HyperScribe™ All in One mRNA Synthesis Kit II

(EZ Cap Reagent AG (3' OMe), T7, poly(A))

Introductions

HyperScribe™ All in One mRNA Synthesis Kit II (EZ Cap Reagent AG (3' OMe), T7, poly(A)) is designed to synthesize capped and tailed mRNA in vitro. Capped mRNA is synthesized by co-transcriptional incorporation of capping reagent (EZ Cap Reagent AG (3' OMe), Catalog No. B8178) using T7 RNA Polymerase. The capping of EZ Cap Reagent AG (3' OMe) results in a Cap 1 structure and ensures high translation efficacy. After a brief DNase I treatment to remove the template DNA, capped mRNA is poly(A) tailed with Poly(A) Polymerase. Polyadenylation (i.e. addition of Poly(A) tail) plays an important role in the stabilization of RNA in eukaryotes and enhances the efficiency of translation initiation.

RNA synthesized using this kit has many applications in biological experiments, such as *in vitro* translation, antisense RNA and RNAi experiments, RNA vaccines, RNA structure and function studies, ribozyme biochemistry, RNase protein experiments and probe-based hybridization blots.

The kit contains sufficient reagents to carry out 25 reactions, 20 μL each time. Up to 20-180 μg of RNA can be generated with 1 μg of control template per standard reaction.

Materials

1. Components in the kit

(1) Capped RNA Synthesis with EZ Cap Reagent AG (3' OMe)

Components	25 rxn
T7 RNA Polymerase Mix	50 μL
10 × Reaction Buffer	50 μL
ATP (20 mM)	50 μL
UTP (20 mM)	50 μL
CTP (20 mM)	50 μL
GTP (20 mM)	37.5 μL
EZ Cap Reagent AG (3' OMe) (60 mM)	50 μL
Control Template (0.5 μg/μL)	5 μL

RNase-free H_2O	0.5 mL
Store the components at -20°C.	

(2) Tailing reaction

Components	25 rxn
E-PAP (2 units/μL)	100 μL
5 x E-PAP Buffer	600 μL
ATP Solution (100 mM)	100 μL
25 mM $MnCl_2$	250 μL
Nuclease - free Water	2 x 1mL
Store the components at -20°C.	

2. Materials not supplied

DNA Template

The DNA Template can be plasmid DNA, oligonucleotides, PCR products, cDNA and so on. The DNA template must be linear and contain a T7 RNA polymerase promoter sequence that determines the transcriptional start position of the target sequence. our company can offer Biotin-NTP, Fluorescein-NTP, Digoxigenin-NTP, Aminoallyl-NTP, ARCA(B8175), Pseudo-UTP (B7972), 5mCTP(B7967), mCAP(B8174) and 5-Methoxy-UTP(B8061). For more reagents related to RNA synthesis *in vitro*, please refer to our website.

Protocols

1. DNA Template Preparation

PCR DNA product, linearized plasmid DNA, cDNA and oligonucleotides can be used as templates for *in vitro* transcription. Many cloning vectors carry two opposite T7 phage polymerase promoter sequences that bind T7 polymerase to initiate the transcription process. To obtain a purified linearized plasmid, the plasmid as a transcription template by digestion with restriction endonuclease treatment must be cleaned up. Figure 1 interpret how the T7 RNA Polymerase transcript to produce RNA with T7 promoter.

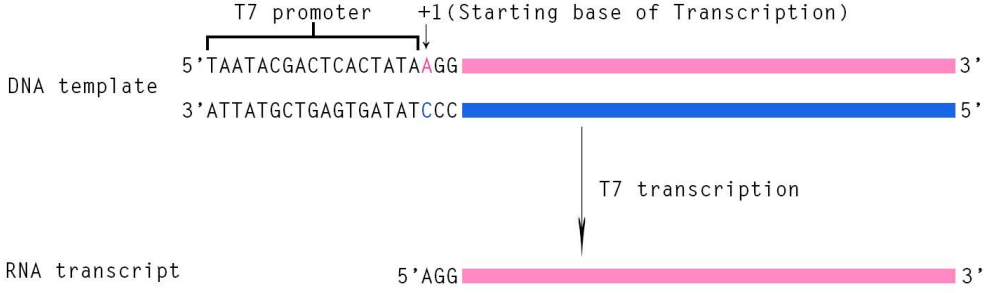


Figure 1: Transcription using T7 RNA Polymerase

■ Plasmid Templates

Many plasmid cloning vectors carry two phage polymerase promoters in different directions, one on each side of the multiple cloning sites, allowing transcription of any strand of the inserted sequence. Such double reverse promoter vectors include pDP vector (Ambion), pGEM vector (Promega), pBluescript vector (Stratagene), pCRII vector (Invitrogen) and so on. The plasmid vector used as a transcription template must be linearized by restriction endonuclease digestion. Since the transcription reaction continues until the end of the DNA template, linearization ensures to obtain RNA transcripts of the defined length and sequences. Restriction sites are not necessarily unique and as long as the promoter is kept adjacent to the transcription template, the vector itself can be digested repeatedly. Purification should be carried out after restriction enzyme digestion, as residues in the digestion reaction may inhibit the transcription reaction.

■ PCR products

The PCR product can also be used as a transcription template for *in vitro* transcription. PCR products with a T7 promoter can be obtained by adding a T7 promoter sequence to the 5' end of the upstream or downstream PCR primer. These sequences form a double-stranded product with a promoter sequence by PCR reaction. Figure 2 interpret how to add T7 promoter to PCR products.

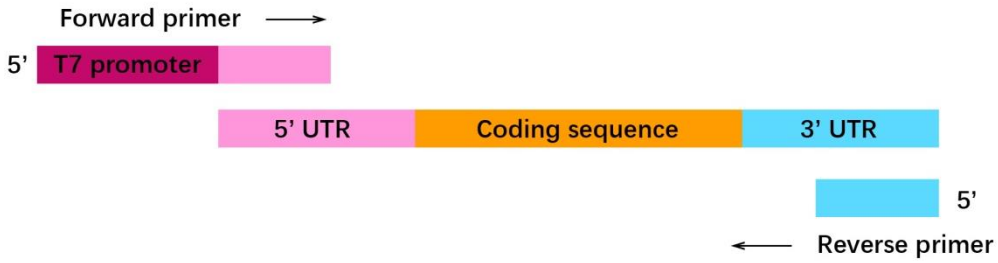


Figure 2: PCR primer design for T7 RNA Polymerase template

■ Synthetic DNA Oligonucleotides

Two oligonucleotides can also be used to construct short transcription templates. A double-stranded DNA template can be formed by simply annealing the two complementary oligonucleotides carrying the phage promoter sequence. In fact, as long as part of the DNA templates form a double-stranded DNA; the *in vitro* RNA transcriptional experiment can be performed.

■ cDNA

In recent years, RNA transcription *in vitro* procedures have been gradually applied to RNA amplification reactions: the oligo(dT)-T7 promoter primers can be used in the reverse transcription process to obtain a transcription template using RNA as an initial template. A double-stranded transcription template will be acquired by a second strand synthesis reaction.

2. Capped RNA Synthesis

The recommended ratio of EZ Cap Reagent AG (3' OMe) to GTP is 4:1.

(1) Thaw corresponding components on ice.

(2) Assemble the reaction system at room temperature in the following order.

Nuclease-free water	X μL	
10xReaction Buffer	2 μL	
ATP (20 mM)	2 μL	2 mM final
UTP (20 mM)	2 μL	2 mM final
CTP (20 mM)	2 μL	2 mM final
GTP (20 mM)	1.5 μL	1.5 mM final
EZ Cap Reagent AG (3' OMe) (60 mM)	2 μL	6 mM final
Template DNA	X μL	1 μg
T7 RNA Polymerase Mix	2 μL	
Total Reaction Volume	20 μL	

(3) Mix thoroughly. Incubate for 2 hours at 37°C. For short (< 300nt) transcripts incubate for 4 hours at 37°C. Increasing the ratio of cap analog to GTP will increase the proportion of capped RNA transcripts, however it also significantly decreases the yield of the transcription reaction. A ratio of cap analog to GTP of 4:1 is preferably used.

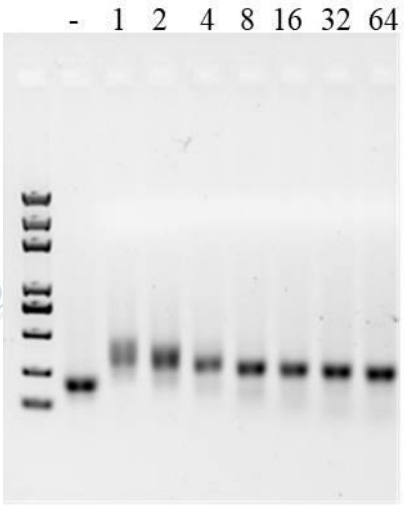
Table 1. Effect of cap analog: GTP ratios on % capped RNA.

Cap Analog: GTP Ratio	Concentration of Cap Analog: GTP (mM)	% Capped RNA
0:1	0:10	0
1:1	5:5	50
2:1	6.7:3.3	67
4:1	8:2	80
8:1	8.9:1.1	89

(4) To remove template DNA, add 2 μL of DNase I (RNase-free), mix and incubate at 37°C for 15 minutes.

3. Poly(A) Tailing Procedure

The reactions described below add a ≥ 150 base poly (A) tail to transcripts generated with the HyperScribe™ All in One mRNA Synthesis Kit II (EZ Cap Reagent AG (3' OMe), T7, poly(A)). To produce a tail shorter than ~150 bases, dilute E-PAP in 1X E-PAP buffer and use less E-PAP in the reaction. (For 1X E-PAP buffer, dilute 5X E-PAP buffer with nuclease - free water.)



The Control DNA Template was transcribed in a HyperScribe™ All in One mRNA Synthesis II reaction. The resulting 130 nt transcript was tailed with decreasing amounts of E-PAP. - indicates untailed transcripts; 1, 2, 4, 8, 16, 32 and 64 represent the dilution factor.

- Tailing reaction

(1) Start with a completed, DNase-treated HyperScribe™ All in One mRNA Synthesis II reaction (20 µL in a 1.5 mL tube) at room temperature. Do not add EDTA to the reaction as is sometimes recommended to inactivate DNase.

(2) At room temperature, add the tailing reagents in the order shown to a 20 µL HyperScribe™ All in One mRNA Synthesis II reaction:

Component	Amount
HyperScribe™ All in One mRNA Synthesis II reaction	20 µL
Nuclease-free Water	42 µL
5 x E-PAP Buffer	20 µL
25 mM $MnCl_2$	10 µL
100 mM ATP	4 µL

(3) Remove 0.5 µL of the reaction mixture above; this minus-enzyme control will be run on a gel next to the tailed RNA at the end of the experiment.

(4) Add 4 µL of E-PAP, and mix gently. The final reaction volume is 100 µL.

(5) Incubate at 37°C for 1 h.

(6) Keep reaction on ice or store at –20°C.

- Denaturing agarose gel electrophoresis

(1) During the 1 h incubation, pour a denaturing agarose-formaldehyde gel of the appropriate percentage for the size of your original (untailed) transcript. Use a 0.75 mm (or thinner) comb for optimal resolution.

Transcription size	Agarose concentration
Larger than 500bp	1% agarose
Smaller than 500bp	1.5% agarose

(2) Prepare an aliquot of each tailing reaction, and the corresponding minus-enzyme control. Also plan to run an RNA size marker. In a 0.5 mL RNase-free microcentrifuge tube, mix 4 μL gel loading dye containing 50 μg/mL Hyper Gel Red with 0.5 μL RNA sample.

NOTE: The gel loading dye must contain ~20 mM EDTA to chelate divalent cations from the tailing reaction reagent. Without EDTA, divalent cations can cause RNA degradation when RNA is denatured by heating.

(3) Heat samples at 75°C for 10 min.

(4) Load the samples, and run the gel in 1 x MOPS buffer at~5 volts/cm until the bromophenol blue dye is near the bottom of the gel.

(5) Examine the gel on a UV light box. The tailed RNA should be ≥ 150 bases longer than the corresponding RNA that was not tailed (minus-enzyme control).

4. Purification of Synthesized RNA

Typically, unmodified RNA transcription products derived from standard RNA synthesis can be purified by phenol-chloroform extraction and ethanol precipitation or by using a spin column-based method. However, Non-radiolabeled RNA or high specific activity radiolabeled RNA probes, spin column chromatography is the most suitable method for capping RNA synthesis. If absolute full-length RNA is required, we recommend gel purification.

- Phenol-chloroform Extraction and Ethanol Precipitation

Phenol: Chloroform extraction and ethanol precipitation of RNA transcripts are preferred methods for removing proteins and most free nucleotides.

(1) The reaction volume was adjusted to 180 μL by the addition of 160 μL of nuclease-free water. Add 20 μL of 3M sodium acetate, pH 5.2, or 20 μL 5M ammonium acetate, mix thoroughly.

(2) The volume ratio was 1:1 phenol/chloroform mixture extraction, followed by extraction twice with chloroform. Collect the aqueous phase and transfer to a new tube.

(3) RNA was precipitated with 2 volumes of ethanol. Incubate at -20°C for at least 30 minutes, and then collect the pellet by centrifugation.

(4) The supernatant was removed and the pellet was washed with 500 μL of cold 70% ethanol.

(5) The RNA was resuspended in 50 μL of 0.1 mM EDTA. Store the RNA at -20°C or lower.

- Spin Column Chromatography

The spin column can remove unincorporated nucleotides, proteins, and salts. The volume of the reaction mixture was adjusted to 100 μL by adding 80 μL of nuclease-free water, and mixed thoroughly. Because each reaction can produce up to 180 μg of RNA, which can exceed column capacity, additional columns are required. Purify RNA according to the manufacturer's instruction manual.

- Gel Purification

When high-purity RNA transcripts such as labeled RNA probes for RNase protection assays or footprint assays are required, we recommend gel purification of the transcription products.

5. Evaluation of Reaction Products

- Quantification by UV Light Absorbance

Measuring the UV spectrophotometry at 260 nm can easily obtain RNA concentration, but any uncombined nucleotides and template DNA in the mixture will affect the reading. Free nucleotides in transcriptional reactions must be removed prior to quantification of RNA concentrations. The purified RNA sample is diluted 1:200 and the absorbance reading should be given in the linear range of the spectrophotometer. RNA dilution may not be required with a NanoDrop™ spectrophotometer. RNA concentrations of 10 ng/μL to 3000 ng/μL can be directly read by NanoDrop spectrophotometer. For single-stranded RNA, 1 A_{260} corresponds to an RNA concentration of 40 μg/mL. RNA concentration can be calculated as follows:

A_{260} x dilution factor x 40 = ___ μg/mL RNA

- Analysis of Transcription Products by Gel Electrophoresis

To assess transcript length, completeness, and quantity, transcriptional reactions should be equated with appropriate denature-altered agarose or polyacrylamide gels. Transcripts larger than 0.3 kb can be run on agarose gels, while denature-polyacrylamide gels (5-15%) are required for smaller transcripts. The gel should be run under denatured conditions to minimize transcription to form secondary structures.

- Preparation of denatured gels

(1) Denatured agarose gel:

To prepare 100mL 1% denature-agarose gel, 1 g agarose powder was added to 72mL nuclease-free water. Melt agarose and add 10mL 10X MOPS buffer. Then in the fume hood, add 18 mL of fresh formaldehyde (37%) and mix thoroughly. Pour in the gel.

10X MOPS gel running buffer: 0.4M MOPS (pH 7.0), 0.1m sodium Acetate, 10mM EDTA.

(2) Denatured PAGE / urea gel:

5-15% PAGE / urea gel.

We recommend the use of commercial premade gels. Standard TBE gel buffer was used.

10 x TBE buffer: 0.9 M Tris base, 0.9 M boric acid, 20mM EDTA.

- Gel electrophoresis of non-radiolabeled RNA

(1) 0.2-1 μg RNA samples were mixed with equal volume RNA Loading Dye.

(2) RNA samples and RNA markers were denatured by heating at 65-70°C for 5-10 minutes.

(3) Pulse rotation, then load onto the gel.

(4) RNA was detected using SYBR Gold or ethidium bromide stained gel.

- Gel electrophoresis of radiolabeled RNA

(1) Aliquots of labeled RNA were mixed with an equal volume of RNA Loading Dye.

(2) RNA samples were denatured by heating at 65-70°C for 5-10 minutes.

(3) Pulse rotation is performed before loading onto the gel.

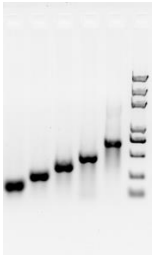
(4) Visualize RNA by autoradiography.

The agarose gel should be dried and then exposed to X-ray film, but a thin polyacrylamide gel (<1mM thickness) can be transferred to the filter paper and covered with a plastic wrap and directly exposed to X-ray film (when using ^{32}P). The exposure time can be from 20 minutes to overnight depending on the specific activity of the RNA probe and the type of intensifying screen used. If the gel is exposed to a Storage Phosphor screen (GE or equivalent), the exposure time may be much shorter.

Notes

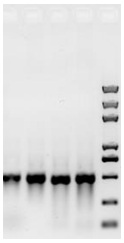
1. Control Reaction

(1) Standard RNA synthesis using templates in different size



37ºC, 2h

(2) RNA Synthesis with Modified Nucleotides



37°C, 2h

Arca+cy5+5moU, EGFP/ Arca+5moU, EGFP/ Arca+5mC+Pseudo, EGFP/ EGFP

2. Low Yield of Full-length RNA

If the transcription of the template produces full-length RNA, but the yield is significantly lower than expected, it may be that the contaminants in the DNA template inhibit the activity of the RNA polymerase, or the DNA concentration may not be correct. Alternatively, DNA templates may require additional purification. It is recommended to use phenol chloroform extraction (see template DNA preparation section).

3. Low Yield of Short Transcript

Short transcripts (<0.3 kb) of high yields can be obtained by extending the incubation time and increasing the amount of the template. The incubation reaction for up to 16 hours (overnight) or the use of up to 2 μg of a template will be beneficial to achieve maximum yield.

4. RNA Transcript Smearing on Denaturing Gel

If the RNA begins to appear on denaturing polyacrylamide or agarose gel (e.g. smeared), this means that RNase contaminates the DNA template. DNA templates contaminated with RNase affect the length and yield of the synthesized transcript (lower than the expected transcript length). Before processing the plasmid DNA template with the T7 High Yield RNA Synthesis Kit, we recommend using the RNase contamination assay kit to assess the quality of the plasmid DNA template. If the plasmid DNA template is contaminated with RNase, it is necessary to extract with phenol/chloroform, then precipitate the DNA and dissolve the DNA in nuclease-free water (see template DNA preparation section).

5. RNA Transcript of Larger Size than Expected

If the yield of the RNA transcript appears to be larger than expected on the denaturing gel, the plasmid template DNA may not be fully digested. Even a small amount of undigested circular DNA can produce a large number of long transcripts. Check that the template is completely digested, if the plasmid is not completely digested, restriction digestion should be repeated. Larger bands can also be observed when the RNA transcript is not completely denatured due to the presence of a stronger secondary structure.

6. RNA Transcript of Smaller Size than Expected

If the denaturation gel analysis indicates a smaller band than expected, it is most likely due to early termination of the polymerase. Some sequences similar to the T7 RNA polymerase termination signal will result in early termination of the RNA transcription reaction. Incubating the transcription reaction at a lower temperature (e.g.at 30°C) may increase the content of the full-length transcript, but the yield will decrease. Incubation at 42°C may increase the yield of full-length transcripts for GC-rich templates or templates with secondary structures.

If the transcription process is prematurely terminated in the synthesis of highly specific active radiolabeled RNA probes, the concentration of "restricted NTP" should be increased. Additional "cold" NTPs can be added to the reaction to increase the proportion of full-length transcripts, however an increase in the yield of the full-length product will compromise the specific activity of the probe.

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