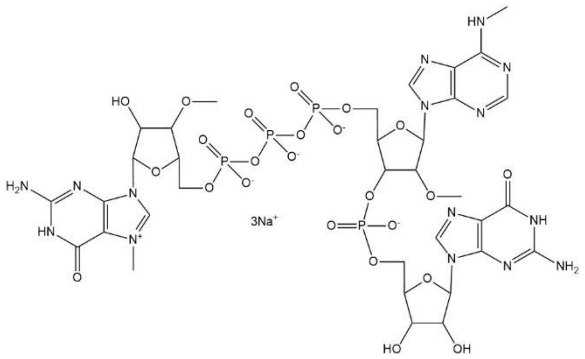
CleanCap® Reagent M6

CleanCap m6AG 3'OMe for Co-transcriptional Capping of mRNA
Catalog No. N-7453

Description

CleanCap Reagent M6, otherwise known as CleanCap m6AG 3'OMe, is designed for the co-transcriptional capping of mRNA to produce a base-modified Cap 1 mRNA. Cap 1 mRNAs have superior in vivo activity compared to Cap 0 mRNA produced by legacy capping methods such as mCap or anti-reverse cap analog (ARCA). CleanCap M6 may further increase protein expression relative to previous generations of cap analogs, such as CleanCap AG or CleanCap AG (3'OMe), or mRNAs produced by enzymatic capping strategies [1]. CleanCap M6 can be used in conjunction with wildtype bases or TriLink's catalog of modified NTPs.



CleanCap Reagent M6 may be ordered using the following catalog numbers:
N-7453-1 (1 µmol)
N-7453-5 (5 µmol)
N-7453-10 (10 µmol)
N-7453-100 (100 µmol)

For larger quantities, or to inquire about GMP-grade CleanCap M6 please visit our website.

Using the conditions described here, transcription with CleanCap M6 results in > 95%[1] capped material, generating a Cap 1 structure and crude yields of 4 mg to 5 mg per mL of transcription. This user guide also describes an optional 2x RNA mass increase through a pulse feed reaction.

Use & Handling

100 mM in H_2O | Store at or below -20 °C. | Upon first use, prepare single-use aliquots. | Use only certified RNase-free reagents and consumables with proper RNase-free technique.

QC Analysis

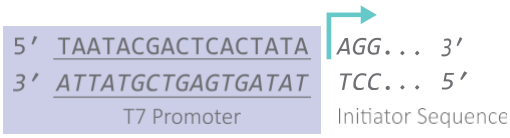
AX-HPLC	Mass Spec
^{31}P NMR	Concentration
^{1}H NMR	Conductivity
pH	

Product released by Quality Assurance. TriLink is certified ISO 9001:2015.

[1]Final capping is dependent upon the CleanCap Reagent, DNA template, and final mRNA sequence. Secondary structure due to RNA length and base composition can affect final capping efficiency, mRNA yield, and translation efficiency.

Template Design

Template design is an integral part of any transcription. CleanCap M6 is to be used with the initiating sequence 5′ AG 3′. The figure below shows the correct T7 promoter sequence (underlined) and initiator sequence (italics) for CleanCap M6.



Customer Supplied Materials

NOTE: All reagents must be RNase free. Use recommended source or equivalent grade.

Required Reagents

- DNA Template
- Nucleoside-5′-Triphosphate (NTP) Set (TriLink cat. no. N-1505)
  Also available individually for use with modified NTPs. See Related Products for commonly used modified NTPs.
- T7 RNA polymerase (New England BioLabs cat. no. M0251S)
- Inorganic Pyrophosphatase (yeast) (New England BioLabs cat. no. M2403S)
- Murine RNase Inhibitor (New England BioLabs cat. no. M0314S)
- 1 M Tris-HCl (pH 7.5), RNase Free (Invitrogen cat. no. 15567-027)
- Dithiothreitol (DTT) (EMD Millipore cat. no. 3860-5GM)
- Spermidine (Sigma Aldrich cat. no. 85558-1G)
- 6N HCl (Sigma-Aldrich no. H1758)
- 1 M Magnesium Chloride (Sigma-Aldrich no. 63069)
- UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific cat. no. 10977015)

Optional Reagents

- RNaseZap™ RNase Decontamination Solution (Thermo Fisher Scientific cat. no. AM9780)
- DNase I (RNase-free) (New England BioLabs cat. no. M0303S)
- $CaCl_2$ (Calcium chloride solution, BioUltra, 1M) (Sigma Alrdich cat. No. 21115)
- RNeasy Kits (QIAGEN cat. no. 74104 or 75144)

Protocol

RNase-Free Techniques

It is essential that all reagents be rigorously RNase-free. Use disposable RNase-free tubes and bottles. Surfaces and pipettes can be wiped down with RNaseZap to destroy RNases. When possible, use dedicated RNase-free pipettes. Avoid using pipettes that have been used for plasmid preparation using RNase A.

10X Transcription Buffer w/ HCl

400 mM Tris-HCl (pH 7.5)
100 mM DTT
21.2 mM Spermidine
160 mM $MgCl_2$
150 mM HCl
DNase/RNase-Free Water

NOTE: To ensure highest capping efficiency, this 10X reaction buffer Ŏ2yűl·ƛyã ŜÈúNl· l /t ƨdzúăƛŘŜ 2Ŧ űKŜ ŌdzŦŦŜNƛyƎ Nl·yƎŜ 2Ŧ ¢NƛåΦ Do not titrate the 10x buffer to achieve a target pH. Preparing tKƛå 10x reaction buffer with HCl NŜădzfűå ƛy l· f2ø LJ l óғHú, K2øŜØŜNΣ 2yŎŜ l·ăåŜY ōfŜŘ ƛyú2 Ŧdzff м· IVT NŜl·Ŏűƛ2y with all components űKŜ LJ l is close to neutral (~6.8).

10x Transcription buffer can be premixed and aliquoted into single use volumes for -20 °C storage up to 1 month.

Protocol

Transcription Reaction

Add reagents in the prescribed order to ensure efficient transcription and capping. Ensure each component is homogenous before use. Store thawed enzymes on ice. These reaction conditions have been tested with templates up to 6 kb in length.

1. Add RNase-free water and NTPs to the reaction tube.
2. Add CleanCap M6 to the tube and vortex to mix. Spin briefly to collect liquid.
3. Add 10X Transcription Buffer w/ HCl as prepared on page 2. Vortex. Spin briefly to collect liquid.
4. Add DNA template.
5. Add Murine RNase Inhibitor, Inorganic Pyrophosphatase, and T7 RNA Polymerase.
6. Mix well by flicking or inverting the tube 10 times and spin briefly to collect liquid.
7. Incubate at 37 °C for 3 hours*.
   *OPTIONAL: After 2 hours of incubation at 37 °C proceed to Pulse Feed IVT protocol below to nearly double the mass of RNA synthesized.
   NOTE: If the optional pulse feed protocol is used, we recommend keeping the 10x reaction buffer and each NTP at 4 °C after the initial reaction set up to avoid an extra freeze thaw. Prepare the Spike-In mix ~5 minutes before use.

Table 2: Reaction Components

Component	Final Concentration	100 µL Rxn
DNase/RNase-Free Water	Up to 100 µL	Up to 100 µL
ATP (100 mM)	5 mM	5 µL
CTP[2] (100 mM)	5 mM	5 µL
GTP (100 mM)	5 mM	5 µL
UTP[2] (100 mM)	5 mM	5 µL
CleanCap M6 (100 mM)	10 mM	10 µL
10X Transcription Buffer w/ HCl	1X	10 µL
DNA template	50 or 25 µg/mL[3]	5 µg or 2.5 µg[3]
Murine RNase Inhibitor (40 units/µL)	1 unit/µL	2.5 µL
Inorganic Pyrophosphatase (0.1 units/µL)	0.002 units/µL	2 µL
T7 RNA Polymerase (50 units/µL)	15 units/µL	30 µL
Total Volume		**100 µL**

[2] Modified NTP can be used in place of wild-type. If using a modified NTP, use at the same concentration as the replaced wild-type NTP.

[3] Final Concentration of DNA template should be 50 µg/mL for a plasmid template or 25 µg/mL for a PCR template.

*Optional Protocol

Pulse Feed Transcription Reaction (continued from Step 7 above)

8. Prepare a spike-in mix + 10% overage as described in Table 3 in a new tube.

   NOTE: For best results prepare the Spike-In mix ~ 5 minutes before use.
9. Vortex to mix. Spin briefly to collect liquid.
10. Temporarily remove 100 µL reaction tube from heat. Mix well by flicking or inverting 10 times and spin briefly to collect the liquid.
11. Add 35 µL of Spike-In mix to the original 100 µL reaction tube. Pipet to mix. Cap the tube and mix well by flicking or inverting 10 times. Spin briefly to collect liquid.
12. Incubate 135 µL pulse-fed reaction at 37°C for an additional 2 hours.

Table 3: Pulse Feed Reaction Components (Spike-In Mix)

Component	Final Concentration after addition to IVT	Spike-In + 10% overage for a 100 µL IVT	Volumes delivered to a 100 µL IVT
ATP (100 mM)	4 mM	5.94 µL	5.4 µL
CTP[4] (100 mM)	4 mM	5.94 µL	5.4 µL
GTP (100 mM)	4 mM	5.94 µL	5.4 µL
UTP[4] (100 mM)	4 mM	5.94 µL	5.4 µL
10X Transcription Buffer w/ HCl[5]	1X	14.85 µL	13.5 µL
Total Volume		**38.61 µL**	**35 µL**

[4] Modified NTP can be used in place of wild-type. If using a modified NTP, use at the same concentration as the replaced wild-type NTP.

[5] Final reaction buffer after spike-in is added will be 1.74x

Post-transcriptional Options

Purifications

IVT reactions may be purified by any traditional methods such as lithium chloride precipitation or spin columns (for example, QIAGEN RNeasy mini or midi kit) for higher purity at small scales. A fixed reaction will typically result in 4-5 mg of RNA per mL of reaction using wildtype NTPs or N1-methylpsuedoUTP whereas a pulse feed reaction typically produces 8-10 mg of RNA per starting mL of reaction at approximately 7.5 mg/mL concentration.

DNase Treatment

DNase treatment may be used per vendor-recommended protocol following first purification (above) and followed with a second small-scale RNA clean up OR by recommended one-pot IVT/DNase reaction below.
For best one-pot DNase results formulate the final IVT reaction in 2 mM $CaCl_2$ with 20 U/µg of template DNA used in IVT at a final dilution of 4.8x the ending IVT volume by water. Mix by inverting or gently flicking the tube and pulse spin to collect liquid. Incubate for 20 minutes at 37 °C.

Example DNase Reaction Set up:

Component	Final Concentration	Fixed 100 µL IVT	Pulse-Fed 135 µL IVT
IVT Reaction	N/A	100 µL	135 µL
$CaCl_2$ (200 mM)	2 mM	4.8 µL	6.5 µL
DNase (2 U/µL)	20 U/µg of template DNA	13.7 µL[6]	18.5 µL[6]
Water	Variable	361.5 µL	488 µL
Total Volume	**4.8x IVT volume**	**480 µL**	**648 µL**

[6] Volumes shown here based on IVT reaction using 25µg/mL DNA template

Troubleshooting

5-methoxy-UTP is known to slow the reaction kinetics in this transcription recipe and may result in 3-4 mg/mL crude mRNA with proportionally lower reaction yields after pulse feed.

If lower than expected yields are observed after the pulse feed method but fixed IVT protocol results in ~5 mg/mL crude mRNA the timing of NTP/1x reaction buffer spike-in may need optimizations. Consider comparing 2 and 3 hour yields and extending pulse-feed incubations accordingly.

For other FAQ's please see our website: TriLink BioTechnologies – CleanCap M6

Related TriLink Products

Nucleoside-5'-Triphosphate (NTP) Set (cat. no. N-1505)
- Adenosine-5'-Triphosphate, ATP (cat. no. N-1510)
- Cytidine-5'-Triphosphate, CTP (cat. no. N-1511)
- Guanosine-5'-Triphosphate, GTP (cat. no. N-1512)
- Uridine-5'-Triphosphate, UTP (cat. no. N-1513)

5-Methylcytidine-5'-Triphosphate (cat. no. N-1014)
Pseudouridine-5'-Triphosphate (cat. no. N-1019)
N^1-Methylpseudouridine-5'-Triphosphate (cat. no. N-1081)
5-Methoxyuridine-5'-Triphosphate (cat. no. N-1093)
CleanCap Reagent AG (cat. no. N-7113)
CleanCap Reagent AG (3' OMe) (cat. no. N-7413)
CleanCap Reagent AU (cat. no. N-7114)

Related TriLink Services

TriLink offers custom CleanCap Cap 1 mRNA production services in addition to the CleanCap Reagents. Select CleanCap Reagents are also available in a GMP-grade format. Visit our website or contact us at sales@trilinkbiotech.com to learn more.

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7. Termination. Your right to have and use the Products will terminate immediately if Buyer fails to comply with the terms and conditions of this Agreement. Upon such termination of rights, Buyer shall destroy all Products, or any components or derivatives thereof, and notify TriLink BioTechnologies, LLC of such in writing.
8. Miscellaneous. This Agreement sets forth the complete and entire agreement of the Parties with respect to the subject matter hereof and supersedes and terminates all prior agreements and understandings between the Parties. No subsequent amendment or addition to this Agreement shall be binding upon the Parties unless reduced to writing and signed by the respective authorized officers of the Parties. This Agreement shall not be assigned or otherwise transferred by the buyer.