

Standard Operating Procedure (S.O.P) for High Yield Capped RNA *in vitro* Transcription using the SP6 Promoter and the mMESSAGE mMACHINE Kit

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The purpose of this S.O.P. is to perform *in vitro* synthesis of large amounts of capped RNA under the control of the SP6 or T7 promoters using the mMESSAGE mMACHINE kit from Ambion/Invitrogen (cat. no. AM1340 and AM1344). Capped RNA mimics most eukaryotic mRNA found *in vivo*. The mMESSAGE mMACHINE have a simplified reaction format in which all four ribonucleotides and cap analog are mixed in a single solution. The DNA template to be transcribed could be linearized plasmids or PCR products. This kit is ideal for the routine synthesis of capped RNAs for oocyte microinjection, *in vitro* translation, transfection, and other applications.

SP6 Promoter: 5'-ATTTAGGTGACTACTATA**G**AAGNG-3'

T7 promoter: 5'-TAATACGACTCACTATA**G**GGAGA-3'

where the **G in bold** in the Sp6 promotor sequence is the first nucleotide incorporated into the cRNA during transcription.

Different factors could affect the cRNA transcripts includes,

- cRNA size to be synthesized. The kit function best with templates that code for cRNA ranging between 0.3 to 5 kb. The kit could be optimized for shorter or longer transcripts.
- Orientation of the cRNA. If sense cRNA is required to express a protein of interest, be sure the promoter is upstream to the 5'-coding region of the protein. If the cDNA for the protein is contained in a plasmid, it must be linearized downstream to the polyA sequence in the 3'-coding region. For antisense cRNA, the promoter must be located downstream to the polyA sequence.
- DNA template. The DNA sample should be free of contaminants, including RNA and Proteins. It is preferable to purify the DNA sample with a commercially available purification kit before proceeding with this S.O.P. If RNase or other protein is still suspected to be in the DNA template after purification, treatment with Proteinase K may be helpful.
- Linearization of the DNA template. It is recommendable to check by gel electrophoresis that the template is completely linearized. Because the RNA polymerase is very processive, circular DNA templates generate long and heterogenous RNA transcripts. Even small amount of circular DNA will produce a heterogenous population of RNA transcripts.
 - It has been reported low levels of transcription when the template is linearized with restriction enzymes that leave 3' hanging heads.
 - Restriction enzymes occasionally introduce RNase or other inhibitors of transcription. Purify the linearized DNA sample with a commercially available purification kit before proceeding with this S.O.P.

Important:

- **If the incubators and bath have not been recently used, check that the temperature is correctly set by using a calibrated thermometer.**
- **Be sure to use the appropriate kit. mMESSAGE mMACHINE kit for other promoters (SP6, T7, and T3) are available.**
- **Tips and microtubes must be autoclaved using RNase-free parameters. If RNase-free tips are not available, use tips with filters.**

- The DNA template must have the correct RNA polymerase promoter site upstream of the sequence to be transcribed.
- All reagents, except the 10X reaction Buffer, must be kept on ice while using the kit, especially the nucleotides and enzyme mixes.
- Enzyme should be the last component added to the reaction and should be taken from the freezer at the right moment of adding it to the reaction. *Keep the enzyme on ice all the time.*
- Let the 10X Reaction Buffer to reach room temperature before assembling the transcription reaction.
- Strictly, follow the order in which they are added to the transcription reaction and keep it at room temperature to avoid coprecipitating the DNA with the spermidine in the 10X Reaction Buffer.
- This S.O.P. is recommended for RNA between 300 to 5000 base pairs.
- Usually, the yield of the transcription reaction reaches 80% after 1 hour incubation, but incubations times up to 6 hours are allowed, especially when using no optimal DNA templates.
- The transcription reaction can be scaled up or down if desired.
- All volumes are given on a per tube basis.

Materials:

- 0.5 µL Microtube
- 2 and 10 µL Micropipettes and Tips
- Linearized DNA Containing the Appropriate Promoter (in water or TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 7-8])
- 70% Ethanol for Disinfection
- Nuclease-free Water (room temperature)
- RNA Polymerase Enzyme Mix (SP6 or T7; Stored at -20°C)
- 10X Reaction Buffer (Stored at -20°C)
- 2X NTP/CAP (Stored at -20°C)
- Turbo DNase (Stored at -20°C)
- Non-shaker Incubator at 37 °C
- Dry Bath at 37 °C (optional for Turbo DNase incubation)
- Ice Bucket
- Centrifuge for 0.5 µL Tubes
- Vortex

Procedure:

This procedure is designed for setting-up a transcription reaction in a volume of 20 µL. *If more cRNA is required, the reaction can be scaled-up by multiplying each component of the reaction by n-fold. E.g., if you need to double the reaction, multiply each component by 2 (2-fold).*

- a) Equilibrate the non-shaker incubator/dry bath at 37 °C for at least 30 minutes before placing the transcription reaction mixture.
- b) Sanitize working area with 70% ethanol before starting procedure. Prepare ice bucket for reagents and label all the microtubes to be use properly.
- c) Thaw the frozen reagents. Vortex the **10X Reaction Buffer** and **2X NTP/CAP** until they are completely in solution. Once thawed, keep **2X NTP/CAP** on ice and the **10X Reaction Buffer** at

room temperature. Keep **Enzyme Mix** on ice, it does not need to be thawed. **All reagents should be centrifuged briefly before opening to prevent loss and contamination of material that may be present around the rim of the tube.**

- d) Calculate the volume of DNA template required to add 1 µg of the linearized DNA template and the volume nuclease-free water needed to complete the 20 µL of the Reaction mix. The sum of the volumes for DNA and water must not exceed 6 µL; therefore, for each µL of DNA that is required 1 µL of water must be subtracted.

Assemble transcription reaction at room temperature. In a 0.5 µL microtube add the reactants in the following order. **The 10X Reaction Buffer must be added after water and 2X NTP/CAP.**

1. 10 µL of 2X NTP/CAP and wait 10 minutes to allow the solution to reach room temperature.
 2. Nuclease-free water
 3. 2 µL 10X Reaction Buffer
 4. Linearized DNA template
 5. 2 µL Enzyme Mix
- e) Mix thoroughly by pipetting up and down and centrifuge briefly to collect the reaction mixture at the bottom of the tube.
- f) Incubate at 37°C in a non-shaker incubator for 4 hours to optimize synthesis yield.
- g) Add 1 µL of **TURBO DNase**, mix well by pipetting, centrifuge, and incubate for another 15 minutes at 37°C in a dry bath or non-shaker incubator. This treatment removes the template DNA.
- h) The working area and any material should be sanitized using 70% ethanol at the end.
- i) Be sure all equipment are shut down: centrifuges and incubator. Return any material and equipment to its proper place. The pipettes must be returned to their maximal volume.
- j) The RNA must be purified before use using the MEGAclean kit (Cat. No. AM1908) or lithium chloride precipitation.