CELLSCRIPT™
RNA for Translation in Cells

AmpliCap™ SP6 High Yield Message Maker Kit

Cat. No. **C-AC0706**

INTRODUCTION

The AmpliCap™ SP6 High Yield Message Maker Kit produces capped RNA by *in vitro* transcription using SP6 RNA polymerase and the standard cap analog m^7G[5']ppp[5']G. A 2 hour, 20 µl AmpliCap reaction yields up to 35 µg of m^7G-capped RNA from 1 µg of the 1.4 kb standard SP6 control template DNA.

The m^7G[5']ppp[5']G cap analog/NTP PreMix contains all four ribonucleotides and the monomethylated cap analog. The PreMix ensures the optimal concentration of each NTP and ratio of methylated cap analog to GTP (4:1), maximizing transcript capping (~80%) and yield. Because the concentration of GTP in the reaction is limiting, the cap analog is preferentially incorporated as the first or 5'-terminal G of the transcript.

CELLSRIPT also offers the AmpliCap-Max™ T7 High Yield Message Maker Kit and the MessageMAX™ T7 ARCA-Capped Message Transcription Kit, both of which produce unsurpassed yields of capped *in vitro* transcripts (up to **60 µg** of RNA in a **30 minute** standard control reaction with an 80% capping efficiency). The AmpliCap-Max Kit uses the standard RNA Cap Analog m^7G[5']ppp[5']G and the MessageMAX Kit uses the anti-reverse cap analog $m_2^{7,3'\text{-}O}$G[5']ppp[5'].

MATERIALS

Materials Supplied

Store at –20°C in a freezer without a defrost cycle. Do not store at –70°C.

AmpliCap™ SP6 High Yield Message Maker Kit Contents (25 reactions)	
Kit Component	Volume
AmpliCap™ SP6 Enzyme Solution	50 µl
AmpliCap™ Cap/NTP PreMix (18.75 mM ATP, CTP & UTP; 3.75 mM GTP & 15 mM m^7G[5']ppp[5']G)	132.5 µl
20 mM GTP	50 µl
10X AmpliCap™ SP6 Transcription Buffer	50 µl
100 mM Dithiothreitol (DTT)	50 µl
ScriptGuard™ RNase Inhibitor	15 µl
RNase-Free Water	1.4 ml
SP6 Control Template DNA, 0.5 µg/µl	10 µl
RNase-Free DNase I, 1 U/µl	25 µl

Inquire about custom kit sizes at 608-442-6484 or sales@cellscript.com.

SP6 Control Template DNA: Is a linearized 4.1 kb plasmid that contains an SP6 promoter followed by a phage lambda dsDNA insert that encodes a 1,375 base runoff transcript. The Control Template DNA is provided at a concentration of 0.5 µg/µl in $T_{10}E_1$ Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

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Materials Required, but not Supplied

- A DNA template for transcription of your RNA of interest
- Materials or kits for purification of the RNA product
  (For suggestions, see Section C "Purification of the Transcription Product")
- RNase-free TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- Optional: TE saturated phenol/chloroform

SPECIFICATIONS

Storage Buffers

RNase-Free DNase I is provided in a 50% glycerol solution containing 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$ and 10 mM $MgCl_2$. All other enzymes are provided in a 50% glycerol solution containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 0.1% Triton® X-100.

Unit Definitions

One unit of RNase-Free DNase I digests 1 μg of pUC19 DNA to oligodeoxynucleotides in 10 minutes at 37°C.

One unit of ScriptGuard RNase Inhibitor results in 50% inhibition of 5 ng of RNase A. Activity is measured by the inhibition of hydrolysis of cyclic 2',3'-CMP by RNase A.

Functional Testing

The AmpliCap SP6 High Yield Message Maker Kit is functionally tested under standard reaction conditions using the SP6 Control Template DNA. The kit must produce at least 30 μg of RNA from 1 μg of the SP6 Control Template DNA in 2 hours at 37°C.

Contaminating Activity Assays

All components of the AmpliCap SP6 High Yield Message Maker Kit are free of detectable RNase and DNase activity, except for the inherent activity of the RNase-Free DNase I component.

BEFORE YOU START: IMPORTANT TIPS FOR OPTIMAL TRANSCRIPTION AND CAPPING

♦ **Template Requirements**:

The optimal templates for *in vitro* transcription are linear double-stranded DNA (dsDNA) molecules with 5'-protruding ends. DNA templates with blunt ends are less preferable and **templates with 3'-protruding ends should not be used**.

Transcription templates can be prepared from clones of the DNA to be transcribed in plasmids or other circular dsDNA vectors by linearizing the vectors downstream of the cloned DNA using a suitable restriction endonuclease or other means.

Alternatively, transcription templates can be generated by PCR amplification of RNA or DNA of interest using a strategy that results in joining of an SP6 promoter to the appropriate end of the PCR product (e.g., wherein, the SP6 promoter is either joined to the DNA or RNA that is amplified or is incorporated into one of the PCR primers).

- **Template Efficiency**:

  In vitro transcription of 1 μg of the SP6 Control Template DNA using the AmpliCap SP6 High Yield Message Maker Kit yields approximately 35 μg of ~1.4-kb RNA in 2 hours at 37°C in a standard 20 μl reaction.

  However, yields vary for different templates based on the template sequence, structure, length, purity and the sequence and length of the particular RNA polymerase promoter. Examples of contaminants that can affect transcription yield include RNase or contaminants such as phenol, trace metals and SDS. See the Technical Appendix for suggestions related to template purification.

- **Reaction Yields**:

  The standard reaction conditions give excellent results with most templates. The protocol may need to be modified to increase yields of some templates. To increase yields of some templates the protocol can be modified as follows:

  1) Extend the incubation to four hours. **Do not** extend the reaction time beyond 4 hours.
  2) Raise the template concentration.
  3) Increase the reaction temperature to 42°C.

- **Optimizing Yields for Long Templates**:

  Synthesis of transcripts ≥5 kb may require the addition of 1-2 μl of 20 mM GTP to the standard protocol. While this may decrease the percentage of capped transcript to 50-60%, it will increase the yield of full length transcript.

- **Maintaining an RNase-Free Environment**:

  Highly stable RNases are ubiquitous, including on human skin.

  Creating an RNase-free work environment and maintaining RNase-free solutions is critical for successful work with RNA.

  We strongly recommend to:

  – Use RNase-free tubes and pipette tips.

  – Always wear gloves when handling kit components or samples containing RNA and change gloves frequently, especially after touching potential sources of RNase contamination such as door knobs, pens, pencils and human skin. Do not touch any kit component or tube containing RNA with an ungloved hand.

  – Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.

PROCEDURE

A. Synthesis of Capped RNA

1. Set up the transcription reaction **at room temperature** by adding the reagents **in the order indicated below**:

Standard AmpliCap SP6 Reaction	
Component	Amount
RNase-Free Water	x µl
Linearized template DNA with SP6 RNAP promoter	1 µg
10X AmpliCap SP6 Transcription Buffer	2 µl
AmpliCap Cap/NTP PreMix	5.3 µl
100 mM DTT	2 µl
ScriptGuard RNase Inhibitor	0.5 µl
AmpliCap SP6 Enzyme Solution	2 µl
Total Reaction Volume	20 µl

! Assemble transcription reactions at room temperature in the order indicated at left. Assembly of transcription reactions at <22°C or in an alternate order, can result in the formation of an insoluble precipitate.

10X AmpliCap SP6 Transcription Buffer stored at –70°C may result in the formation of a white precipitate. To dissolve it, heat the tube at 37°C for 5 minutes and mix thoroughly.

One microgram of DNA template is recommended for most reactions. If the DNA template is <0.12 µg/µl, concentrate it, then resuspend in the appropriate amount of RNase-Free Water.

2. Incubate at 37°C for 2 hours.

B. DNase I Treatment of IVT Reaction

1. DNase I treatment is used to remove the DNA template from the IVT reaction.

Standard DNase I Treatment of IVT Reaction	
Component	Amount
IVT Reaction (from Step A)	20 µl
RNase-Free DNase I	1 µl
Total Reaction Volume	21 µl

2. Incubate for 15 minutes at 37°C.

3. Proceed to RNA Purification.

C. Purification of the Transcription Product

Purify the RNA using your preferred method. The method chosen should remove residual proteins and unincorporated NTPs from the RNA. Several options are listed below. RNA can be stored at –20°C or –70°C. For long-term storage, RNA can be stored as an ethanol pellet.

I) **Ammonium Acetate Precipitation**: Selectively precipitates RNA, while leaving most of the protein, DNA and unincorporated NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Add one volume of 5 M ammonium acetate (21 µl for the standard reaction), mix well.
2) Incubate for 15 minutes on ice.
3) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
4) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
5) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
6) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.
7) While usually unnecessary, steps 1-6 may be repeated a second time for even cleaner RNA.
8) Allow the pellet to dry, then resuspend in 30-50 µl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap1 RNA (e.g., with CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase Kit).
9) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

II) **Organic Extraction / Ammonium Acetate Precipitation**: Removes all proteins and selectively precipitates RNA, while leaving most of the DNA and unincorporated NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Adjust reaction volume to 50 µl total using RNase-Free Water (add 29 µl to the reaction).
2) Add one volume (50 µl) of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
3) Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
4) Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
5) Add one volume (50 µl) of 5 M ammonium acetate, mix well then incubate for 15 minutes on ice.
6) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
7) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
8) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
9) Allow the pellet to dry, then resuspend in 30-50 µl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap1 RNA (e.g., with CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase Kit).
10) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

Continued on next page.

III) **Organic Extraction / Chromatography / Ethanol Precipitation**: Removes all proteins, digested DNA, and unincorporated NTPs from the RNA.

1) Adjust reaction volume to 50 µl total using RNase-Free Water (add 29 µl to the reaction).
2) Add one volume (50 µl) of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
3) Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
4) Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
5) Remove digested DNA and unincorporated NTPs by spin column chromatography.[1] For commercially-available columns, follow the manufacturer's instructions for this step. Recover the RNA in 50-100 µl.
6) Add one-tenth volume (5-10 µl) of 3 M sodium acetate and 2.5 volumes (125-250 µl) of 95% ethanol to the tube, mix well.
7) Incubate for 15 minutes on ice.
8) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
9) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
10) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
11) Allow the pellet to dry, then resuspend in 30-50 µl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap1 RNA (e.g., with CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase Kit).
12) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

IV) **RNA-Binding Purification Column**: Several options are available commercially from multiple vendors. Follow the manufacture's recommended protocol.

1) Follow the manufacture's recommended protocol.
2) The final resuspension of RNA should be in 30-50 µl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap1 RNA (e.g., with CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase Kit).
3) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

TROUBLESHOOTING

Symptom	Solution
Low yields or less than full-length transcripts	Cleanup the templates to remove any RNase or other contaminants (see Technical Appendix for procedure).
	Verify that ScriptGuard RNase Inhibitor was added to the reaction.
	Extend the incubation to four hours. Do not extend the reaction time beyond 4 hours.
	Increase the template concentration.
	Increase the reaction temperature to 42°C.
Assembled reaction formed an insoluble precipitate	Repeat assembly of the reaction at >22°C.
White precipitate in reaction buffer	Incubate the reaction buffer at 37°C for 5 minutes then mix thoroughly to dissolve the precipitate.
	Do not store the kit at –70°C.

RELATED PRODUCTS

– A-Plus™ Poly(A) Polymerase Tailing Kit
– AmpliCap™ T7 High Yield Message Maker Kit
– AmpliCap-Max™ T7 High Yield Message Maker Kit
– ARCA, Anti-Reverse Cap Analog
– MessageMAX™ T7 ARCA-Capped Message Transcription Kit
– mScript™ mRNA Production Systems
– ScriptCap™ 2'-O-Methyltransferase Kit
– ScriptCap™ m^7G Capping System
– ScriptGuard™ RNase Inhibitor
– SP6 RNA Polymerase

REFERENCE

1. Sambrook, J. et al., (1989) Molecular Cloning: A Laboratory Manual (2nd ed.), New York, Cold Spring Harbor Laboratory Press.

TECHNICAL APPENDIX

A. Clean-up of Problematic Templates

Templates that give low yields or less than full-length transcripts may contain RNase or other contaminants. Such templates usually give better results after the following treatment:[1] See Purification of the Transcription Product Section III, skip step 4.

1) Add Proteinase K to 100-200 μg/ml and SDS to 0.5%.
2) Incubate for 30-60 minutes at 37°C.
3) Extract with an equal volume of a 1:1 mixture of TE-saturated phenol/chloroform.
4) Ethanol precipitate.
5) Gently remove the supernatant and rinse the pellet with 70% ethanol.
6) Resuspend in RNase-Free TE Buffer.

The performance of this product is guaranteed for one year from the date of purchase.

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