Capped mRNA Synthesis using Modified Nucleotides and the HiScribe® T7 ARCA mRNA Kit (NEB #E2065)

Materials Required but not Supplied

HiScribe® T7 ARCA mRNA Kit

- Nuclease-free Water (NEB #B1500)

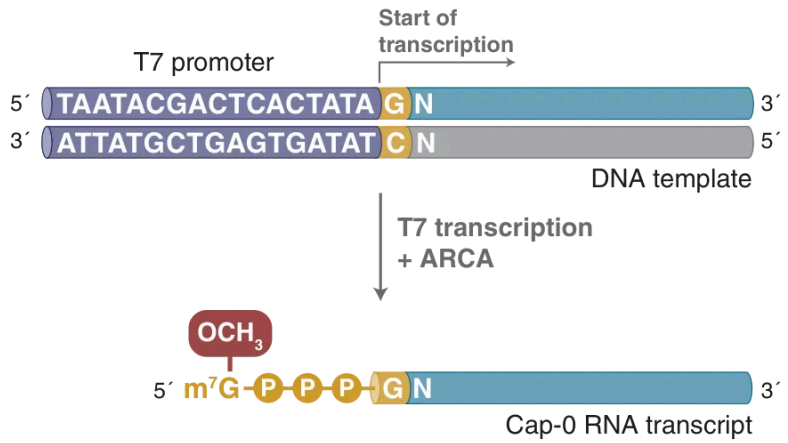
Overview

This example protocol can be used to synthesize cap-0 mRNA containing 50% 5mCTP and 50% N1-M-Pseudo-UTP from a linear DNA template containing the T7 RNA Polymerase promoter sequence immediately followed by a guanosine using the HiScribe® T7 ARCA mRNA Kit (NEB #E2065).

The provided cap structure, Anti-Reverse Cap Analog (ARCA) (NEB #S1411), is incorporated co-transcriptionally by T7 RNA Polymerase exclusively in the correct orientation due to a methyl group at the 3' position of the m7G cap.

This kit is not supplied with modified nucleotides, which must be purchased separately. Complete substitution with base-modified nucleotides is not possible due to the premixed ARCA/NTP solution provided. Up to 2.5 mM total base-modified UTP and/or CTP can be added into the transcription reaction without significantly impacting the mRNA yield. Modified GTP and ATP should not be used because they will interfere with capping and tailing efficiency. For complete modified nucleotide substitution to generate cap-1 mRNA, we recommended using the HiScribe® T7 mRNA Kit with CleanCap® Reagent AG (NEB #E2080), in which the cap analog and all four nucleotides are supplied separately.

Figure 1: T7 RNA Polymerase co-transcriptionally caps RNA with anti-reverse cap analog



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:

- 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:

COMPONENTS	20 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	-
2X ARCA/NTP Mix	10 µl	1 mM GTP, 4 mM ARCA, 1.25 mM CTP, 1.25 mM UTP, >1.25 mM ATP final
10 mM N1-Methyl-Pseudo-UTP (NEB #N0431)	2.5 µl	1.25 mM
10 mM 5-Methyl-CTP (NEB #N0432)	2.5 µl	1.25 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 30 minutes in a dry air incubator or thermocycler to prevent evaporation.

   Reactions for short RNA transcripts (< 0.3 kb) should be incubated for 1 hour or longer. It is safe to incubate the reaction for 16 hours (overnight).

5. To remove template DNA, add 2 µl of DNase I (RNase-free) (NEB #M0303), mix, and incubate for 15 minutes at 37°C.

6. Proceed with purification of synthesized RNA and/or evaluation of transcription product yield and/or length. For purification, we recommend the 50 µg capacity Monarch RNA Spin Cleanup Kit (NEB #T2040).

   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.

Related Resources

- 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
- A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers