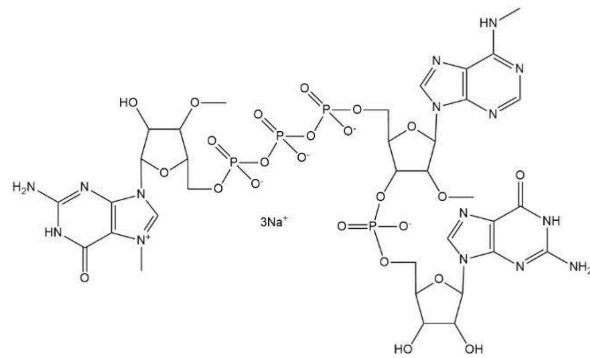
CleanCap® Reagent M6

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

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. CleanCap M6 can be used in conjunction with wildtype bases or TriLink’s catalog of modified NTPs.



MODIFIED IVT PROTOCOL REQUIRED

For the best results, use the M6-specific IVT transcription buffer described here. Transcription with CleanCap M6 and these conditions typically results in > 95%[1] capped material, generating a Cap 1 structure with approximate crude yields of 4 to 5 mg/mL of RNA.

CAUTION: using this cap analog in conventional IVT reactions will likely result in significantly lower capping efficiency and/or low mRNA yields.

ADDITIONAL HIGH-YIELD PULSE FEED PROTOCOL INCLUDED

The pulse feed IVT reaction can result in up to 2x RNA yield improvements without impacting mRNA quality attributes by adding additional NTPs and buffer without additional cap analog.

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.

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

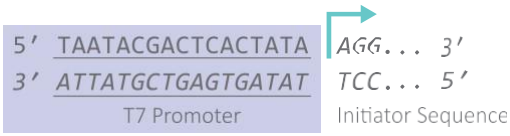
AX-HPLC	Mass Spec
^{31}P NMR	Concentration
^{1}H NMR	Conductivity
pH	

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)*
- 1N HCl (Fisher Scientific cat. no. SA48)
- 1 M Magnesium Chloride (Sigma-Aldrich cat. 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-Aldrich cat. no. 21115)
- RNeasy Kits (QIAGEN cat. no. 74104 or 75144)

***NOTE: Preparation of 5M Spermidine Stock Solution**

Undiluted spermidine stock (~6.37M) is a viscous liquid that is difficult to pipette. To facilitate easier handling, we recommend making a 5M dilution by adding 296 µL of nuclease-free water to a new 1g bottle of 85558-1G and vortexing for a minimum of 60 seconds. This 5M stock solution can then be aliquoted and stored at -20°C for at least 6 months. If using a larger starting mass than 1g, scale the amount of water added accordingly.

Protocol

RNase-Free Techniques

It is essential that all reagents be 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 M6 IVT Transcription Buffer Preparation

Reagent Preparation

Add reagents in the prescribed order to ensure efficient transcription and capping. Ensure each component is well-mixed and homogenous before use, which is especially important for viscous reagents like spermidine.

Table 1: 10x M6 IVT Transcription Buffer

Component	10x Concentration	Example 1000 µL 10x M6 Transcription Buffer
DNase/RNase-Free Water	N/A	185.8 µL
Tris pH 7.5 (1M)	400 mM	400 µL
HCl (1M)	150 mM	150 µL
MgCl2 (1M)	160 mM	160 µL
DTT (1M)	100 mM	100 µL
Spermidine (5M)	21.2 mM	4.24 µL

NOTE: To enable the highest capping efficiency, this 10X M6 transcription buffer contains extra HCl outside the buffering range of Tris. Do not titrate the 10x M6 transcription buffer to achieve a target pH. Preparing this 10x M6 transcription buffer with HCl results in a low pH (<2), however, once assembled into full 1X IVT reaction with all components the pH is close to neutral (~6.8).

Standard-Yield Protocol

Reagent Preparation

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 M6 Transcription Buffer as prepared according to Table 1. 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.

Table 2: Standard-Yield 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 M6 Transcription Buffer	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.

High-Yield Pulse Feed Transcription Protocol

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 M6 Transcription Buffer as prepared according to Table 1. 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 2 hours. We recommend keeping the 10x M6 transcription buffer and NTPs at 4 °C after the initial reaction set up to avoid an additional freeze thaw.
8. Approximately 5 minutes before the 2-hour incubation point, prepare the Spike-In mix in a separate tube, making sure to include 10% overage to account for pipetting error.
9. Vortex to mix. Spin briefly to collect liquid.
10. Add the appropriate volume of Spike-In mix (35 µL of Spike-In per 100 µL initial volume IVT) to the original reaction tube. Pipette to mix. Cap the tube and mix well by flicking or inverting 10 times. Spin briefly to collect liquid.
11. Incubate pulse-fed reaction at 37°C for an additional 2 hours (4 hours total).

Table 3: High-Yield Pulse Feed Transcription Protocol

	Component	Initial IVT Mixture Concentration	Volume
Initial IVT Mixture	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 M6 Transcription Buffer	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**
Incubate at 37 °C, 2 hours			
	Component	**Concentration Change After Pulse Feed**	**Volume**
Spike-In Mixture	ATP (100 mM)	Additional 4 mM	5.94 µL
	CTP[2] (100 mM)	Additional 4 mM	5.94 µL
	GTP (100 mM)	Additional 4 mM	5.94 µL
	UTP[2] (100 mM)	Additional 4 mM	5.94 µL
	10X M6 Transcription Buffer	Additional 0.74X[4]	14.85 µL
	Spike-In + 10% overage for 100 µL IVT		**38.61**
	Volume of Spike-In to add to 100 µL IVT		**35 µL**
	Final High-Yield Transcription Volume		**135 µL**
Incubate at 37 °C, 2 hours			

[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

[4]The final Reaction Buffer will be 1.74X after addition of the Spike-In Mix

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-methylpseudoUTP whereas a pulse feed reaction typically produces 8-10 mg of RNA per starting mL of reaction at approximately 7.5 mg/mL concentration (crude).

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.

Table 4: Example DNase Reaction Setup

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	25 µL[6]	25 µL[6]
Water	Variable	350.2 µL	481.5 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

Using this cap analog in conventional IVT reaction conditions will likely result in significantly lower capping efficiency and/or low mRNA yields. For the best results, use the M6-specific IVT transcription buffer described here.

Undiluted spermidine is viscous, use slow and careful pipetting to ensure the most accurate volume measurements.

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 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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