Protocol for Co-transcriptional capping using CleanCap® Reagent AG from TriLink and HiScribe™ T7 High Yield RNA Synthesis Kit (NEB #E2040)

Materials

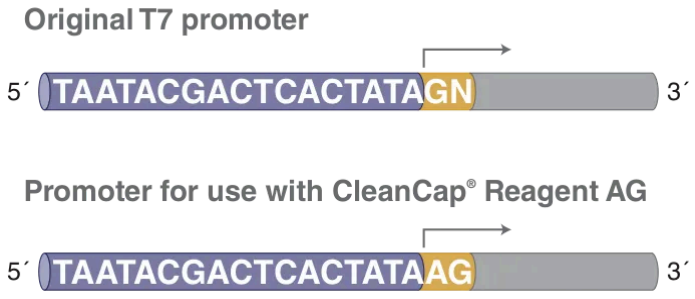
- HiScribe™ T7 High Yield RNA Synthesis Kit (NEB #E2040)
  - Includes: T7 Reaction Buffer (10X), ATP (100 mM), GTP (100 mM), UTP (100 mM), CTP (100 mM), FLuc Control Template (0.5 µg/µl), T7 RNA Polymerase Mix, Dithiothreitol (DTT) (0.1 M)

Overview

This protocol can be used to co-transcriptionally incorporate user-supplied trinucleotide cap analogs, such as CleanCap® AG or AG (3' OMe) from TriLink, into IVT RNA using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB #E2040). Unlike standard RNA synthesis with NEB #E2040, reactions with this protocol are typically 40 µl with 5mM each NTP. NEB does offer a separate HiScribe® T7 mRNA synthesis kit that is supplied with CleanCap® Reagent AG (NEB #E2080), which has its own dedicated protocol.

By using a DNA template with a T7 promoter sequence followed by an AG initiation sequence and an encoded poly(A) tail, mRNAs can be transcribed with a 5´-m7G Cap-1 structure that is polyadenylated, translationally competent, and able to evade the cellular innate immune response.

****IMPORTANT NOTE ABOUT TEMPLATE SEQUENCE:** CleanCap AG requires modification to the sequence immediately downstream of the T7 promoter sequence, replacing the "GG" that follows the T7 promoter sequence with an "AG" as follows:



The use of CleanCap Reagent AG with templates containing the standard T7 promoter sequence with GG will result in RNA with triphosphorylated 5' ends. If you need to change your template plasmid DNA sequence to "AG", we recommend using our Q5 Site-Directed Mutagenesis Kit (NEB #E0554).

General Guidelines:

We strongly recommend wearing gloves and using nuclease-free tubes (microfuge tubes or PCR strip tubes) and reagents to avoid RNase contamination. This reaction can be scaled as needed; please see our application note, Scaling of High-Yield In vitro Transcription Reactions for Linear Increase of RNA Production.

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 following order:

COMPONENTS	40 µl REACTION	FINAL CONCENTRATION
Nuclease-free Water	X µl	
10X T7 Reaction Buffer	2 µl	0.5X
100 mM ATP	2 µl	5 mM
100 mM GTP	2 µl	5 mM
100 mM UTP *	2 µl	5 mM
100 mM CTP **	2 µl	5 mM
100 mM CleanCap® AG (N-7113) **or** AG (3' OMe) (N-7413)	1.6 µl	4 mM
Linear Template DNA	X µl	1 µg
DTT (0.1M) ***	2 µl	5 mM
T7 RNA Polymerase Mix	4 µl	

*UTP can be completely substituted with N1-Methyl-Pseudouridine-5´-Triphosphate (NEB #N0431) and Pseudouridine-5'-Triphosphate (NEB #N0433).

** CTP can be completely substituted with 5-Methyl-Cytidine-5´-Triphosphate (NEB #N0432) and 5-Methoxy-Uridine-5'-Triphosphate (NEB #N0434).

*** Addition of DTT (5mM final) to the reaction is optional but recommended. The RNA polymerase in the kit is sensitive to oxidation and could result in lower RNA yield over time due to repeated handling, etc., and adding DTT to the reaction may help restore the kit performance in such cases. Adding DTT will not compromise the reaction in any situation.

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.

   Optional: DNase treatment to remove DNA template. To remove template DNA, add 50 µl nuclease-free water, 10 µl of 10X DNase I Buffer, 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

5. Proceed with purification of synthesized RNA an/or analysis of transcription products by gel electrophoresis. For purification, we recommend the 500 µg capacity Monarch RNA Cleanup Kit (NEB# T2050).

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

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