CHAPTER FOURTEEN

RNA Radiolabeling

Rishi Porecha, Daniel Herschlag[1]
Department of Biochemistry, Stanford University, Stanford, CA, USA
[1]Corresponding author: e-mail address: herschla@stanford.edu

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Abstract

Radioactive end-labeling is useful for visualizing and allowing the detection of nucleic acids at trace concentrations. Radioactive end-labeling can be carried out on RNA, DNA, or other modified nucleic acids. For RNA, the uses of end-labeling extend beyond simple detection of the intact RNA. A number of RNA molecules studied by biologists form three-dimensional structures in solution, and many of the techniques used to study these structures depend on the ability to visualize the RNA after fragmentation. Labeling at either the 5′- or 3′-end serves as a gateway into these structural analysis techniques (see Structural Analysis of RNA Backbone Using In-Line Probing), and protocols for these labeling procedures are described below (for a nonradiactive labeling protocol, see Fluorescently Labeling Synthetic RNAs).

1. THEORY

For 5′-labeling of transcribed RNAs, the unlabeled triphosphate moiety on the 5′-end of the RNA must be removed prior to replacement with a labeled phosphate. For oligonucleotides synthesized without a 5′-phosphate and RNAs transcribed using excess guanosine (lacking phosphate), this step is unnecessary and the RNA can be directly radiolabeled. For removal of the 5′-triphosphate, an alkaline phosphatase is used. Although there are many types of alkaline phosphatases, the protocol here uses a phosphatase that can be heat inactivated, as this can simplify the labeling protocol. Shrimp alkaline phosphatase is used, as it is readily heat inactivated, unlike the enzyme from calf intestine (Nilsen et al, 2001; Sambrook and Russell, 2001). There are other heat-sensitive alkaline phosphatases, such as antarctic phosphatase (New England Biolabs), that can be used for the following protocol with slight modifications. After inactivation of the phosphatase, the RNA is incubated with T4 polynucleotide kinase and [γ-^{32}P]-ATP (Richardson, 1981). Afterward, the

RNA is typically PAGE purified to ensure that degradative products, which can interfere with downstream analyses, are not included in the final sample. If ultrapure RNA is not required, then spin column purification can be carried out instead of PAGE purification.

There are two commonly used procedures for 3′-labeling of RNA. First, there is untemplated end-labeling using terminal deoxynucleotidyl transferase from calf thymus and a labeled dideoxy- or cordycepin-nucleoside triphosphate. The second is templated end-labeling using *E. coli* Klenow fragment and a labeled deoxynucleoside triphosphate (Klenow and Hennington, 1970; Huang and Szostak, 1996). Compared to terminal transferase labeling, Klenow labeling usually yields RNA of higher specific activity and the radionucleosides required are considerably cheaper. Klenow labeling is described in the protocol below. For 3′-labeling, there is often no need to dephosphorylate the RNA, as the 3′-hydroxyl is not phosphorylated and is already free to accept a labeled nucleoside. However, 2′, 3′-cyclic phosphates may sometimes have to be removed prior to labeling, as numerous workers in the RNA biochemistry field induce ribozyme/DNAzyme cleavage *in cis*, or *in trans* at the 3′-ends of their RNAs of interest to eliminate 3′-heterogeneity. Because the 2′, 3′-cyclic phosphate group blocks addition of the labeling group onto the 3′-end, the RNA is dephosphorylated using the 2′, 3′-cyclic phosphodiesterase activity of T4 polynucleotide kinase prior to the labeling reaction (Cameron and Uhlenbeck, 1977; Morse and Bass, 1997). For Klenow labeling, the RNA is hybridized to a template DNA oligonucleotide that is complementary to the 3′-end of the RNA. The first overhanging base serves as the template for the labeled nucleotide that will be added to the 3′-end of the RNA. After hybridization, the RNA/DNA hybrid is incubated with Klenow fragment and the [α-^{32}P]-labeled deoxynucleoside triphosphate complementary to the first overhanging base. After the 3′-labeling reaction is complete, the RNA is PAGE purified just as 5′-labeled material. Again, if ultrapure material is not needed, the RNA may be spin column purified to remove the unincorporated radionucleotides.

For each type of labeling, a flowchart of required steps is shown in Fig. 14.1.

2. EQUIPMENT

General (Basic Equipment Needed for Nearly all Procedures in This Protocol)

1.5-ml nonstick polypropylene microcentrifuge tubes
Micropipettors

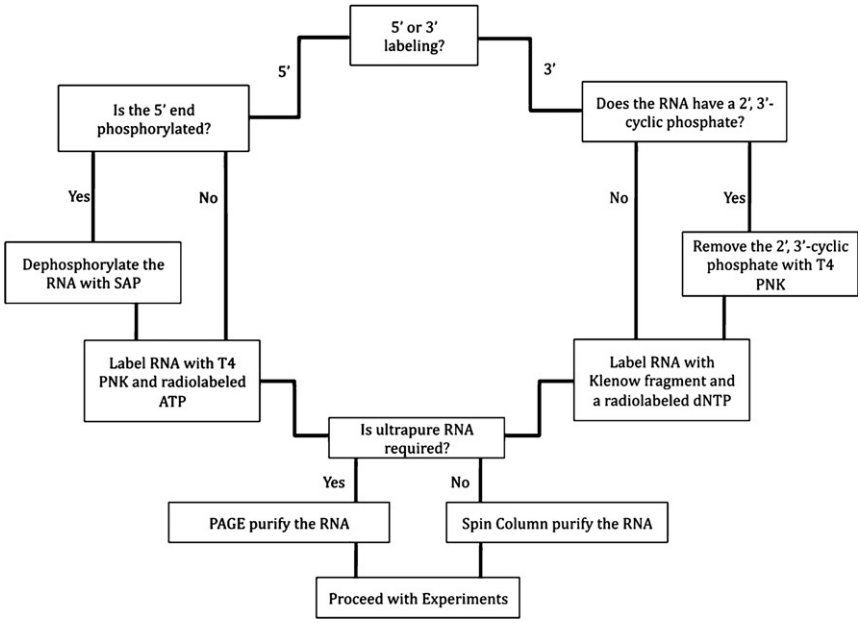

Figure 14.1 This flowchart is provided to assist the reader in deciding what steps are necessary for successful radiolabeling of RNA. Phosphorylated RNAs must always undergo dephosphorylation steps prior to radiolabeling. If not working with phosphorylated RNAs, one may proceed straight to the labeling reactions. For either 5′-, or 3′-labeling, the RNA should be spin column- or PAGE-purified prior to experiments.

Disposable micropipettor tips
Disposable gloves

For Buffers and Solutions

25–1,000-ml beakers (for mixing solutions)
10–1,000-ml graduated cylinders (for measuring accurate volumes)
RNase-free bottles/tubes (to store solutions)
Magnetic stir plate
Magnetic stir bars

For Gel Casting and Running

30 × 27-cm gel plates
0.5–1-mm width gel plate spacers
0.5–1-mm width gel comb with 1.5 × 1 cm lanes
Polyacrylamide gel electrophoresis apparatus
Power supply and leads
Kimwipes

For Radiolabeling and Gel Excision

Labcoat
Calibrated Geiger counter
4–5 Plexiglass radiation shields
Adsorbent bench pads
Paper towels
Water baths or heat blocks (37 °C and 65 °C)
Plastic wrap
Microcon YM-30 spin columns
RNase-free micro bio-spin columns (BioRAD), or other RNase-free desalting columns
Plexiglass box with lid ($L \times W \times H = 35 \times 30 \times 2.5$ cm)
Irregularly shaped piece of plexiglass (that fits in the above box – a trapezoid works well)
Disposable syringe needles
Autoradiography film
Cart
Film developer
Light box
Radioactive liquid waste container
Small plastic bags for all radioactive waste
Radioactive solid waste container
Radioactive waste container for sharps
Sterilized scalpels or razor blades
Scintillation tubes
Scintillation counter
Speed-vac

3. MATERIALS

General (Basic Materials Needed for Nearly all Procedures in This Protocol)

RNase-free water
Tris base
Hydrochloric acid (HCl)
Sodium hydroxide pellets (NaOH)

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
Sodium acetate trihydrate ($NaOAc \cdot H_2O$)
Ethylenediaminetetraacetic acid, disodium salt (EDTA)
Ethanol
Dry ice
Dithiothreitol (DTT)

For 2′, 3′-Cyclic Phosphate Removal

Imidazole
β-Mercaptoethanol
Bovine serum albumin (BSA)
T4 polynucleotide kinase (NEB, 10 U μl^{-1})
Buffer-saturated phenol/chloroform/isoamylalcohol (25/24/1) (Sigma)
Chloroform

For Gel Casting and Running

Glacial acetic acid
Boric acid (H_3BO_3)
40% Acrylamide/bisacrylamide solution (19:1)
Urea
Ammonium persulfate (APS)
Tetramethylethylenediamine (TEMED)
SigmaCoat, or other siliconizing solution
Formamide
Bromophenol blue
Xylene cyanol

For 5′-Labeling Procedures

[γ-^{32}P]-ATP (>7,000 Ci $mmol^{-1}$; 166.67 mCi ml^{-1})
T4 polynucleotide kinase (NEB, 10 U μl^{-1})
Shrimp alkaline phosphatase (Fermentas, 10 U μl^{-1})

For 3′-Labeling Procedures

Oligo complementary to the 3′-end of the RNA and a 2 base overhang
[α-^{32}P]-dNTP (The protocol below uses [α-^{32}P]-dATP) (3,000 Ci $mmol^{-1}$; 10 mCi ml^{-1})
Klenow fragment (NEB, 5 U μl^{-1})

For General Radiolabeling Procedures

Scintillation fluid
Radiac Wash or other radiation-sequestering reagent

3.1. Solutions & buffers

Step 1 10× Shrimp alkaline phosphatase buffer

Component	Final concentration	Stock	Amount
Tris–HCl (pH 7.6)	200 mM	1 M	10 ml
$MgCl_2$	100 mM	1 M	5 ml

Add water to 50 ml.

10× PNK buffer

Component	Final concentration	Stock	Amount
Tris–HCl (pH 7.6)	700 mM	1 M	7 ml
$MgCl_2$	100 mM	1 M	1 ml
DTT	100 mM	1 M	1 ml

Add water to 10 ml.

Gel loading buffer

Component	Final concentration	Stock	Amount
Formamide	90%	–	9 ml
TBE	1×	10×	1 ml
Bromophenol blue	0.05%	–	5 mg
Xylene cyanol	0.05%	–	5 mg

Step 2 5× Phosphate removal buffer

Component	Final concentration	Stock	Amount
Imidazole (pH 6.0)	500 mM	1 M	5 ml
$MgCl_2$	50 mM	1 M	500 µl
β-mercaptoethanol	50 mM	14 M (Neat)	36 µl
BSA	100 µg ml^{-1}	10 mg ml^{-1}	100 µl

Pipette β-mercaptoethanol in the fume hood as it has a strong odor
Adjust the final volume of the buffer to 10 ml with water

Step 3 10× Klenow annealing buffer

Component	Final concentration	Stock	Amount
Tris–HCl (pH 7.6)	140 mM	1 M	1.4 ml
NaCl	400 mM	5 M	800 µl
EDTA	2 mM	0.25 M	80 µl

Add water to 10 ml

20× Klenow reaction supplement

Component	Final concentration	Stock	Amount
$MgCl_2$	140 mM	1 M	1.4 ml
DTT	20 mM	1 M	800 µl

Add water to 10 ml

Step 4 10× Tris–Borate EDTA buffer (TBE)

Component	Final concentration	Amount
Tris base	1 M	121 g
Boric acid	831 mM	51.4 g
EDTA*Na_2	10 mM	3.72 g

Add water to 1 l. Dissolve the components by autoclaving

7 M Urea 8% Polyacrylamide Gel Solution

Component	Final concentration	Stock	Amount
TBE	1×	10×	100 ml
40% Acryl/Bis solution	8%	40%	200 ml
Urea	7 M	–	420 g

Add water to 1 l

4. PROTOCOL

4.1. Preparation

Transcribe (see *In Vitro* Transcription from Plasmid or PCR amplified DNA) and purify RNA for labeling. For 3′-labeling, dephosphorylate and purify RNAs that had homogeneous 3′-ends generated by *cis*- or *trans*-ribozyme/DNAzyme cleavage. Also for 3′-labeling, design and order an oligonucleotide complementary to the 3′-end of the RNA with a two base overhang (see Explanatory chapter: PCR -Primer design). For example, for the *Tetrahymena thermophila* group I intron, the sequence of the oligo is shown below:

5′-dGdTdAdCdTdCdCdAdAdAdAdCdTdAdAdTdCdAdAdTdAdTdAddC-3′

The ‘d’ ’s in the above sequence denote deoxyribosyl residues. The final nucleotide on the template is dideoxy to ensure that it is not labeled during

the Klenow reaction. The second residue from the 5′-end is the template for the added labeled nucleoside. Klenow will also incorporate other labeled nucleosides other than dATP if the correct template is used.

Make stock solutions and buffers according to the recipes provided. If possible, cast the polyacrylamide gel for PAGE purification the day before labeling.

Thaw radionucleotides just before the labeling reaction is carried out. Avoid mixing radionucleotide stocks by inverting or shaking, as minute amounts of this material on lids can lead to contamination.

4.2. Duration

Preparation	Approximately 1–3 days
Protocol	Approximately 1.5 days, including overnight elution of labeled RNA

Note: The preparation time is longer for 3′-labeling than for 5′-labeling because of the lengthy dephosphorylation step for 3′-labeling.

4.3. Caution

Wear gloves for all of the procedures described below. This is done not only to prevent exposing you to harmful substances, but also to protect the RNA you are labeling from the ribonucleases present on your skin.

Take care when working with phenol, as it is corrosive and can cause chemical burns. Clean up promptly any spillage that occurs and dispose of waste as instructed by your institution's guidelines for hazardous materials.

Be cautious when working with acrylamide; it is a potent neurotoxin. Clean up promptly any spillage that occurs and dispose of waste as instructed by your institution's guidelines for hazardous materials.

Be cautious when running the polyacrylamide gel. Do not touch the apparatus while the power supply is on. The current running through the gel is close to the amount needed for a fatal electrical shock.

Be careful when working with scalpels, needles, and other sharp objects.

Consult your institution's radiation safety officer for proper ordering, handling, and disposal of radioactive materials.

When working with radioactivity, wear a labcoat. Survey yourself and your workspace frequently with the Geiger counter. Clean up contamination promptly and dispose it of in the appropriate waste containers. Change gloves frequently to avoid

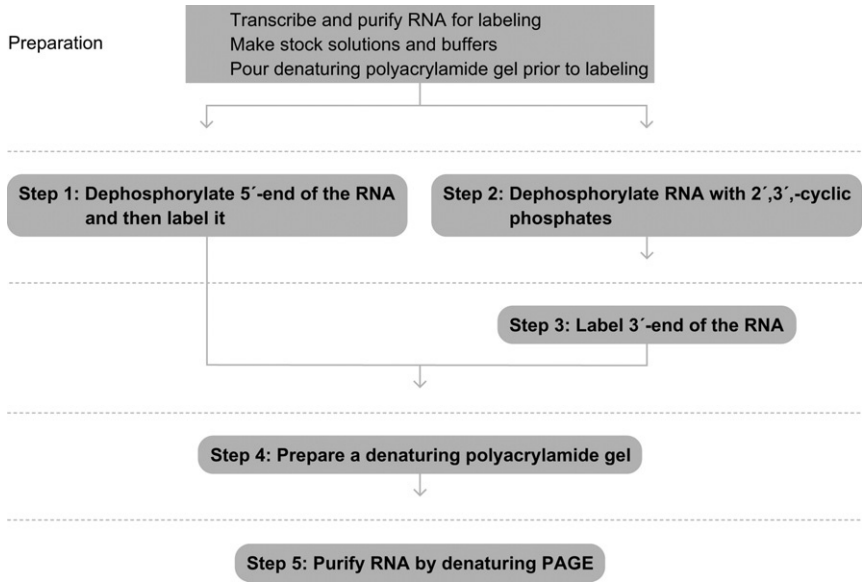

Figure 14.2 Flowchart of the complete protocol, including preparation.

unnecessary radioactive contamination. Finally, take care to limit your exposure by using plexiglass shields whenever possible.

See Fig. 14.2 for the flowchart of the complete protocol, including preparation.

5. STEP 1 5′-DEPHOSPHORYLATION OF TRANSCRIBED RNAs USING SHRIMP ALKALINE PHOSPHATASE AND 5′-LABELING WITH T4 POLYNUCLEOTIDE KINASE

5.1. Overview

Remove the triphosphate group from the 5′-end of a transcribed RNA using shrimp alkaline phosphatase. Heat-inactivate the enzyme, and then use T4 polynucleotide kinase to add a radiolabeled phosphate to the 5′-end.

5.2. Duration

1.5–2 h

1.1 Set up two water baths: one at 37 °C and the other at 65 °C. The first will be used for the alkaline phosphatase and polynucleotide kinase reactions, while the second will be used to heat-inactivate the alkaline phosphatase.

1.2 To dephosphorylate the 5′-end add to a 1.5-ml microcentrifuge tube:

RNA	~100 pmoles
10× shrimp alkaline phosphatase buffer	1 µl
Shrimp alkaline phosphatase (10 U μl^{-1})	1 µl
RNase-free water	to 10 µl

1.3 Incubate at 37 °C for 30 min.

1.4 Stop the phosphatase reaction by adding 0.8 µl of 250 mM EDTA. This will chelate the divalent metal ions in solution.

1.5 Heat the solution at 65 °C for 20 min. This will inactivate the shrimp alkaline phosphatase.

1.6 Phosphorylate the 5′ end using T4 polynucleotide kinase. Add to the reaction:

100 mM $MgCl_2$	1 µl
10× PNK buffer	2 µl
RNase-free H_2O	4.2 µl
[γ-^{32}P]-ATP	1 µl
Mix by pipetting.	
Add: T4 PNK (10 U μl^{-1})	1 µl
Mix again.	

1.7 Incubate the reaction at 37 °C for 30 min.

1.8 After the reaction is complete, add 20 µl of gel loading buffer. The sample is now ready to load onto the polyacrylamide gel.

5.3. Tip

Shrimp alkaline phosphatase treatment is not necessary for synthetic RNA oligonucleotides, unless they were synthesized to include a 5′-phosphate. Also, this step is not necessary for RNAs transcribed with excess guanosine.

5.4. Tip

Be aware that the EDTA added after the phosphatase treatment will stop the reaction and will prevent metal-dependent degradation of the RNA when the temperature is raised to heat-inactivate the phosphatase. The final concentration of EDTA after this step is ~19 mM, ~9 mM over that of the $MgCl_2$ in solution.

5.5. Tip

Carry out Steps 1.6 to 1.8 with the protection of a plexiglass shield.

5.6. Tip

The radiolabeling reaction will be purified on the polyacrylamide gel. Because there is a significant risk of contaminating your workspace with radioactivity, place adsorbent bench pads underneath the gel, so any spill can be dealt with by simply disposing the pads as solid radioactive waste.

5.7. Tip

While the reaction is incubating, start prerunning the polyacrylamide gel (prerun the gel for greater than 20 min).

See Fig. 14.3 for the flowchart of Step 1.

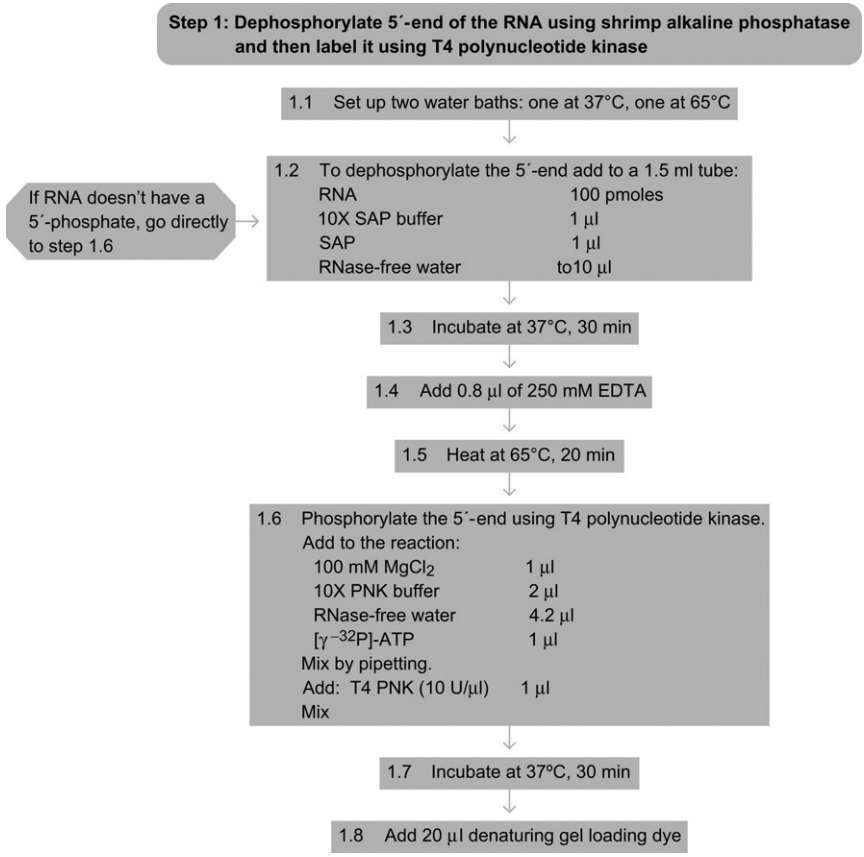

Figure 14.3 Flowchart of Step 1.

6. STEP 2 DEPHOSPHORYLATION OF RNAs WITH 2′, 3′-CYCLIC PHOSPHATES USING T4 POLYNUCLEOTIDE KINASE FOLLOWED BY PHENOL/CHLOROFORM EXTRACTION

6.1. Overview

Prepare and incubate the dephosphorylation reaction. Then, carry out phenol/chloroform extraction and ethanol precipitation (see RNA purification – precipitation methods).

6.2. Duration

7 h

2.1 Treat 10 nmoles of RNA harboring a 2′, 3′-cyclic phosphate end with T4 polynucleotide kinase. Add to a 1.5-ml microcentrifuge tube:

RNA	10 nmoles
5× Phosphatase removal buffer	100 µl
T4 PNK (10 U μl^{-1})	10 µl
RNase-free water	to 500 µl

2.2 Incubate at 37 °C for 5 h.

2.3 Add 500 µl of buffer-saturated phenol/chloroform/isoamyl alcohol (25/24/1) and vortex. Centrifuge the tube briefly to separate the organic (bottom) and aqueous phase (top). Transfer the aqueous fraction to a clean 1.5-ml tube.

2.4 Add 500 µl of chloroform to the aqueous fraction and vortex. Centrifuge the tube briefly to separate the organic (bottom) and aqueous phase (top). Transfer and split the aqueous fraction into two tubes.

2.5 Add 25 µl of 3 M sodium acetate to each tube. Then add 700 µl of 100% ethanol to each tube. Chill this on dry ice for 20 min.

2.6 Centrifuge the samples at 15,000×g for 20 min at room temperature. The RNA should appear as a translucent pellet at the bottom of the tube. Carefully remove the supernatant. Add 500 µl of 70% ethanol to each tube and vortex to resuspend the pellet. Centrifuge again at 15,000×g for 10 min at room temperature. Carefully remove the supernatant again and allow the pellet to air-dry. Resuspend the pellet in ~100 µl of water. Store the RNA at −20 °C.

6.3. Tip

This dephosphorylation step is unnecessary for RNAs that have not been cleaved in cis or in trans to generate a homogeneous 3′-end. If the RNA has been cleaved, the remaining cyclic 2′, 3′-phosphate product on the end of the RNA must be removed before any base additions can take place. Dephosphorylation should be carried out before the day that the RNA will be radiolabeled. As described in the introduction, this step uses the 2′, 3′-phosphodiesterase activity of T4 polynucleotide kinase. This activity is distinct from the kinase activity.

See Fig. 14.4 for the flowchart of Step 2.

7. STEP 3 3′-LABELING WITH *E. COLI* DNA POLYMERASE KLENOW FRAGMENT

7.1. Overview

Hybridize a DNA oligonucleotide to the 3′-end of the RNA that is to be radiolabeled. Klenow fragment will label the RNA by adding a radio-deoxynucleotide to the 3′-end in a templated extension reaction.

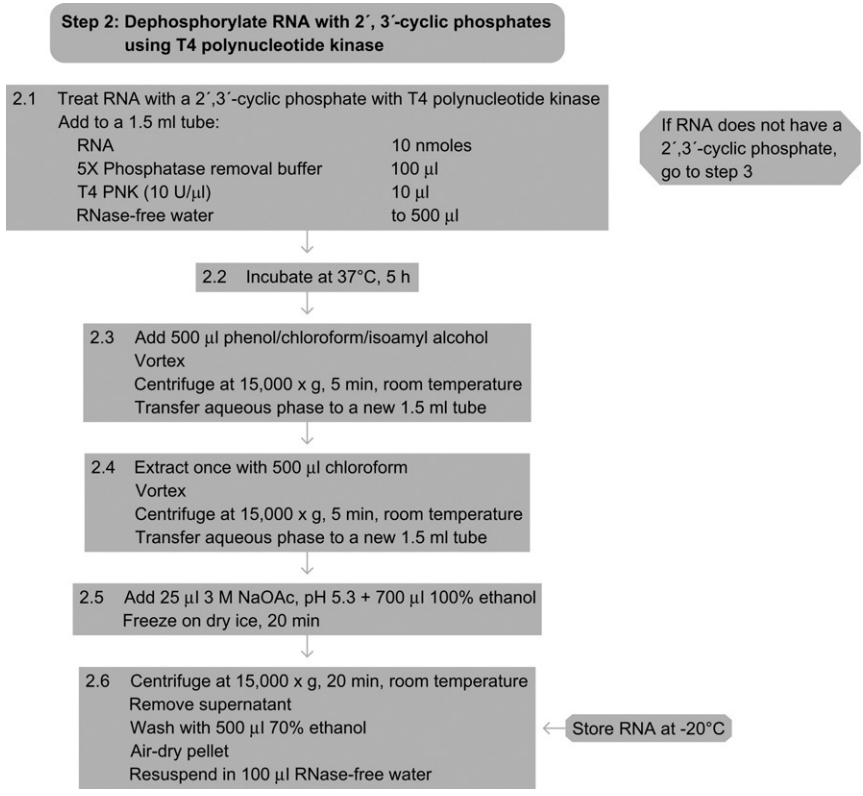

Figure 14.4 Flowchart of Step 2.

7.2. Duration

1.5–2 h

3.1 Set up two water baths, one at 37 °C (for the enzymatic reaction) and one at 60 °C (for the annealing).

3.2 To anneal the template oligonucleotide to the RNA, add to a 1.5-ml tube:
100 pmole of RNA
125 pmole of DNA oligo that is complementary to the 3′-end of the RNA
4 μl 10× Klenow annealing buffer
to 40 μl RNase-free water

3.3 Heat the solution at 60 °C for 1 min and then take the reaction out of the water bath and cool at room temperature for 10 min. Spin the reaction down briefly in a microcentrifuge as there may be condensation on the top of the tube.

3.4 Prepare the labeling reaction. Add to the annealing reaction:
4 μl of 20× Klenow reaction supplement
20 μl of water
8 μl of [α-^{32}P]-dATP
Pipette up and down to mix the solution.
Add 8 μl of Klenow fragment (5 U μl^{-1}).

3.5 Incubate the mixture at 37 °C for 60 min.

3.6 Reduce the volume of the labeling reaction (now at 80 μl) by passing it through a Microcon YM-30 spin column according to the manufacturer's instructions. Spin at 14,000×g for 15 min.

3.7 Recover the sample and add 1 volume (10-20 μl) of gel loading buffer. The sample is now ready to load onto the polyacrylamide gel. Dispose of the used spin column and collection tube as your institution's radiation safety guidelines instruct.

7.3. Tip

Different annealing temperatures might be needed for different pairs of RNA and oligonucleotide.

7.4. Tip

Carry out Steps 3.4 to 3.7 with the protection of a plexiglass shield.

7.5. Tip

The radiolabeling reaction will be purified on the polyacrylamide gel. Because there is a significant risk of contaminating your workspace with radiation, place adsorbent bench

pads underneath the gel, so any spill can be dealt with by simply disposing the pads as solid radioactive waste.

7.6. Tip

While the reaction is incubating, start prerunning the polyacrylamide gel (prerun the gel for greater than 20 min).

See Fig. 14.5 for the flowchart of Step 3.

8. STEP 4 PREPARING THE DENATURING POLYACRYLAMIDE GEL

8.1. Overview

Prepare the gel plates. Initiate the polymerization of the acrylamide gel solution. Pour the activated gel solution between the plates and allow the gel to polymerize.

8.2. Duration

40 min

4.1 Rinse the 30 by 27 cm gel plates, spacers, and comb with deionized water followed by absolute ethanol. Wipe the plates, spacers, and comb dry with a Kimwipe.

4.2 Pipette ~20 μl of siliconizing solution onto the shorter plate. Using a Kimwipe, coat the plate with the solution. This will prevent the gel from sticking to both plates when the plates are separated for gel fragment excision.

4.3 Assemble the spacers and gel plates in the manner appropriate for pouring the gel.

4.4 Take the appropriate measures to prevent the acrylamide solution from leaking out from between the gel plates. For some gel casting systems, this means sealing the plate edges with electrical tape.

4.5 Pour ~50 ml of room temperature 7 M urea/8% polyacrylamide gel solution into an appropriately sized beaker. Add 50 μl of TEMED to the solution and mix. Then add 250 μl of 10% ammonium persulfate to the solution and mix again. At this point, the free-radical polymerization of acrylamide is proceeding, so you need to pour the gel fairly quickly. Pour the gel solution between the plates. If bubbles form between the plates, remove them by tilting the gel plates nearly upright, and tapping the plates. Using the siliconizing solution helps eliminate

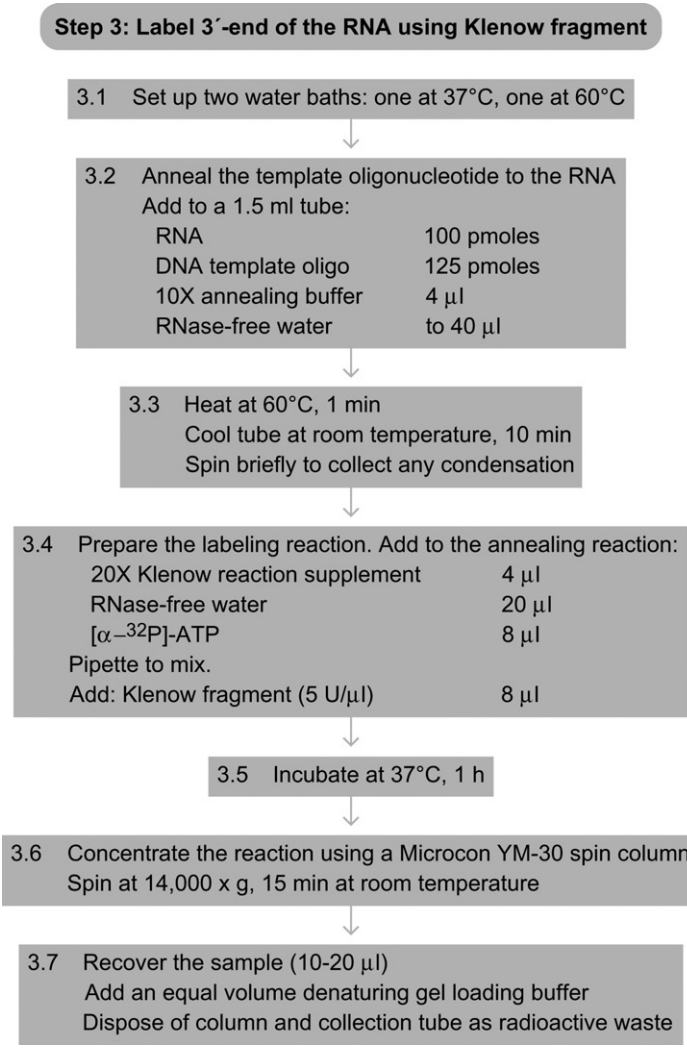

Figure 14.5 Flowchart of Step 3.

bubbles when pouring the gel, as it decreases the adherence of bubbles to the glass plate.

4.6 After you have filled the plates with the gel solution, place the gel comb between the plates to form the appropriately sized wells.

4.7 Allow the gel to polymerize ~30 min.

4.8 Assemble your gel running apparatus as per the manufacturer's instructions. Pour 1× TBE buffer into the top and bottom tanks of the

apparatus and make sure there is minimal buffer leakage. Take out the gel comb carefully, and using the 1× TBE running buffer, rinse the urea out of the wells with a needle and syringe. Connect the apparatus to the power supply and prerun the gel at an appropriate wattage. For our gels, which are 30 cm by 27 cm, we run the gel at 45 W for at least 20 min.

8.3. Tip

Do not siliconize both plates, as the gel will fail to adhere to either plate when the plates are opened.

8.4. Tip

There is great diversity in gel casting equipment between laboratories. Consequently, a detailed description of how a gel is cast in our laboratory may not be the optimal method for all laboratories. Therefore, the following steps of assembling the plates for pouring are purposefully general.

8.5. Tip

In this protocol, a recipe for 7 M urea/8% acrylamide gel mix has been included. This percentage of acrylamide is used for the PAGE purification of the Group I ribozyme (~400 nucleotides). For RNAs of different sizes, consult published data to determine the acrylamide percentage required for the optimal resolution of the target RNA from unincorporated radionucleotides and degradative products (Maniatis et al., 1975; Ellington and Pollard, 2001).

See Fig. 14.6 for the flowchart of Step 4.

9. STEP 5 PAGE PURIFICATION AND RNA EXTRACTION

9.1. Overview

Run the RNA on a polyacrylamide gel to separate the full-length RNA from RNA degradation products and unincorporated nucleotides. Then, use autoradiography to visualize where the RNA migrates on the gel. Finally, cut out the gel fragment that corresponds to the full-length RNA and elute the RNA from the gel (see RNA purification by preparative polyacrylamide gel electrophoresis).

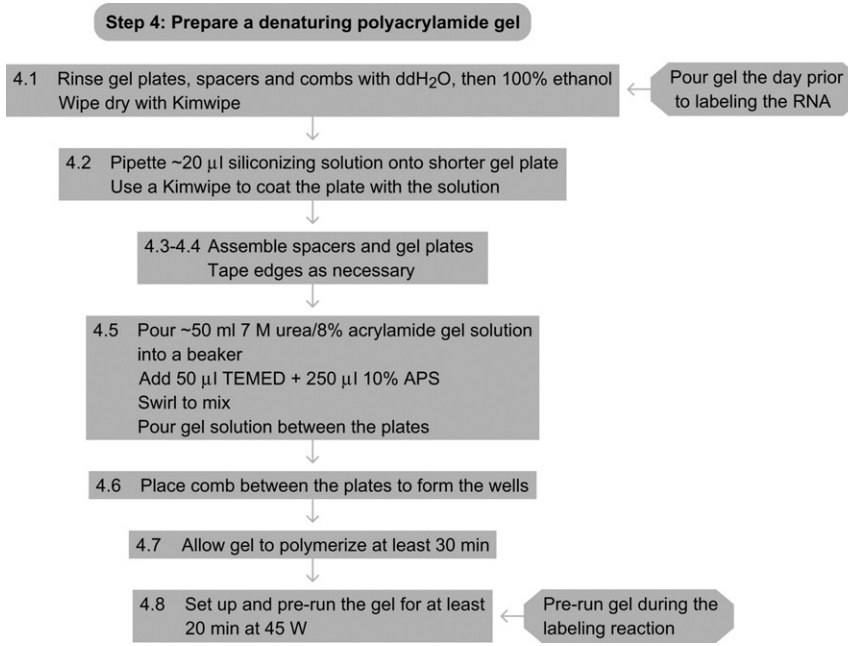

Figure 14.6 Flowchart of Step 4.

9.2. Duration

2.5–4 h

5.1 Turn off the power supply to the prerunning gel and detach the connecting electrodes. Rinse the wells out once more before the sample is loaded. Place a plexiglass shield between you and the gel while loading the sample. Using a gel loading pipette tip, carefully dispense the labeling reaction to the bottom of a well. If you cannot fit the entire sample into one lane, load the remainder into the next lane. Try not to load different samples in adjacent lanes, as the possibility of sample mixing becomes greater.

5.2 Start running the gel at 45 W. For the group I intron (~400 nucleotides) the gel is run 3 h, while for shorter RNAs, such as the P4–P6 domain of the group I intron (~160 nucleotides), the running time is ~1.5 h.

5.3 While the gel is running, prepare the materials needed for gel imaging. Place adsorbent bench pads onto a cart (to prevent radioactive contamination). Then place autoradiography film, Geiger counter, needles,

and a plexiglass shield onto the cart. Cover the interior of the plexiglass container with plastic wrap so that the interior surface of the container is protected from contamination.

5.4 After the gel has run for the appropriate amount of time, take it off the apparatus and carefully open it. The gel should remain on the larger plate as the shorter plate was siliconized. Place a plastic wrap over the gel, and place the gel and glass plate into the plexiglass container. Close the plexiglass container and put the container onto the cart. Cover the plexiglass box with the plexiglass shield to protect yourself from the radiation that escapes from the box.

5.5 Go to the dark room to expose the gel to autoradiography film. Place the plexiglass shield down on a flat surface, and then place the plexiglass gel container behind this. Align your gel with one corner of the plexiglass container. Turn off the regular light, and turn on the red safety light. Place a piece of film over the plastic wrap-covered gel for ~30 s, and then develop the film. You should see an intense black band corresponding to the radiolabeled RNA. If you do not see this band, place another piece of film onto the gel for 30–300 s and then develop the film again. Be careful not to expose the film for too long, as the signal will saturate and locating the position of the RNA will be difficult. Survey your surroundings intermittently to ensure that the area is not contaminated with radioactivity.

5.6 Repeat the procedure above, but place the irregularly shaped piece of plexiglass over the plastic wrap-covered gel. Use a syringe needle to poke around the irregularly shaped piece of plexiglass, through the film and into the gel (Fig. 14.8(a)). The holes made around the irregularly shaped plexiglass will help in orienting the film in respect to the gel. By aligning the holes in the film with the appropriate holes in the gel, you can deduce where your radiolabeled RNA is on the gel. The ability to visualize the bands is crucial in excising the correct gel fragments. Survey the area for radioactivity and then leave the darkroom.

5.7 Place the developed, punctured film onto a plastic wrap covered-lightbox and the plexiglass box, containing the gel (still on the plate and covered with plastic wrap) over the film. Place a plexiglass shield in front of the lightbox. Align the film and gel according to the puncture holes (Fig. 14.8(b)). The dark bands on the film should align with positions of the gel containing radiolabeled RNA (Fig. 14.8(c)). Using sterile scalpels (one for each RNA) cut out the fragment of the gel (through the plastic wrap) corresponding to the desired bands on

the film. Separate the plastic wrap from the gel slice and place the slice into its own tube.

5.8 Take the gel back to the dark room and check the gel again by autoradiography to ensure that the correct bands were excised.

5.9 Cover the gel slices with ~100 μl of RNase free water. Freeze this suspension on dry ice and then allow it to thaw. Repeat the freeze–thaw Steps 1–3 times. Start cleaning up the work area (Step 5.10) at this stage. Store the thawed suspensions overnight at 4 °C. The supernatant eluate can be collected after 2 h if needed.

5.10 Clean up the work area. Sequester radioactivity using paper towels and a sequestering reagent, such as Radiac wash. Place the unused portions of the gel, contaminated bench pads, and contaminated paper towels into a radioactive waste container. The scalpels and needles should be placed in a radioactive sharps container. The gel running buffer, which contains unincorporated radionucleotides, should be stored in a radioactive liquid waste container. All radioactive waste generated should be disposed of as per your institution's guidelines. The gel running apparatus and plates will be extremely radioactive after this protocol; therefore, clean them with Radiac wash and paper towels. If you cannot completely decontaminate the apparatus (or plates), place it in an area of your lab where the radioisotope can safely decay to reasonable levels.

5.11 The labeled RNA is usually radioactive enough to be used without any further purification; however, residual salts, urea, and acrylamide can be removed by spin column purification, or ethanol precipitation. For spin column purification (RNase-free p30 micro bio-spin columns work well, BioRAD), follow the guidelines from the appropriate manufacturer. For the ethanol precipitation, add 1/10 volume of 3 M sodium acetate (pH 5.2), and 2.5 volumes of absolute ethanol. Chill this solution on dry ice for 20 min and then centrifuge at >15,000 g for 20 min. Remove the supernatant, and using the Geiger counter, make sure you did not remove the radiolabeled sample. Wash the sample with 200 μl 70% ethanol and centrifuge again for 10 min. Remove the supernatant and evaporate the residual ethanol using a speed-vac. Resuspend the dried sample with 100 μl of RNase-free water.

5.12 Count 1–5 μl of your sample (in ~5 ml of scintillation fluid) in a scintillation counter to obtain counts per minute per microliter of sample (cpm μl^{-1}). If the sample is >200,000 cpm μl^{-1}, dilute it with

RNase-free water to 200,000 cpm μl^{-1} to prevent autoradiolysis during storage.

5.13 Store the final sample at −20 °C until use.

9.3. Tip

If ultrapure RNA is not required, then spin column purification (e.g., using RNase-free p30 micro bio-spin columns, BioRAD) can be carried out instead of PAGE purification. This will separate the unincorporated radionucleotides from the final sample, but not potential degradative products. Consult the appropriate manufacturer's protocol if you want to do this.

9.4. Tip

To minimize the chances of electrocution, avoid touching the gel apparatus unless it is disconnected from the power source. Disconnect the gel from the power source before rinsing out and loading the wells and when disassembling the gel apparatus.

9.5. Tip

If you place a metal plate over the gel while it is running, heat will distribute more evenly over the gel plates and gel smiling or frowning will be significantly reduced. If you do this, make sure the plate is not touching the buffer in the bottom tank.

9.6. Tip

While the gel is running, surround it with 3–4 plexiglass shields. This will minimize your exposure to radioactivity.

9.7. Tip

The gel is extremely radioactive and is exposed to the environment when the plates are separated. Always keep a plexiglass shield between you and the gel.

9.8. Tip

Ethanol precipitation adds an unknown amount of sodium acetate to the final sample. If you want to minimize the concentration of this salt, repeat the 70% ethanol wash 1–2 times.

See Fig. 14.7 for the flowchart of Step 5.

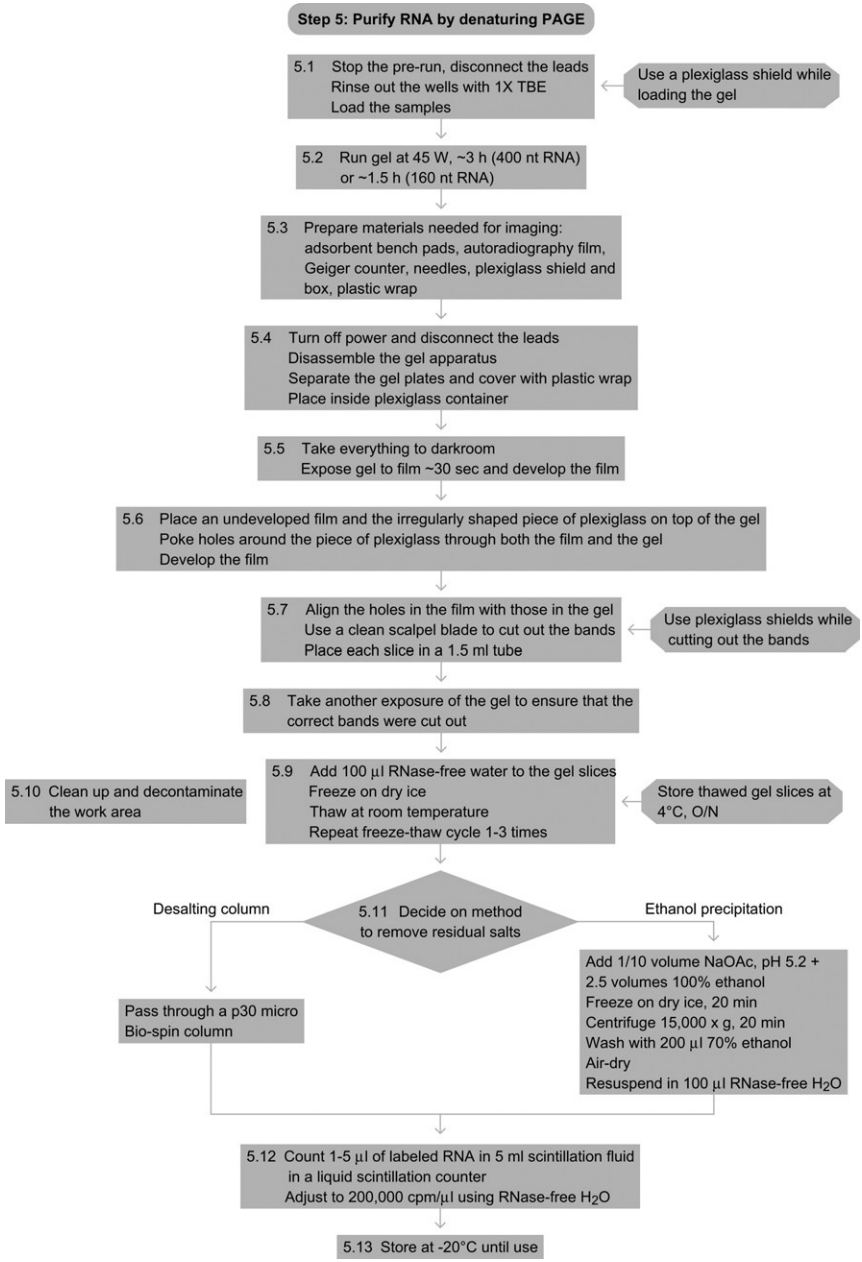

Figure 14.7 Flowchart of Step 5.

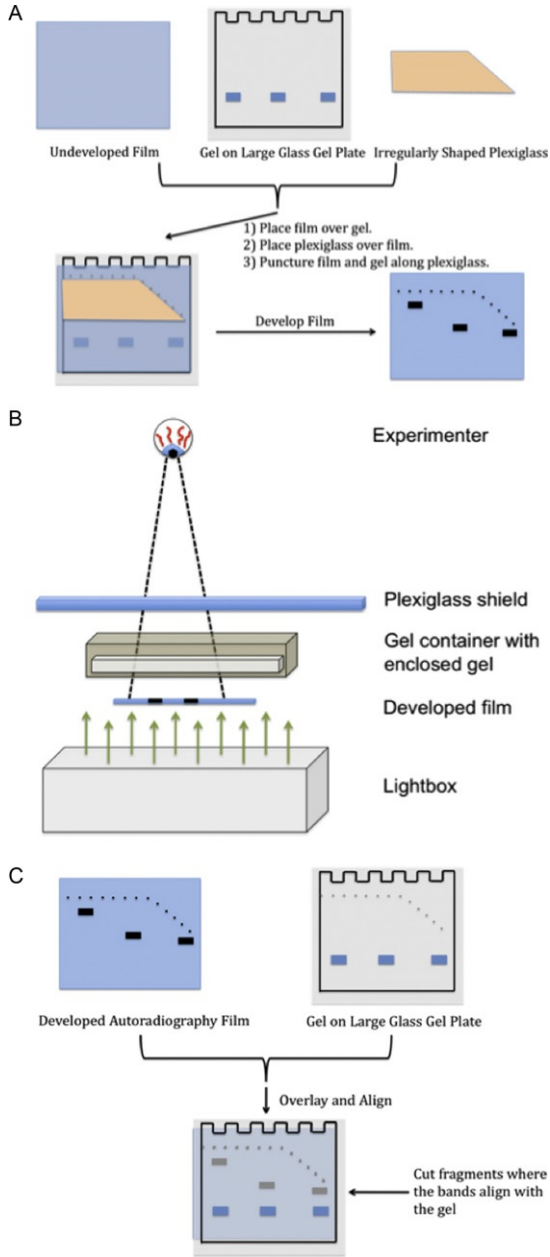

Figure 14.8 (a) Depicted above is the technique to puncture holes in the acrylamide gel and film so that the position of the labeled RNA on the gel can be determined. Push the syringe needle hard through the film and gel so puncture marks clearly are visible. (b) The gel and developed film are taken to a standard light box where the holes in the gel and film are aligned prior to excising the desired gel fragments. Note that the experimenter's field of vision is perpendicular to the position of the gel and film; viewing at this angle negates parallax effects and allows the best alignment of film and gel. (c) The black bands on the film arise through the absorption of radioparticles from the labeled RNA. By aligning the film below the gel, the black bands on the film superimpose onto regions of the gel containing the radiolabeled sample. At this point, the gel fragment is cut out through the plastic wrap and placed into its own tube.

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RNA purification – precipitation methods.
RNA purification by preparative polyacrylamide gel electrophoresis.