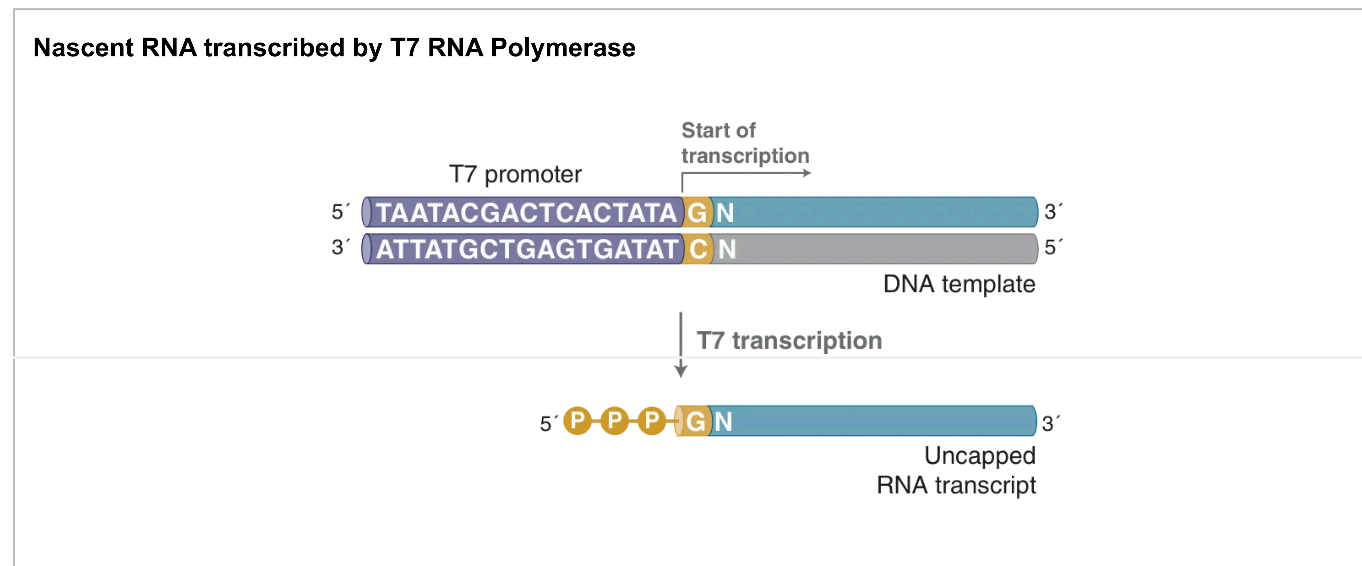
RNA Synthesis with Modified Nucleotides using the HiScribe® T7 High-Yield RNA Synthesis Kit (NEB #E2040)

PDF Copy Email

Overview

Incorporation of chemically modified ribonucleotides can improve the utility of mRNA by increasing stability, reducing immunogenicity, and enhancing translatability. This protocol can be used to synthesize 5' triphosphorylated RNA containing partial or complete substitutions of canonical nucleotides with base-modified nucleotides from a DNA template containing the T7 RNA Polymerase promoter sequence immediately followed by a guanosine. Modified ribonucleotides reduce transcription efficiency; therefore, lower transcription yields should be expected as compared to transcription using unmodified NTPs.



Materials Required but not Supplied

HiScribe® T7 High Yield RNA Synthesis Kit

- Nuclease-free Water (NEB #B1500)

T1

T2

T3

Related Resources

- Nucleotide Solutions for RNA
- Minding your caps and Poly A tails – Strategies for synthesizing *in vitro* transcribed (IVT) mRNA
- Scaling of High-Yield *In vitro* Transcription Reactions for Linear Increase of RNA Production
- Avoiding Ribonuclease Contamination
- Monarch RNA Purification Brochure
- A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers

Before starting

Template Considerations:

- DNA template should be purified prior to use and suspended in nuclease-free water. EDTA should not be present, and the solution should be free of salts.
- For RNA longer than 2kb, we recommend using linearized plasmid DNA as template. Completely linearized plasmid template of the highest purity is critical for successful IVT. NEB has a large selection of restriction enzymes; we recommend selecting restriction enzymes that generate blunt ends or 5' overhangs. For very long RNA, use high-fidelity (HF) restriction enzymes to minimize star activity, if possible.
- PCR products can be used as template but we recommend using a high-fidelity DNA polymerase, such as Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493/M0494). Though the PCR product can be directly used as template, better yields will be obtained with purified PCR products. PCR products should be examined on an agarose gel to confirm the presence of a single, robust band of the expected size. 0.1–0.5 µg of PCR product can be used in a standard 20 µl reaction.

Reaction Considerations:

- The ratio of base-modified nucleotides to canonical nucleotides may need to be determined empirically.
- We strongly recommend wearing gloves and using nuclease-free tubes (microfuge tubes or PCR strip tubes) and reagents to avoid RNase contamination.
- For reaction times of 60 minutes or less, a water bath or heating block may be used. For reaction times longer than 60 minutes, we recommend using a dry air incubator or a thermocycler to prevent evaporation.

Protocol

1. Thaw the necessary components at room temperature. Keep the T7 RNA Polymerase Mix on ice.
2. Mix and pulse-spin in a microfuge to collect the solutions to the bottom of the tubes.
3. Set up the reaction at **room temperature** in the order listed in the table below:

   3a. **Follow the Standard RNA Synthesis protocol for complete substitution with a base-modified NTP**.

   Base-modified nucleotides that NEB has tested that can fully substitute for the canonical NTP: N1-Methyl-Pseudo-UTP (NEB #N0431), 5-Methyl-CTP (NEB #N0432), Pseudo-UTP (NEB #N0433), 5-Methoxy-UTP (NEB #N0434).

3b. **An example of a 1/3 partial substitution with a base-modified UTP**

Base-modified nucleotides that NEB has tested that require partial substitution: Biotin-, Fluorescein-, Digoxigenin-, Aminoallyl-NTP, N6-methyladenosine

COMPONENT	20 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	
10X T7 Reaction Buffer	1.5 µl	0.75X
100 mM ATP	1.5 µl	7.5 mM
100 mM GTP	1.5 µl	7.5 mM
100 mM CTP	1.5 µl	7.5 mM
100 mM UTP	1 µl	5 mM
10 mM Modified UTP	5 µl	2.5 mM
Linear Template DNA	X µl	1 µg
DTT (0.1M)	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	

4. Mix thoroughly by pipetting and pulse-spin in a microfuge. Incubate at 37°C for 2 hours in a dry air incubator or thermocycler to prevent evaporation.
5. Reactions for short RNA transcripts (< 0.3 kb) should be incubated for 4 hours or longer. It is safe to incubate the reaction for 16 hours (overnight).

   Optional: To remove template DNA, add 30 µl nuclease-free water and 2 µl of DNase I (RNase-free) (NEB #M0303), mix, and incubate for 15 minutes at 37°C. Alternatively, 2 µl of DNase I-XT (NEB #M0570) can be added directly to the IVT product and incubated for 15 minutes at 37°C.
6. Proceed with purification of synthesized RNA and/or analysis of transcription products by gel electrophoresis. For purification, we recommend the 500 µg capacity Monarch RNA Spin Cleanup Kit (NEB #T2050).

   Optional: If a poly(A) tail is desired and is not encoded in the template plasmid, you can add one post-transcriptionally with *E. coli* Poly(A) Polymerase (NEB #M0276) using this protocol.

Videos

Tips for successful RNA Cleanup using the Monarch® RNA Cleanup Kits

Avoiding RNase Contamination

Product(s) utilizing this protocol:

- HiScribe® T7 High Yield RNA Synthesis Kit
- N1-Methyl-Pseudouridine-5´-Triphosphate (N1-Methyl-Pseudo-UTP)
- 5-Methyl-Cytidine-5´-Triphosphate (5-Methyl-CTP)
- Pseudouridine-5´-Triphosphate (Pseudo-UTP)
- 5-Methoxy-Uridine-5´-Triphosphate (5-Methoxy-UTP)