INSTRUCTION MANUAL

HiScribe™ T7 mRNA Kit with CleanCap® Reagent AG

NEB #E2080S/L

20/100 reactions

Version 5.1_10/25

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The HiScribe T7 mRNA Kit with CleanCap Reagent AG Includes:

All kit components should be stored at –20°C. Each kit contains sufficient reagents for 20 x 20 µl reactions (S size) or 100 x 20 µl reactions (L size).

T7 RNA Polymerase Mix

T7 CleanCap Reagent AG Reaction Buffer (10X)

ATP (60 mM)

GTP (50 mM)

UTP (50 mM)

CTP (50 mM)

CleanCap Reagent AG (40 mM)

CLuc AG Control Template (0.25 mg/ml)

DNase I (RNase-free) (2,000 units/ml)

LiCl Solution (7.5 M LiCl, 10 mM EDTA)

Dithiothreitol (DTT) (0.1 M)

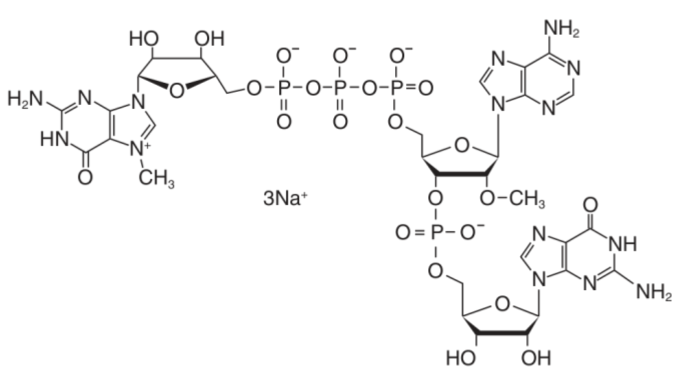
Required Materials Not Included:

DNA Template:	The DNA template must be linear and contain the T7 RNA Polymerase promoter with correct orientation in relation to target sequence to be transcribed, followed by an AG initiation sequence
Modified-NTP:	N1-Methyl-Pseudouridine-5'-Triphosphate (NEB #N0431) 5-Methyl-Cytidine-5'-Triphosphate (NEB #N0432) Pseudouridine-5'-Triphosphate (NEB #N0433) 5-Methoxy-Uridine-5'-Triphosphate (NEB# N0434) Biotin-, Fluorescein-, Digoxigenin-, or Aminoallyl-NTP
General:	Thermal cycler, microcentrifuge, nuclease-free water, nuclease-free tubes and tips
Purification:	Phenol, chloroform, ethanol and 3 M sodium acetate, pH 5.2 or Ammonium Acetate, Monarch® RNA Cleanup Kit (500 µg; NEB #T2050), equipment and reagents for RNA quantitation
Gel Analysis:	Gels, running buffers, loading dye, nucleic acid ladders, gel apparatus, power supply

Introduction

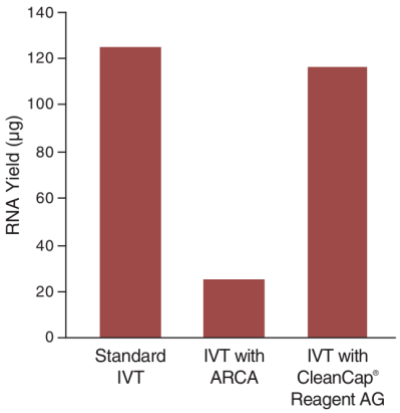
Most eukaryotic mRNAs require a 5’ 7-methyl guanosine (m7G) cap structure and a 3’ poly(A) tail for efficient translation. The HiScribe T7 mRNA kit with CleanCap Reagent AG utilizes an optimized RNA synthesis formulation and trinucleotide cap analog technology for co-transcriptionally capping mRNAs that contain a natural Cap-1 structure (Figure 1) in a single simplified reaction without compromising RNA yield. 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.

Figure 1. Structure of CleanCap Reagent AG.



The HiScribe T7 mRNA kit with CleanCap Reagent AG is formatted with individual vials of NTPs and CleanCap Reagent AG to allow for partial or complete substitution of base-modified NTPs. By using a DNA template encoding a poly(A) tail, this kit can be used to generate capped and tailed mRNAs. Cap-1 mRNA synthesized from this kit is suitable for many applications, including transfections, microinjections, *in vitro* translation, preclinical mRNA therapeutic mRNA studies, as well as RNA structure and function analysis. Unlike *in vitro* transcription reactions that utilize Anti-Reverse Cap Analog (ARCA) for co-transcriptional capping, where expected yields are significantly decreased due to lower concentration of GTP to accommodate ARCA incorporation, the HiScribe T7 mRNA kit with CleanCap Reagent AG results in no loss of yield when compared to a standard synthesis reaction where no cap analog is present (Figure 2).

Figure 2. Comparison of RNA Yields from *In Vitro* Transcription Reactions with no cap analog, ARCA, or CleanCap Reagent AG.



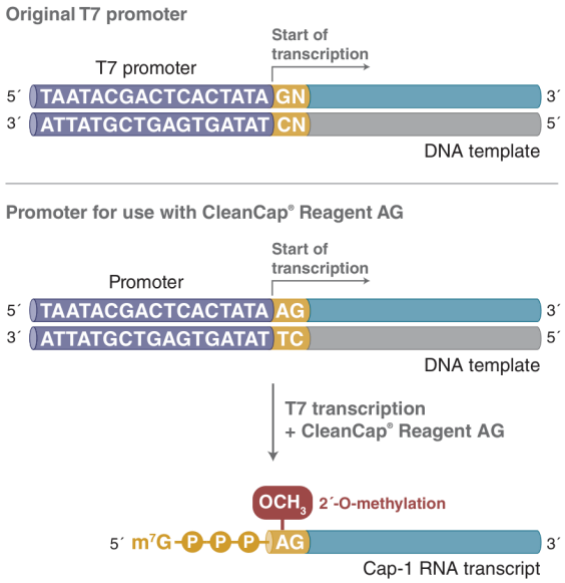
All reactions were performed with 5 mM CTP, 5 mM UTP and 6 mM ATP. Standard IVT reactions included 5 mM GTP and no cap analog. ARCA reactions contained a 4:1 ratio of ARCA:GTP (4 mM:1 mM). IVT with CleanCap Reagent AG contained 5 mM GTP and 4 mM CleanCap Reagent AG and was performed as described below (Standard mRNA Synthesis). Reactions were incubated for 2 hours at 37°C, purified and quantified by NanoDrop®.

This kit includes DNase I (RNase-free) and Lithium Chloride Solution for template removal and quick mRNA purification. Each standard reaction yields ≥ 90 µg of Cap-1 unmodified RNA from 1 µg CLuc AG Control Template DNA. Each kit can yield ≥ 1.8 mg RNA (S size) or ≥ 9 mg RNA (L size). Reactions may be scaled linearly; please see our application note.

DNA Template Preparation

Linearized plasmid DNA, PCR products or synthetic DNA oligonucleotides can be used as templates for *in vitro* transcription with the HiScribe T7 mRNA Kit with CleanCap Reagent AG. These sequences must contain a double-stranded T7 promoter region (5′ TAATACGACTCACTATA 3′), immediately followed by an AG initiation sequence, upstream of the sequence to be transcribed (Figure 3).

Figure 3. T7 promoter sequence and changes to initiating bases for compatibility with CleanCap Reagent AG.



Plasmid Templates

Completely linearized plasmid templates of the highest purity are critical for the successful use of the HiScribe T7 mRNA Kit with CleanCap AG Reagent. The quality of the DNA template affects the yield and integrity of the RNA as the highest yield is achieved with the highest purity template.

Plasmids purified by many laboratory methods can be successfully used, provided the preparations contain mostly supercoiled DNA and are free from contaminating RNases, protein, RNA and salts.

Plasmids normally used for *in vitro* transcription with T7 RNA Polymerase using standard NTPs or cap analogs will need to be modified to contain the +1 A and +2 G just downstream of the promoter. We recommend using the Q5® Site-Directed Mutagenesis Kit (NEB #E0554) to create the site-specific mutation(s).

To produce RNA transcript of a defined length, plasmid DNA must be completely linearized with a restriction enzyme downstream of the insert to be transcribed. Circular plasmid templates will generate long heterogeneous RNA transcripts in higher quantities because of the high processivity of T7 RNA polymerase. NEB has a large selection of restriction enzymes; we recommend selecting restriction enzymes that generate blunt ends or 5′-overhangs. If possible, we also recommend using High-Fidelity (HF) restriction enzymes to limit star activity, especially for long RNA.

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.

PCR Templates

PCR products containing T7 RNA Polymerase promoter with an AG initiation sequence in the correct orientation can be transcribed with the HiScribe T7 mRNA Kit with CleanCap Reagent AG. We recommend using Q5® Hot Start High-Fidelity DNA Polymerase (NEB #M0493/M0494). Though PCR mixture can be used directly, better yields will be obtained with purified PCR products. PCR products can be purified according to the protocol for plasmid restriction digests above, or by using commercially available spin columns (we recommend Monarch Spin PCR & DNA Cleanup Kit, NEB #T1130). PCR products should be examined on an agarose gel to estimate concentration and to confirm amplicon size prior to its use as a template. Depending on the PCR products, 0.1–0.5 µg of PCR fragments can be used in a 20 µl *in vitro* transcription reaction.

Synthetic DNA Oligonucleotides

Synthetic DNA Oligonucleotides which are either entirely double-stranded or mostly single-stranded with a double-stranded T7 promoter sequence can be used with the HiScribe T7 mRNA Kit with CleanCap Reagent AG. In general, the yields are relatively low and also variable depending upon the sequence, purity, and preparation of the synthetic oligonucleotides. We do not recommend using synthetic oligonucleotides directly as template for IVT RNA greater than 100 nucleotides.

Co-transcriptional Cap-1 mRNA Synthesis With CleanCap Reagent AG

We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 20 µl but can be scaled up linearly as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

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	
10X T7 CleanCap Reagent AG Reaction Buffer	2 µl	1X
60 mM ATP	2 µl	6 mM
50 mM GTP	2 µl	5 mM
50 mM CTP*	2 µl	5 mM
50 mM UTP*	2 µl	5 mM
40 mM CleanCap Reagent AG	2 µl	4 mM
Linear Template DNA	X µl	1 µg
DTT (0.1M)	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	

* *The following base-modified nucleotides can completely replace the canonical NTP: N1-Methyl-Pseudo-UTP (NEB #N0431), 5-Methyl-CTP (NEB #N0432), Pseudo-UTP (NEB #N0433), 5-Methoxy-UTP (NEB #N0434).*

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. For reaction times of 60 minutes or less, a water bath or heating block may be used. The yield will not be compromised if the incubation temperature is within the range of 35–40°C.

   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.

5. Proceed with purification of synthesized RNA and/or evaluation of transcription products yield and/or length.

   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).

Purification of Synthesized RNA

In general, RNA synthesized by *in vitro* transcription can be purified by LiCl precipitation, phenol-chloroform extraction followed by ethanol precipitation, or by using a spin column-based method (*preferred*).

LiCl Precipitation

LiCl precipitation can be used for quick recovery of the synthesized RNA and is an effective method for removing the majority of unincorporated NTPs and enzymes. However, RNAs shorter than 300 bases or at concentrations lower than 0.1 mg/ml do not precipitate well. In such cases, other purification methods may be used.

Protocol

1. Adjust the reaction volume to 50 µl by adding nuclease-free water.
2. To the 50 µl reaction, add 25 µl LiCl solution and mix well.
3. Incubate at –20°C for 30 minutes.
4. Centrifuge at 4°C for 15 minutes at top speed to pellet the RNA.
5. Remove the supernatant carefully.
6. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.
7. Remove the ethanol carefully. Spin the tube briefly to bring down any liquid on the wall.
8. Remove residual liquid carefully using a sharp tip (e.g., loading tip).
9. Air dry the pellet and resuspend the mRNA in 50 µl of 0.1 mM EDTA or a suitable RNA storage solution.
10. Heat the RNA at 65°C for 5-10 minutes to completely dissolve the RNA. Mix well.
11. Store the RNA at –20°C or below.

Phenol-chloroform Extraction and Ethanol Precipitation

For removal of proteins and most of the free nucleotides, phenol-chloroform extraction and ethanol precipitation of RNA transcripts is the preferred method.

Protocol

1. Adjust the reaction volume to 180 µl by adding nuclease-free water. Add 20 µl of 3 M sodium acetate (pH 5.2) or 20 µl of 5 M ammonium acetate and mix thoroughly.
2. Extract with an equal volume of 1:1 phenol:chloroform mixture, followed by two extractions with chloroform. Collect the aqueous phase and transfer it to a new tube.
3. Precipitate the RNA by adding 2 volumes of ethanol. Incubate at –20°C for at least 30 minutes and collect the pellet by centrifugation.
4. Remove the supernatant carefully.
5. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.
6. Remove the ethanol carefully. Spin the tube briefly to bring down any liquid on the wall.
7. Remove residual liquid carefully using a sharp tip (e.g., loading tip).
8. Air dry the pellet and resuspend the mRNA in 50 µl of 0.1 mM EDTA or a suitable RNA storage solution.
9. Store the mRNA at –20°C or below.

Spin Column Chromatography

Spin columns will remove unincorporated nucleotides, proteins, and salts. We recommend using a Monarch Spin RNA Cleanup Kit (10 µg capacity NEB #T2030, 50 µg capacity #T2040, or 500 µg capacity #T2050) with the Monarch® Spin RNA Cleanup Kit Protocol.

Adjust the volume of the reaction mixture to 100 µl by adding nuclease-free water to the IVT product and mix well. Purify the RNA by following the manufacturer's instructions. Be aware that some reactions could produce 90 µg of RNA, which may exceed individual column capacity and require additional columns.

Evaluation of Reaction Products

Quantification by UV Light Absorbance

RNA concentration can be determined by measuring the ultraviolet light absorbance at 260 nm. However, any unincorporated nucleotides, CleanCap Reagent AG, and template DNA in the mixture will affect the reading, so must be removed before the RNA concentration can be quantified.

A Nanodrop spectrophotometer can directly read RNA concentrations from 10 ng/µl to 3000 ng/µl; it may be necessary to dilute your RNA prior to measurement. For single-stranded RNA, 1 A_{260} is equivalent to an RNA concentration of 40 µg/ml. The RNA concentration can be calculated as follows: A_{260} x dilution factor x 40 = __ µg/ml RNA.

Analysis of Transcription Products by Gel Electrophoresis

To evaluate transcript length, integrity and quantity, an aliquot of the transcription reaction should be run on an appropriate denaturing agarose or polyacrylamide gel. Transcripts larger than 0.3 kb can be run on agarose gels, whereas denaturing polyacrylamide gels (5–15%) are necessary for smaller transcripts. The gels should be run under denaturing conditions to minimize formation of secondary structures by the transcript.

Sample preparation

1. Prepare denatured samples by mixing 0.05–200 ng RNA sample with 10-20 µl of RNA Loading Dye, 2X (NEB #B0363).
2. Denature the RNA sample and an aliquot of RNA marker by heating at 70°C for 10 minutes.
3. Pulse-spin prior to loading onto the gel.

Preparation of denatured samples and gels

4a. **Denaturing agarose gel:**

Load 100-200ng denatured RNA sample.

It is common practice to electrophorese RNA on a fully denaturing agarose gel, such as one containing formaldehyde. However, in many cases it is possible to run RNA on a native agarose gel and obtain suitable results. In fact, it has been demonstrated that treatment of RNA samples in a denaturing buffer (outlined below) maintains the RNA molecules in a denatured state for at least 3 hours during electrophoresis. The use of native agarose gels eliminates problems associated with toxic chemicals and the difficulties encountered when staining and blotting formaldehyde gels.

4b. **Denaturing PAGE/Urea Gel:**

Load 50-100ng denatured RNA sample.

We recommend using commercially available premade gels and standard TBE gel running buffer (10X TBE buffer: 0.9 M Tris Base, 0.9 M Boric Acid, 20 mM EDTA).

Visualize RNA

5. Stain the gel post-electrophoresis with SYBR® Gold (preferred) or ethidium bromide.

mRNA Quality Analysis by BioAnalyzer or Capillary Electrophoresis

RNA quality can also be assessed by Capillary Electrophoresis, BioAnalyzer or comparable technologies. Please refer to the manufacturers' protocols.

Troubleshooting

Control Reaction

The CLuc AG control template is a linearized plasmid containing the *Cypridina lucifierase* gene under the transcriptional control of the T7 promoter. The initiating sequence has been changed to an AG by site-directed mutagenesis to be compatible with CleanCap Reagent AG. The size of the run-off transcript is ~1.76 kb. The control reaction, following the standard reaction protocol, should yield ≥ 90 µg of RNA in 2 hours at 37°C.

If the control reaction is not working, there may be technical problems during reaction set up. Repeat the reaction by following the protocol carefully and supplement with DTT to a final concentration of 5 mM; take any precaution to avoid RNase contamination. Contact NEB for technical assistance.

The control plasmid sequence can be found within the DNA Sequences and Maps Tool under the name "pCMV-CLuc2 AG Control Plasmid". The CLuc AG control template is generated by linearizing the plasmid with the restriction enzyme XbaI.

Low Yield of Full-length RNA

If the transcription reaction with your template generates full-length RNA, but the yield is significantly lower than expected, it is possible that contaminants in the DNA template are inhibiting the RNA polymerase, or the DNA concentration may be incorrect. Alternatively, additional purification of DNA template may be required. Phenol-chloroform extraction is recommended (see template DNA preparation section).

Addition of DTT

The RNA polymerase in the kit is sensitive to oxidation and could result in lower RNA yield over time due to repeated handling etc. Adding DTT to the reaction may help restore the kit performance in such cases. Adding DTT will not compromise the reaction in any situation.

Low Yield of Short Transcript

High yields of short transcripts (< 0.3 kb) are achieved by extending incubation time and increasing the amount of template. Incubation of reactions up to 16 hours (overnight) or using up to 2 µg of template will help to achieve maximum yield.

RNA Transcript Smearing on Denaturing Gel

If the RNA appears degraded (e.g. smeared) on a denaturing agarose or polyacrylamide gel, DNA template is contaminated with RNase. DNA templates contaminated with RNase can affect the length and yield of RNA synthesized (a smear below the expected transcript length). If the plasmid DNA template is contaminated with RNase, perform phenol/chloroform extraction, then ethanol precipitate and dissolve the DNA in nuclease-free water (see template DNA preparation section).

RNA Transcript of Larger Size than Expected

If the RNA transcript appears larger than expected on a denaturing gel, template plasmid DNA may be incompletely digested. Even small amounts of undigested circular DNA can produce large amounts of long transcripts. Check template for complete digestion and if undigested plasmid is confirmed, repeat restriction enzyme digestion.

Larger size bands may also be observed when the RNA transcript is not completely denatured due to the presence of strong secondary structures.

RNA Transcript of Smaller Size than Expected

If denaturing gel analysis shows the presence of smaller bands than the expected size, it is most likely due to premature termination by the polymerase. Some sequences which resemble T7 RNA Polymerase termination signals will cause premature termination. Incubating the transcription reaction at lower temperatures, for example at 30°C, may increase the proportion of full-length transcript, however the yield will be decreased. For GC rich templates, or templates with secondary structures, incubation at 42°C may improve yield of full-length transcript.

Ordering Information

NEB #	PRODUCT	SIZE
E2080S/L	HiScribe T7 mRNA Kit with CleanCap Reagent AG	20/100 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
T2050S/L	Monarch Spin RNA Cleanup Kit (500 µg)	10/100 preps
T2040S/L	Monarch Spin RNA Cleanup Kit (50 µg)	10/100 preps
T2030S/L	Monarch Spin RNA Cleanup Kit (10 µg)	10/100 preps
B0363S	RNA Loading Dye (2X)	4 x 1 ml
M0303S/L	DNase I (RNase-Free)	1,000/5,000 units
M0570S/L	DNase-XT	1,000/5,000 units
M0493S/L	Q5 Hot Start High-Fidelity DNA Polymerase	100/500 units
M0494S/L	Q5 Hot Start High-Fidelity 2X Master Mix	100/500 units
N0362S	ssRNA Ladder	25 gel lanes
E0554S	Q5 Site-Directed Mutagenesis Kit	10 reactions
E0552S	Q5 Site-Directed Mutagenesis Kit (Without Competent Cells)	10 reactions
N0364S	Low Range ssRNA Ladder	25 gel lanes
M0276S/L	*E. coli* Poly(A) Polymerase	100/500 units
N0450S/L	Ribonucleotide Solution Set	10/50 µmol of each
N0431S	N1-Methyl-Pseudouridine-5′-Triphosphate	0.1 ml
N0432S	5-Methyl-Cytidine-5′-Triphosphate	0.1 ml
N0433S	Pseudouridine-5′-Triphosphate	0.1 ml
N0434S	5-Methoxy-Uridine-5′-Triphosphate	0.1 ml
C2987H/I	NEB 5-alpha Competent *E. coli* (High Efficiency)	20 x 0.05 ml/ 6 x 0.2 ml
B9020S	SOC Outgrowth Medium	100 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	10/21
2.0	Change > 0.3 kb incubations in Step 4 on page 5 and 6 to < 0.3 kb incubations.	2/22
2.1	Updated location to find control plasmid to DNA Sequences and Maps Tool (page 8).	10/22
3.0	Updated to include addition of DTT. Also updated table formatting and legal footer.	7/23
4.0	Updated to include new L size.	1/24
4.1	Updated Step 8, page 7, to 0.1 mM EDTA.	2/24
5.0	Updated standard protocol with details on full substitution with modified nucleotides and removed "optional" note about DTT. Removed modified nucleotide protocol. Updated other protocols for consistency.	10/25
5.1	Added Figure 2, bar graph comparing yield.	10/25