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Synthesis and Labeling of RNA In Vitro

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Abstract

This unit discusses several methods for generating large amounts of uniformly labeled, end-labeled, and site-specifically labeled RNAs in vitro. The methods involve a number of experimental procedures, including RNA transcription, 5' dephosphorylation and rephosphorylation, 3' terminal nucleotide addition (via ligation), site-specific RNase H cleavage directed by 2'-O-methyl RNA-DNA chimeras, and 2-piece splint ligation. The applications of these RNA radiolabeling approaches are also discussed.

Keywords

in vitro transcription; radiolabeled RNAs; RNA research

INTRODUCTION

DNA-dependent phage T7, T3, and SP6 RNA polymerases are widely used to synthesize a large quantity of RNAs. These enzymes are highly processive and are thus capable of generating long RNA molecules (thousands of nucleotides in length) with low probability of falling off DNA templates during transcription. Phage RNA polymerases specifically recognize their 18-bp promoter sequences (T7, 5'-TAATACGACTCACTATAG; T3, 5'-AATTAACCCTCACTAAAG; and SP6, 5'-ATTTAGGTGACACTATAG) and initiate transcription precisely at the 18th nucleotide guanosine. With a T7, T3, or SP6 promoter fused to the 5' end of a DNA template, the transcription reaction is expected to generate an RNA molecule with the predicted sequence.

To facilitate experimental analyses, in vitro-synthesized RNAs are often radiolabeled with ³²P (incorporated into specific nucleotides). There are several benefits to using ³²P as an RNA radiolabeling agent. First, compared to other common isotopes, ³²P has the highest emission energy (1.7 MeV), thus increasing the experimental sensitivity. Second, ³²P has a short half-life (14.3 days), making it easy to dispose of radioactive waste after experiments. Third, due to the fact that the presence of ³²P does not change the chemical properties of a nucleotide, RNAs containing ³²P-nucleotides behave exactly the same as their unlabeled RNA counterparts in virtually all reactions tested.

The experimental procedures for RNA transcription and RNA radiolabeling are detailed below. Basic Protocol 1 describes in vitro synthesis of RNA using [α -³²P]CTP so that the resultant molecule is uniformly labeled with ³²P. Basic Protocol 2 describes in vitro labeling of RNA using T4 PNK and [γ -³²P]ATP so that the resultant molecule is 5' end-labeled with ³²P. Basic Protocol 3 describes in vitro labeling of RNA using T4 RNA ligase and 5'-[³²P]pCp so that the resultant molecule is 3' end-labeled with ³²P. Basic Protocol 4 describes in vitro labeling of RNA using a series of methods, including RNase H digestion, 5' end-

radiolabeling, and splint ligation, so that the resultant molecule is site-specifically labeled with ^{32}P

CAUTION: When handling radioactive materials, one should always wear personal protective equipment, including lab coat, gloves, goggles, etc. Investigators should also be shielded by a thick plastic or glass plate from direct exposure to any vials containing radioactive isotope. Working areas, as well as the investigator, should be routinely checked for contamination using a hand-held counter. Also see *APPENDIX 1F*.

IN VITRO SYNTHESIS OF UNIFORMLY RADIOLABELED RNAs

The DNA template dictates the sequence of RNAs to be transcribed, and can be prepared using three different methods as listed below.

1. *Preparing DNA template using plasmid construction and amplification.* To start, a vector with a T7 promoter positioned upstream of the polylinker region is used. A DNA template (for transcription) is inserted into the polylinker region (restriction enzyme cleavage sites), and the transcription of the DNA into RNA is under the control of the T7 promoter (Fig. 4.15.1). For a “runoff” transcription, restriction enzymes are used to cleave the plasmid at the restriction enzyme cleavage sites that are downstream of the DNA template, thus generating an end for polymerase to run off. Restriction enzymes that generate blunt ends or 5′ overhangs are preferred, as a 3′ overhang leads to aberrant transcript (Schenborn and Mierendorf, 1985). If restriction digestion is far less than 100%, linearized plasmid should be purified using gel electrophoresis and phenol/chloroform/amy alcohol extraction. The purity of DNA template has a great impact on RNA transcription.
2. *Preparing DNA template using PCR.* If the DNA template does not contain a T7/T3/SP6 promoter sequence, it can be added to the DNA template via PCR. To this end, a sense primer should be designed that locates the T7 promoter sequence at the 5′ end of the template. When performing the PCR reaction, non-proofreading polymerases (e.g., *Taq*) should be avoided, as they generate double-stranded DNA with 3′ overhangs.
3. *Preparing DNA template using oligodeoxynucleotide annealing.* This approach is practical only if the RNA to be transcribed is less than 100 bp (Milligan et al., 1987). Two pieces of DNA oligonucleotide are prepared: a short sense strand containing only the promoter sequence, and a long antisense strand consisting of the complementary DNA template to be transcribed and the complementary promoter sequence at the 3′ end. After annealing, the promoter region becomes double-stranded, whereas the template region is single-stranded antisense sequence.

Materials

- 5 × buffer for transcription (see recipe)
- 3 NTP mix (see recipe)
- 100 μM CTP (Thermo Scientific)
- 10 μCi/μl [α - ^{32}P]CTP (sp. act. 800 Ci/mmol; PerkinElmer)
- 1 μg/μl DNA template (from linearized plasmid, PCR, or oligodeoxynucleotides)
- 40 U/μl RNase inhibitor (Thermo Scientific)
- 20 U/μl T7 RNA polymerase (Thermo Scientific)
- DNase I (RNase-free; Thermo Scientific)

G50 buffer (see recipe)

25:24:1 phenol/chloroform/isoamyl alcohol

100% ethanol

Additional reagents and equipment for urea-PAGE (Williams and Chaput, 2010) and autoradiography (*APPENDIX 3A*)

1. Prepare the following reaction mixture at room temperature in a 1.5-ml microcentrifuge tube with the components added in the indicated order (total reaction volume, 20 μ l):

4 μ l 5 $\times$ buffer for transcription

4.6 μ l distilled deionized H₂O

1 μ l 3 NTP mix, 10 mM each of ATP, GTP, UTP (0.5 mM each NTP final concentration)

2.4 μ l 100 μ M CTP (12 μ M final concentration)

5 μ l 10 μ Ci/ μ l [α -³²P]CTP (sp. act. 800 Ci/mmol) 1 μ l 1 μ g/ μ l linear DNA template

1 μ l 40 U/ μ l RNase inhibitor

1 μ l 20 U/ μ l T7 RNA polymerase.

If doing multiple different reactions, a cocktail containing the first five ingredients can be prepared and aliquotted before addition of the template, RNA inhibitor, and enzyme. The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

2. Incubate at 37°C for 2 hr.

The optimal reaction time is 2 hr. After 2 hr, the RNA yield will not increase appreciably.

3. Add 2 U of DNase I (RNase-free) and incubate at 37°C for another 15 min to degrade the DNA template.
4. Stop the reaction by adding 230 μ l of G50 buffer and 500 μ l of 25:24:1 phenol/chloroform/isoamyl alcohol.
5. Centrifuge the mixture 5 min at 14,000 $\times g$, 4°C.
6. Transfer the upper aqueous phase to a new microcentrifuge tube and mix it with 600 μ l of 100% ethanol.
7. Microcentrifuge the mixture 10 min at 14,000 $\times g$, 4°C.
8. Keep the precipitated pellet and air dry it for 10 min at room temperature.
9. Re-suspend the air-dried pellet in 10 μ l of distilled, deionized water.
10. For high purity, separate the resuspended radiolabeled RNA product on an 8 M urea-PAGE gel (Williams and Chaput, 2010) and visualize by autoradiography (*APPENDIX 3A*).
11. Excise the RNA band from the gel, and place it in a microcentrifuge tube containing 400 μ l of G50 buffer for “freeze-thaw” elution. Specifically, place the

microcentrifuge tube containing the RNA gel slice (in 400 μ l of G50 buffer) on dry ice for 5 min, then transfer to bench top at room temperature for overnight elution.

12. Transfer the gel-free supernatant to a new microcentrifuge tube, and perform phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation as described in steps 4 to 9.

Here, 1 ml of the ethanol should be used for precipitation.

13. Resuspend the air-dried pellet in an appropriate amount of distilled, deionized water.

To achieve a high concentration, the radiolabeled RNA pellet is usually resuspended in 10 μ l of distilled, deionized water. If low concentrations are needed, this solution can be diluted accordingly. The resuspended RNA should be used immediately. The radiolabeled RNA can be stored at -70°C for a brief period (~1 week) without compromising quality.

IN VITRO SYNTHESIS OF 5' END-RADIOLABELED RNAs

To label an in vitro-transcribed RNA at its 5' end, it is recommended to dephosphorylate the 5' terminal nucleotide of the RNA using calf intestine phosphatase (CIP). The dephosphorylated RNA is then rephosphorylated in a forward reaction catalyzed by T4 polynucleotide kinase (PNK) in the presence of [$\gamma^{32}\text{P}$]ATP (Fig. 4.15.2). The simple exchange reaction by T4 PNK is not recommended here, as it usually results in low labeling efficiency. The RNA substrate could be prepared either from in vitro transcription or in vivo purification. To ensure the success of 5' end-labeling, the RNA substrate should be prepared to its highest purity, which can usually be achieved by gel purification (8 M urea-PAGE gel electrophoresis). The length of the RNA substrate will not significantly interfere with the labeling; however, it is sometimes more efficient to label a shorter RNA than it is to label a longer RNA.

Materials

10 $\times$ buffer for CIP (see recipe)

RNA substrate from in vitro transcription (Basic Protocol 1) or purified directly from cells (endogenous RNA; Huang and Yu, 2010)

40 U/ μ l RNase inhibitor (Thermo Scientific)

1 U/ μ l calf intestine phosphatase (CIP; Thermo Scientific)

G50 buffer (see recipe)

10 $\times$ buffer for T4 PNK forward reaction (see recipe)

10 μ Ci/ μ l [$\gamma^{32}\text{P}$]ATP (3000 Ci/mmol; PerkinElmer)

10 U/ μ l T4 polynucleotide kinase (PNK; Thermo Scientific)

Additional reagents and equipment for phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation of RNA (Basic Protocol 1, steps 4 to 9), urea-PAGE (Williams and Chaput, 2010), autoradiography (*APPENDIX 3A*), and “freeze-thaw” elution/ethanol precipitation (Basic Protocol 1, steps 10 to 13)

Dephosphorylation of RNA at the 5' end

1. Prepare the following reaction mixture at room temperature in a microcentrifuge tube by combining the reagents in the indicated order (total reaction volume, 20 μ l):

- 2 µl 10× buffer for CIP
- 7 µl distilled deionized H₂O
- 8 µl (10 µg) RNA substrate
- 1 µl 40 U/µl RNase inhibitor
- 2 µl 1 U/µl CIP.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

2. Incubate at 37°C for 30 min.
3. Stop the reaction by adding 230 µl of G50 buffer, followed by phenol/chloroform/ amyl alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
4. Resuspend the dephosphorylated RNA pellet in 10 µl distilled, deionized water.

Rephosphorylation of RNA at the 5' end

1. Prepare the following reaction mixture at room temperature in a microcentrifuge tube by combining the reagents in the indicated order (total reaction volume, 20 µl):
 - 2 µl 10× buffer for T4 PNK forward reaction
 - 2 µl distilled, deionized H₂O
 - 10 µl dephosphorylated RNA substrate (from step 4)
 - 3 µl 10 µCi/µl [γ -³²P]ATP (3000 Ci/mmol)
 - 1 µl 40 U/µl RNase inhibitor
 - 2 µl 10 U/µl T4 PNK.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

2. Incubate at 37°C for 30 min and stop the reaction by adding 230 µl of G50 buffer, followed by phenol/chloroform/amyl alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
3. Resuspend the rephosphorylated RNA pellet in 10 µl distilled, deionized water.
4. For high purity, separate the 5' end-radiolabeled RNA on an 8 M urea-PAGE gel (Williams and Chaput, 2010), followed by autoradiography (APPENDIX 3A), and “freeze-thaw” elution and ethanol precipitation as described in Basic Protocol 1, steps 10 to 13.
5. Resuspend the air-dried pellet (5' end-radiolabeled RNA) in an appropriate amount of distilled, deionized water.

To achieve a high concentration, the radiolabeled RNA pellet is usually resuspended in 10 µl of distilled, deionized water. If low concentrations are needed, this solution can be diluted accordingly. The resuspended RNA should be used immediately. The radiolabeled RNA can be stored at -70°C for a brief period (~1 week) without compromising quality.

IN VITRO SYNTHESIS OF 3' END-RADIOLABELED RNAs

RNAs can also be radiolabeled at the 3' end (Fig. 4.15.3). The 3' end-labeling technique described here requires the RNA to have a hydroxyl group at its 3' end (in vitro transcription naturally generates an RNA with a 3' hydroxyl group). 5' [³²P]pCp, a commercially available radiolabeled 3',5'-bisphosphate cytidine, can then be ligated to the 3' end of the RNA by T4 RNA ligase, increasing RNA length by 1 nucleotide. If the RNA substrate does not have a free 3'-hydroxyl terminus, a CIP reaction, as described in Basic Protocol 2, is needed before 3' end-labeling. The RNA substrate can be prepared either from in vitro transcription or from in vivo purification. To ensure the success of 5' end-labeling, the RNA substrate should be prepared to its highest purity, which can usually be achieved by gel purification (8 M urea-PAGE gel electrophoresis). The length of the RNA substrate will not significantly interfere with the labeling; however, it is sometimes more efficient to label a shorter RNA than it is to label a longer RNA.

Materials

10 × buffer for T4 RNA ligase (see recipe)

10 mM ATP (Thermo Scientific)

RNA substrate with 3' hydroxyl end derived from in vitro transcription (Basic Protocol 1) or purified directly from cells (endogenous RNA; Huang and Yu, 2010)

5' 10 μCi/μl [³²P]pCp (3000 Ci/mmol; PerkinElmer)

10 U/μl T4 RNA ligase (Thermo Scientific)

G50 buffer (see recipe)

Additional reagents and equipment for phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation of RNA (Basic Protocol 1, steps 4 to 9), urea-PAGE (Williams and Chaput, 2010), autoradiography (*APPENDIX 3A*), and “freeze-thaw” elution/ethanol precipitation (Basic Protocol 1, steps 10 to 13)

1. Prepare the following reaction mixture at room temperature in a microcentrifuge tube by combining the reagents in the indicated order (total reaction volume, 20 μl):

2 μl 10× buffer for T4 RNA ligase

1 μl distilled, deionized H₂O

1 μl 10 mM ATP

5 μl RNA substrate with a 3'-hydroxyl end (30 pmol)

10 μl 10 μCi/μl 5' [³²P]pCp (3000 Ci/mmol)

1 μl 10 U/μl T4 RNA ligase.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

2. Incubate at 4°C overnight and stop the reaction by adding 230 μl of G50 buffer, followed by phenol/chloroform/amy alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
3. If high purity is required for the subsequent experiment, further purify 3' end-radiolabeled RNA by 8 M urea-PAGE gel electrophoresis followed by

autoradiography (*APPENDIX 3A*), and “freeze-thaw” elution and ethanol precipitation as described in Basic Protocol 1, steps 10 to 13.

4. Resuspend the air-dried pellet (3′ end-radiolabeled RNA) in an appropriate amount of distilled, deionized water.

To achieve a high concentration, the radiolabeled RNA pellet is usually resuspended in 10 μl of distilled, deionized water. If low concentrations are needed, this solution can be diluted accordingly. The resuspended RNA should be used immediately. The radiolabeled RNA can be stored at −70°C for a brief period (~1 week) without compromising quality.

IN VITRO SYNTHESIS OF SITE-SPECIFICALLY RADIOLABELED RNAs

In addition to RNA end labeling, in certain applications it is desirable to produce RNAs containing a [³²P]nucleotide specifically at an internal site(s). Site-specific internal labeling can be accomplished through four reactions: (1) site-specific cleavage of RNA substrate by RNase H directed by a 2′-O-methyl RNA-DNA chimera (this step generates two cleaved fragments—the 5′ fragment and the 3′ fragment); (2) dephosphorylation of the 3′ fragment at its 5′ end by CIP; (3) rephosphorylation of the 3′ fragment at its 5′ end by T4 PNK in the presence of [³²P]ATP; (4) joining of the 5′ fragment and the 3′ fragment by T4 DNA ligase in the presence of a complementary bridging DNA oligonucleotide. The protocol can be interrupted briefly (less than a week) between each phase by freezing the RNA sample at −70°C. However, to achieve the best result, it is highly recommended to proceed to the next phase as soon as possible.

Materials

200 pmol/μl 2′-O-methyl RNA-DNA chimera (Integrated DNA Technologies, Inc.)

RNA substrate from in vitro transcription (Basic Protocol 1) or in vivo purification

10 × buffer for RNase H (see recipe)

2 U/μl RNase H (Amersham)

40 U/μl RNase inhibitor (Thermo Scientific)

G50 buffer (see recipe)

10 × buffer for CIP (see recipe)

1 U/μl calf intestine phosphatase (CIP; Thermo Scientific)

10 × buffer for T4 PNK forward reaction (see recipe)

[³²P]ATP (3000 Ci/mmol, 10 μCi/μl) (PerkinElmer)

10 U/μl T4 polynucleotide kinase (PNK; Thermo Scientific)

Bridging DNA oligo (Integrated DNA Technologies, Inc.)

10 × buffer for T4 DNA ligase (see recipe)

5 U/μl T4 DNA ligase (Thermo Scientific)

95°C heat block

Additional reagents and equipment for phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation of RNA (Basic Protocol 1, steps 4 to 9), urea-PAGE (Williams and Chaput, 2010), autoradiography (*APPENDIX 3A*), and “freeze-thaw” elution/ethanol precipitation (Basic Protocol 1, steps 10 to 13)

Site-specific RNase H cleavage of RNA directed by 2'-O-methyl RNA-DNA chimeras

1. Design a 2'-O-methyl RNA-DNA chimera using the following criteria: (1) the chimera is usually 18 nt long; (2) it should consist of three 2'-O-methyl RNA nucleotides at the 5' end, four deoxynucleotides in the middle, and eleven 2'-O-methyl RNA nucleotides at the 3' end (Yu, 1999); (3) it should be complementary to the RNA sequences to be cleaved; (4) the cleavage site is the phosphodiester bond 3' of the substrate nucleotide that base pairs with the 5'-most deoxynucleotide of the chimera (Fig. 4.15.4).

The 2'-O-methyl RNA-DNA chimera is purchased from Integrated DNA Technologies, Inc.

2. Prepare the following reaction mixture at room temperature in a microcentrifuge tube by combining the reagents in the indicated order:

6 μ l (100 pmol) RNA substrate

1 μ l 200 pmol/ μ l 2'-O-methyl RNA-DNA chimera (200 pmol).

3. Heat mixture 1 at 95°C for 3 min in a heat block and gradually cool to room temperature (~10 min) to allow annealing of the 2'-O-methyl RNA-DNA chimera to the RNA substrate.

4. Add the following reagents to the mixture:

1 μ l 10 $\times$ buffer for RNase H

1 μ l 40 U/ μ l RNase inhibitor

1 μ l 2 U/ μ l RNase H.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

5. Incubate at 37°C for 1 hr and stop reaction by adding 230 μ l of G50 buffer, followed by phenol/chloroform/amy alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
6. Separate cleaved 5' and 3' RNA fragments on an 8 M urea-PAGE gel (Williams and Chaput, 2010) and recover them by "freeze-thaw" elution and ethanol precipitation as described in Basic Protocol 1, steps 10 to 13.
7. Resuspend the precipitated pellets of both 5' RNA fragment and 3' RNA fragment in 10 μ l of distilled deionized water, respectively.

The protocol could be interrupted here by freezing the RNA samples at -70°C for less than a week. However, to achieve the best result, it is highly recommended to proceed to the next phase as soon as possible.

Dephosphorylation of the 3' RNA fragment to generate a 5'-hydroxyl end

In the second phase of this protocol, the 3' RNA fragment needs to be dephosphorylated by calf intestine phosphatase (CIP) to generate a free 5'-hydroxyl terminus.

8. Prepare the following reaction mixture, adding the reagents in the order indicated (total reaction volume, 20 μ l):

2 μ l 10 $\times$ buffer for CIP

5 μ l distilled, deionized H₂O

10 µl cleaved 3' RNA fragment (from step 7)

1 µl 40 U/µl RNase inhibitor

2 µl 1 U/µl CIP.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

9. Incubate at 37°C for 30 min and stop the reaction by adding 230 µl of G50 buffer, followed by phenol/chloroform/ethyl alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
10. Resuspend the precipitated pellet (dephosphorylated 3' RNA fragment) in 10 µl of distilled, deionized water.

The protocol could be interrupted here by freezing the RNA samples at –70°C for less than a week. However, to achieve the best result, it is highly recommended to proceed to the next phase as soon as possible.

Rephosphorylation of the 3' fragment to generate a 5'-³²P-radiolabeled end

In the third phase of the protocol, the dephosphorylated 3' RNA fragment is rephosphorylated by T4 polynucleotide kinase (PNK) in the presence of [γ ³²P]ATP

11. Prepare the following reaction mixture, adding the reagents in the order indicated (total reaction volume, 20 µl).

2 µl 10× buffer for T4 PNK forward reaction

3 µl distilled, deionized H₂O

10 µl dephosphorylated 3' RNA fragment (from step 10)

2 µl 10 µCi/µl [γ ³²P]ATP (3000 Ci/mmol)

1 µl 40 U/µl RNase inhibitor

2 µl 10 U/µl T4 PNK.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

12. Incubate at 37°C for 30 min and stop reaction by adding 230 µl of G50 buffer, followed by phenol/chloroform/ethyl alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
13. Purify the 5' radiolabeled 3' RNA fragment by urea-PAGE (Williams and Chaput, 2010), followed by “freeze-thaw” elution and ethanol precipitation as described in Basic Protocol 1, steps 10 to 13.
14. Resuspend the precipitated pellet in 10 µl of distilled, deionized water.

The protocol could be stopped here by freezing the RNA samples at –70°C for less than a week. However, to achieve the best result, it is highly recommended to proceed to the next phase as soon as possible.

Two-piece splint ligation to rejoin the 5' and 3' RNA fragments

In this last phase of the protocol, the 5' RNA fragment and the radiolabeled 3' RNA fragment are precisely aligned together through hybridization with a complementary

bridging DNA oligonucleotide, and ligated by T4 DNA ligase. The bridging DNA oligo is purchased by standard order from Integrated DNA Technologies (IDT). Except for its specific sequence (depending on the two fragments to be ligated), the oligo is a 30-nucleotide-long DNA oligo without any base or sugar ring modification. The bridging DNA oligo sequence is dependent on the sequences of the two RNA fragments (5' RNA fragment and 3' RNA fragment) that need to be ligated. The bridging DNA oligo uses its 5' end half (15 nt) to basepair with 3' RNA fragment and its 3' end half (15-nt) to basepair with 5' RNA fragment.

15. Prepare the following reaction mixture at room temperature in a microcentrifuge tube by combining the reagents in the indicated order:

10 μ l radiolabeled 3' RNA fragment (~25 pmol; from step 14)

1 μ l 5' RNA fragment (50 pmol; from step 7)

1 μ l bridging DNA oligo (50 pmol).

16. Heat mixture at 95°C for 3 min and gradually cool down to room temperature (~10 min) to allow the annealing of the bridging DNA oligo with both the 5' and 3' RNA fragments.

17. Add the following reagents to the mixture in the order indicated:

2 μ l 10 $\times$ buffer for T4 DNA ligase

1 μ l distilled, deionized H₂O

1 μ l 40 U/ μ l RNase inhibitor

4 μ l 5 U/ μ l T4 DNA ligase.

The enzyme is added last to ensure that the reaction mixture reaches the optimal conditions (proper buffer and reagent concentrations, etc.) for the enzyme activity.

18. Incubate at 37°C for ~2 hr and stop the reaction by adding 230 μ l of G50 buffer, followed by phenol/chloroform/amy alcohol extraction and ethanol precipitation as described in Basic Protocol 1, steps 4 to 9.
19. Purify the ligated product using 8 M urea-PAGE gel electrophoresis, followed by "freeze-thaw" elution and ethanol precipitation as described in Basic Protocol 1, steps 10 to 13.
20. Resuspend the precipitated pellet in appropriate amount of distilled, deionized water.

To achieve a high concentration, the radiolabeled RNA pellet is usually resuspended in 10 μ l of distilled, deionized water. If low concentrations are needed, this solution can be diluted accordingly. The resuspended RNA should be used immediately. The radiolabeled RNA can be stored at -70°C for a brief period (~1 week) without compromising quality.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Buffer for CIP, 10 $\times$

100 mM Tris-Cl, pH 7.5 (APPENDIX 2)

100 mM MgCl₂
1 mg/ml bovine serum albumin (BSA)
Store up to 1 month at −20°C

Buffer for RNase H, 10×

200 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
100 mM MgCl₂
1000 mM KCl
250 mM dithiothreitol (DTT)
50% (w/v) sucrose
Store up to 1 month at −20°C

Buffer for T4 DNA Ligase, 10×

400 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
100 mM MgCl₂
100 mM dithiothreitol (DTT)
5 mM ATP
Store up to 1 month at −20°C

Buffer for T4 PNK forward reaction, 10×

500 mM Tris·Cl, pH 7.6 (*APPENDIX 2*)
100 mM MgCl₂
50 mM dithiothreitol (DTT)
1 mM spermidine
Store up to 1 month at −20°C

Buffer for T4 RNA ligase, 10×

500 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
100 mM MgCl₂
100 mM dithiothreitol (DTT)
10 mM ATP
Store up to 1 month at −20°C

Buffer for transcription, 5×

200 mM Tris·Cl, pH 7.9 (*APPENDIX 2*)
30 mM MgCl₂
50 mM dithiothreitol (DTT)
50 mM NaCl
10 mM spermidine

Store up to 1 month at -20°C

G50 buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)

300 mM sodium acetate

2 mM EDTA

0.25% sodium dodecyl sulfate (SDS)

Store up to 1 year at -20°C

3 NTP mix

For Basic Protocol 1 this consists of 10 mM each of nonlabeled ATP, GTP, and UTP. Combine 1 μl of 100 mM ATP, 1 μl of 100 mM GTP, and 1 μl of 100 mM UTP (all from Thermo Scientific) with 7 μl of distilled, deionized water. Store the mix up to 1 month at -20°C .

COMMENTARY

Background Information

RNAs can be ^{32}P -radiolabeled in many different ways. For instance, since the radioactive nucleotides are readily available, phage RNA polymerase-catalyzed transcription (in the presence of ^{32}P -nucleotides) can easily produce uniformly (and randomly) radiolabeled RNA molecules. RNAs can also be radiolabeled at any specific site(s)—the 5' terminal site, the 3' terminal site, and/or any internal site(s).

Uniformly radiolabeled RNAs (antisense) can be used as probes to detect and quantify DNA or RNA (northern blot analysis). One benefit of using RNA probes in northern blotting is the better sensitivity achieved, as RNA-DNA duplexes are more thermodynamically favorable than DNA-DNA duplexes. Uniformly radiolabeled RNAs (antisense) can also be used to map the termini of RNA or DNA of interest in RNase protection assays (*UNIT 4.7*). Following hybridization of the uniformly radiolabeled complementary RNA to its target RNA (or DNA), single-strand-specific RNases cleave off the unpaired region of the radiolabeled RNA, allowing precise mapping of the termini of target RNA (or DNA). In addition, uniformly radiolabeled small RNAs, such as tRNAs, snRNAs, and ribozymes, can be used directly in in vitro biochemical assays (Schenborn and Mierendorf, 1985), and uniformly radiolabeled mRNA precursors and rRNA precursors can be used directly in in vitro processing experiments (Moon et al., 2006).

Like uniformly radiolabeled RNAs, end-radiolabeled RNAs can also be used as probes to detect and quantify DNA or RNA (northern blot analysis; *unit 4.9*). In addition, end-radiolabeled RNAs can be used to study biological processes in vitro. For instance, 5' end-radiolabeled tRNA can be used for modification analysis and 5' end-radiolabeled mRNA can be used for 5' capping analysis. In some instances, it is advantageous to use end-radiolabeled pre-mRNA for splicing analysis. Furthermore, end-radiolabeled RNAs can be used for chemical modification interference assays (Yu and Nilsen, 1992; Yu et al., 1995; Suydam and Strobel, 2009). Importantly, the end-radiolabeling technique can be used to label endogenous RNAs isolated from cells, thus making it convenient to study in vivo RNA-RNA and RNA-protein interactions (Wu et al., 1999; Romfo et al., 2001; Ma et al., 2003; also see Chapter 27 of this manual).

Similarly, the approach of site-specific internally radiolabeling RNAs allows radiolabeling of endogenous RNA as well as in vitro transcribed RNA at a specific internal site, thus facilitating research in RNA-RNA and RNA-protein interactions (binding-site determination; Zhao et al. 2002; also see Chapter 27 in this manual). Site-specific internal radiolabeling, when coupled with thin-layer chromatography, also facilitates RNA nucleotide modification research (Zhao and Yu, 2004; Ma et al., 2005).

Critical Parameters and Troubleshooting

For uniformly radiolabeled RNAs, about 95% of cytidine residues in the RNA transcript are radiolabeled. When [$\alpha^{32}\text{P}$]CTP is unavailable or impractical, other [$\alpha^{32}\text{P}$]NTPs ([$\alpha^{32}\text{P}$]ATP, [$\alpha^{32}\text{P}$]UTP, or [$\alpha^{32}\text{P}$]GTP) can be used for labeling, as well. As a rule of thumb, when a [$\alpha^{32}\text{P}$]NTP is used as labeling agent, it should be omitted from the 3NTP mix. However, its nonradioactive form is still needed to be no less than 12 μM (final concentration) to avoid transcription stalling and premature termination.

For site-specifically and internally radiolabeled RNAs, in order to obtain the best yield, the 5' RNA fragment and the bridging DNA oligo should be in excess relative to the 3' RNA fragment, with the molar ratio being 2:2:1 (5' RNA fragment:bridging DNA oligo:3' RNA fragment). Alternatively, the 3' RNA fragment and the bridging DNA oligo can be in excess relative to the 5' RNA fragment (2:2:1).

The reaction volume in each protocol could be scaled up and down as desired. However, no less than 10 μl should be used, because evaporation could significantly change the reaction conditions, resulting in unsuccessful transcription. On the other hand, no more than 500 μl is recommended because the large volume hinders heat transfer during the relative short (1-to 2-hr) incubation.

Anticipated Results

For uniformly radiolabeled RNAs, it is necessary to quantify the radioactive isotope incorporated. The measurement is usually done by liquid scintillation counter, and the specific radioactivity of an RNA is reported in units of cpm/ μg . Usually, a successful transcription generates a large quantity of highly radioactive RNA transcript (greater than 10 μg per 1 μg DNA template).

Time Considerations

For generating uniformly radiolabeled RNAs, it usually takes 2 days to finish, provided that the DNA template is prepared beforehand and is ready to use. The DNA template preparation time is dependent on the type of DNA template, from 2 to 3 days for plasmid DNA template, to 1 day for PCR DNA template, to a couple of hours for annealed oligo DNA template. The transcription reaction (steps 1 to 9 in Basic Protocol 1) and subsequent 8 M urea-PAGE gel purification (steps 10 to 11 in Basic Protocol 1) can be done on Day 1. The recovery of RNA transcript (steps 12 to 13 in Basic Protocol 1) can be completed on Day 2.

For generating either 5' end-radiolabeled or 3' end-radiolabeled RNAs, it usually takes 2 days to finish, provided that the RNA substrate is prepared beforehand and is ready to use. The RNA substrate preparation time is dependent on the type of RNA substrate, from 2 to 3 days for purification of endogenous RNA, to 1 to 2 days for synthesis and purification of in vitro transcribed RNA. The labeling reaction and subsequent 8 M urea-PAGE gel purification (steps 1 to 8 in Basic Protocol 2, and steps 1 to 3 in Basic Protocol 3) can be done on Day 1. The recovery of RNA transcript (steps 8 to 9 in Basic Protocol 2, and steps 4 in Basic Protocol 3) can be completed on Day 2.

For generating site-specifically and internally radiolabeled RNAs, it usually takes 4 days to finish, provided that the RNA substrate for RNase H cleavage is prepared beforehand and is ready to use. The RNA substrate preparation time is dependent on the type of RNA substrate, from 2 to 3 days for purification of endogenous RNA, to 1 to 2 days for synthesis and purification of in vitro transcribed RNA. The site-specific cleavage of RNA substrate by RNase H (steps 1 to 6 in Basic Protocol 4) can be done on Day 1. The dephosphorylation and re-phosphorylation of the 3' RNA fragment (steps 7 to 13 in Basic Protocol 4) can be done on Day 2. The ligation of the 5' RNA fragment to the 3' RNA fragment (steps 14 to 19 in Basic Protocol 4) can be done on Day 3. The recovery of ligation product (steps 19 to 20 in Basic Protocol 4) can be done on Day 4.

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Figure 4.15.1.

Schematic representation of T7 RNA polymerase runoff transcription to generate uniformly radiolabeled RNA. T7 RNA polymerase first recognizes and binds to the double-stranded T7 promoter (white box, 18 nucleotides), and then initiates transcription at the start site (G₁₈, see text). When polymerase travels along the DNA template (black bars), radiolabeled ribonucleotides (empty circles) incorporate into the nascent RNA chain. At the end, polymerase runs off the DNA template and the newly transcribed RNA is released. The runoff T7 RNA polymerase enters another round of transcription.

Figure 4.15.2.

Schematic representation of radiolabeling of RNA at its 5' end. The single-stranded RNA substrate with a 5' phosphate end is dephosphorylated by calf intestine phosphatase (CIP), generating a 5' hydroxyl terminus. In the following rephosphorylation reaction, T4 polynucleotide kinase (PNK) transfers a radioactive phosphate (a circled P) from [$\gamma^{32}\text{P}$]ATP to the 5' end of RNA substrate.

Figure 4.15.3.

Schematic representation of radiolabeling of RNA at its 3' end. T4 RNA ligase catalyzes the ligation reaction where 5' [³²P]pCp is covalently attached to the 3' end of the single-stranded RNA substrate. The radiolabeled RNA molecule is one nucleotide longer than the RNA substrate.

Figure 4.15.4.

Schematic representation of site-specific internal radiolabeling of RNA. The procedure includes four steps as described in the text. (1) The RNA substrate hybridizes with the complementary 2'-O-methyl RNA-DNA chimera, which, in turn, directs RNase H site-specific cleavage (black wedge), generating two RNA fragments, the 5' fragment and the 3' fragment. The 5' terminal phosphate of the 3' fragment is the target for ^{32}P -replacement. (2) The 3' fragment is dephosphorylated in a CIP-catalyzed dephosphorylation reaction, generating a free 5' hydroxyl terminus. (3) The dephosphorylated 3' fragment is rephosphorylated in a T4 PNK-catalyzed phosphorylation reaction, where a ^{32}P (circled P) is transferred from $[\gamma^{32}\text{P}]\text{ATP}$ to the RNA 5' terminal nucleotide. (4) The radiolabeled 3' fragment is ligated to the 5' fragment by T4 DNA ligase in the presence of a bridging DNA oligo.