

Ribo m⁷G Cap Analog: A Reagent for Preparing In Vitro Capped Transcripts



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Promega's Ribo m⁷G Cap Analog can be used with the Riboprobe[®] Systems^(a) and the RiboMAX[™] Large Scale RNA Production Systems^(a,b,c) to prepare RNA transcripts with 5' m⁷G cap structures. These cap structures provide an anchor for attachment of cap binding protein (translation initiation factor or eIF4E), thus facilitating translation.

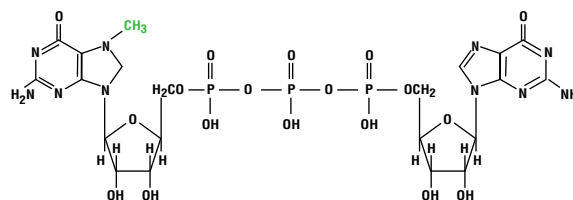
INTRODUCTION

A majority of eukaryotic mRNA molecules have a specialized 5' structure containing 5'-5' triphosphate-linked guanine modified with a 5' 7-methyl group (m⁷G). This cap structure is added to RNA polymerase II-produced transcripts by the nuclear enzymes guanylyl transferase and methyltransferase (1). The capped RNA structure influences several cellular functions, the most studied being translation. A key step of translation initiation is recognition and binding to the RNA m⁷G cap structure by cap binding protein, also referred to as eukaryotic translation initiation factor 4E (eIF4E; reviewed in reference 2). Early studies using reovirus RNA with a naturally occurring m⁷G cap demonstrated that these capped structures are necessary for efficient translation in wheat germ extracts (3,4). Other roles for the RNA m⁷G cap are to stabilize and increase the half-life of nuclear and cytoplasmic mRNA (3,5), to aid pre-mRNA splicing (5-7) and to transport RNA from nucleus to cytoplasm (8-10).

CAP STRUCTURES FOR IN VITRO TRANSCRIPTS

RNA transcripts prepared in vitro by T7, SP6 or T3 phage RNA polymerase can be synthesized with a cap structure when a cap analog, such as m⁷G(5')ppp(5')G (Figure 1), is included in the reaction (11,12). These phage polymerases require GTP as the initiating nucleotide (13) and readily incorporate the m⁷G analog as the 5' nucleotide, particularly when present at a higher molar concentration than GTP in the reaction (11). Incorporation of the cap dinucleotide provides RNA analogous to native capped RNA, as well as some RNA species with a cap in the opposite orientation (14).

RNA transcripts prepared in vitro with the m⁷G cap analog are useful for many different applications. For example, in vitro translation extracts prepared from reticulocytes or from wheat germ have been reported to synthesize more protein from capped than from uncapped transcripts (15,16). A recent study illustrated the importance of a 5' cap structure in recognition of open reading frame 2 of genomic RNA for southern bean mosaic virus added to cell-free extracts (17). Other studies of viral function have correlated infectivity with capped, but not with uncapped, RNA (18). RNA transcripts prepared in vitro with a m⁷G cap analog have also been injected into *Xenopus* oocytes to study RNA stability, translation (19) and nucleocytoplasmic transport (9,10,19).



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Figure 1. Chemical structure of Ribo m⁷G Cap Analog. The cap analog, m⁷G(5')ppp(5')G, is depicted with the methyl group highlighted (green) on position 7 of the guanosine moiety.

Promega's Ribo m⁷G Cap Analog (Cat.# P1711, P1712) can be used with the Riboprobe[®] Systems (Cat.# P1420, P1430, P1440, P1450, P1460) and RiboMAX[™] Large Scale RNA Production Systems (Cat.# P1280, P1290, P1300) to prepare RNA transcripts with a 5' m⁷G cap structure. The Riboprobe[®] Systems are designed to produce RNA that can be labeled with radioisotopes for use as probes. The RiboMAX[™] Systems are designed to produce larger amounts of RNA per unit of DNA template.

Figure 2 shows the separation of capped and uncapped RNA transcripts prepared using the Ribo m⁷G Cap Analog with the Riboprobe[®] System (Cat.# P1440). The transcripts were separated by denaturing polyacrylamide gel electrophoresis. Capped transcripts that incorporated the dinucleotide cap analog were larger and migrated slower than uncapped transcripts. Increased percentages of capped transcripts were synthesized as the ratio of cap analog increased relative to the concentration of GTP.

EFFECT OF CAP ANALOG:GTP RATIO ON RNA YIELD

When using cap analog, lower GTP concentrations, relative to ATP, CTP and UTP, are used for the in vitro transcription reaction. Figure 3 illustrates the effect of varying cap analog:GTP ratios on the yield of a 1,916 nucleotide transcript prepared using the RiboMAX[™] System under conditions of decreasing GTP concentrations and various molar ratios of cap analog:GTP. As shown by these data, decreasing GTP concentrations correspond to decreasing RNA yields. The molar ratio of cap analog at a given GTP concentration had minimal impact upon RNA yield.

If the cap dinucleotide analog was incorporated into the body of the transcript to the same extent as GTP, one would expect the yield of RNA to be similar between the sample with 7.5mM GTP and the sample with a 10:1 ratio of cap analog:GTP, using 7.5mM cap analog and 0.75mM GTP. These data, however, suggest the cap analog is not a direct substitute for GTP in these in vitro transcription reactions.

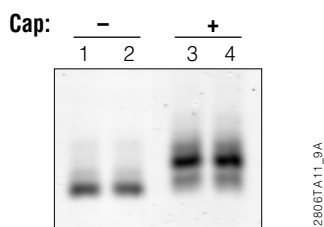


Figure 2. Separation of capped and uncapped RNA transcripts. RNA transcripts were synthesized with T7 RNA polymerase using the Riboprobe® System (Cat.# P1440) under standard conditions. pCI-neo Mammalian Expression Vector^(d,e) (Cat.# E1841) template DNA was linearized with restriction enzyme *Xba* I before use. Uncapped transcript was synthesized in the presence of 0.5mM (final concentration) GTP. Capped transcript was synthesized in the presence of 0.05mM (final) GTP and 0.5mM Ribo m⁷G Cap Analog. RNA samples (2µl of the capped reaction volume and 2µl of a 1:10 dilution of the uncapped reaction) were heat denatured, loaded and separated by electrophoresis on a 15% polyacrylamide/7M urea gel. The gel was stained using GelStar® (FMC BioProducts) and visualized with a FluorImager® instrument and associated software (Amersham Pharmacia Biotech). Approximately 66% of the transcripts synthesized in the presence of the cap analog migrated with slower mobility due to presence of the dinucleotide analog in the transcript. Lanes 1 and 2, uncapped RNA; lanes 3 and 4, capped RNA.

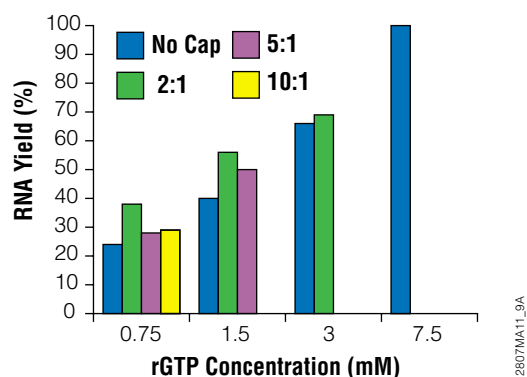


Figure 3. Effect of Ribo m⁷G Cap Analog:GTP ratio on RNA yield. The RiboMAX™ System (Cat.# P1300) and T7 RNA Polymerase were used under standard assay conditions (20) with either no Cap Analog or the Cap Analog:GTP ratios indicated, to generate RNA transcripts. A linearized T7 luciferase vector DNA was used as template. After the transcription reaction, samples were treated with RQ1 RNase-Free DNase (Cat.# M6101), centrifuged through Sephadex® G-50 spin columns (Amersham Pharmacia Biotech) and quantified by absorbance at 260nm. The yields are expressed as a percentage of the RNA concentration obtained from the reaction using 7.5mM GTP and no cap analog.

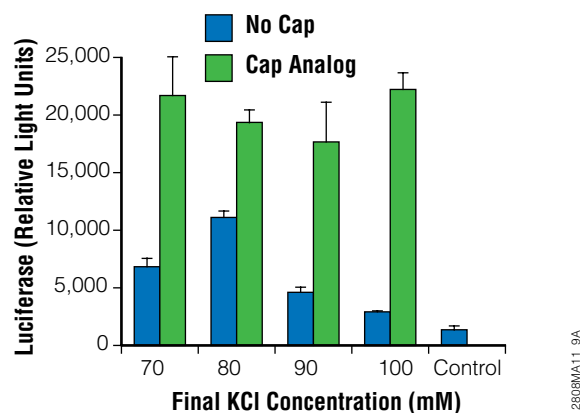


Figure 4. Efficiency of translation is increased with capped luciferase RNA in combination with reticulocyte lysate extracts. The RiboMAX™ System with T7 RNA Polymerase (Cat.# P1300) was used under standard assay conditions (20) to generate RNA transcripts. A linearized T7-luciferase vector DNA was used as a template. The “No Cap” reaction had a final concentration of 7.5mM GTP and the “Cap” reaction had final concentrations of 0.6mM GTP and 3mM Ribo m⁷G Cap Analog. After transcription, samples were treated with RQ1 RNase-Free DNase (Cat.# M6101), centrifuged through Sephadex® G-50 spin columns (Amersham Pharmacia Biotech) and quantified by absorbance at 260nm. One microgram of each RNA was added to each in vitro translation reaction using the Flexi® Rabbit Reticulocyte Lysate Translation System (Cat.# L4540; 23). KCl (2.5M) was added to yield final concentrations of 70mM, 80mM, 90mM and 100mM. Aliquots (5µl of each 50µl translation reaction) were assayed for luciferase activity using the Luciferase Assay System^(f) (Cat.# E1500). Light unit values were obtained using a Luminoskan® luminometer (LabSystems). The graph depicts values in relative light units, showing luciferase protein activity. The Control column shows the relative light units obtained with 1µg of the Luciferase Control RNA^(b) (Cat.# L4561, provided with the in vitro translation system), added to reticulocyte lysate with 100mM KCl.

CAP ANALOG, KCL INFLUENCE IN VITRO TRANSLATION

Capped and uncapped luciferase RNAs were tested with the Flexi® Rabbit Reticulocyte Lysate System^(b,f) (Cat.# L4540) to determine whether the presence of the m⁷G cap structure influenced the amount of protein expressed. Rabbit reticulocyte- and wheat germ extract-based translation systems have demonstrated increased translation efficiency with some capped transcripts. In this experiment, a DNA template encoding the luciferase gene was transcribed in the RiboMAX™ System with T7 RNA polymerase, under standard RiboMAX™ System conditions (20), in the presence or absence of Ribo m⁷G Cap Analog. RNA concentration was determined by absorbance at 260nm.

Several steps were taken to ensure reliable RNA concentration determinations. Following transcription and prior to absorbance determinations, both samples were treated with RNase-Free DNase (Cat.# M6101) to eliminate template DNA. Free nucleotides were preferentially removed with Sephadex® G-50 spin columns (Amersham Pharmacia Biotech). Sephadex® G-50 separation also had the effect of removing free cap analog from the RNA sample that would otherwise carry over to the in vitro translation reaction. Free cap analog has been reported to inhibit capped mRNA in vitro translation (21). One microgram of RNA sample was added per reticulocyte lysate reaction and standard reaction conditions

were used, varying the KCl per reaction. It has been reported that the KCl concentration in the in vitro translation reaction influences the translation efficiency of capped and uncapped transcripts, with higher KCl concentrations favoring capped transcripts (22).

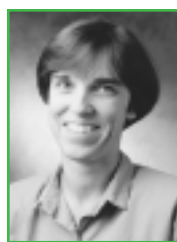
Luciferase was produced during the in vitro translation reactions and was quantified by measuring the relative light units (RLU) in a luciferase assay (Figure 4). The results illustrate higher overall protein production from capped than from uncapped transcripts. In addition, the amount of translated protein from this particular capped transcript was relatively constant over the tested KCl range, whereas the amount of translated protein from the corresponding uncapped transcript decreased with increasing KCl concentrations. For each particular transcript, we recommend testing the effect of capped and uncapped transcripts, and testing various concentrations of KCl in the translation extract to obtain maximal translation efficiency.

SUMMARY

The Ribo m⁷G Cap Analog reagent can be used in combination with the Riboprobe® Systems and RiboMAX™ Large Scale RNA Production Systems to prepare RNA transcripts with a 5' m⁷G cap structure. Capped RNA transcripts can be used for in vitro translation applications, as well as for studying cellular RNA functionality in processes such as splicing, RNA processing, intracellular transport, viral infectivity and translation.

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Ordering Information

Product	Size	Cat.#
Ribo m ⁷ G Cap Analog	10 × A ₂₅₄ units	P1711
	25 × A ₂₅₄ units	P1712
Riboprobe® Combination System - T3/T7 RNA Polymerase	1 system	P1450
Riboprobe® Combination System - SP6/T7 RNA Polymerase	1 system	P1460
Riboprobe® System - SP6	1 system	P1420
Riboprobe® System - T3	1 system	P1430
Riboprobe® System - T7	1 system	P1440
RiboMAX™ Large Scale RNA Production System - SP6	1 system	P1280
RiboMAX™ Large Scale RNA Production System - T3	1 system	P1290
RiboMAX™ Large Scale RNA Production System - T7	1 system	P1300
Flexi® Rabbit Reticulocyte Lysate System	1 system	L4540
Luciferase Assay System	100 assays	E1500

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^(b)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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