Chapter 13

In Vitro Transcription of Modified RNAs

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Abstract

RNAs containing a variety of terminal and internal modifications can be produced using bacteriophage polymerases often with a few simple adjustments to standard transcription protocols. RNAs containing a single phosphate or a cap structure at their 5′ ends can readily be generated either co-transcriptionally or through enzymatic treatments of transcription products. Likewise, a variety of modified bases, including fluorescent or biotinylated species, can be effectively incorporated co-transcriptionally. The key to effective co-transcriptional incorporation lies in determining the efficiency of incorporation of modified base relative to its standard counterpart. Finally, an approach to place a poly(A) tail at the exact 3′ end of a desired transcription product is presented. Collectively, these protocols allow one to synthesize RNAs with a variety of modifications to serve as versatile molecules to analyze biological questions.

Key words: Transcription, Capping, 5′ Monophosphate, Fluorescent nucleotides, Poly(A) tail, Modified bases

1. Introduction

The efficient production of RNA by bacteriophage polymerases (e.g., T7, SP6, T3) using commercially available vectors or oligonucleotide-derived templates has provided an enormous boost to posttranscriptional research and molecular biology in general over the last 25 years (1, 2). A key aspect of this technology is the ability to produce RNAs containing directed physiological or synthetic modifications for specific applications. The goal of this chapter is to provide a discussion of key methodologies to produce transcripts with 5′, 3′, or internal modifications.

RNA substrates containing a *bona fide* 5′ cap structure and/or a 3′ poly(A) tail, for example, are invaluable for translation, RNA stability, and other mRNA functional analyses. RNAs with a 5′ monophosphate at their 5′ ends are extremely useful as efficient ligation substrates and for exonuclease assays. Transcripts with

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internally modified nucleotides have a plethora of applications. Internal biotin modifications, for example, can be used for affinity purification of RNA-binding proteins. The incorporation of radioactive rNTPs is used routinely for detection probes, protein–RNA interaction analyses, and substrates for a variety of cell-free RNA processing assays. The substitution with fluorescent bases creates opportunities for non-isotopic detection for a variety of assays, including protein–RNA interaction, RNA structural perturbations, and drug screening. Finally, the inclusion of a poly(A) tail at the precise 3′ end of an RNA creates a physiologically relevant substrate molecule for translation, deadenylation, and mRNA structure/function assays. Therefore, collectively the protocols described below should provide the RNA researcher with the ability to produce a variety of RNA modification to tailor the transcript to the desired application.

2. Materials

2.1. Production of RNAs with a Modified 5′ End

1. rNTP solution: 0.5 mM rGTP and 5 mM each of rCTP, rATP, rUTP (see Note 1). Store at –20°C or colder.
2. Guanosine-5′-monophosphate (5′-GMP) solution: 5 mM 5′-GMP dissolved in dd H_2O. Store at –20°C or colder.
3. Double-distilled H_2O (dd H_2O).
4. 5× Transcription Buffer: 200 mM Tris–HCl (pH 7.9), 30 mM $MgCl_2$, 10 mM spermidine, and 50 mM dithiothreitol.
5. Commercial RNase inhibitor, e.g., Ribolock RNAse Inhibitor, 40 u/μL (Fermentas).
6. SP6 or T7 RNA Polymerase, 50 u/μL.
7. Phenol–chloroform–isoamyl alcohol. Mix components in a 25:24:1 ratio.
8. 20 mg/mL glycogen in ddH_2O.
9. 10 M ammonium acetate.
10. Ethanol. Stock solutions of 100% (200 proof) and 80% (prepared with water) are required.
11. RNA Loading Dye: Mix 12 g urea, 0.185 g EDTA, 125 μL 1 M Tris–HCl (pH 7.5 at 25°C), 0.125 g bromophenol blue, and 0.125 g xylene cyanol. Dissolve all to a final volume of 25 mL with dd H_2O.
12. 40% acrylamide/bisacrylamide mix: Bisacylamide represents 5% of the total acrylamide in this solution prepared in water and filtered to remove any particulates (see Note 2).
13. TBE Gel Running Buffer: 450 mM Tris base, 445 mM boric acid, and 10 mM EDTA.

14. Urea/TBE Gel Mix: Mix 240 g urea (final concentration 7 M), 50 mL 10× TBE running buffer, and 200 mL distilled water.
15. 10% ammonium persulfate solution. Prepare in water and store at 4°C (see Note 3).
16. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED). Store at 4°C.
17. HSCB Buffer: 25 mM Tris–HCl (pH. 7.6 at 25°C), 400 mM NaCl, and 0.1% SDS.
18. 1 mg/mL proteinase K solution in water. Store at −80°C.

2.2. Production of RNAs with Internal Modifications

1. rNTP-flATP Labeling Solution: 0.5 mM rATP and 5 mM each of rCTP, rGTP, rUTP (see Note 4). Store at −20°C or colder.
2. Fluorescein-ATP Solution: 5 mM Fluorescein-12-ATP dissolved in ddH_2O. Store at −20°C or colder.
3. Other transcription-related reagents (items 3–18) described in Subheading 2.1.

2.3. Production of RNAs with a poly(A) Tail at Precisely the 3′ End

1. DNA oligonucleotides.
2. T4 polynucleotide kinase (10 U/μL).
3. 10× T4 kinase reaction buffer: 700 mM Tris–HCl (pH 7.6 at 25°C), 100 mM $MgCl_2$, and 50 mM dithiothreitol.
4. Oligonucleotide hybridization buffer: 10 mM Tris–HCl (pH 8.0 at 25°C), 50 mM NaCl, and 1 mM EDTA.
5. Ligation buffer: 20 mM Tris–Cl (pH 7.6 at 25°C), 10 mM $MgCl_2$, 10 mM dithiotreitol, and 0.6 mM ATP.
6. T4 DNA Ligase (400 U/μL).
7. 10 mM dNTPs dissolved in water. Store at −20°C.
8. 10× PCR reaction buffer: 100 mM Tris–HCl (pH 8.3 at 25°C), 15 mM $MgCl_2$, and 500 mM KCl.
9. Taq DNA polymerase.
10. *Nsi*I restriction enzyme.
11. Other transcription-related reagents (items 3–18) described in Subheading 2.1.

3. Methods

3.1. Production of RNAs Containing a Modified 5′ End

While standard transcription reactions using phage polymerases yield RNAs with a 5′ triphosphate, it is often desirable to generate RNAs with alternative 5′ ends. The production of an RNA with a 5′ m^7GTP cap, for example, is necessary for efficient in vitro translation (3), polyadenylation (4), RNA decay assays (5), and for

many RNA transfection studies (6). The capping of the 5′ end of a transcript can readily be performed co-transcriptionally. Alternatively, an RNA containing a 5′ triphosphate can be capped using GTP and recombinant vaccinia capping enzyme (7), albeit at a much lower efficiency than what can be obtained by adding cap analogs directly to transcription reactions.

One common 5′ end modification that we have used for a variety of applications is the production of RNAs containing a 5′ monophosphate. These transcripts can be used directly in ligation reactions (8) and exonuclease assays (9). This protocol is presented below to serve as a model for the co-transcriptional modification of RNA 5′ ends.

1. In a 1.5 mL microfuge tube, mix together 3.5 μL of ddH_2O, 2 μL of 5× transcription buffer, 1 μL of rNTP solution, 1 μL of DNA template (1 μg/μL of plasmid or ~0.5 pmol of oligonucleotide template), 1 μL 5′ GMP solution, 0.5 μL (~20 units) of RNase inhibitor, and 1 μL of SP6 or T7 polymerase. Give the mixture a brief spin in a tabletop microfuge (e.g. ~500 × *g* for 3 s) to ensure that all of the reaction contents are at the bottom of the tube (see Notes 5 and 6).
2. Incubate reaction at 37°C for 1–3 h.
3. Add 150 μL of ddH_2O and 150 μL of phenol–chloroform–isoamyl alcohol (25:24:1). Vortex until the mixture goes from clear to white. Centrifuge in a table top microcentrifuge at 16,000 × *g* room temperature for 1 min, and pipet the aqueous top layer into a fresh 1.5 mL tube.
4. Add 1 μL glycogen (20 mg/mL) and 40 μL 10 M ammonium acetate (see Note 7). Add 500 μL ice cold 100% ethanol and vortex. Incubate at least 10 min at −80°C.
5. Spin in a microcentrifuge at full speed for 10 min at room temperature to pellet the RNA. Carefully remove supernatant without disturbing the white pellet.
6. Wash the pellet with 150 μL ice cold 80% ethanol. Remove as much of the ethanol as possible without disturbing the pellet and dry the pellet either under vacuum in a Speed Vac system or for 5–10 min on the bench top.
7. Resuspend the RNA either in 5 μL of RNA loading dye and proceed to purification (see step 8) or in 20 μL of ddH_2O for storage at −80°C if desired (see Note 8).
8. To gel purify the RNA, electrophorese the pellet resuspended in RNA loading dye on a vertical 5% denaturing acrylamide gel containing 7 M urea. Set up the glass plates and spacers to form the gel mold. In a 50 mL beaker, add 3.75 mL 40% acrylamide/bisacrylamide mix, 21.25 mL urea/TBE gel mix, 300 μL 10% ammonium persulfate, and 30 μL TEMED. Quickly pour the solution between the glass plates and insert the desired gel comb.

9. Pre-run the gel in 1× TBE buffer for 20–30 min before loading (see Note 9).
10. Heat the RNA sample to 95°C for 30 s, quick chill on ice and load onto the gel using a standard micropipette tip. Run the gel approximately 1 h using the migration of the marker dyes to determine the approximate position of the RNA transcript (see Note 10).
11. Visualize the position of the RNA by either UV shadowing the gel (see Note 11) or using X-ray film or a phosphorimager if the RNA product is labeled with radioactivity or a fluorescent dye.
12. Cut out desired products, and let the gel slice incubate at room temperature in 400 μL HSCB buffer overnight to allow for passive diffusion of the RNA from the gel fragment.
13. Pipet the supernatant into a fresh tube. Add 400 μL of phenol–chloroform–isoamyl alcohol (25:24:1). Vortex until the mixture goes from clear to white. Centrifuge in a table top microcentrifuge at 16,000 × *g* room temperature for 1 min, and pipet the aqueous top layer into a fresh 1.5 mL tube.
14. Add 1 μL glycogen (20 mg/mL) and 1 mL of ice cold 100% ethanol and vortex. Precipitate and wash the RNA pellet as described in steps 5–7 above, and resuspend the pellet in 20 μL of ddH_2O.

3.2. Production of RNAs with Internal Modifications

The incorporation of modified ribonucleotides into transcripts generated by conventional run-off transcriptions is an effective approach to produce large amounts of RNA for a variety of applications. A co-transcriptional incorporation procedure is described below. Note that a variety of modifications can also be added by splinted ligation approaches (8) that are described elsewhere in this book. The incorporation of fluorescently labeled ribonucleic acids into reporter transcripts using in vitro transcriptions is particularly useful in determining changes in RNA structure and binding affinity to particular ligands, including therapeutic compounds, using fluorescence spectroscopy (10). Although the attachment of fluorescent analogs to pyrimidines has traditionally not been pursued in favor of the use of purine analogues (particularly 2-aminopurine), the efficient incorporation of furan-containing ribonucleic acids via T7 run-off transcription has been described (10). Similarly, modified UTP containing positively charged, hydrophobic groups at the 5-position have also been shown to be permissive to T7-mediated incorporation into run-off transcription products (11). Interestingly, efforts are being made to expand the capacity of the T7 polymerase to incorporate modified nucleotides into nascent RNA transcripts by manipulating the enzyme itself (12). Chelliserrykattil and Ellington reported the generation of a variant T7 RNA polymerase with improved ability to incorporate 2′-*O*-methyl ribose nucleotides with the goal of enhancing RNA stability with removal of the 2′ hydroxyl group (12, 13).

Table 1
A survey of commercially available modified nucleotides that can be incorporated into RNA co-transcriptionally

Company	Hapten nucleotide analogs	Fluorescent analogs
PerkinElmer	Haptens: Biotin-ATP, -CTP, -GTP, -UTP Dinitrophenol-UTP	Fluorescent-labeled: Fluorescein-ATP, -CTP, -GTP,-UTP Cyanine-3 (UTP, GTP, ATP, CTP) Cyanine-5 (UTP, GTP, ATP, CTP)
Invitrogen (Life Technologies)		ChromaTide™ UTPs: Alexa Fluor 488, AlexaFluor 546, Fluorescein, Rhodamine Green
Applied Biosystems (Life Technologies)		Aminoallyl-UTP (transcript can be labeled subsequently with amine-reactive fluor or hapten of choice)
Fermentas (Thermo Fisher Scientific)		Aminoallyl-UTP (transcript can be labeled subsequently with amine-reactive fluor or hapten of choice)
Roche Applied Science	Haptens: Biotin-UTP Dinitrophenol-UTP	

Although the following protocol is described using fluorescein-ATP, it can be readily adapted for the incorporation of many other modified NTPs. Table 1 outlines some of the modified rNTPs that can be added by simply substituting the desired modified base for fluorescein-ATP. Note that if one chooses to incorporate aminoallyl-UTP using T7, T3, or SP6 polymerase, the RNA can subsequently be labeled with any amine-reactive fluor or hapten.

1. In a 1.5 mL microfuge tube, mix together 3.5 μL of ddH_2O, 2 μL of 5× transcription buffer, 1 μL of rNTP-flATP labeling solution, 1 μL of DNA template (1 μg/μL of plasmid or ~0.5 pmol of oligonucleotide template), 1 μL Fluorescein-ATP solution, 0.5 μL (~20 units) of RNase inhibitor and 1 μL of SP6 or T7 polymerase. Give the mixture a brief spin in a tabletop microfuge (e.g. ~500 × *g* for 3 s) to ensure that all of the reaction contents are at the bottom of the tube.
2. Proceed as described in the protocol of Subheading 3.1, steps 2–13.

3.3. Production of RNAs with a poly(A) Tail at Precisely the 3′ End

Commercial vectors for in vitro transcription reactions do not provide a means to generate an RNA with a poly(A) tail at precisely the 3′ end of the transcript. The production of such a transcript could be highly desirable since it makes the in vitro synthesized RNA as biologically relevant as possible. Plasmid vectors have been described that encode a 60 base poly(A) tail followed immediately by an *Nsi*I site (14). Cleavage using this restriction enzyme and

subsequent run off transcription allows the poly(A) stretch to be located at precisely the 3′ end of the RNA. Alternatively, one can add poly(A) stretches posttranscriptionally using yeast or bacterial poly(A) polymerases (see Note 12). However if one desires to avoid extra cloning and RNA modification steps, below is a straightforward PCR-based approach that will allow a fragment encoding a poly(A) tail to be attached onto any DNA fragment such that the adenosine stretch will appear at the precise 3′ end of the subsequent run-off transcription product.

1. Synthesize a complementary pair of oligonucleotides containing the following structure:

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XXXXAAAAA(n)AAAAATGCATTACCTCGAGCACTC
     TTTTT(n)TTTTTACGTAATGGAGCTCGTGAG
_____|_____|_____|_____|_____|_____|
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RE site poly(A) tract *Nsi*I site primer binding site

See Note 13 for a complete description of the roles of the four regions of this oligonucleotide.

2. Change the 5′ end of the oligonucleotides from a 5′ hydroxyl to a 5′ phosphate using 1 μL of T4 polynucleotide kinase and 1 μL 10× kinase reaction buffer in a 10 μL reaction for 1 h at 37°C.
3. Hybridize the synthetic oligonucleotides by adding 100 pmol of each strand in 100 μL of oligo hybridization buffer. Place the tube in a heating block at 100°C, turn off the block and allow it to slowly cool to room temperature to ensure that the most stable hybrid is formed.
4. Ligate the hybridized oligonucleotides to the target DNA fragment. Mix 1 pmol of restriction enzyme-cut target DNA (or 1 pmol of oligonucleotide transcription template containing a four base single-stranded overhang) with 1 pmol of hybridized oligos in a 10 μL reaction containing 1 μL of ligation buffer and 10 units of T4 DNA ligase. Incubate at room temperature for at least 1 h or overnight at 15°C.
5. To amplify the ligated products by PCR, mix 5 μL of the ligation reaction, 50 pmol of an SP6 or T7 promoter primer (e.g. SP6 = 5′-CATACGATTTAGGTGACACTATAG), 50 pmol of a downstream primer (5′-GAGTGCTCGAGGTA); note—this is specific for the primer binding site region of the ligated oligonucleotide), 10 mM dNTPs, 10 mM Tris–Cl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 20–25 units of Taq polymerase in a 500 μL reaction. Perform at least 30 cycles of amplification in a thermocycler: denaturation at 94°C for 1 min; annealing at 43°C for 1 min; and extension at 72°C for 1 min, for the example presented here.
6. Extract the PCR reaction mixture with phenol–chloroform–isoamyl alcohol (25:24:1) and concentrate the DNA by ethanol

precipitation as described in Subheading 3.1, steps 3–7. Alternatively, amplified products can also be purified from agarose gels or using spin columns if desired.

7. Resuspend the DNA in 100 μL of TE buffer. Treat the DNA with *Nsi*I according to the manufacturer's recommendations in order to place the A/T tract at the precise 3′ end of the transcription template.
8. Following digestion, add 400 μL of HSCB buffer along with 20–30 μg of proteinase K. Incubate at 37°C for 15 min (see Note 14).
9. Phenol–choroform–isoamyl alcohol extract and ethanol precipitate the DNA as described in Subheading 3.1, steps 3–7. Resuspend the DNA in 100 μL of TE buffer.
10. Transcribe the DNA template as described in Subheading 3.1 or 3.2 depending on the application of the RNA that will be generated.

4. Notes

1. The amount of rGTP is reduced tenfold relative to other rNTPs to allow for efficient incorporation of the 5′GMP at the initiation step of transcription. Since phage polymerases strongly prefer to begin transcription with a G residue, this nucleotide mixture is generally applicable for all 5′ modifications. Note that for internal modifications, the relative concentrations of rNTPs are adjusted to accommodate the efficient incorporation of modified bases.
2. Unpolymerized acrylamide is a neurotoxin and should be handled carefully.
3. Solution will lose potency over time. Best results are obtained with solutions less than a week old.
4. The concentration of rGTP in the solution should be lowered to 0.5 mM if the transcript is to be capped co-transcriptionally.
5. The two largest sources of problems that affect the yield of transcription reactions in our hands has been the quality of the reagents used and the quality of the DNA template. These can be minimized by purchasing high-quality chemicals from a reputable supplier, using double-distilled water and ensuring the purity and concentration of the DNA template.
6. Ten times more 5′ GMP (or m7GpppG cap analog) than 5′ GTP is added to the reaction since it is utilized less efficiently than the nucleotide triphosphate by the phage polymerase.

This is common with modified bases and we use the 10× concentration as an empirical starting point when coaxing the polymerase to add modified bases.

7. The use of ammonium acetate is preferred over sodium salts in this step since unincorporated rNTPs will not be effectively precipitated in the presence of ammonium salts. This aids in the purification of the RNA product away from the reaction components.
8. Note that the reaction can be stored at –80°C for extended periods of time in ethanol without loss of RNA quality.
9. The acrylamide gel is pre-run to allow the ion front that develops during the preparation of the gel to move down the gel. If this is not done, it could influence the migration of RNAs of interest.
10. On a 5% denaturing gel, the bromophenol blue dye runs approximately at the position of a 27–30 base RNA. The xylene cyanol runs at approximately the site of a 120 base RNA.
11. To UV shadow RNA on a gel, carefully place the gel on a piece of Saran Wrap and overlay this onto a TLC plate that contains a fluor which will absorb at 260 nm light. When the gel is irradiated with short wave UV light, the RNA will appear as a black shadow on the gel as it absorbs the light and prevents it from hitting the fluor below it.
12. In our hands, it has been very difficult to control the size of the poly(A) tail added to the ends of RNA using yeast poly(A) polymerase. This product heterogeneity requires an extra gel purification step which often results in low yields of RNAs containing defined lengths of poly(A).
13. The sequence of the restriction enzyme overhang in the oligo is used to hybridize with a convenient restriction enzyme site at the downstream end of the target DNA fragment for ligation purposes. If oligonucleotides instead of plasmid DNA are being used as the parent template, then any 4 base sequence can be used here as long as it hybridizes to the template. The A/T tract will serve as template for the poly(A) tail upon transcription. The length of the tract used can be determined by the researcher. We have successfully used A/T tracts up to 120 nucleotides long in this procedure. *Nsi*I is used to cleave the template so that the run off transcription product ends precisely with the poly(A) tail. The primer binding site is used for amplification of ligation products via PCR. The sequence of this primer binding site can be varied if necessary.
14. This step will degrade any proteins (including ribonucleases) in your reaction mixture and allow for transcription templates that will give consistently good results.

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