (5 to 15 µg/µl) to 0.5 µg/µl with 50 mM Tris (pH 7.5), 10 mM $MgCl_2$.
11. 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 (15).
12. Add 10 µl of 0.1 M Na_2EDTA (pH 8.0) to terminate the DNase I reaction.
13. 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.
14. Extract with 110 µl of buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] as described in step 13.
15. 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.
    WARNING: The ethanol supernate contains radiolabeled material and should be disposed of properly.
16. Wash the pellet in 70% (v/v) ethanol and centrifuge briefly. Remove the supernate.
17. Evaporate residual ethanol. Dissolve the RNA in TE.
18. 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. In the following procedure, SP6 RNA Polymerase is used to prepare full-length RNA transcripts, especially from DNA templates of 6 kb or longer (15). 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 5 to 10 µg of RNA transcripts from 1 µg of DNA template. The reaction may be scaled up linearly.

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

Component	Amount	Final Concentration
5X SP6 RNA Polymerase Reaction Buffer	10 µl	1X
NTP stock of 10mM each ATP, CTP, GTP	2 µl	400 µM
10 mM UTP	2 µl	400 µM
10 mM DTT	5 µl	1 mM
3000 Ci/mmol [α-^{32}P] UTP	1 µl	--
Linearized DNA template	1 µg	20 µg/ml
Autoclaved distilled water	up to 49 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.

2. Equilibrate the reaction mixture at 37°C for 1 h.
3. Add 1 µl (15 units) of SP6 RNA Polymerase. Mix by gentle pipetting.
4. Incubate at 37°C for 1 h.
5. Add 5 µl of 0.1 M Na_2EDTA to stop the reaction.
6. Add 45 µl autoclaved, distilled water.
7. 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.
8. Extract with 100 µl of buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] as described in step 7.
9. 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. (17). Carefully remove the supernate.
10. Wash the pellet in 70% ethanol and centrifuge briefly. Remove the supernate.
11. Evaporate residual ethanol. Dissolve the RNA in TE and store at –70°C.

Troubleshooting

Some possible causes of synthesizing incomplete RNA transcripts with SP6 RNA Polymerase are described below and accompanied by suggested solutions.

Possible Causes	Suggested Solutions
DNA template is too long	For high specific activity hybridization probes, only templates < 2 kb will produce full-length transcripts using the recommended nucleotide concentrations. 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 (15).
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.
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, SP6 RNA Polymerase stops transcribing at the end of the linear DNA.
Sample contaminated with RNases	Be sure solutions and microcentrifuge tubes are sterile. Treat tubes and solutions with DEPC. Add RNaseOUT™ Recombinant Ribonuclease Inhibitor.
SP6 RNA Polymerase handled incorrectly	Store SP6 RNA Polymerase at –20°C. Do not vortex solutions containing SP6 RNA Polymerase. Do not dilute the enzyme.

Additional information

Storage and stability

SP6 RNA Polymerase is shipped on wet ice and should be stored at –20°C. The enzyme can be completely inactivated by heating it at 65°C for 10 min.

SP6 RNA Polymerase is susceptible to denaturation when exposed to air. Handle the enzyme very gently. Shaking or vortexing the vial results in denaturation and aggregation. SP6 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 SP6 promoter flanking one side of the polylinker region and the T7 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 SP6 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 is minimized by using restriction endonucleases that generate blunt or 5´ protruding ends.

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 (18-20).

RNase inhibitors

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

Ordering information

Description	Concentration	Quantity	Cat. No.
SP6 RNA Polymerase	15 units/µl	500 units	18018-010
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
DNase I	50-375 units/µl	20,000 units	18047-019
Yeast + RNA		25 mg	15401-011
		50 mg	15401-029

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