miRNA 1st Strand cDNA Synthesis Kit (by stem-loop)

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50 rxns

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Intended for research use only

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Introduction

Intended Use

The T7 High Yield RNA Transcription Kit optimizes the transcription reaction system and enables users to efficiently obtain a large amount of RNA product through in vitro transcription reactions.

Principle of the Assay

The kit employs T7 RNA polymerases to transcribe the DNA sequence downstream of the T7 promoter in linear double-stranded DNA (dsDNA) to single-stranded RNA (ssRNA), using NTPs as substrates. The kit can also produce dye, biotin or radioactive-labeled RNA using modified nucleotides as substrates and also add cap structures or cap-like structures produce capped RNA.

One reaction (20 µl system) with this kit can produce 150 - 200 µg of RNA products, and the reaction can be amplified to produce milligram-grade RNA. RNA synthesized through transcription can be used for applications such as RNA structure and function studies, RNase I protection, probe hybridization, and RNA interference, and can also be capped with the vaccinia capping system or 2'-O-Methyltransferase. In addition, mRNA can be tailed with the E. coli Poly(A) Polymerase for in vitro translation, transfection, microinjection, or other downstream applications.

- ✓ Applications

  In vitro synthesis of ssRNA.

General Information

Materials Supplied

List of component

Component	Amount
T7 RNA Polymerase Mix	100 µL
10 × Transcription Buffer	100 µL
UTP Solution (100 mM)	100 µL
ATP Solution (100 mM)	100 µL
CTP Solution (100 mM)	100 µL
GTP Solution (100 mM)	100 µL
DNase I (1 U/µL)	50 µL
Control Template (0.5 µg/µL)	10 µL
RNase-free ddH2O	1 mL

Storage Instruction

Store at -30 ~ -15℃ and transport at ≤ 0℃.

Precautions for Use

For Research Use Only. Not for use in diagnostic procedures.

Assay Protocol

Reagent Preparation

1. Linearized plasmids with double-stranded T7 promoters, PCR products, or synthesized DNA fragments with double-stranded T7 promoters can be used as in vitro transcription templates for the T7 High Yield RNA Transcription Kit and can be dissolved in TE buffer or RNase-free ddH_2O.
2. Plasmid template

   The linearized plasmid containing the T7 promoter and GG initiation sequence can be used as a transcription template. The linearization and purity of the plasmid will affect the yield and integrity of the transcribed RNA product. Circular plasmids will get RNA products of different lengths in the process of transcription due to failure of effective termination. To obtain RNA with specific length, plasmids must be fully linearized. For the linearized plasmids, please make sure that double-stranded have blunt end or the end protruding 5' end of coding strand. It is recommended to add 1μg of template to each reaction.

   After the plasmid is linearized, it is recommended to be purified and then used as a template for in vitro transcription to avoid the influence of RNase, protein, RNA and salt residues in the system.
3. PCR product template

   PCR products with T7 promoters can be used as in vitro transcription templates. The T7 promoter (TAATACGACTCACTATAGGG) is added to the 5' end of the upstream primer of the non-template chain during PCR amplification of the template. PCR products can be used as templates directly without purification, although higher RNA yields will be obtained after purification.

   PCR products are transcriptional templates, and the uniformity of the products needs to be confirmed by electrophoresis. It is recommended to add 0.3-2 μg templates to each reaction system.
4. Synthesized DNA template

   Synthesized DNA fragments containing the T7 promoter and GG initiation sequence can also serve as transcription templates.

   It is recommended to add 0.3-2 μg templates to each reaction system.

Sample Preparation

1. Linearized plasmids, PCR products or synthetic DNA fragments with T7 promoters can be used as in vitro transcription templates for T7 High Yield RNA Transcription Kit 2.0, which can be dissolved by TE buffer or RNase-free Water.
2. Plasmid template (It is recommended to add 1μg linearized plasmid per reaction as template) Plasmids with T7 promoters can be used as transcriptional templates, and the linearization and purity of plasmids can affect the yield of transcription and the integrity of RNA. Circular plasmids templates will produce RNA products of different lengths because there is no termination. In order to obtain RNA of a specific length, the plasmid must be completely linearized, and the linearized plasmid must ensure that the double strand has a flat end or the 5 'end is a prominent structure.

3. PCR product template (it is recommended to add 0.1 μg to 1 μg per reaction as template) PCR products with T7 promoters can be used as transcriptional templates in vitro. In the PCR amplification of the template, the T7 promoter was added to the 5 'end of the upstream primer of the sense chain. PCR products need to be purified before used as templates.
4. Synthetic DNA template (it is recommended to add 0.1 μg to 0.5 μg per reaction as template) Synthetic DNA fragments with T7 promoters can also serve as templates for transcription in vitro.

Assay Procedure

1. In vitro transcription
   - ✓ Mix well the components except the T7 RNA Polymerase Mix by shaking, briefly centrifuge to collect the solution to the bottom of the tube, and place the tube on ice for later use.

   - ✓ Add the following components step-by-step:
     The DNA template needs to be added in the last step, because the 10 × Co-Reaction Buffer contains spermidine, and the high concentration of spermidine will cause the DNA template to precipitate.
     The reaction system should be configured at room temperature to prevent high concentration DNA template from precipitating at low temperature.

   - ✓ Mix the components thoroughly with a pipette. Collect the solution by brief centrifugation, and then incubate it at 37℃ for 2 h. If less than 0.3 kb of RNA is synthesized, the reaction can be extended to 4 h or longer, as the quality of the product is not affected by 16-hour overnight incubation.
     - *In order to avoid the impact of evaporation. it is recommended to react in the PCR instrument.*
     - *The reaction product may contain a white precipitate. It is the reaction process in which pyrophosphoric acid is hydrolyzed to phosphoric acid, and phosphoric acid forms magnesium phosphate with magnesium ions in the reaction solution, which does not affect the subsequent experiment. If you want to remove it, add EDTA to diminish it. If EDTA affects the subsequent experiment, the supernatant can also be recovered by centrifugation.*
     - *Keep RNase-free by using reagents, containers, etc.*

   - ✓ Add 1 μL of DNase I to the reaction system and incubate the solution at 37℃ for 15 min to digest the transcribed DNA template (optional).

2. Product purification

RNA transcription products can be purified with VAHTS RNA Clean Beads or FastPure RNA purification columns, or by phenol/chloroform-based or lithium chloride-based purification. Purified RNA, after determination by electrophoresis, can be used for downstream lab operations or stored at -80℃.

✓ Magnetic bead-based purification

Magnetic bead-based purification can remove proteins and free nucleotides.

Perform purification according to the Instructions for Use (IFU) of the magnetic beads.

✓ Column-based purification

Column-based purification can remove proteins and free nucleotides.

Before purification, add 80 μl of RNase-free ddH2O to dilute the product to 100 μl, and then perform purification according to the IFU of the column.

✓ Phenol/chloroform-based purification

Phenol/chloroform-based purification can remove proteins and most free nucleotides.

- Add 160 μl of RNase-free ddH2O to dilute the product to 180 μl.
- Add 20 μl of 3 M sodium acetate (pH 5.2) to the diluted product, and then mix thoroughly with a pipette.
- Add 200 μl of phenol/chloroform mix (1:1) for extraction. Centrifuge the solution at 10,000 rpm for 5 min at room temperature, and then transfer the upper solution (aqueous phase) to a new RNase-free EP tube.
- Add chloroform at a volume equal to the aqueous phase to extract twice, and then collect the upper aqueous phase.
- Add ethanol absolute at a two-fold volume, mix well, incubate the resultant solution at -20℃ for 30 min, centrifuge it at 15,000 rpm for 15 min at 4℃, and then discard the
- supernatant.
- Add 500 μl of precooled 70% ethanol to wash the RNA precipitate, centrifuge the
- solution at 15,000 rpm for 15 min at 4℃, and then discard the supernatant.
- Air-dry the residue for 2 min with lid open, and then add 20 - 50 μl of RNase-free ddH_2O or other buffers to dissolve the RNA precipitate.

3. Lithium chloride-based purification

   Lithium chloride-based purification can remove proteins and most free nucleotides.

   - Add 30 µl of RNase-free ddH2O to dilute the product to 50 µl.
   - Add 30 µl of the lithium chloride precipitation solution (7.5 M lithium chloride, 50 mM EDTA) into the diluted product.
   - Mix well, incubate the resultant solution at -20℃ for 30 min, centrifuge it at 12,000 rpm for 15 min, and then discard the supernatant.
   - Add 500 µl of precooled 70% ethanol to wash the RNA precipitate, centrifuge the solution at 15,000 rpm for 15 min at 4℃, and then discard the supernatant. Repeat this step three times.
   - Air-dry the residue for 2 min with lid open, and then add 20 - 50 µl of RNase-free ddH_2O or other buffers to dissolve the RNA precipitate.

Data Analysis

Calculation of Results

✓ RNA quantification

1. UV spectrophotometry

   Free nucleotides can affect the accuracy of quantification; thus RNA purification should be performed before quantification using this method. The yield of in vitro transcribed RNA is determined by measuring the A_{260} reading of the product.

   For ssRNA, 1 A_{260} ≈ 40 µg/ml, so the RNA concentration determined by this method is (A_{260} × 40 × dilution factor) µg/ml.

2. Dye-based quantification

   Perform RNA quantification using the RiboGreen dye, which is not affected by free nucleotides and can accurately quantify RNA in purified or unpurified in vitro transcripts. Perform RNA quantification according to the IFU of the dye

This reaction is highly sensitive to RNase, so RNase mixing in reaction system shall be strictly avoided, and it prohibits using RNase-free products as experimental equipment such as tip and EP tube.

Resources

Troubleshooting

1. Low Transcript Yield

   Templates are closely related to the yield. When the yield of the test group is significantly lower than that of the control group, the possible reasons are:

   - There are components inhibiting reactions in the test template;
   - Reasons due to the test template itself. Recommendations: a. re-purify the template; b. determine the template amount and integrity; c. prolong the reaction time at 37℃; d. increase the template input amount; e. try other promoters and RNA polymerases.

2. Low Transcript Yield from Short-Fragment Template

   Short template fragments can inhibit the reaction. Prolonged reaction time or increased template input amount can improve the RNA yield if the transcript is less than 0.3 kb. RNA yields can be maximized by 16-hour overnight reaction or by using templates of 2 μg.

3. Smeared Bands in Product Electrophoresis

   Smearing in product electrophoresis may be caused by:

   - RNase contamination during lab operations;
   - Use of RNase-contaminated DNA templates. RNase inhibitors in the system can only inhibit a trace of residual RNase; it is recommended to re-purify the template DNA, use RNase-free pipette tips and EP tubes, wear disposable latex gloves and masks during the experiment, and prepare all reagents with RNase-free ddH_2O.

4. Larger-Than-Expected RNA Product Fragment

   If electrophoresis results show larger-than-expected product bands, possible reasons may be:

   - The plasmid template may not be completely linearized;
   - Overhang at the 3' end of the sense strand of the linearized template;
   - There is a secondary structure in the RAN that is not completely denatured. It is recommended to check the template structure and verify the transcription results by denaturing electrophoresis.

5. Smaller-Than-Expected RNA Product Fragment

   If electrophoresis results show smaller-than-expected product bands, possible reasons may be:

   - The template sequence contains a sequence similar to the T7 RNA polymerase terminator;
   - The GC content of the template is high, and higher-order structures are formed;
   - RNase contamination. Different polymerases recognize different termination sequences. If the template contains a termination sequence, it is recommended to try different RNA polymerases. If the template has a high GC content, it is recommended to use SSB proteins to improve transcription efficiency.