

In Vitro Transcription of Long RNA Containing Modified Nucleosides

Norbert Pardi, Hiromi Muramatsu, Drew Weissman, and Katalin Karikó

Abstract

The in vitro synthesis of long RNA can be accomplished using phage RNA polymerase and template DNA. However, the in vitro synthesized RNA, unlike those transcribed in vivo in cells, lacks nucleoside modifications. Introducing modified nucleosides into in vitro transcripts is important because they reduce the potential of RNA to activate RNA sensors [1–6] and translation of such nucleoside-modified RNA is increased in cell lines, primary cells, and after in vivo delivery [1, 3, 7–10]. Here, we describe the in vitro synthesis of nucleoside-modified RNA with enhanced translational capacity and reduced ability to activate immune sensors.

Key words: messenger RNA, Immunogenicity, Modified nucleoside, Pseudouridine, 5-methylcytidine, Capping, In vitro transcription

1. Introduction

The in vitro delivery of protein-encoding DNA by transfection was first reported in 1973 (11) and 5 years later, a similar delivery of a protein-encoding viral RNA was also described (12). Over the subsequent years, DNA delivery became a standard laboratory technique, while RNA transfection was limited to viral RNA isolates until 1984 when in vitro transcription utilizing phage RNA polymerase and cloned cDNA was introduced (13). Interestingly, the first in vivo application of an in vitro transcript encoding a physiologically relevant protein was reported in 1992 (14), but RNAs further development beyond use as antigen-encoding vaccine vector (15–17) was delayed until 2011 (9). The potential reasons for the limited interest in applying mRNA as a therapeutic or using it in laboratory techniques for cellular delivery are RNA's immunogenicity, lability, low level and transient translatability, and

difficulty to work with. The recent discovery that incorporating naturally occurring modified nucleosides into RNA reduces its immunogenicity and greatly increases its translatability (3, 4, 6–8, 10, 18) has revolutionized RNA as a therapeutic. Now, in vitro transcripts containing modified nucleosides have been tested in vitro, to express protein at a high efficiency (3, 7, 19); ex vivo, to deliver transcription factors for the generation of induced pluripotent stem (iPS) cells (20–22); and recently, in vivo to express therapeutic proteins in mice and macaques (9, 10, 14).

Generally, eukaryotic mRNAs contain protein-coding sequences flanked by 5' and 3' untranslated regions (UTRs) and 5' cap and 3' poly(A) tails at their extremities. One way to increase or regulate the half-life and translational efficiency of an in vitro transcribed mRNA is to optimize its UTRs. Favorable 5' and 3' UTRs can be obtained from cellular or viral RNAs. We have found that using a 5' UTR derived from the tobacco etch virus 5' leader RNA, which mediates cap-independent translation (23–26), results in rapid and high level translation of the coding sequence (3, 7, 10, 19). Similarly, applying the 3' UTR of *Xenopus* beta-globin mRNA also ensures enhanced translation (27). To create an RNA expression plasmid, the coding sequence is cloned downstream from the promoter of T3, T7, or Sp6 RNA polymerase and flanked by 5' and 3' UTRs of interest. Sequences corresponding to a poly(A) tail can also be encoded. Incorporation of unique restriction enzyme recognition sites downstream of the poly(A) tail-related sequence aids in complete linearization of the plasmid. Linear DNA can also be prepared by PCR using primers containing the appropriate phage RNA polymerase promoter, but the choice of UTRs and encoded poly(A) tails is more limited. In addition, we found that RNA transcribed from PCR template contains more aberrant products.

After in vitro transcription, the newly synthesized mRNA can be further optimized. 7-methylguanylate cap and a poly(A) tail give significant stability and translatability to mRNA. Both can be incorporated into RNA during transcription with the inclusion of an anti-reverse cap analog (ARCA) and by encoding an extended region of polyadenylate, but these approaches have limitations. The use of ARCA reduces the amount of transcribed RNA per reaction, typically up to 50%, and a portion of the RNA remains uncapped and contains a 5'-triphosphate. The length of polyadenylate regions that a plasmid can tolerate without recombination is also limited. Vaccinia virus capping enzyme and 2'-O-methyltransferase (28) modify the 5'-triphosphate end of RNA with a cap structure containing terminal 7-methyl guanosine and 2'-O-methyl group at the penultimate nucleotide of the 5'-end (cap1). If the template plasmid or PCR product does not contain a region coding for poly(A) or the short poly(A) tail of the newly synthesised mRNA requires lengthening, poly(A) tail can be added to the 3' end of the mRNA using yeast poly(A) polymerase.

Different nucleoside modifications can be incorporated into mRNA. We found that T7 RNA polymerase or the double mutant (Y639F/H784A) with superior ability to incorporate noncanonical NTPs (29) were able to incorporate 2-thiouridine (s2U), 5-methyluridine (m5U), 5-methylcytidine (m5C), N6-methyladenosine (m6A), and pseudouridine (Ψ), but were unable to incorporate 2'-O-methylated NTPs into long RNA (6). Other groups have incorporated modified nucleosides that are not naturally found in RNA (30, 31), however these RNAs are translated very poorly or not at all (32). In addition, we believe that the risk of using nonnatural nucleosides is high, due to their metabolism and ability to be reincorporated into cellular or mitochondrial DNA or RNA. We have found that for translation, complete replacement of uridine with pseudouridine results in RNA with the highest level of translation and low levels of immunogenicity. Incorporation of m5C and Ψ into the RNA results in a further decrease in immunogenicity and variable but usually lower levels of translation. The use of HPLC purification further decreases residual immunogenicity from both types of modified mRNA with mRNA containing Ψ resulting in the highest levels of translation with reduced cost (7). Partial replacement of one or multiple nucleosides has been studied, but immunogenicity of the RNA remains (6, 33).

2. Materials

Prepare all the solutions using RNase-free reagents. Store all reagents at -20°C unless otherwise indicated. Follow all waste disposal regulations when disposing waste materials.

2.1. Analysis of DNA and RNA by Non-denaturing Agarose Gel Electrophoresis

1. Sub-Cell GT Agarose Gel Electrophoresis System with 7×10 cm tray and 15-well 0.75 mm thick combs (Cat. number 170-4487) (Bio-Rad, Hercules, CA).
2. Sub-Cell GT/PowerPac Basic System (Cat. number 164-0302) (Bio-Rad).
3. Agarose for molecular biological applications, store at 4°C .
4. Lithium boric acid electrophoresis buffer (LB) (Cat. number #LB10-1) (Faster Better Media, Hunt Valley, MD), store at room temperature (RT).
5. $5\times$ LB sample-loading media with orange G (Cat. number #LB5N-8) (Faster Better Media), store at RT.
6. Ethidium bromide (EtBr) stock: 0.5 mg/ml EtBr, store at RT.
7. UV transilluminator, we use a GelDoc 1000 gel imaging system (Bio-Rad).

2.2. Plasmid Linearization

1. Buffer and restriction enzymes that cleave at the 3' end of desired RNA sequence, if using a plasmid as the template.
2. Phenol–chloroform–isoamyl alcohol (25:24:1), pH 8.0, store at 4°C.
3. Chloroform, store at RT.
4. NaOAc: 3.0 M NaOAc, pH 5.5, store at 4°C.
5. Isopropanol, store at –20°C.
6. Ethanol: 75%, store at 4°C.

2.3. In Vitro RNA Synthesis

1. In vitro transcription kits with or without modified nucleoside triphosphates and Anti-Reverse Cap Analog (ARCA). Kits provide the nucleoside triphosphate solutions, reaction buffer, DNase, and RNA polymerase enzyme (see Note 1).
2. Modified nucleoside triphosphates (NTPs) can be obtained separately for transcription kits that do not include them (see Note 2).
3. DNase- and RNase-free, ultrapure (UP) water, stored at RT.
4. Lithium chloride precipitation solution: 7.5 M LiCl, 50 mM EDTA.
5. Siliconized microcentrifuge tubes.
6. NanoVue (Cat. number 28-9569-62) (GE Healthcare Biosciences, Piscataway, NJ) or similar UV spectrophotometer for determination of RNA and DNA concentrations.

2.4. Cap Modification of In Vitro Transcribed RNA

1. ScriptCap m⁷G Capping System (Cat. number C-SCCE0610) (CellScript, Madison, WI).
2. 2'-O-Methyltransferase kit (Cat. number C-SCMT0610) (CellScript).

2.5. Polyadenylation of RNA

1. Yeast poly(A) polymerase (600 U/μl, Cat. number 74225Y) (USB, Cleveland, OH) provided with 5× reaction buffer.
2. ATP stock: 100 mM ATP.

2.6. Dephosphorylation of RNA

1. APex heat-labile phosphatase provided with 10× buffer, pH 7.8 (Cat. Number APAP4850) (Epicentre, Madison, WI).
2. Acetic acid: 1 M, to adjust the pH of the reaction buffer, store at RT.

3. Methods

Plasmids constructed to contain the protein-coding sequence of interest, flanked by sequences corresponding to 5' and 3' UTRs, and encoded poly(A) tail are linearized prior to in vitro

transcription (see Note 3). The RNA can be capped during transcription by incorporating an Anti-Reverse Cap Analog (ARCA) or after transcription using capping enzymes. If ARCA is incorporated during transcription, the ratio of GTP to ARCA can be varied to increase the percent of RNA transcripts containing cap, but this is done at the expense of the total amount of RNA made in the reaction. Transcription kits with ARCA use a ratio of ARCA to GTP of 4:1, which results in approximately 80% of the transcripts capped and a 50% reduction in the total amount of RNA made. If immune activation is a concern, ARCA-containing RNA can be treated with a phosphatase to remove 5'-triphosphates. A number of factors weight into the decision to cap transcriptionally versus after transcription. We have found that RNA with the highest level of translation and the least overall cost, especially if phosphatase treatment will be used, is generated with posttranscriptional capping. A long poly(A) tail can be added to the 3' end of the RNA to further increase its stability and translatability using poly(A) polymerase.

All work with RNA is compromised by the presence of RNases. A major source of RNase contamination in the laboratory is plasmid DNA purification kits most of which use RNase A to remove bacterial RNA. RNase A renatures after drying or autoclaving, and therefore, special decontaminating procedures are needed. Ideally, a closed area isolated from reagents used for plasmid isolation should be secured for RNA synthesis.

3.1. Analysis of DNA and RNA by Non-denaturing Agarose Gel Electrophoresis

Standard agarose gel electrophoresis with EtBr staining is used to analyze RNA and DNA. Care to avoid RNase contamination of equipment or solutions should be taken.

1. Heat 0.5 g of agarose in 50 ml of 1× LB in a microwave until just boiling. This will make a 1% gel that is good for DNA and RNA above 0.5 kb in length. Higher concentrations of agarose can be used, when needed.
2. Cool to approximately 60°C, add 2 µl of EtBr stock solution, pour into a 7 cm × 10 cm gel tray, and add 1 or 2 15-well combs (0.75 mm thick).
3. Cool to room temperature, remove combs, and place gel tray into an electrophoresis apparatus containing 1× LB.
4. Apply 0.2–0.4 µg of DNA or RNA in 6 µl of 1× LB sample-loading media per well (see Note 4).
5. Apply a standard DNA or RNA ladder, if desired. RNA isolated from human cells can be used. Human 28S and 18S rRNAs are 5.0 and 1.9 kb, respectively.
6. Perform electrophoresis at 10 V/cm.
7. View migration of the nucleic acid intermittently by UV illumination (see Note 4).

3.2. Preparation of Linearized Plasmid DNA for In Vitro Transcription

In vitro RNA synthesis is performed on linear template DNA, preferentially from plasmids rather than PCR products. Template plasmid is linearized using a unique restriction endonuclease that leaves a 5'-overhang (preferred) or blunt end close to the encoded poly(A) sequence, if present (see Note 3). Restriction enzymes that leave a 3' overhang should not be used, as this can result in the polymerase continuing to transcribe off of the opposite strand resulting in double stranded RNAs (34).

1. Digest 50 µg of plasmid DNA with 50–100 units of restriction enzyme(s) in a 100 µl reaction for 4 h or overnight. Use high fidelity restriction endonucleases, if possible, to avoid star activity. Use two enzymes that cleave a sufficient distance apart to allow both to cleave the same plasmid, if possible, to ensure complete cleavage of the plasmid (see Note 5).
2. Analyze by agarose gel electrophoresis with EtBr staining and UV illumination to confirm complete cleavage of the plasmid DNA (Fig. 1).
3. Isolate the linearized plasmid DNA by extracting with 50 µl of phenol–chloroform–isoamyl alcohol following standard techniques.
4. Re-extract the aqueous phase, which contains the linearized plasmid, with chloroform (see Note 6).
5. Precipitate the DNA with 1/10 volume of NaOAc and an equal volume of isopropanol.
6. Incubate at –20°C for at least 30 min.
7. Pellet the precipitated DNA for 2 min at 13,000 × g in a microcentrifuge at RT.
8. Wash three times with cold 75% ethanol (see Note 7).

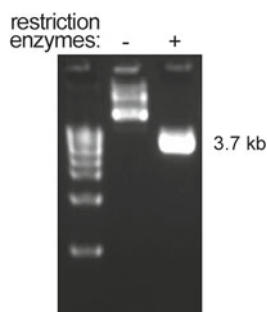


Fig. 1. Linearization of plasmid DNA. Aliquots of reaction mixtures incubated with or without restriction enzymes were separated on a 1% agarose gel. The ethidium bromide-stained bands are visualized after UV illumination. Although equal amounts of plasmids were loaded onto the gel, linearized plasmid, which is more accessible to ethidium bromide than circular plasmid, generates a stronger signal. A 1 kb DNA ladder (first lane) is used as a molecular weight reference.

9. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min.
10. Resuspended in UP water (see Note 8).
11. Measure the concentration of the linearized DNA using a spectrophotometer and reading the absorbance at 260 nm. The concentration is calculated by multiplying the OD₂₆₀ by 0.05 to obtain µg/µl of DNA.

3.3. In Vitro RNA Synthesis

In vitro transcription kits are designed to produce high yields of RNA from linearized plasmid DNA template containing a T3, T7, or Sp6 promoter (see Note 9). One microgram of DNA template is sufficient to generate 50–100 µg of RNA.

1. For generating modified nucleoside-containing RNA with transcription kits not containing a modified nucleoside(s), exchange the basic NTP with the corresponding triphosphate-derivative of the modified nucleoside at the same concentration in the transcription reaction (see Note 10).
2. In a siliconized microcentrifuge tube for a 10 µl reaction volume, add 1 µg template DNA, reaction buffer, nucleoside triphosphate stocks (see Note 11), and RNA polymerase enzyme, per the kit being used.
3. Incubate the in vitro transcription reaction for 4 h at 37°C (see Note 12).
4. Degrade the DNA template with DNase by incubating at 37°C for 15 min.
5. Precipitate the RNA by adding ½ volume (5 µl) of cold LiCl precipitation solution.
6. Incubate overnight at –20°C.
7. Centrifuge the precipitated RNA at 13,000×*g* for 5 min in a microcentrifuge at RT.
8. Wash the firm RNA pellet three times with cold 75% ethanol (see Note 7).
9. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
10. Resuspend the RNA pellet in UP water.
11. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A –80°C or –20°C freezer can also be used, but freezing takes more time.
12. Measure the RNA concentration with a NanoVue.
13. Analyze the quality of the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4).

3.4. Posttranscriptional Capping of In Vitro Transcribed RNA

RNA can be capped posttranscriptionally using an enzyme derived from vaccinia virus, which forms cap0. The addition of a second enzyme that methylates the 2'-OH of the penultimate nucleotide at the 5'-end of the RNA produces cap1. The ScriptCap m⁷G capping enzyme and 2'-O-methyltransferase (CellScript) are used. The enzymes can be used simultaneously in one reaction (see Note 13). Optimizing the 5' sequence of the RNA to avoid formation of a stem-loop structure ensures that the capping enzyme has full access to convert all of the 5' triphosphate ends (3), thereby capping all the RNA.

1. Add 60 µg of in vitro transcribed RNA (see Note 14) and UP water to a siliconized microcentrifuge tube and incubate at 65°C for 5 min to disrupt secondary structures of the RNA.
2. After heating, snap-cool the RNA on ice and add the remaining components of the capping reaction, 10 µl 10× reaction buffer, 10 µl GTP, 5 µl SAM, 2.5 µl ScriptGuard RNase inhibitor, 5 µl ScriptCap m⁷G capping enzyme, and 5 µl 2'-O-methyltransferase in a 100 µl final volume.
3. Incubate at 37°C for 1 h.
4. Precipitate the RNA with 50 µl of cold LiCl precipitation solution.
5. Incubate at -20°C overnight.
6. Centrifuge the precipitated RNA at 13,000×g for 5 min in a microcentrifuge at RT.
7. Wash the firm RNA pellet three times with cold 75% ethanol (see Note 7).
8. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
9. Resuspend the RNA pellet in UP water.
10. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A -80°C or -20°C freezer can also be used, but freezing takes more time.
11. Measure the concentration of the RNA with a NanoVue. The concentration is calculated by multiplying the OD260 by 0.04 to obtain µg/µl of RNA.
12. Analyze the quality of the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4).

3.5. Polyadenylation of In Vitro Transcribed RNA

Poly(A) tails can be encoded in the DNA used to transcribe RNA and/or added posttranscriptionally.

1. In a siliconized microcentrifuge tube for a 25 μ l reaction, add 33 pmol of RNA, which correspond to \sim 10 μ g of a 1 kb-long RNA (see Note 15).
2. Add 1.3 μ l ATP stock, 5 μ l 5 \times reaction buffer, and 2.5 μ l poly(A) polymerase enzyme.
3. Incubate for 3 h at 37°C.
4. Precipitate RNA by adding 12.5 μ l of cold LiCl precipitation solution.
5. Centrifuge the precipitated RNA at 13,000 $\times g$ for 5 min in a microcentrifuge at RT.
6. Wash the RNA pellet three times with cold 75% ethanol (see Note 7).
7. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
8. Resuspend the RNA pellet in UP water.
9. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A -80°C or -20°C freezer can also be used, but freezing takes more time.
10. Measure the concentration of the RNA with a NanoVue.
11. Analyze the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4). Poly(A)-tailed RNA migrates slower relative to the RNA without poly(A) tail addition (see Note 16 and Fig. 2).

3.6. Removal of 5'-Triphosphate with APex Phosphatase

Most phosphatases are active only at \sim pH 8 or higher. Considering that RNA is quickly hydrolyzed at such a high pH, removing 5'-end phosphates from RNA should be performed with APex alkaline phosphatase that is active at pH's as low as 5. APex phosphatase is provided with 10 \times acetate buffer (pH 7.5), but our experience is that RNA is still hydrolyzed in this buffer. Therefore, use acetic acid and UP water to convert the pH 7.5 acetate buffer (10 \times) to pH 6.5 buffer (5 \times).

1. Adjust the pH of the 10 \times buffer to 6.5 using 1 M acetic acid and a pH meter or narrow range (4 to 7) pH test strips.
2. Increase the final volume of the pH adjusted buffer to twice the starting volume to make a 5 \times buffer.

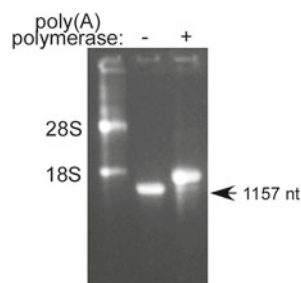


Fig. 2. Poly(A)-tailing of RNA. Aliquots of RNA before and after a 3 h incubation with poly(A) polymerase were separated on a 1.4% agarose gel. The ethidium bromide-stained bands are visualized after UV illumination. RNA isolated from human cells is used as a molecular weight reference. Human 28S and 18S rRNAs are 5.0 and 1.9 kb, respectively. A higher amount of poly(A)-tailed RNA was loaded to the gel to detect any RNA that remained unextended.

3. For a 50 μ l reaction, add 10 μ l of 5 \times acetate buffer (pH 6.5) to 25 μ l of RNA (50 μ g).
4. Add 15 μ l of APex alkaline phosphatase (1 unit per μ g of RNA).
5. Incubate at 37°C for 60 min.
6. Heat-inactivate by incubating at 70°C for 5 min.
7. Precipitate RNA by adding 25 μ l ($\frac{1}{2}$ volume) of cold LiCl precipitation solution.
8. Centrifuge the precipitated RNA at 13,000 $\times g$ for 5 min in a microcentrifuge at RT.
9. Wash the firm RNA pellet three times with cold 75% ethanol (see Note 7).
10. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
11. Resuspend the RNA pellet in UP water.
12. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A -80°C or -20°C freezer can also be used, but freezing takes more time.
13. Measure the concentration of the RNA with a NanoVue.
14. Analyze the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4).

4. Notes

1. RNA transcription kits, including INCOGNITO kits from CellScript (Madison, WI) (T7 \pm ARCA) with Ψ and m5C (Cat. numbers: C-ICTAMY110510 and C-ICTMY110510, respectively), T7 with Ψ (Cat. number: C-ICTY110510), SP6 with Ψ (Cat. number: C-ICSY110510) and kits without modified nucleosides; SP6-Scribe (Cat. number: C-AS3106), T7 mScript (Cat. number: C-MS11610), and T7-Scribe (Cat. number: C-AS2607) (CellScript) and Megascript and mMESSAGE mMACHINE (T7, T3, and SP6) (Cat. numbers: AM1333, AM1338, and AM1339 and AM1334, AM1348, and AM1340, respectively) (Ambion, Grand Island, NY) can be used.
2. Triphosphate-derivatives of modified nucleosides, including m5C (Cat. number N-1014), m5U (Cat. number N-1024), s2U (Cat. number N-1032), m6A (Cat. number N-1013), and Ψ (Cat. number N-1019) (TriLink, San Diego, CA) are prepared at a concentration of 50 mM (Sp6) or 75 mM (T3 or T7).
3. For plasmid linearization, restriction enzymes generating 5'-overhangs should be selected to avoid aberrant transcription leading to dsRNA production. To achieve 100% plasmid linearization, two restriction enzymes should be used that cut close (10–20 nucleotides) to each other.
4. Such a small amount of RNA can only be detected when non-denaturing agarose gel electrophoresis is used for the analysis. Under non-denaturing conditions, the RNA migration can be anomalous due to *cis*- and *trans*-molecular interactions, and thus, additional slower migrating bands might appear. Modified nucleoside-containing RNAs may migrate differently from the corresponding RNA that does not contain modified nucleosides. However, the bands should appear sharp and intact with no smear, a sign of RNA degradation. RNAs can also be analyzed under denaturing conditions to determine their correct length.
5. Incomplete plasmid linearization leaves contaminating circular plasmid templates that will be transcribed into extremely long and heterogeneous RNA due to the processivity of the RNA polymerases. Transcription initiation is a limiting step, thus, the presence of a small amount of circular template will lead to generation of a large amount of unwanted transcripts.
6. A chloroform extraction is performed to remove residual phenol that inhibits transcription.
7. The ethanol wash is performed by adding 100 μ l of 75% ethanol per 10 μ l of reaction volume, dislodging the pellet by using the force of adding the liquid, briefly centrifuging the tube at

high speed, and removing the liquid. Care should be taken when removing liquid, we do not use a vacuum aspirator but prefer a micropipettor, so that if the pellet is aspirated, it can be spun again. The siliconized tube allows the RNA pellet to be slightly less adherent, but the force of expelling the ethanol from a micropipette is needed to dislodge. Avoid breaking the pellet into pieces.

8. Typically, a third of the starting amount of DNA is lost during precipitation. The volume of water used to dissolve the DNA should aim to give a DNA concentration of 1–2 $\mu\text{g}/\mu\text{l}$.
9. The yields of mRNA are lower when transcription is performed with Sp6 RNA polymerase.
10. For RNAs that will contain only a fraction of modified nucleosides, the relative amounts of certain modified and its cognate unmodified NTP can be added to the transcription reaction. We found that for m5C, m6A, and Ψ , the percent of modified nucleotide in the transcription reaction equaled the percent incorporated into the RNA (6). Other modified nucleotides will have to be examined for efficiency of incorporation.
11. Thaw NTP stocks that will be used repeatedly on ice, thus avoiding their hydrolysis to NDP and NMP.
12. The length of the transcription reaction is typically 4 h, but can be optimized for each RNA, however, extended overnight incubation is not recommended because at low NTP concentrations, the T7 RNA polymerase exerts RNase activity (35).
13. Reactions can be scaled up or down depending on how much RNA is needed. We typically do not process more than 200 μl of a reaction in a 1.5 ml Eppendorf tube because of the difficulty of rehydrating the large thick RNA pellet.
14. RNA transcripts can form stable secondary structures involving the 5'-most nucleotides that can severely limit access by the capping enzyme. Heating followed by snap-cooling can partially disrupt the RNA secondary structure resulting in increased capping efficiency. Properly designed sequences in plasmid templates that avoid stem-loop formation at the 5' end of RNA allow access for the capping enzyme, thus ensuring generation of completely capped RNA that can be measured by the loss of γ - ^{32}P label from the RNA (3).
15. The concentration of ends of RNA is used to determine how much RNA can be efficiently poly(A) tailed. We have found that with this poly(A) tailing kit, 33 pmol of RNA can be nearly 100% poly(A) tailed. When calculating molarity for a 1,000 nt long RNA, $\sim 0.32 \mu\text{g}$ RNA equals 1 pmol.
16. After poly(A)-tailing, the RNA length is increased significantly (~ 200 nt) that can be detected by analyzing RNA samples before

and after poly(A)-tailing with agarose gel electrophoresis. A lack of an increase in RNA length or an easily detectable amount of RNA with identical length to untailed RNA can be caused by the inclusion of too many RNA ends (>33 pmol) or the formation of secondary structures at the 3' end of the RNA that obstruct access of the poly(A) polymerase.

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