HyperScribe™ T7 High Yield Cy3 RNA Labeling Kit

Introductions

HyperScribe™ T7 High Yield Cy3 RNA Labeling Kit Plus is designed to generate randomly Cy3-modified RNA probes by in vitro transcription. Such probes are ideally suited for in situ hybridization and Northern blot hybridization experiments. The principle of labeling is similar to the basic labeling principle of a mixture of Cy3 RNA labels. Cy3-UTP is efficiently incorporated into RNA using the optimized reaction buffer and T7 RNA polymerase mixture in place of its natural counterpart UTP. An appropriate Cy3-UTP substitution typically achieves an optimal balance between reaction and labeling efficiency. The resulting Cy3-modified RNA probe can then be detected by fluorescence spectroscopy.

Materials

1. Components and storage

Size / Components	25 rxns	Storage
T7 RNA Polymerase Mix	50 µL	-20°C
10 × Reaction Buffer	50 µL	-20°C
ATP (20 mM)	50 µL	-20°C
GTP (20 mM)	50 µL	-20°C
UTP (20 mM)	37.5 µL	-20°C
CTP (20 mM)	50 µL	-20°C
Cy3-UTP (10 mM)	25 µL	-20°C
Control Template (0.5 µg/µL)	5 µL	-20°C
RNase-free H_2O	1 mL	-20°C

Shipping: Dry Ice **Shelf life:** 2 years

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.

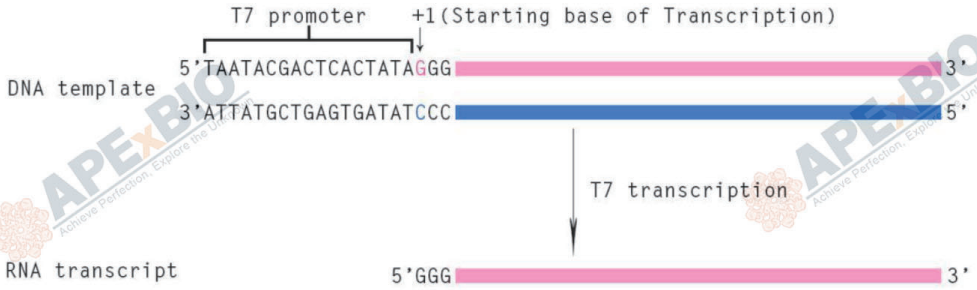

Figure 1: Transcription using T7 RNA Polymerase

1.1. Plasmid Templates

Many plasmid cloning vectors carry two phage polymerase promoters in different directions, one on each side of the multiple cloning site, 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.

After linearization, we recommend purifying the template DNA by phenol/chloroform extraction:

1. Extract DNA with an equal volume of 1:1 phenol/chloroform mixture, repeat if necessary.

2. Extract twice with an equal volume of chloroform to remove residual phenol.

3. Precipitate the DNA by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and two volumes of ethanol. Incubate at –20°C for at least 30 minutes.

4. Pellet the DNA in a microcentrifuge for 15 minutes at top speed. Carefully remove the supernatant.

5. Rinse the pellet by adding 500 µL of 70% ethanol and centrifuging for 15 minutes at top speed. Carefully remove the supernatant.

6. Air dry the pellet and resuspend it in nuclease-free water at a concentration of 0.5–1 µg/µL.

1.2. 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.

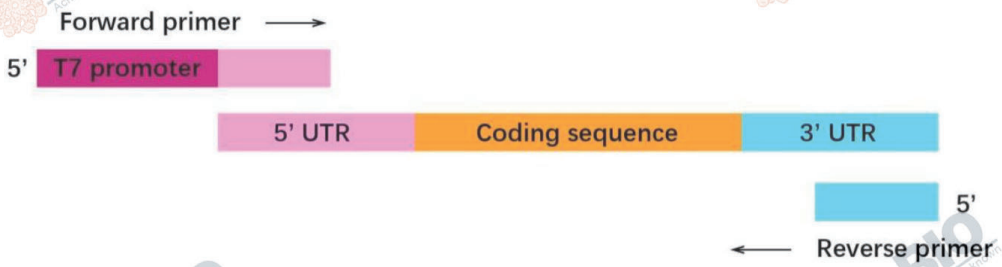

Figure 2: PCR primer design for T7 RNA Polymerase template

1.3. 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.

1.4. cDNA

In recent years, RNA transcription *in vitro* procedures have been gradually applied to aRNA 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. RNA Synthesis

2.1. Standard RNA Synthesis

RNA Synthesis with Cy3-UTP

(1) The recommended molar ratio of Cy3-UTP to standard UTP is 1:3 or 1:2.

(2) Thaw corresponding components on ice.

(3) Assemble the reaction system at room temperature in the following order：

Reagent	Volume	Final Concentration
Nuclease-free water	X μL	
10 x Reaction Buffer	2 μL	
ATP (20 mM)	2 μL	2 mM final
GTP (20 mM)	2 μL	2 mM final
CTP (20 mM)	2 μL	2 mM final
UTP (20 mM)	1.5 μL	1.5 mM final
Cy3-UTP (10 mM)	1 μL	0.5 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. Incubate the transcripts of short fragments (<300nt) for 4h.

(4) (Optional) Add 1μL of DNase I to the reaction system and incubate at 37°C for 15min to digest the DNA template. Compared with the product RNA, the content of template DNA is very low. Generally, it does not need to be removed, and it can also be digested with DNase I.

(5) Continue to purify the synthesized RNA or detect the transcription product by gel electrophoresis.

3. 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.

3.1. 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 μL5M 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 -80°C.

3.2. 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.

3.3. 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.

4. 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

5. Incorporation rate of fluorophore

The efficiency of RNA labeling can be estimated by calculating the ratio of incorporated fluorophores to the number of bases (dye /base).

Note! Blanc correction with probe buffer solution is required.

(a) Measurement of the nucleic acid-dye conjugate absorbance:

Measure the absorbance of the labeled RNA fragment at 260 nm (A_{260}) and at the excitation maximum (λexc) of dye (A_{dye}).

(b) Correction of A260 reading:

To obtain an accurate nucleic acid absorbance measurement, the contribution of the dye at 260 nm needs to be corrected. Use the following equation:

$A_{base} = A_{260} - (A_{dye} \times CF_{260})$

Correction Factor for Cy3: $CF_{260} = 0.08$

(c) Calculation of dye to base ratio by the law of Lambert-Beer (A = c x ε x d):

dye / base ratio = $(A_{dye} \times \varepsilon_{base}) / (A_{base} \times \varepsilon_{dye})$

Extinction coefficients:

Cy3: $\varepsilon_{dye} = 150{,}000\ cm^{-1}\ M^{-1}$

ssRNA: $\varepsilon_{base} = 12{,}030\ cm^{-1}\ M^{-1}$ (average, 50% GC)

(d) Calculation of the degree of labeling (DOL)

The degree of labeling (DOL) indicates the number of dyes per 100 bases. DOL = 100 x dye/base ratio

Example: A dye/base ratio of 0.02 corresponds to a DOL of 2 that corresponds to 2 dyes per 100 bases.

Notes

1. 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).

2. 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.

3. RNA Transcript Smearing on Denaturing Gel

If the RNA begins to appear degraded (e.g. smeared) 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).

4. 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.

5. 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.

APExBIO Technology
www.apexbt.com
7505 Fannin street, Suite 410, Houston, TX 77054.
Tel: +1-832-696-8203 | Fax: +1-832-641-3177 | Email: info@apexbt.com