Promega

# Product Application

# Using RiboMAX™ to Generate Transcripts Containing Modified Nucleotides

*RiboMAX™ Large Scale RNA Production Systems can be used incorporate modified nucleotides into transcripts. Modified nucleotides are often used to alter transcript immunogenicity and/or stability* in vivo.

**Kit:** RiboMAX™ Large Scale RNA Production System – T7 (Cat.# P1300)
RiboMAX™ Large Scale RNA Production System – SP6 (Cat.# P1280)

**Analyses:** TapeStation RNA ScreenTape

**Materials Required:**

- Linear DNA Template
- Modified nucleotides (see TriLink Biotechnologies)
- Heat Block/ThermoMixer®/Thermal Cycler set to 37°C for reaction incubation

This protocol was developed by Promega Applications Scientists and is intended for research use only.

Users are responsible for determining suitability of the protocol for their application.

For further information, see Technical Manual TB166, available at: **www.promega.com/protocols**

or contact Technical Services at: **techserv@promega.com**

**Protocol**:

Please refer to the Technical Bulletin TB166 for guidance in the preparation of DNA template for in vitro transcription.

Note: If the DNA template is generated by PCR amplification or restriction digestion of a plasmid, the DNA should be cleaned up using ReliaPrep™ DNA Clean-Up and Concentration System (Cat.# A2892), or similar.

1. Prepare the nucleotide mix, as shown in the example in Table 1.

    a. For 100% substitution of the modified nucleotide in the transcript, replace the analogous native nucleotide with the modified nucleotide at the same concentration*. (*e.g.* For 100% incorporation of $N^1$-methylpseudo-UTP, omit rUTP in the mix, and replace with the modified nucleotide as shown in the example in Table 1).

    *Note: The relative concentration of the modified nucleotide to be used in the reaction may need to be adjusted to obtain the optimal yield of full-length transcript.

**Table 1. Preparation of nucleotide mix for in vitro transcription**

| | Control: Native Nucleotides | Example: Incorporation of $N^1$-methylpseudo-UTP |
|---|---|---|
| **rATP, 100mM** | 20µl | 20µl |
| **rUTP, 100mM** | 20µl | -- |
| **rCTP, 100mM** | 20µl | 20µl |
| **rGTP, 100mM** | 20µl | 20µl |
| **Other, 100mM** | -- | 20µl 100mM $N^1$-methylpseudo-UTP |

2. Assemble the in vitro transcription reaction according to Table 2. Add reagents in the order listed, and then gently pipette to mix.

    a. Reactions may be scaled according to the required amount of transcript to be recovered. A 1ml reaction will typically produce 2-5mg of RNA in 2-4 hours.

**Table 2. Assembly of complete in vitro transcription reactions**.

| | Volume (µl) | |
|---|---|---|
| | T7 Reaction | SP6 Reaction |
| **Transcription 5X Buffer** | 20 | 20 |
| **Nucleotide Mix (Table 1)** | 30 | 20 |
| **5µg Template plus Nuclease-Free Water** | 40 | 50 |
| **T7 Enzyme Mix** | 10 | 10 |
| **Final Volume** | 100 | |

3. Incubate the reaction at 37°C for 2-4 hours.
4. Add RQ1 RNase-free DNase to a concentration of 1U/µg of template, and mix gently by pipetting.
5. Incubate for 15 minutes at 37°C.
6. Clean up reaction as described in TB166, Section 4B., if desired.

**Results:**

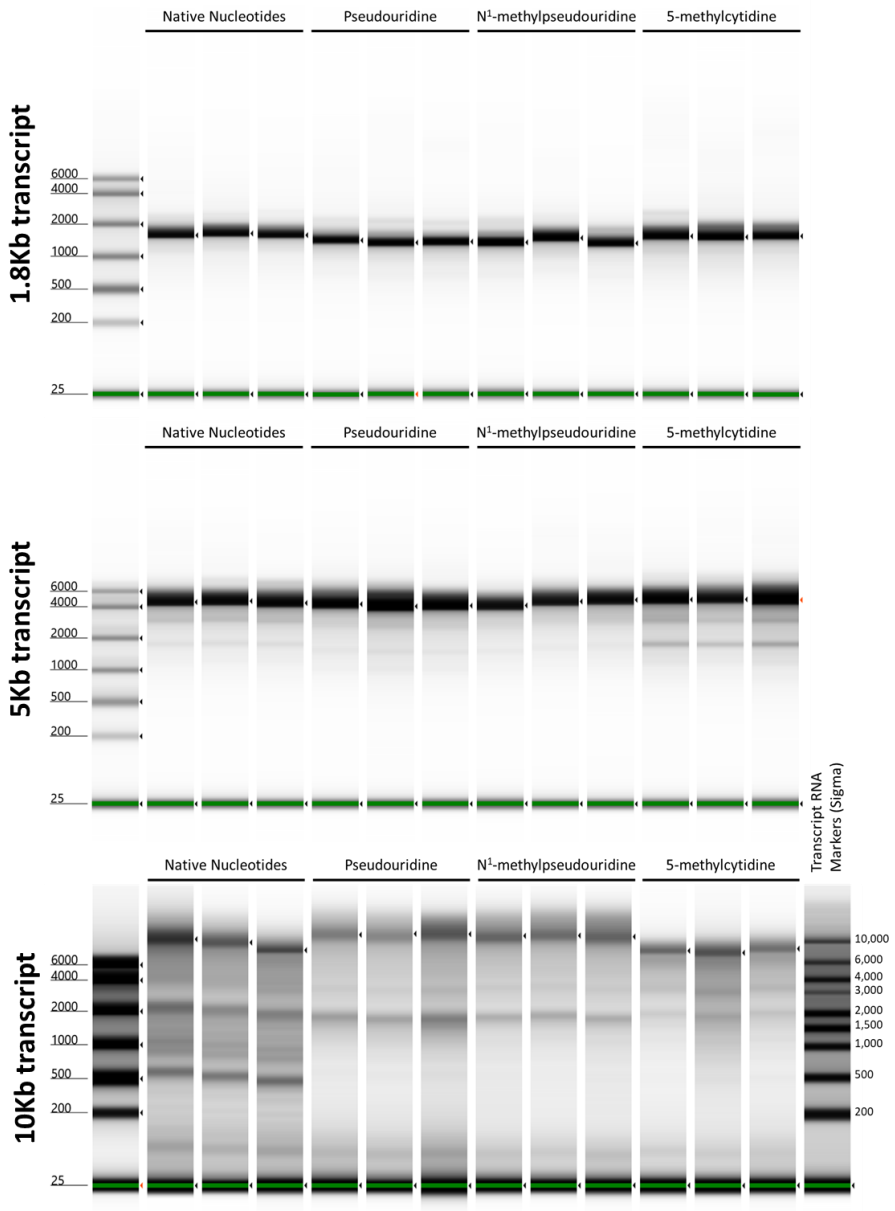


**Figure 1. Analysis of transcripts produced by in vitro transcription with modified nucleotides.** Three DNA templates were transcribed in 20µl reactions with RiboMAX™ Large Scale RNA Production System (Cat.# P1300) containing 1µg of template to generate 1.8Kb, 5Kb, and 10Kb transcipts. Separate reactions were prepared to incorporate no modified nucleotides (native nucleotides), pseudo-UTP (TriLink Biotechnologies, Cat.# N-1019-5), N1-methylpseudo-UTP (TriLink Biotechnologies, Cat.# N-1081-5) or 5-methyl-CTP (TriLink Biotechnologies, Cat.# N-1014-5). For the 1.8Kb and 5Kb transcripts, the modified nucleotides were substituted at 1X concentration (7.5mM) in the reaction (relative to the native nucleotide). For the 10Kb reaction, the modified nucleotides were substituted at 2X concentration (15mM) in the reaction, as reactions containing only 1X of the modified nucleotides produced little to no transcript (data not shown). Template was removed by treatment with RQ1 RNase-Free DNase, then reactions were diluted and prepared for analysis by TapeStation RNA ScreenTape. Small arrows indicate the band that was analyzed for concentration, which was used to determine reaction yield reported in Table 3.

**Table 3. Reaction yields for in vitro transcription with modified nucleotides relative to a control reaction containing only native nucleotides.** The yield of product was calculated based on the concentration of the full-length transcript determined by RNA ScreenTape analysis (Figure 1), which was adjusted for sample dilution and multiplied by the reaction volume (20µl). Percent yield for the reactions containing modified nucleotides was calculated relative to the control reaction, containing only native nucleotides. Data represent the average ± standard deviation for n=3 for each reaction condition and transcript length tested.

| | 1.8Kb Transcript | | 5Kb Transcript | | 10Kb Transcript | |
|---|---|---|---|---|---|---|
| | mg/ml | % control | mg/ml | % control | µg/ml | % control |
| Control | 3.99 ± 0.25 | 100 | 5.47 ± 0.35 | 100 | 252.0 ± 63.0 | 100 |
| Pseudo-UTP | 4.17 ± 0.08 | 104 | 6.95 ± 1.39 | 127 | 167.6 ± 17.4 | 67 |
| $N^1$-methyl pseudo-UTP | 4.92 ± 0.34 | 123 | 4.7 ± 0.67 | 86.0 | 213.2 ± 18.7 | 85 |
| 5-methyl-CTP | 5.22 ± 0.38 | 131 | 6.55 ± 1.05 | 120 | 172.7 ± 34.7 | 69 |