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mRNA synthesis

Improved mRNA synthesis with TheraPure products

Keywords
mRNA synthesis, *in vitro* transcription, mRNA therapeutics, post-transcriptional modification, transfection, capping analysis, transcription efficiency, TheraPure

The efficacy of messenger RNA (mRNA)-based technology was prominently showcased during the COVID-19 vaccination campaign [1]. mRNA became an increasingly influential platform not only for combating emerging infectious diseases but also for helping to advance cancer therapy, enabling gene editing to treat other diseases, and developing new forms of cell and gene therapy. mRNA is produced using *in vitro* transcription (IVT), the trusted process for mRNA synthesis. IVT is a procedure that enables DNA template–directed synthesis of RNA molecules of any sequence, from short oligonucleotides to those several kilobases long. *In vitro* transcripts need to be capped and poly(A)-tailed for protection from nucleases and for promotion of translation by engaging critical translation factors to recruit ribosomes to mRNA. Cap structures can be added in two ways: after transcription by using capping enzymes, or during transcription by including cap analogs. Poly(A) tails usually are incorporated into the DNA template or added as a separate step using poly(A) polymerase. In the last step, DNase I is used to remove the DNA template from the synthesized mRNA [4].

To develop and produce therapeutics using mRNA, the process and reagents should fit the needs and requirements of each development stage to facilitate easy and quick transition from proof-of-concept and research to preclinical studies and scaling to clinical and commercial CGMP manufacturing. Therefore, raw materials used for mRNA synthesis for therapeutic development should be of appropriate quality, consistent, and scalable to help ensure low-risk transfer through process development to the clinical-trial phases.

Here we present the catalog line of Thermo Scientific™ TheraPure™ reagents for IVT reactions and demonstrate efficient mRNA synthesis with post-transcriptional enzymatic capping or co-transcriptional capping.

The catalog line of TheraPure reagents is the connecting link between general catalog Thermo Scientific™ reagents and custom TheraPure and TheraPure GMP raw materials for higher-scale mRNA production (**mRNA Manufacturing Process**). Either general Thermo Scientific reagents or catalog TheraPure products could be used in initial research or proof-of-concept stages. However, moving to preclinical studies, focusing on process research, the usage of TheraPure materials provides higher flexibility and capabilities—through availability of custom TheraPure reagents for process development, optimization, and scaling, to progress quickly and smoothly toward ramping up GMP manufacturing for clinical trials and commercial phase with custom TheraPure GMP reagents. All TheraPure reagents are manufactured with stringent process and quality controls. Moreover, TheraPure GMP reagents are accompanied by comprehensive documentation to support regulatory submissions.

For Research Use Only. Not for use in diagnostic procedures.

Important notes

To minimize the risk of environmentally-borne contamination, thoroughly clean the laboratory workspace and all equipment using a 70% ethanol solution and Invitrogen™ RNase*Zap*™ RNase Decontamination Solution (Cat. No. AM9780).

Prior to handling RNA samples, spray RNase*Zap* solution on gloved hands and wipe down instruments, pipettors, and other surfaces using Invitrogen™ RNase*Zap*™ RNase Decontamination Wipes (Cat. No. AM9786) to avoid liquid contact with sensitive parts (e.g., electronic control modules).

Methods

DNA template preparation

DNA templates were prepared by PCR from a plasmid containing the 868 bp eGFP gene with 5′ and 3′ UTR sequences, downstream of a T7 promoter (Figure 1). Linearized plasmid was used in the PCR reaction, with the forward primer binding to the T7 promoter sequence and the reverse primer annealing downstream of the 3′ UTR region. The forward primer contained either a GGG or AGG transcription start, while the reverse primer was designed with and without a 120-nucleotide poly(T) tail.

PCR reaction setup and cycling conditions are shown in Tables 1 and 2. PCR products were purified using the Thermo Scientific™ GeneJET™ Gel Extraction and DNA Cleanup Micro Kit (Cat. No. K0831).

Table 1. PCR reaction setup.

Component	Final concentration	Volume (µL)
2X Invitrogen™ Platinum™ SuperFi™ PCR Master Mix	1X	25
Forward primer, 10 µM	0.5 µM	2.5
Reverse primer, 10 µM	0.5 µM	2.5
Plasmid DNA, 1 ng/µL	1 ng per reaction	1
Water, nuclease-free	–	19

Table 2. PCR cycling conditions.

Temperature	Time	Number of cycles
98°C	30 sec	1
98°C	10 sec	
60°C	10 sec	30
72°C	30 sec	
72°C	5 min	1

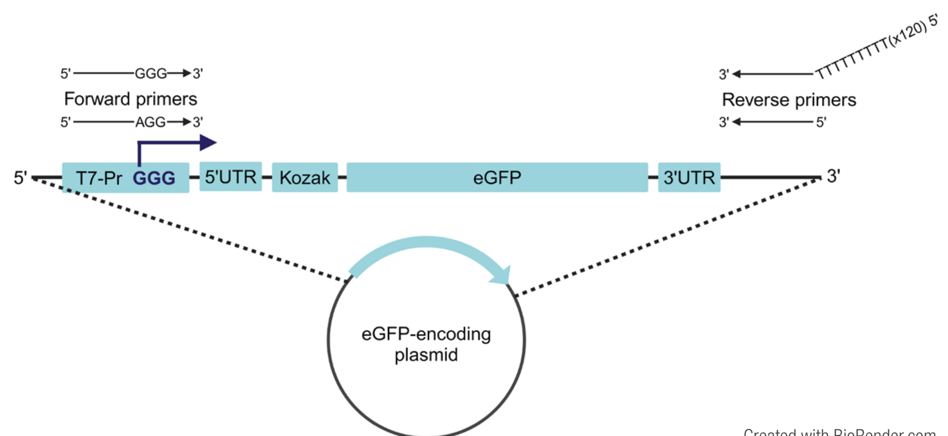


Figure 1. DNA template structure. A PCR fragment was amplified from a linearized plasmid with Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase, using a forward primer binding to the T7 promoter sequence and a reverse primer annealing downstream of the 3′ UTR region. The forward primer contained either a GGG or AGG transcription start, while the reverse primer was designed with and without a 120-nucleotide poly(T) tail.

mRNA synthesis

Table 3 provides a summary of reaction setup and incubation conditions for the IVT reaction, enzymatic capping, and DNA removal in one pot without intermediate purification steps. The IVT reactions were set up in Thermo Scientific™ EasyStrip™ Plus Tube Strips with Attached Flat Caps (Cat. No. AB2000) at room temperature and run in an Applied Biosystems™ ProFlex™ 3 x 32-Well PCR System (Cat. No. 4484073). RNA synthesis was carried out for 2 hr at 37°C. Afterwards, the IVT reaction was diluted 2-fold and denatured at 70°C for 10 min to increase the accessibility of the RNA's 5′ end for capping, and to inactivate the RNA synthesis enzymes. Capping reagents were then added to the reaction to cap the RNA transcript. After 1 hr of incubation, Thermo Scientific™ TheraPure™ DNase I was added to degrade the DNA template for 15 min.

To synthesize mRNA without capping, the capping step was omitted and the DNA template was removed right after IVT reaction as described in Table 6.

Table 3. Reaction setup and conditions for mRNA synthesis with enzymatic capping.

IVT reaction		
Component	**Volume (µL)**	**Final concentration**
10X Transcription buffer	2	1X
TheraPure™ ATP, sodium solution, 100 mM	2	10 mM
TheraPure™ CTP, sodium solution, 100 mM	2	10 mM
TheraPure™ GTP, sodium solution, 100 mM	2	10 mM
TheraPure™ UTP, sodium solution, 100 mM	2	10 mM
TheraPure™ T7 RNA polymerase (200 U/µL)	1	10 U/µL
TheraPure™ RNase inhibitor (40 U/µL)	0.5	1 U/µL
TheraPure™ pyrophosphatase (0.1 U/µL)	1	0.005 U/µL
PCR fragment with or without poly(A) stretch	Dependent on concentration	0.3 µg/reaction
Water, nuclease-free	Up to 20 µL	–
Total volume	20	–
Incubation conditions		
2 hr at 37°C		
RNA denaturation		
Component	**Volume (µL)**	
IVT reaction	20	
Water, nuclease-free	20	
Total volume	40	
Incubation conditions		
10 min at 70°C, then 5 min on ice		

Table 3. Reaction setup and conditions for mRNA synthesis with enzymatic capping. (continued)

Transcript capping using vaccinia capping enzyme and 2´-O-methyltransferase*		
Component	Volume (µL)	Final concentration
Denatured 2-fold diluted IVT reaction	40	–
10X capping buffer	8	1X
TheraPure™ GTP, sodium solution, 10 mM	8	1 mM
TheraPure™ S-adenosyl-methionine (SAM), 32 mM**,†	1.25	0.5 mM
TheraPure™ vaccinia capping enzyme (10 U/µL)	4	0.5 U/µL
TheraPure™ vaccinia cap 2´-O-methyltransferase (50 U/µL)†,‡	4	2.5 U/µL
TheraPure™ RNase inhibitor (40 U/µL)	1.5	1 U/µL
Water, nuclease-free	Up to 80 µL	–
Total volume	80	–
Incubation conditions		
1 hr at 37°C		
DNA template degradation		
Component	Volume (µL)	Final concentration
Capping reaction	80	–
TheraPure™ DNase I (50 U/µL)	1	50 U/reaction
Total volume	81	–
Incubation conditions		
15 min at 37°C		

* To manufacture uncapped RNA, this step was omitted. In such cases, DNA template was degraded as described in co-transcriptional capping (Table 4).

** Thawed TheraPure SAM solutions were kept on ice.

† When synthesizing the unmethylated control (CapG), TheraPure SAM and TheraPure 2´-O-methyltransferase were not added to the reaction.

‡ When synthesizing cap 0, 2´-O-methyltransferase was not added to the reaction.

Tables 4 and 5 provide summaries of the reaction setup and incubation conditions for co-transcriptional capping with Invitrogen™ Anti-Reverse Cap Analog (ARCA) (Cat. No. AM8045) or with CleanCap™ Reagent AG (TriLink BioTechnologies). The IVT reactions were set up in EasyStrip Plus Tube Strips with Attached Flat Caps (Cat. No. AB2000) and run in the ProFlex 3 x 32-Well PCR System (Cat. No. 4484073). DNA degradation with TheraPure DNase I was done immediately after IVT reactions, as described in Table 6.

The prepared mRNA can be purified immediately after the DNA degradation step or stored at –20°C until purification.

Table 4. IVT reaction setup for co-transcriptional capping with ARCA.

Co-transcriptional capping with ARCA		
Component	**Volume (µL)**	**Final concentration**
10X transcription buffer	2	1X
TheraPure™ ATP, sodium solution, 100 mM	2	10 mM
TheraPure™ CTP, sodium solution, 100 mM	2	10 mM
TheraPure™ GTP, sodium solution, 20 mM*	2	2 mM
TheraPure™ UTP, sodium solution, 100 mM	2	10 mM
ARCA (Anti-Reverse Cap Analog), 40 mM	5	10 mM
TheraPure™ T7 RNA polymerase (200 U/µL)	1	10 U/µL
TheraPure™ RNase inhibitor (40 U/µL)	0.5	1 U/µL
TheraPure™ pyrophosphatase (0.1 U/µL)	1	0.005 U/µL
PCR fragment with poly(A) stretch	Dependent on concentration	0.3 µg/reaction
Water, nuclease-free	Up to 20 µL	–
Total volume	20	–
Incubation conditions		
2 hr at 37°C		

* TheraPure GTP, sodium solution, 100 mM is diluted with nuclease-free water to a final concentration of 20 mM.

Table 5. IVT reaction setup for co-transcriptional capping with CleanCap Reagent AG.

Co-transcriptional capping with CleanCap Reagent AG		
Component	**Volume (µL)**	**Final concentration**
10X transcription buffer	2	1X
TheraPure™ ATP, sodium solution, 100 mM	2	10 mM
TheraPure™ CTP, sodium solution, 100 mM	2	10 mM
TheraPure™ GTP, sodium solution, 100 mM	2	10 mM
TheraPure™ UTP, sodium solution, 100 mM	2	10 mM
CleanCap Reagent AG, 100 mM	1.6	8 mM
TheraPure™ T7 RNA polymerase (200 U/µL)	1	10 U/µL
TheraPure™ RNase inhibitor (40 U/µL)	0.5	1 U/µL
TheraPure™ Pyrophosphatase (0.1 U/µL)	1	0.005 U/µL
PCR fragment with poly(A) stretch	Dependent on concentration	0.3 µg/reaction
Water, nuclease-free	Up to 20 µL	–
Total volume	20	–
Incubation conditions		
2 hr at 37°C		

Table 6. Reaction setup for DNA template degradation.

DNA template degradation		
Component	**Volume (µL)**	**Final concentration**
Co-transcriptional capping reaction	20	–
10X reaction buffer with $MgCl_2$ for DNase I	8	1X
TheraPure™ DNase I (50 U/µL)	1	50 U/reaction
Water, nuclease-free	51	–
Total volume	80	–
Incubation conditions		
15 min at 37°C		

RNA purification

RNA transcripts were purified using the Invitrogen™ MEGAclear™ Transcription Clean-Up Kit (Cat. No. AM1908). Each RNA sample was processed using a microcentrifuge and recovered with 50 µL of preheated elution solution.

Nucleic acid quantification

After purification, DNA or RNA concentration was determined using a Thermo Scientific™ NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Cat. No. ND-ONE-W).

Nucleic acid visualization

DNA and RNA were visualized using Invitrogen™ E-Gel™ agarose gels and the E-Gel™ Power Snap Plus Electrophoresis System

RNA samples and Thermo Scientific™ RiboRuler™ High Range RNA Ladder (Cat. No. SM1821) were diluted with nuclease-free water to a volume of 10 µL, then mixed with 10 µL of formamide for a final volume of 20 µL. RNA samples and ladders were heated at 70°C for 5 min and then placed on ice for 2 min.

DNA samples and Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder (Cat. No. 10488091) were diluted with water to a final volume of 20 µL.

Samples (20 µL) were loaded onto a 1% Invitrogen™ E-Gel™ EX Double Comb Gel (Cat. No. A42345) and allowed to settle in the wells for 2 min before starting the run on the E-Gel Power Snap Plus Electrophoresis System (Cat. No. G9301) using a pre-programmed protocol. Sample loads were 40 ng of RNA or 20 ng of DNA per well. For the DNA and RNA ladders, 80 ng and 500 ng were loaded per well, respectively.

mRNA integrity analysis

The integrity and quality of synthesized mRNA were analyzed using an Agilent™ 5200 Fragment Analyzer System and HS RNA Kit (15 nt) (Agilent, Cat. No. DNF-472-1000) following the manufacturer's protocol. Briefly, RNA was diluted to 2 ng/µL, and 2 µL of the diluted RNA sample and HS RNA Ladder were heated for 2 min at 70°C, and immediately cooled. The RNA samples and ladder were then diluted with 18 µL of HS RNA Diluent Marker (15 nt). Samples were analyzed following the instructions provided by Agilent, and data were analyzed using ProSize™ Data Analysis Software 3.0. RNA integrity was defined as the percentage of the main corresponding peak relative to any smear peaks.

Capping efficiency determination

Current available methods for identifying cap presence or different cap structures are limited to short RNA fragments. Therefore, for capping efficiency measurements, 5′ RNA fragment cleavage was performed with deoxyribozyme (DNAzyme) specific to the 5′ UTR region [2,3].

A mixture comprising DNAzyme and RNA sample was set up as described in Table 7. The mixture was preheated at 85°C, equilibrated to 37°C, and incubated for 5 min to denature RNA and hybridize the DNAzyme to the RNA. Then $MgCl_2$ was added to initiate digestion. The reaction was stopped by DNAzyme removal using DNase I.

Table 7. Reaction setup and conditions for 5′ RNA fragment cleavage.

RNA fragmentation		
Component	**Volume (µL)**	**Final concentration**
1 M Tris-HCl, pH 7.5	1	50 mM
RNA, 100 ng/µL	10	1 µg
DNAzyme, 10 µM	1	0.5 µM
Water, nuclease-free	4	–
Total volume	16	–
Incubation conditions		
Hybridization: 30 sec at 85°C, then 5 min at 37°C		
Component	**Volume (µL)**	**Final concentration**
Hybridized mix	16	–
50 mM $MgCl_2$	4	10 mM
Total volume	20	–
Incubation conditions		
RNA fragmentation: 1 hr at 37°C		
Component	**Volume (µL)**	**Final concentration**
RNA fragmentation reaction	20	–
DNase I, 1 U/µL	10	10 U/reaction
Total volume	30	–
Incubation conditions		
Degradation of DNAzyme: 45 min at 37°C		

5′ RNA fragments were purified with the GeneJET RNA Cleanup and Concentration Micro Kit (Cat. No. K0841) using a modified protocol to reduce the amount of long RNA produced by 5′ RNA fragment cleavage, which may interfere with capillary electrophoresis results.

Modified RNA cleanup protocol for reducing long RNA fragments

1. 30 µL of the DNAzyme reaction was mixed with 100 µL of Binding Buffer. Then 20 µL of 96% ethanol was added and mixed.
2. 50 µL of the mixture was transferred to a GeneJET RNA purification micro column and centrifuged at 14,000 x *g* for 30 sec. The column was discarded and the flow-through was used for further purification.
3. 100 µL of Binding Buffer was added to the flow-through and mixed. Then 280 µL of 96% ethanol was added and mixed.
4. The mixture was added to a new column and centrifuged at 14,000 x *g* for 30 sec. The flow-through was discarded.
5. 700 µL of Wash Buffer 1 was added and the column was centrifuged at 14,000 x *g* for 30 sec. The flow-through was discarded.
6. 700 µL of Wash Buffer 2 was added and the column was centrifuged at 14,000 x *g* for 30 sec. The flow-through was discarded.
7. The remaining wash buffer was removed by additional centrifuging at 14,000 x *g* for 1 min. The flow-through was discarded.
8. The column was transferred to a 1.5 mL collection tube, and 10–20 µL of nuclease-free water was added to the column and incubated at room temperature for 2 min to elute the RNA. The column was then centrifuged at 14,000 x *g* for 1 min.

Eluted RNA samples can be used for further analysis or stored at –20°C.

Purified RNA fragments (2 ng) were analyzed on an Agilent 2100 Bioanalyzer instrument using the Agilent™ small RNA assay kit (Agilent, Cat. No. 5067-1548) that allows differentiation between capped and uncapped 5′ fragments of RNA. RNA capping efficiency was defined as the percentage of the capped RNA peak relative to the sum of capped and uncapped RNA peaks.

For LC-MS analysis, 10 µL of water was added to 10 µL of purified RNA samples, which were then injected into an LC-MS system without processing. UHPLC was performed on a Thermo Scientific™ Vanquish™ Horizon UHPLC System (Cat. No. IQLAAAGABHFAPUMZZZ) using a 2.1 x 100 mm, 4 µm Thermo Scientific™ DNAPac™ RP column (Cat. No. 088923), with an ion-pairing buffer system where mobile phase A consisted of 100 mM HFIP and 10 mM TEA, and mobile phase B consisted of 50% mobile phase A and 50% methanol. Column temperature was set to 40°C. Samples were acquired on a Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer (Cat. No. BRE725535) in negative polarity using intact protein mode. Data processing and sequences and modifications of RNA were calculated using BioPharma Finder™ software.

Transfection with mRNA

HEK293T cells were cultivated in a medium (Table 8) prepared in Gibco™ DMEM, high glucose, no glutamine (Cat. No. 11960044). Cells were kept in a CO_2 incubator at 37°C.

HEK293T cells were seeded into a 96-well plate at 1.2×10^4 cells/well. After 24 hr, cells were transfected with 25 ng RNA/well using 0.3 µL/well of Invitrogen™ Lipofectamine™ MessengerMAX™ Transfection Reagent (Cat. No. LMRNA001). Lipofectamine MessengerMAX reagent and RNA or plasmid DNA were diluted with Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985070) and mixed together and transferred to the wells containing cells (Table 8).

After another 24 hr, transfection efficiency of eGFP RNA was measured using flow cytometry. Briefly, cells were lifted using 20 µL/well of Gibco™ Trypsin-EDTA (0.05%), phenol red (Cat. No. 25300104), and 100 µL/well of Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer (Cat. No. 00-4222-26) was added; then 100 µL of cell suspension was mixed with 100 µL Invitrogen™ eBioscience™ IC Fixation Buffer (Cat. No. 00-8222-49). Fluorescence was then measured on an Invitrogen™ Attune™ NxT Flow Cytometer (Cat. No. A51848) (Figure 2). Three separate transfection experiments were repeated using all three synthesis replicates of each RNA.

Table 8. Reagent preparation for cell culture and transfection.

Medium for HEK293T cell cultivation

Component	Final concentration
DMEM	–
FBS	10% of volume
L-glutamine (200 mM)	1% of volume
Gentamicin (10 mg/mL)*	50 µg/mL

* When seeding cells for transfection, medium without antibiotic is used.

Lipofectamine MessengerMAX reagent diluted in Opti-MEM medium**

Component	Volume
Opti-MEM	5 µL
Lipofectamine MessengerMAX reagent	0.3 µL

**After dilution, the mixture is gently mixed and incubated at room temperature for 10 min.

RNA or plasmid DNA sample diluted in Opti-MEM medium

Component	Volume
Opti-MEM medium	4 µL
RNA or plasmid DNA sample (25 ng/µL)	1 µL

Transfection mixture for 1 well†

Component	Volume
Diluted Lipofectamine MessengerMAX solution	5 µL
Diluted RNA or plasmid DNA solution	5 µL

† After mixing, the solution is incubated at room temperature for 5 min.

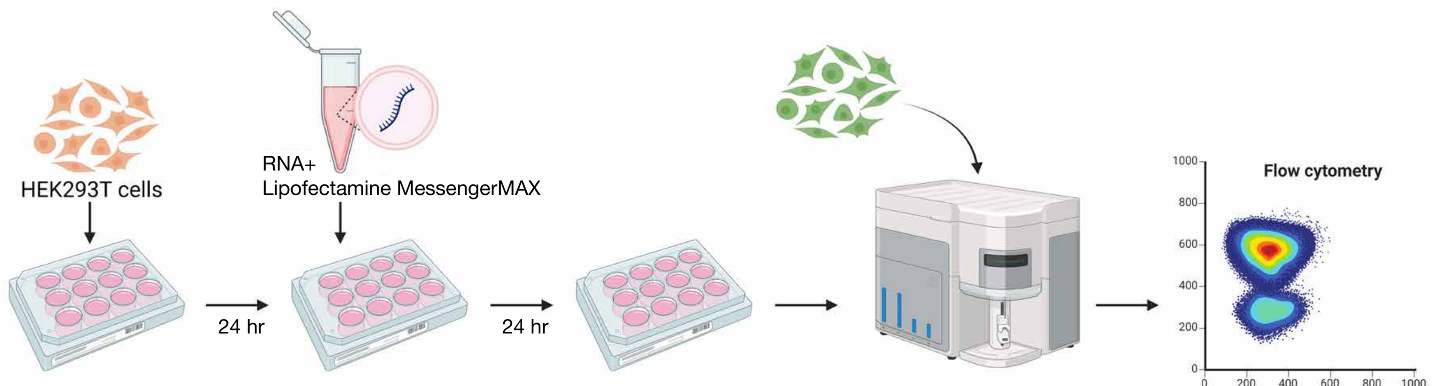


Figure 2. eGFP RNA workflow for transfection of HEK293T cells.

Results

mRNA synthesis, yield, and integrity

High-quality starting materials are required to achieve successful high-yield transcription.

One of the main components for mRNA synthesis is the DNA template—double-stranded linear DNA (dsDNA) containing a T7 promoter upstream of the target sequence and other optional elements, like 3´ and 5´ UTRs and a poly(A) stretch. Two different strategies are commonly used for IVT template preparation. One utilizes linearization of the plasmid DNA immediately after the transcript coding sequence (usually after the poly(A) stretch) using a restriction enzyme, while the other uses high-fidelity PCR to generate dsDNA. PCR provides flexibility to introduce or modify the promoter sequence on a forward primer or to add a poly(A) sequence on the reverse primer.

Therefore, we used PCR to prepare several variations of templates (Figure 1): with and without a poly(A) sequence and with a standard GGG start codon for uncapped RNA, for post-transcriptional enzymatic capping and for co-transcriptional capping with ARCA; or with a poly(A) tail and alternative AGG start codon for co-transcriptional capping with CleanCap Reagent AG. Proofreading Platinum SuperFi II DNA Polymerase (Cat. No. 12361010) of the highest fidelity was used to avoid errors in the amplified dsDNA and minimize optimization efforts for high-yield amplification. To avoid any inhibition of the IVT reactions, PCR fragments were purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Cat. No. K0832), which allows purification of DNA template free of contaminants and inhibitors in 3.5 min. The concentration of PCR fragments was determined by spectrophotometry, and the quality was evaluated by electrophoresis on E-Gel agarose gels (Figure 4, bottom lane).

RNA requires a cap structure at the 5´ end and a poly(A) tail at the 3´ end to be efficiently translated in human cells. The 5´ mRNA cap is particularly important, as it facilitates molecular transport and protein translation, adds resistance to exonuclease degradation, and serves as a differentiator between self and foreign RNA *in vivo*. The cap can be added co-transcriptionally in the IVT reaction using a cap analog (ARCA or CleanCap Reagent AG) or enzymatically after transcription using vaccinia capping enzyme and 2´-O-methyltransferase. Enzymatic capping provides capabilities to add different cap structures. Vaccinia capping enzyme carries three enzymatic activities (RNA triphosphatase, guanylyltransferase, and guanine-7-methyltransferase). If only GTP is included in the capping reaction with vaccinia capping enzyme (no S-adenosylmethionine (SAM)), the RNA triphosphatase and guanylyltransferase activities produce a cap G structure. If SAM is added to the capping reaction, the guanine-7-methyltransferase activity of the capping enzyme adds a methyl group to generate a cap 0 structure. Both 2´-O-methyltransferase and SAM must be added to the capping reaction to methylate ribose of the first nucleotide to produce cap 1 (Figure 3).

In this experiment, 8 eGFP RNAs with different modifications were synthesized in triplicate using prepared PCR templates. A PCR fragment with poly(A) stretch and TheraPure enzymes and nucleotides were used to synthesize different mRNA variants with a poly(A) tail and cap G (CapG-eGFP-polyA), cap 0 (Cap0-eGFP-polyA), and cap 1 (Cap1-eGFP-polyA) structures by enzymatic capping, while the capping step was omitted for poly(A)-tailed RNA without cap (eGFP-polyA). In addition, poly(A)-tailed RNAs with cap 0 and cap 1 were prepared by co-transcriptional capping. ARCA and a PCR fragment with a poly(A) stretch were used to synthesize Cap0-eGFP-polyA (ARCA), while a PCR fragment with a poly(A) stretch and the alternative AGG start codon was used for co-transcriptional capping using CleanCap Reagent AG to prepare Cap1-eGFP-poly(A) (CleanCap Reagent). A PCR fragment without poly(A) was used for eGFP RNA synthesis, and the cap was added using TheraPure vaccinia enzyme to obtain RNA with cap 1 and without a poly(A) tail (Cap1-eGFP).

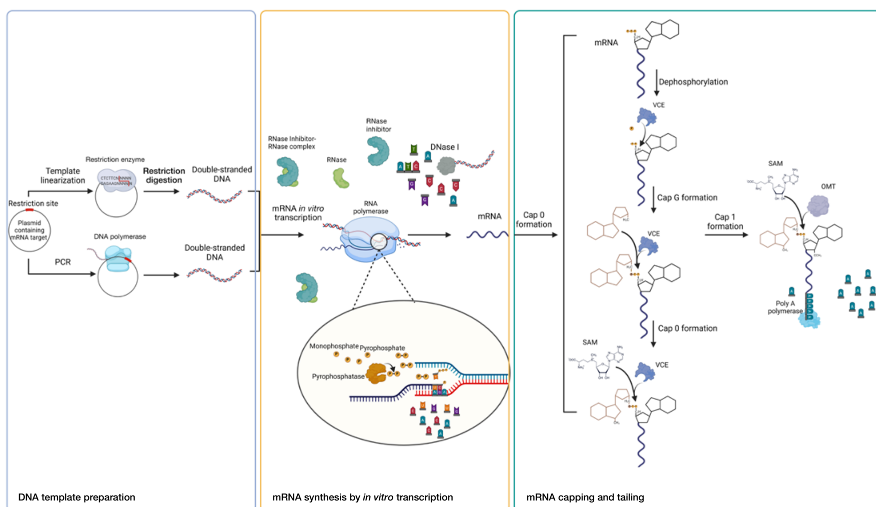


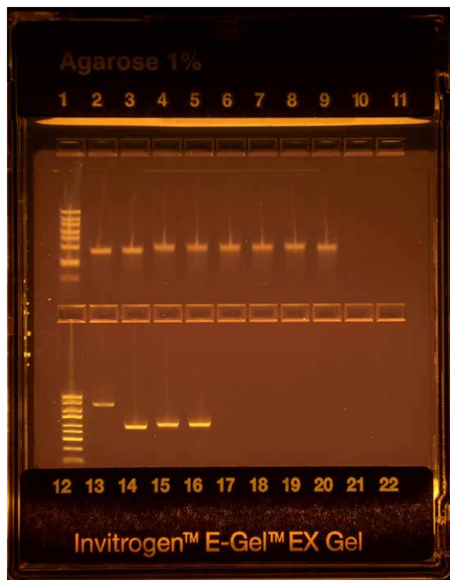
Figure 3. Enzymatic capping cascade. Schematic representations of cap G, cap 0, and cap 1 synthesis using vaccinia capping enzyme (VCE) and 2´-O-methyltransferase (OMT).

The yields of uncapped RNA, enzymatically capped RNA, and RNA co-transcriptionally capped with CleanCap Reagent AG were similar, ranging from 56 to 82 µg (Table 9). However, the yield of RNA capped co-transcriptionally with ARCA was significantly lower (~25 µg) due to the reduced amount of GTP nucleotides used to optimize capping efficiency. The integrity and quality of the mRNAs were analyzed visually by electrophoresis on E-Gel agarose gels (Figure 4, upper row). The gel showed a single band at the expected transcript size in all samples tested, and the band size difference corresponded to the addition of 120 nt for RNA samples with poly(A) tails. Additionally, RNA integrity was measured using an Agilent 5200 Fragment Analyzer System with Small RNA assay kit. Most of the synthesized modified and unmodified RNA displayed one clearly defined RNA peak (Figure 5A), indicating an integrity of ~80% (Table 9). An exception was the co-transcriptionally capped RNA with CleanCap Reagent AG, which had an integrity of only ~40% due to additional lower-size peaks adjacent to the main RNA peak in the electropherogram (Figure 5B). However, these additional peaks were not visible in the gel image (Figure 4, lane 8). Moreover, higher variability between synthesis replicates and technical repeats was observed, which had no impact on transfection efficiency (see Figure 9). This makes it unclear whether these additional peaks are artifacts of electrophoresis on Agilent instruments of the co-transcriptionally capped RNA with CleanCap Reagent AG, or shortened RNA forms.

Table 9. RNA yield and integrity.*

Synthesized RNA sample	Yield (µg)	Integrity (%)
eGFP	82 ± 3	85 ± 4
Cap1-eGFP	62 ± 11	84 ± 2
eGFP-polyA	74 ± 7	77 ± 4
CapG-eGFP-polyA	56 ± 15	80 ± 4
Cap0-eGFP-polyA	64 ± 13	82 ± 2
Cap1-eGFP-polyA	64 ± 20	81 ± 2
Cap1-eGFP-polyA (CleanCap Reagent)	78 ± 33	40 ± 12
Cap0-eGFP-polyA (ARCA)	25 ± 12	82 ± 5

* Average of three RNA synthesis replicates.



1: Thermo Scientific™ RiboRuler™ High Range RNA Ladder

2: eGFP RNA

3: Cap1-eGFP RNA

4: eGFP-polyA RNA

5: CapG-eGFP-polyA RNA

6: Cap0-eGFP-polyA RNA

7: Cap1-eGFP-polyA RNA

8: Cap1(CleanCap Reagent)-eGFP-polyA RNA

9: Cap0(ARCA)-eGFP-polyA RNA

12: Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder

13: Linearized eGFP gene containing plasmid

14: PCR fragment containing eGFP gene with standard promoter GGG

15: PCR fragment containing eGFP gene with standard promoter GGG and poly(A) stretch

16: PCR fragment containing eGFP gene with poly(A) stretch and modified promoter AGG for IVT reactions with CleanCap Reagent AG

Figure 4. Products of eGFP mRNA synthesis. Both RNA and DNA samples were fractionated on an E-Gel EX 1% agarose double comb gel.

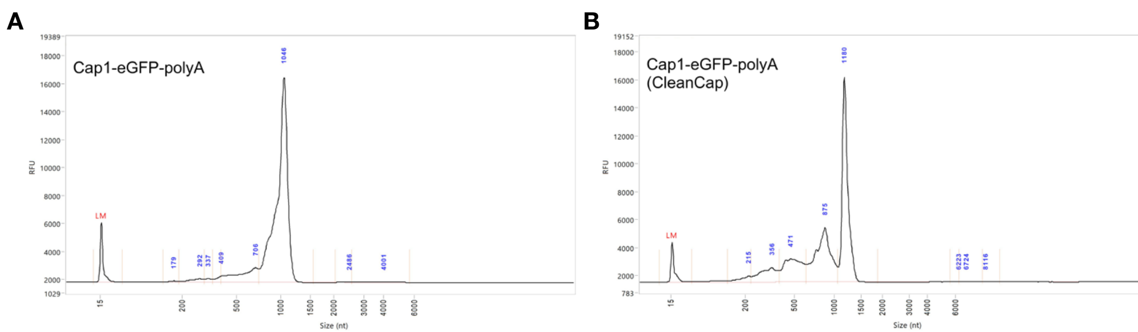


Figure 5. Example electropherograms of RNA integrity analysis. Electropherograms of **(A)** eGFP RNA capped enzymatically using vaccinia enzyme, and **(B)** eGFP RNA capped co-transcriptionally using CleanCap Reagent AG.

Capping efficiency

Electrophoretic analysis allows differentiation of RNA polyadenylation status; however, different capped forms of long RNA migrate identically. Therefore, cleavage of a short 5´ part of the RNA is required for capping analysis to differentiate between capped and uncapped fractions by capillary electrophoresis or liquid chromatography coupled with mass spectrometry (LC-MS) (Figure 6).

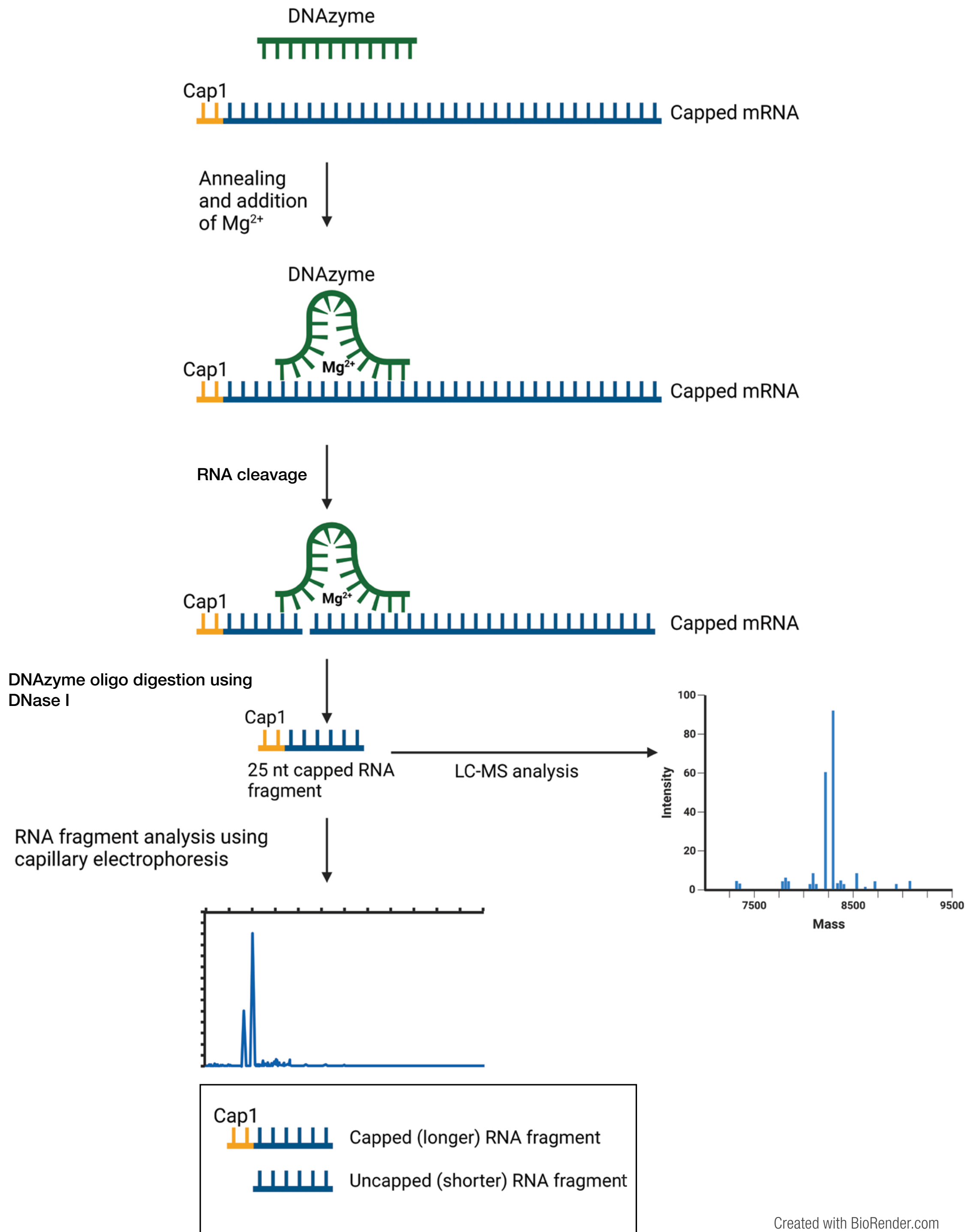


Figure 6. Schematic representation of capping efficiency analysis. RNA digestion with a DNAzyme oligonucleotide, and cap analysis on an Agilent Bioanalyzer 2100 or LC-MS.

DNAzyme complementary to the 5′ UTR sequence was used to cleave a 25 nt 5′ part of RNA. Short 5′ RNA fragments were purified to remove long 3′ RNA fragments, using the GeneJET RNA Cleanup and Concentration Micro Kit with a modified protocol. Capping efficiency was measured by separating capped and uncapped fractions of the RNA on an Agilent Bioanalyzer using a small RNA chip (Figure 6). Capped and uncapped fractions differ by a cap nucleotide and can be differentiated in electropherograms (Figure 7).

For most enzymatic capping samples, capping efficiency was greater than 95%, except with CapG-eGFP-polyA, for which it was nearly 95%. Similar capping efficiency was attained by co-transcriptional capping using CleanCap Reagent AG, while co-transcriptional capping with ARCA produced only ~74% of capped mRNA (Figure 8, red bars). It is worth noting that this method does not provide resolution to differentiate between different cap forms. Hence, values declared are the total cap structure—the sum of cap G, cap 0, and cap 1.

Further LC-MS analysis of short 5′ RNA fragments was done to differentiate these different cap structures. Total capping efficiencies (sum of identified cap species in each sample) were comparable to those obtained by analysis on the Agilent Bioanalyzer: 96–100% for capped RNA except for RNA prepared with ARCA, which was ~87%, slightly higher than the value determined by capillary electrophoresis. The cap form determined by mass spectrometry aligned with what was expected from the experimental design (Figure 8). In the case of enzymatic capping with a full vaccinia system (vaccinia capping enzyme and vaccinia O-methyltransferase) or co-transcriptional capping with CleanCap Reagent AG, only the cap 1 structure was found in the samples: **m^{7}Gppp(m$^{2'-O}$G)**pGpG(…) or **m^{7}Gppp(m$^{2'-O}$A)**pGpG(…), respectively. If RNA was capped only with the vaccinia capping enzyme using SAM (no 2-O′-MTase used) or co-transcriptionally capped with ARCA, only the cap 0 structure was identified: **m^{7}GpppG**pGpG(…) or **m$_2^{7,3'-O}$GpppG**pGpG(…), respectively. If RNA was capped using the vaccinia capping enzyme without SAM (and without 2-O′-MTase), only the cap G structure was found: **GpppG**pGpG(…). In the case of uncapped RNA, no capped RNA was determined and only **pppG**pGpG(…) and small amounts of **ppG**pGpG(…) at the 5′ end of the RNA were found, indicating that spontaneous dephosphorylation of the 5′ triphosphate occurs to some extent during IVT/DNase I reactions or purification.

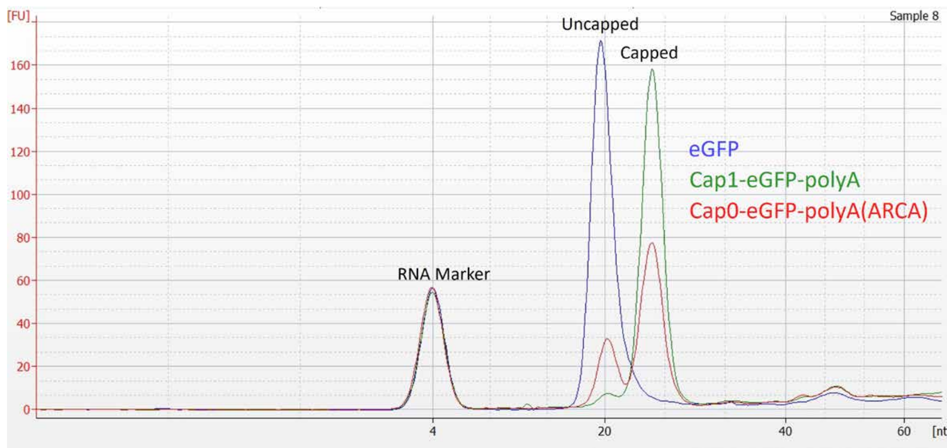


Figure 7. Electropherogram of 5′ RNA fragments cleaved with a DNAzyme specific to the 5′ UTR. Traces from fragments uncapped (eGFP), capped with vaccinia capping enzyme (Cap1-eGFP-polyA), and capped with ARCA (Cap0-eGFP-polyA (ARCA)) are shown.

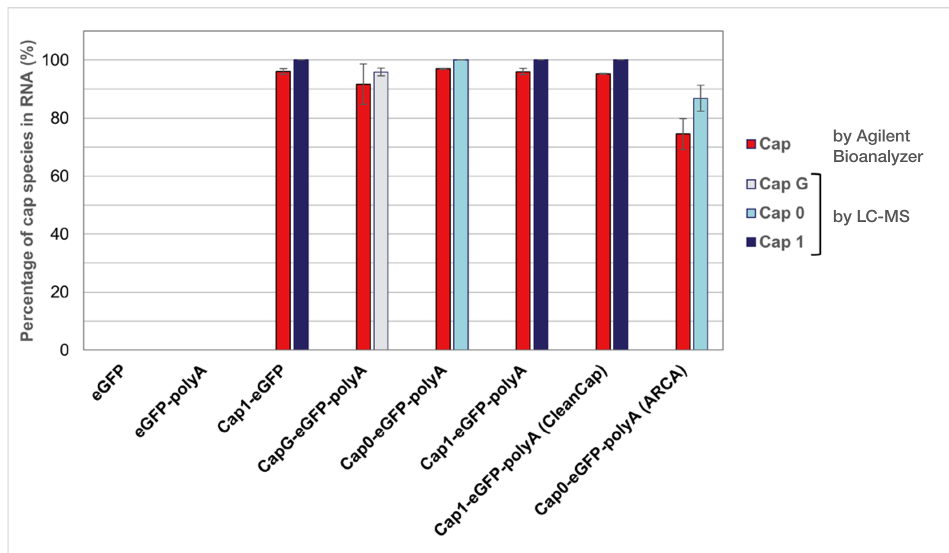


Figure 8. Capping efficiency and cap species of different RNA variants. The bars show the percentage of capped species measured in RNA samples. Red bars indicate the total capped species determined by capillary electrophoresis. The other bars represent capping species determined by LC-MS, with the color indicating the exact cap form. Error bars represent standard deviation of three RNA synthesis replicates.

Transfection of cells using synthesized RNA with different 3′ and 5′ end modifications

RNAs carrying different modifications were transfected into HEK293T cells (Figure 2). mRNAs that had a full structure with cap 1 and poly(A) tail (Cap1-eGFP-polyA and Cap1-eGFP-polyA (CleanCap Reagent)) resulted in the highest translational response (measured by eGFP fluorescence), as shown in Figure 9. There was no difference in transfection between RNAs enzymatically capped with vaccinia enzyme (Cap1-eGFP-polyA) or co-transcriptionally capped using CleanCap AG Reagent (Cap1-eGFP-polyA (CleanCap Reagent)). Additionally, the difference in transcription start codon (GGG or AGG, respectively) did not affect eGFP translation efficiency.

Unexpectedly, poly(A) RNA with cap 0, but only enzymatically capped (Cap0-eGFP-polyA), had a translational response very similar to those of poly(A) RNA with cap 1 (Cap1-eGFP-polyA and Cap1-eGFP-polyA (CleanCap Reagent)). In contrast, only ~1/3 of that translational response was obtained after transfection with cap 0 RNA prepared by co-transcriptional capping with ARCA (Cap0-eGFP-polyA (ARCA)). This difference could be due to the presence of uncapped RNA in Cap0-eGFP-polyA (ARCA), which reduces the actual amount of capped RNA used for transfection and may activate enzymatic RNA decay [4]. The cap G form of poly(A) RNA (CapG-eGFP-polyA) also resulted in ~1/3 of the signal of the more efficiently translated variants, while RNA samples without a cap structure and/or a poly(A) tail (eGFP, Cap1-eGFP, and eGFP-polyA) resulted in dramatically diminished eGFP signal. This indicates the importance of a proper cap structure and poly(A) tail for efficient translation in eukaryotic cells.

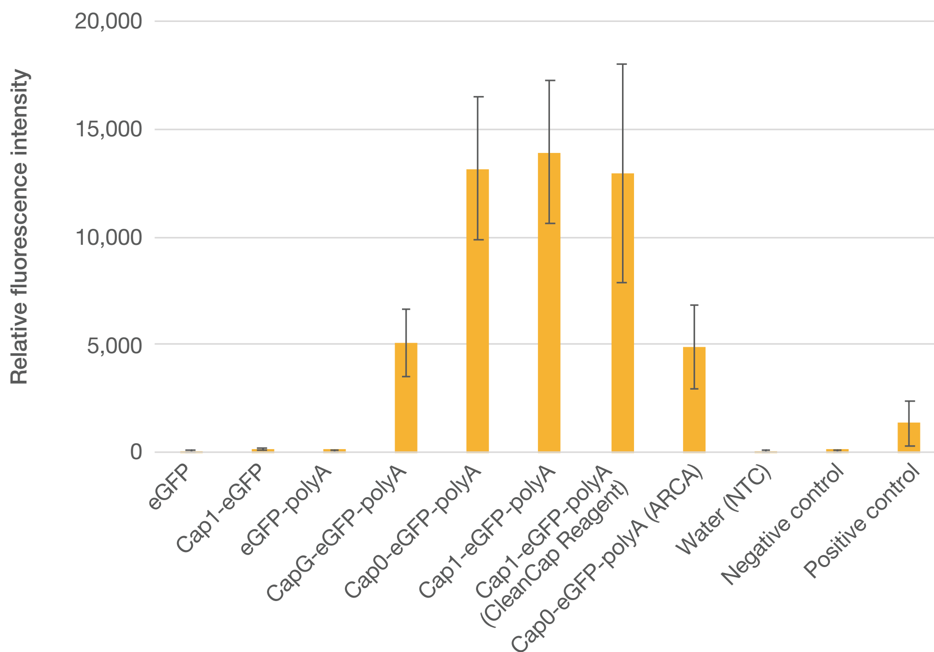


Figure 9. Translational efficiency of HEK293T cells transfected with eGFP RNA variants. eGFP relative fluorescence intensity is shown, and error bars represent standard deviations from two separate transfection experiments, each performed with three RNA synthesis replicates in triplicate repeats. A plasmid that can be expressed in eukaryotic cells, and carrying the eGFP gene, was used as a positive control. A plasmid that can only be expressed in *E. coli* bacteria, also carrying the eGFP gene, was used as a negative control.

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Summary

We demonstrate RNA synthesis using TheraPure enzymes, employing either enzymatic post-transcriptional or co-transcriptional capping. The TheraPure reagent portfolio includes provide all necessary enzymes and nucleotides to synthesize capped RNA and remove the DNA template. We show that RNA synthesis, enzymatic capping, and DNA removal can be performed in consecutive reactions without an intermediate purification step, resulting in capped, high-quality RNA in less than 4 hours. The transfection potency of mRNA was strongly dependent on the presence of proper cap 1 structure and poly(A) tail. However, transfection efficiency was independent of the method (enzymatic or co-transcriptional) used to produce cap 1.

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Ordering information

Description	Cat. No.
TheraPure T7 RNA Polymerase	EP011T1
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TheraPure RNase Inhibitor	EO038T1
TheraPure DNase I, RNase-free	EN052T1
TheraPure ATP, 100 mM, Sodium Solution	R044T1
TheraPure CTP, 100 mM, Sodium Solution	R045T1
TheraPure GTP, 100 mM, Sodium Solution	R046T1
TheraPure UTP, 100 mM, Sodium Solution	R047T1
TheraPure ATP, 200 mM, Tris Solution	R00044T1
TheraPure CTP, 200 mM, Tris Solution	R00045T1
TheraPure GTP, 200 mM, Tris Solution	R00046T1
TheraPure UTP, 200 mM, Tris Solution	R00047T1
10X Transcription Buffer	F8156AF
10X Capping Buffer	B23AF
TheraPure Vaccinia mRNA Cap 2′-O-Methyltransferase, 50 U/µL	Available as OEM only
TheraPure Vaccinia Capping Enzyme, 10 U/µL	Available as OEM only
50 mM $MgCl_2$ (polymerase co-factor)	F-5101
TheraPure S-Adenosyl-Methionine (SAM), 32 mM	Available as OEM only

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