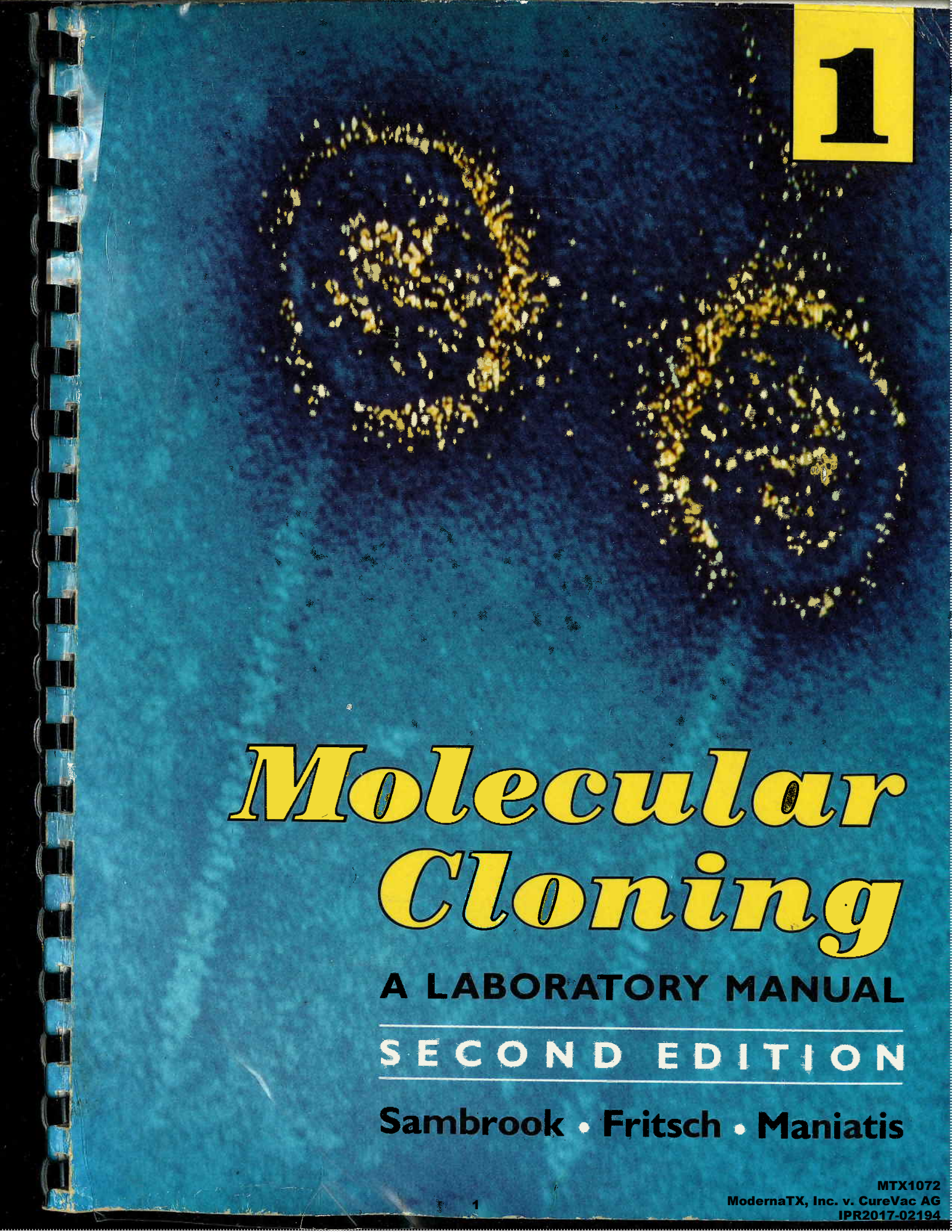


Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

Printed in the United States of America

9 8 7 6 5 4 3 2

Book and cover design by Emily Harste

Cover: The electron micrograph of bacteriophage λ particles stained with uranyl acetate was digitized and assigned false color by computer. (Thomas R. Broker, Louise T. Chow, and James I. Garrels)

Cataloging in Publications data

Sambrook, Joseph
Molecular cloning : a laboratory manual / E.F. Fritsch, T. Maniatis—2nd ed.
p. cm.
Bibliography: p.
Includes index.
ISBN 0-87969-309-6
1. Molecular cloning—Laboratory manuals. 2. Eukaryotic cells--Laboratory manuals. I. Fritsch, Edward F. II. Maniatis, Thomas III. Title.
QH442.2.M26 1987
574.87'3224—dc19 87-35464

All Cold Spring Harbor Laboratory Press publications may be ordered directly from Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, New York 11724. Phone: 1-800-843-4388. In New York (516)367-8423.

7
Extraction, Purification, and Analysis of Messenger RNA from Eukaryotic Cells

A typical mammalian cell contains about 10^{-5} μg of RNA, 80–85% of which is rRNA (chiefly 28S, 18S, and 5S). Most of the remaining 15–20% consists of a variety of low-molecular-weight species (tRNAs, small nuclear RNAs, etc.). These RNAs are of defined size and sequence and can be isolated in virtually pure form by gel electrophoresis, density gradient centrifugation, or anion-exchange or high-performance liquid chromatography. In contrast, mRNA, which makes up between 1% and 5% of the total cellular RNA, is heterogeneous in both size (from a few hundred bases to many kilobases in length) and sequence. However, most eukaryotic mRNAs carry at their 3′ termini a tract of polyadenylic acid residues that is generally long enough to allow mRNAs to be purified by affinity chromatography on oligo(dT)-cellulose. The resulting heterogeneous population of molecules collectively encodes virtually all of the polypeptides synthesized by the cell.

CONTROLLING RIBONUCLEASE ACTIVITY

To obtain good preparations of eukaryotic mRNA, it is necessary to minimize the activity of RNAases liberated during cell lysis by using inhibitors of RNAases or methods that disrupt cells and inactivate RNAases simultaneously as discussed below. Consequently, it is also important to avoid the accidental introduction of trace amounts of RNAase from other potential sources in the laboratory. A number of precautions that can be used to avoid problems with RNAases are listed below. Most experienced investigators do not rigorously adhere to these precautions but may employ one or more of them when difficulties are encountered. This list is intended to be used as a guide when contamination with RNAase is a problem.

Laboratory Procedures

If proper care is not taken, preparations of RNA can be contaminated with RNAases from outside sources including:

- *Glassware, plasticware, and electrophoresis tanks.* Sterile, disposable plasticware is essentially free of RNAases and can be used for the preparation and storage of RNA without pretreatment. General laboratory glassware and plasticware, however, are often contaminated with RNAases and should be treated by baking at 180°C for 8 hours or more (glassware) or by rinsing with chloroform (plasticware). Alternatively, some workers fill beakers, tubes, and other items that are to be used for the preparation of RNA with diethyl pyrocarbonate (DEPC) (0.1% in water), which is a strong, but not absolute, inhibitor of RNAases (Fedorcsak and Ehrenberg 1966). After the DEPC-filled glassware or plasticware has been allowed to stand for 2 hours at 37°C, it is rinsed several times with sterile water and then heated to 100°C for 15 minutes (Kumar and Lindberg 1972) or autoclaved for 15 minutes at 15 lb/sq. in. on liquid cycle. These treatments remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation.

  Note: Carboxymethylated RNA is translated with very low efficiency in cell-free systems; however, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified.

  Electrophoresis tanks used for electrophoresis of RNA should be cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H_2O_2. After 10 minutes at room temperature, the electrophoresis tank should be rinsed thoroughly with water that has been treated with 0.1% DEPC (see below).

  It is a good idea to set aside items of glassware, batches of plasticware, and electrophoresis tanks that are to be used only for experiments with RNA, to mark them distinctively, and to store them in a designated place.

  Caution: DEPC is suspected to be a carcinogen and should be handled with care.

- *Contamination by workers.* A potentially major source of contamination with RNAase is the hands of the investigator. Disposable gloves should therefore be worn during the preparation of materials and solutions used for the isolation and analysis of RNA and during manipulations involving RNA. Because gloves remain RNAase-free only if they do not come into contact with "dirty" glassware and surfaces, it is usually necessary to change gloves frequently when working with RNA.
- *Contaminated solutions.* All solutions should be prepared using RNAase-free glassware, autoclaved water, and chemicals reserved for work with RNA that are handled with baked spatulas. Wherever possible, the solutions should be treated with 0.1% DEPC for at least 12 hours at 37°C and then heated to 100°C for 15 minutes or autoclaved for 15 minutes at 15 lb/sq. in. on liquid cycle.

  Note: DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers such as Tris. Reserve a fresh, unopened bottle of Tris crystals for preparation of RNAase-free solutions.

Inhibitors of Ribonucleases

The following three types of specific inhibitors of RNAases are widely used:

- *Protein inhibitor of RNAases.* Many RNAases bind tightly ($K_i \cong 3 \times 10^{10}$) to a protein isolated from human placenta (Blackburn et al. 1977), forming equimolar, noncovalent complexes that are enzymatically inactive. In vivo, the protein is probably an inhibitor of angiogenin, an angiogenic factor whose amino acid sequence and predicted tertiary structure are similar to those of pancreatic RNAase (Kurachi et al. 1985; Strydom et al. 1985). The inhibitor, which is sold by several manufacturers under various trade names, should be stored at −20°C in 50% glycerol solutions containing 5 mM dithiothreitol. Preparations of the inhibitor that have been frozen and thawed several times or stored under oxidizing conditions should not be used; these treatments may denature the protein and release bound RNAases. The inhibitor is therefore not used when denaturing agents are used to lyse mammalian cells in the initial stages of extraction of RNA. However, it should be included when more gentle methods of lysis are used and should be present at all stages during the subsequent purification of RNA. Fresh inhibitor should be added several times during the purification procedure, since it is removed by extraction with phenol. The inhibitor requires sulfhydryl reagents for maximal activity and does not interfere with reverse transcription (de Martynoff et al. 1980) or cell-free translation of mRNA (Scheele and Blackburn 1979).
- *Vanadyl-ribonucleoside complexes.* The complexes formed between the oxovanadium IV ion and any of the four ribonucleosides are transition-state analogs that bind to many RNAases and inhibit their activity almost completely (Berger and Birkenmeier 1979). The four vanadyl-ribonucleoside complexes are added to intact cells and used at a concentration of 10 mM during all stages of RNA extraction and purification. The resulting mRNA is isolated in a form that can be directly translated in frog oocytes

and can be used as a template in some in vitro enzymatic reactions (e.g., reverse transcription of mRNA). However, vanadyl-ribonucleoside complexes strongly inhibit translation of mRNA in cell-free systems and must be removed from the mRNA by multiple extractions with phenol (equilibrated with 0.01 M Tris·Cl [pH 7.8]) containing 0.1% hydroxyquinoline. Vanadyl-ribonucleoside complexes are available from several commercial suppliers.

- *Macaloid.* Macaloid is a clay that has been known for many years to adsorb RNAase. The clay is prepared as a slurry (see Appendix B) that is used at a final concentration of 0.015% (w/v) in buffers used to lyse cells (Favaloro et al. 1980). The clay, together with its adsorbed RNAase, is removed by centrifugation at some stage during the subsequent purification of the RNA (e.g., after extraction with phenol).

Methods That Disrupt Cells and Inactivate Ribonucleases Simultaneously

Proteins dissolve readily in solutions of potent denaturing agents such as guanidine HCl and guanidinium thiocyanate (Cox 1968). Cellular structures disintegrate and nucleoproteins dissociate from nucleic acids as protein secondary structure is lost. RNAases can recover activity after many forms of treatment (such as boiling) but are inactivated by 4 M guanidinium thiocyanate and reducing agents such as β-mercaptoethanol (Sela et al. 1957). This combination of reagents can therefore be used to isolate intact RNA from tissues, such as the pancreas, that are rich in RNAase (Chirgwin et al. 1979).

The protocols presented below use inhibitors of RNAase and/or methods that lead to the rapid inactivation of RNAases for the isolation of total, nuclear, and cytoplasmic RNAs from tissues and cultured cells.

ISOLATION OF RNAs

Isolation of Total RNA from Mammalian Cells

This procedure is a modification of the method described by Favaloro et al. (1980) for the isolation of RNA from monolayers of mammalian cells grown in tissue culture. However, it can also be used to isolate RNA from mammalian cells grown in suspension or from mammalian tissues that can be readily dispersed into single cells. This method is not suitable for extraction of RNA from solid tissue because the method used to lyse the cells (digestion with proteinase K in the presence of SDS) is slow. Endogenous RNAases therefore have time to act before they are digested by protease or inactivated by inhibitors. The lysis buffer used in the original method (Favaloro et al. 1980) contained 0.015% (w/v) of a diatomaceous earth, "Macaloid," which adsorbed and inactivated RNAases. Although Macaloid is still available (NL Chemicals), vanadyl-ribonucleoside complexes or a protein inhibitor of RNAases is now more commonly used. The advantages of the following procedure are its speed and the ability to process many samples simultaneously.

1. *Lysis of cells growing in monolayers:*

   a. Remove the medium by aspiration, and wash each monolayer twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B). Stand the plates on a bed of ice until all of the monolayers have been washed.

      The plates may be placed on an aluminum plate on top of a tray of ice.

   b. Add 0.5 ml of RNA extraction buffer per 90-mm plate. Allow the extraction buffer to spread across the surface of the plate.

RNA extraction buffer

0.14 M NaCl
1.5 mM $MgCl_2$
10 mM Tris · Cl (pH 8.6)
0.5% Nonidet P-40 (NP-40)
1 mM dithiothreitol
1000 units/ml placental RNAase inhibitor *or* 20 mM vanadyl-ribonucleoside complexes

   c. Add 0.5 ml of proteinase digestion buffer. Mix the viscous lysate with a policeman, and scrape it to the edge of the plate.

Proteinase digestion buffer

0.2 M Tris · Cl (pH 8.0)
25 mM EDTA (pH 8.0)
0.3 M NaCl
2% SDS

d. Draw the lysate into a hypodermic syringe fitted with a 21-gauge needle, and then expel it into a polypropylene tube. Repeat three or four times to shear the cellular DNA.

e. Add proteinase K to a final concentration of 200 μg/ml. Mix the solution well and incubate for 30 minutes at 37°C.

Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B).

Lysis of cells growing in suspension or single-cell suspensions of tissues:

a. Collect the cells by centrifugation at 2000*g* for 5 minutes at 4°C. Wash the cell pellet three times by resuspension in 10 volumes of ice-cold PBS lacking calcium and magnesium ions; use a wide-bore pipette to disperse the cell pellet gently, but completely, each time.

b. Estimate the volume of the packed cells, and resuspend them in 10–20 volumes of RNA extraction buffer.

c. Add a volume of proteinase digestion buffer equal to the volume of RNA extraction buffer added in b. Mix the solution rapidly by vortexing. Draw the lysate into a hypodermic syringe fitted with a 21-gauge needle, and then expel it into a polypropylene tube. Repeat three or four times to shear the cellular DNA.

d. Add proteinase K to a final concentration of 200 μg/ml. Mix the solution well and incubate for 30 minutes at 37°C.

Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B).

2. Remove the proteins by extracting once with an equal volume of phenol:chloroform.

3. Separate the aqueous and organic phases by centrifugation at 5000*g* for 10 minutes at room temperature in a swinging-bucket rotor. Transfer the aqueous phase to a fresh tube, and add 2.5 volumes of ice-cold ethanol. Mix the solution well and chill to 0°C for 1 hour.

4. Recover the RNA by centrifugation at 5000*g* for 10 minutes at 0°C. Discard the supernatant, and wash the pellet with 70% ethanol containing 0.1 M sodium acetate (pH 5.2). Use an automatic micropipettor to remove as much of the ethanol as possible, and then allow the pellet to dry at room temperature for a few minutes.

It is important not to desiccate the pellet. Dried pellets of nucleic acid are very difficult to redissolve.

5. Redissolve the pellet in a small volume (200 μl per 90-mm plate or 10^7 cells) of 50 mM Tris · Cl (pH 7.8), 1 mM EDTA (pH 8.0).

6. Add $MgCl_2$ and dithiothreitol to final concentrations of 10 mM and 0.1 mM, respectively, and then add placental RNAase inhibitor or vanadyl-ribonucleoside complexes to a final concentration of 1000 units/ml or 10 mM, respectively.

7. Add RNAase-free pancreatic DNAase I (see Appendix B) to a final concentration of 2 μg/ml. Incubate the mixture for 60 minutes at 37°C.

8. Add EDTA and SDS to final concentrations of 10 mM and 0.2%, respectively.

9. Extract the solution once with an equal volume of phenol:chloroform.

10. Separate the aqueous and organic phases by centrifugation at 5000*g* for 10 minutes at room temperature. Transfer the aqueous phase to a fresh tube, and add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2.5 volumes of ice-cold ethanol, mix the solution well, and chill for 2 hours on ice.

11. Collect the RNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

12. Remove all of the ethanol. Stand the open tube on the bench for a few minutes to allow the last traces of ethanol to evaporate.

13. Redissolve the pellet in 200 μl of TE (pH 7.6). Add 500 μl of ethanol, and store the preparation at −70°C until it is needed.

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i. The concentration of the RNA can be determined by measuring the OD_{260} of an aliquot of the final preparation. Recover the RNA from 10 μl of the ethanol/TE mixture (step 13) and redissolve the pellet in 400 μl of H_2O. Measure the OD_{260}. A solution of RNA whose $OD_{260} = 1$ contains approximately 40 μg of RNA per milliliter. If you wish to use this RNA dilution sample, use cuvettes that have been soaked for 1 hour in concentrated HCl:methanol (1:1) and then washed extensively in water that has been treated with diethyl pyrocarbonate (DEPC) and autoclaved (see page 7.4).

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

ii. If desired, poly(A)$^+$ RNA can be purified from the preparation of total cellular RNA and freed from contaminating oligodeoxyribonucleotides by chromatography on oligo(dT)-cellulose (see pages 7.26–7.29).

iii. In some cases (e.g., when preparing RNA from cells infected with DNA viruses or from cells transfected with DNA), it is necessary to remove oligodeoxyribonucleotides from the preparation of total cellular RNA. If this is not done, problems may arise when the RNA is used to generate cDNA libraries or in primer-extension reactions. Contaminating fragments of template DNA may hybridize to the RNA and serve as primers during reverse transcription. This can lead to erroneous mapping of the 5′ termini of specific mRNAs and to the generation of truncated cDNA clones.

   a. After step 12, resuspend the pellet of nucleic acid in 200 μl of 3 M sodium acetate (pH 5.2) by repeated pipetting with an automatic micropipettor. Transfer the suspension to a fresh, sterile microfuge tube.

   b. Centrifuge the suspension at 12,000*g* for 10 minutes at room temperature in a microfuge. The RNA sediments to the bottom of the tube, while the great majority of the oligodeoxyribonucleotides remain in solution.

   c. Discard the supernatant, and redissolve the pellet in 200 μl of TE (pH 7.6). Add 20 μl of 3 M sodium acetate (pH 5.2), mix well, and add 550 μl of ice-cold ethanol. Mix the solution and chill for 30 minutes on ice. Recover the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Carefully remove the supernatant by aspiration.

   d. Redissolve the pellet in 300 μl of TE (pH 7.6), and add 1 ml of ethanol. Store the preparation at −70°C until it is needed.

      To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

      If necessary, vanadyl-ribonucleoside complexes can be removed by extracting the final preparation of RNA several times with phenol (equilibrated with 0.01 M Tris · Cl [pH 7.8]) containing 0.1% hydroxyquinoline.

iv. The yield of RNA from most lines of cultured cells is 100–200 μg per 90-mm plate.

Rapid Isolation of Total RNA from Mammalian Cells

In recent years, a number of rapid methods have been developed to isolate RNA from mammalian cells grown in culture. These methods fall into two classes: those that depend on differential extraction of RNA by organic solvents (e.g., phenol at acid pH; Stallcup and Washington 1983) and those that utilize differential precipitation to separate high-molecular-weight RNA from other types of nucleic acid (e.g., Birnboim 1988). Both classes of method work well even with types of cultured cells (e.g., macrophages and granulocytes) that contain relatively little mRNA and have high endogenous levels of RNAase. Many samples can be handled simultaneously and there are minimal losses of RNA. The method given below is an adaptation (D. Israel, unpubl.) of a protocol developed by Stallcup and Washington (1983).

1. Remove the medium by aspiration, and wash each monolayer twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B). Stand the plates on a bed of ice until all of the monolayers have been washed.

   The plates can be placed on an aluminum plate on top of a tray of ice.

2. Add 2 ml of 10 mM EDTA (pH 8.0), 0.5% SDS to each 90-mm plate of cells. Using a policeman, scrape the lysate into a 15-ml disposable polypropylene tube (Falcon 2053 or equivalent).

3. Rinse the plate with 2 ml of 0.1 M sodium acetate (pH 5.2), 10 mM EDTA (pH 8.0). Transfer the solution to the tube containing the cell lysate.

4. Add 4 ml of phenol (equilibrated with water), and mix the contents of the tube by shaking for 2 minutes at room temperature. The cellular DNA precipitates as a white, stringy mass.

5. Separate the phases by centrifugation at 5000 rpm for 10 minutes at 4°C in a Sorvall HB-4 or SS34 rotor (or equivalent). The DNA should form a tight cushion at the interface of the two phases.

6. Using a sterile pasteur pipette, transfer the upper (aqueous) phase to a fresh tube containing 440 μl of ice-cold 1 M Tris · Cl (pH 8.0) and 180 μl of 5 M NaCl.

7. Add 2 volumes of ice-cold ethanol. Mix the contents of the tube and store it for at least 30 minutes at 0°C.

8. Collect the RNA by centrifugation at 5000 rpm for 10 minutes at 4°C in a Sorvall HB-4 or SS34 rotor (or equivalent). Remove the ethanol, and store the tubes in an inverted position until all of the ethanol has drained away.

9. Redissolve the RNA in 200 μl of ice-cold TE (pH 8.0). Transfer the solution to a sterile microfuge tube, and add 4 μl of 5 M NaCl and 500 μl of ice-cold ethanol.

10. Collect the RNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

11. Remove all of the ethanol. Stand the open tube on the bench for a few minutes to allow the last traces of ethanol to evaporate.

12. Redissolve the RNA in the desired buffer.

Notes

i. The yield of RNA from most lines of cultured cells is 100–200 μg per 90-mm plate.

ii. Several plates can be harvested simultaneously by transferring the lysate and acetate wash sequentially from plate to plate.

iii. The yield of RNA can be increased slightly by extracting the organic phase at the end of step 6 with 2 ml of a solution containing equal amounts of the EDTA/SDS lysis solution (step 2) and the sodium acetate/EDTA solution (step 3). Pool this aqueous phase with the original aqueous phase. Adjust the volumes of the reagents used in step 6 to maintain the same ratios of aqueous phase to reagents.

Isolation of Cytoplasmic RNA from Mammalian Cells

This is a convenient and well-tested procedure for purifying cytoplasmic RNA from cells grown in tissue culture. The method is similar to that described previously to isolate total cellular RNA, except that the nuclei are removed by centrifugation at an early stage. The procedure is rapid, many samples can be processed simultaneously, and most of the steps can be carried out at room temperature. RNA prepared in this way is an excellent template for the preparation of cDNA libraries, for cell-free translation, and for primer extension and nuclease-S1 protection assays.

1. *To harvest cells growing in monolayers:*

   a. Remove the medium by aspiration, and wash each monolayer twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B). Stand the plates on a bed of ice until all of the monolayers have been washed.

      The plates may be placed on an aluminum plate on top of a tray of ice.

   b. Using a policeman, scrape the cells into the small amount of residual PBS. Transfer the cells to a microfuge tube.

   To harvest cells growing in suspension or single-cell suspensions of tissues:

   a. Collect the cells by centrifugation at 2000*g* for 5 minutes at 4°C. Wash the cell pellet twice by resuspension in 10 volumes of ice-cold PBS lacking calcium and magnesium ions; use a wide-bore pipette to disperse the cell pellet gently, but completely, each time.

   b. Estimate the volume of the packed cells, and resuspend them in 10–20 volumes of PBS. Transfer 1 ml of the suspension to a microfuge tube.

2. Centrifuge the cells at 12,000*g* for 30 seconds at 4°C in a microfuge. Discard the supernatant, and resuspend the cell pellet in 200 μl of RNA extraction buffer. Vortex the suspension for 15 seconds, and then stand the microfuge tube on ice for 5 minutes.

RNA extraction buffer

0.14 M NaCl
1.5 mM $MgCl_2$
10 mM Tris · Cl (pH 8.6)
0.5% Nonidet P-40 (NP-40)
1 mM dithiothreitol
1000 units/ml placental RNAase inhibitor *or* 20 mM vanadyl-ribonucleoside complexes

3. Centrifuge at 12,000*g* for 90 seconds at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube. Discard the pellet, which consists of unlysed cells and nuclei.

4. Add 200 μl of proteinase digestion buffer. Mix by vortexing. Add proteinase K to a final concentration of 50 μg/ml. Mix the solution well and incubate for 30 minutes at 37°C.

Proteinase digestion buffer

0.2 M Tris · Cl (pH 8.0)
25 mM EDTA (pH 8.0)
0.3 M NaCl
2% SDS

Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B).

Older versions of this protocol do not call for digestion with proteinase K. However, if this step is omitted, a bulky precipitate forms at the interface of the organic and aqueous phases in step 5. The size of this precipitate is affected by the type of cells used and the conditions under which they are grown. In the worst cases, it can completely occupy the aqueous phase and make recovery of RNA very difficult.

5. Remove the proteins by extracting once with an equal volume of phenol:chloroform.

6. Separate the aqueous and organic phases by centrifugation at 5000*g* for 10 minutes at room temperature.

It is best to centrifuge the microfuge tubes in a swinging-bucket rotor (e.g., in a Sorvall HB-4 rotor or its equivalent) rather than in a fixed-angle microfuge. In the latter case, particulate material sticks to the side of the tube and contaminates the aqueous phase.

7. Transfer the aqueous phase to a fresh microfuge tube, and add 400 μl of ice-cold isopropanol. Mix well, and chill for 30 minutes on ice.

8. Collect the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Use an automatic micropipettor fitted with a 1-ml disposable tip to remove the supernatant. Add 1 ml of 70% ethanol at room temperature, vortex briefly, and recentrifuge.

9. Carefully remove as much as possible of the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated. Proceed to step 18 or continue with steps 10–17 if necessary as noted below.

It is important not to desiccate the pellet. Dried pellets of nucleic acid are very difficult to redissolve.

Steps 10–17 need to be carried out only when isolating cytoplasmic RNA from cells that have been transfected with DNA or from cells infected with DNA viruses. Cytoplasm prepared from such cells invariably contains a large quantity of template DNA, which must be removed before the RNA can be used for translation, northern hybridization, or synthesis of cDNA. Cytoplasm prepared from uninfected or untransfected cells generally contains only a small quantity of cellular DNA that generally does not compromise experiments with the RNA.

10. Redissolve the pellet in a small volume (200 μl per 90-mm plate or 10^7 cells) of 50 mM Tris · Cl (pH 7.8), 1 mM EDTA (pH 8.0).

11. Add $MgCl_2$ and dithiothreitol to final concentrations of 10 mM and 0.1 mM, respectively, and then add placental RNAase inhibitor or vanadyl-ribonucleoside complexes to a final concentration of 1000 units/ml or 10 mM, respectively.

12. Add RNAase-free pancreatic DNAase I (see Appendix B) to a final concentration of 2 μg/ml. Incubate for 60 minutes at 37°C.

13. Add EDTA and SDS to final concentrations of 10 mM and 0.2%, respectively.

14. Extract the solution once with an equal volume of phenol:chloroform.

15. Separate the aqueous and organic phases by centrifugation at 12,000*g* for 5 minutes at room temperature in a microfuge. Transfer the aqueous phase to a fresh microfuge tube, and add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2.5 volumes of ice-cold ethanol, mix well, and chill for at least 30 minutes on ice.

16. Collect the RNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

17. Remove all of the ethanol. Stand the open tube on the bench for a few minutes to allow the last traces of ethanol to evaporate.

18. Redissolve the pellet in 200 μl of TE (pH 7.6). Add 500 μl of ethanol, and store the preparation at −70°C until it is needed.

To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i. The concentration of the RNA can be determined by measuring the OD_{260} of an aliquot of the final preparation. Recover the RNA from 10 μl of the ethanol/TE mixture (step 18) and redissolve it in 400 μl of H_2O. Measure the OD_{260}. A solution of RNA whose $OD_{260} = 1$ contains approximately 40 μg of RNA per milliliter. If you wish to use this RNA dilution sample, use cuvettes that have been soaked for 1 hour in concentrated HCl:methanol

(1:1) and then washed extensively in water that has been treated with diethyl pyrocarbonate (DEPC) and autoclaved (see page 7.4).

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

ii. If desired, poly(A)$^+$ RNA can be purified from the preparation of total cytoplasmic RNA and freed from contaminating oligodeoxyribonucleotides by chromatography on oligo(dT)-cellulose (see pages 7.26–7.29).

iii. The yield of cytoplasmic RNA from different types of mammalian cells varies greatly—from 30 μg to 500 μg of RNA per 10^7 cells—depending on their sizes and states of differentiation. The amount of RNA obtained per plate of cells is even more variable, since it is greatly influenced by the density of the cells at the time of harvest.

Isolation of Total RNA from Eggs and Embryos

This simple method works very well for oocytes, fertilized eggs, and embryos of frogs, sea urchins, tunicates, worms, and flies. Precipitation of the RNA with lithium chloride is helpful in removing glycoproteins and yolky components from the preparations. No attempt is made to remove DNA from the preparation, since the amount of RNA obtained greatly exceeds the amount of contaminating DNA. Although these genomic DNA sequences do not interfere with hybridization or translation, they might cause complications if the RNA is to be used to generate cDNA libraries. In this case, we recommend that the DNA be removed by digestion with RNAase-free pancreatic DNAase I as described on page 7.8.

1. Place the tissue in a baked glass homogenizer and remove all extraneous fluid. Fly embryos should be washed with 0.5% Triton X-100 to remove medium.

2. Add about 10 volumes of homogenization buffer, and immediately homogenize until the tissue is thoroughly dispersed.

Homogenization buffer

50 mM NaCl
50 mM Tris · Cl (pH 7.5)
5 mM EDTA (pH 8.0)
0.5% SDS
200 μg/ml proteinase K

Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in water (see Appendix B).

3. Incubate the homogenate for 1 hour at 37°C. Mix occasionally.

4. Transfer the homogenate to a polypropylene tube, add an equal volume of phenol:chloroform, and vortex vigorously for 1 minute. Separate the organic and aqueous phases by centrifugation at 5000*g* for 10 minutes at room temperature in a swinging-bucket rotor.

5. Transfer the aqueous (upper) phase to a fresh tube, and reextract with phenol:chloroform as described in step 4.

6. Transfer the aqueous phase to a fresh tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2). Mix well, and add 2.5 volumes of ice-cold ethanol. Store for 2 hours on ice.

7. Centrifuge at 5000*g* for 15 minutes at 4°C. Carefully discard the supernatant, and allow the pellet of nucleic acid to dry at room temperature. Proceed to step 11 or continue with steps 8–10 if necessary as noted below.

Many embryonic tissues contain large amounts of yolk and glycoproteins that are not removed from preparations of RNA by extraction with organic solvents. In these cases, the RNA should be purified further by precipitation with LiCl (steps 8–10).

8. Redissolve the pellet in a small volume of water. Add an equal volume of 8 M LiCl that has been sterilized by autoclaving, and mix well. Store at −20°C for at least 3 hours.

9. Recover the RNA by centrifugation at 10,000*g* for 30 minutes at 4°C. Carefully discard the supernatant.

10. Wash the pellet with 70% ethanol, recentrifuge briefly, discard the supernatant, and allow the pellet of nucleic acid to dry in the air.

11. Redissolve the RNA in a small volume of water. Add 3 volumes of ethanol, and store the preparation at −70°C until it is needed.

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Note

The concentration of the RNA can be determined by measuring the OD_{260} of an aliquot of the final preparation. Recover the RNA from 10 μl of the ethanol/water mixture (step 11) and redissolve it in 400 μl of H_2O. Measure the OD_{260}. A solution of RNA whose $OD_{260} = 1$ contains approximately 40 μg of RNA per milliliter. If you wish to use this RNA dilution sample, use cuvettes that have been soaked for 1 hour in concentrated HCl:methanol (1:1) and then washed extensively in water that has been treated with diethyl pyrocarbonate (DEPC) and autoclaved (see page 7.4).

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

Isolation of Total RNA by Extraction with Strong Denaturants

The two methods given below were devised originally to isolate RNA from cells that cannot be fractionated easily into cytoplasm and nuclei (e.g., frozen fragments of tissue) or from cells that are particularly rich in RNAases (e.g., pancreatic cells) (Chirgwin et al. 1979). Because of their success in obtaining RNA from these "difficult" sources, the methods have become widely adopted and are now routinely used to isolate RNA from common mammalian cell lines. Both the yield and quality of RNA prepared with chaotropic agents are consistently high, and we therefore recommend that these methods be used whenever possible. However, both procedures suffer from the disadvantage of being rather cumbersome and therefore are not well-suited to the isolation of RNA from large numbers of different samples of cultured cells. For this purpose, one of the methods described previously might be more suitable.

Tissues should be freshly harvested, weighed, cut into small pieces (1 cm^2) with a sterile scalpel, and placed directly into homogenization buffer (see below) or, if not to be used immediately, into liquid nitrogen and stored at −70°C.

Cells grown in suspension should be recovered by centrifugation, washed once with phosphate-buffered saline (PBS) (see Appendix B), recentrifuged, drained well, and either used immmediately or quick-frozen in liquid nitrogen and stored at −70°C. Cells grown in monolayers should be washed once with PBS, drained well, and either used immediately or quick-frozen in liquid nitrogen and stored at −70°C. This can be achieved as follows:

1. Make a shallow tray from aluminum foil and arrange the tissue-culture plates (with their covers on) in it. Do not stack the plates on top of one another.
2. Slowly pour liquid nitrogen into the tray, so that it runs underneath the bottom of the plates. After the residual liquid in the plates has frozen, quickly wrap the plates in aluminum foil and store them at −70°C.

Frozen monolayers of cells stored at −70°C should be thawed directly in homogenization buffer at room temperature. Frozen tissues and pellets of cells should be powdered in a chilled homogenizer; homogenization buffer should then be added to the powdered cells while they are still in the frozen state.

EXTRACTION OF RNA WITH GUANIDINIUM THIOCYANATE FOLLOWED BY CENTRIFUGATION IN CESIUM CHLORIDE SOLUTIONS

This is a modification of methods described by Glišin et al. (1974) and Ullrich et al. (1977). Cells and tissues are prepared as described on pages 7.6–7.7. Guanidinium thiocyanate is used to disrupt the cells, and the resulting homogenate is then layered on a cushion of a dense solution of CsCl. The buoyant density of RNA in CsCl (>1.8 g/ml) is much greater than that of other cellular components. During centrifugation, the RNA forms a pellet on the bottom of the tube, while the DNA and protein float in the supernatant solution.

Small RNAs (e.g., 5S RNA and tRNAs), which do not sediment efficiently during centrifugation through CsCl, should not be prepared by this method.

1. *Either*

   a. To a fragment of tissue or a pellet of cells, add 5 volumes of guanidinium thiocyanate homogenization buffer.

   or

   b. To each confluent monolayer of cells, add 1 ml of guanidinium thiocyanate homogenization buffer. Allow the solution to spread across the plate until all of the cells are covered. Scrape the viscous lysate to the edge of the plate with a policeman, and transfer it to a tube for homogenization.

Guanidinium thiocyanate homogenization buffer

4.0 M guanidinium thiocyanate ($M_r = 118.1$)
0.1 M Tris · Cl (pH 7.5)
1% β-mercaptoethanol

Dissolve 50 g of guanidinium thiocyanate in 10 ml of 1 M Tris · Cl (pH 7.5), and add H_2O to 100 ml. Filter the solution through a Whatman No. 1 filter or equivalent. This solution is stable and can be stored indefinitely at room temperature. Just before use, add β-mercaptoethanol to a final concentration of 1% (0.14 M).

2. Homogenize the cell lysates with a Con-Torque tissue grinder (made by Eberbach and available from Fisher) or a Polytron homogenizer (Brinkmann) (RNAase-free) at high speed for 1–2 minutes. Homogenization shears the nuclear DNA and prevents the formation of an impenetrable mat on the top of the cushion of CsCl, which might block sedimentation of the RNA to the bottom of the centrifuge tube. Homogenization increases the yield of RNA by a factor of approximately 2.

   If commercial homogenizers are not available, the nuclear DNA can be sheared by drawing the preparation into a sterile hypodermic syringe and expelling it through a 23-gauge needle. Repeat this procedure several times until the preparation is no longer viscous.

3. After homogenization, add sodium lauryl sarcosinate to a final concentration of 0.5% and mix the suspension well. Centrifuge at 5000*g* for 10 minutes at room temperature.

   Sodium lauryl sarcosinate is used as a detergent because SDS is insoluble in concentrated salt solutions.

   The centrifugation step can usually be omitted when processing lysates of cultured cells.

4. Transfer the supernatant (or the lysate of cultured cells) to a fresh tube, and draw it into a hypodermic syringe fitted with a 23-gauge needle. Layer the samples onto a cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) in a clear ultracentrifuge tube. Using a waterproof marker, mark the position of the top of the cushion on the outside of the tube. Use guanidinium thiocyanate homogenization buffer to fill the tubes and to equalize their weights.

   The CsCl/EDTA solution is made in 100-ml batches by dissolving 96.0 g of CsCl in 90 ml of 0.01 M EDTA (pH 7.5) and adding diethyl pyrocarbonate (DEPC) to a final concentration of 0.1%. Allow the solution to stand for 30 minutes, and then autoclave for 20 minutes at 15 lb/sq. in. on liquid cycle. When the solution has cooled, adjust the volume to 100 ml with DEPC-treated water (see page 7.4).

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

   For convenience in later steps, the tubes should be labeled on the bottom.

5. Centrifuge at 20°C for the time and at the speed indicated in the table below.

   A swinging-bucket rotor is preferred to a fixed-angle rotor because the RNA is deposited at the bottom of the centrifuge tube rather than along the wall (where it comes into contact with the cell lysate).

   Turn off the centrifuge brake before decelerating the rotor to prevent disturbing the contents of the tube.

Rotor	Volume of homogenate (ml)	Volume of CsCl/ EDTA cushion (ml)	Time (hours)	Speed (rpm)
SW60 (7/16″ × 2⅜″)	1.2	3.1	12	40,000
SW41 (9/16″ × 3½″)	3.5	9.7	24	32,000
SW28 (1½″ × 3½″)	12.0	26.5	24	25,000

6. Take care not to disturb the gradients when removing the tubes from the centrifuge. Draw a line on the outside of each tube approximately 0.5 cm from the bottom. Using a pasteur pipette, carefully remove the fluid above the level of the cushion (upper mark on the outside of the tube). This part of the gradient contains the viscous cellular DNA, which is usually visible as a white band. With a fresh pipette, remove the fluid above the lower mark on the outside of the tube.

7. Using a red-hot razor blade held in a hemostat, cut off the bottom of the tube just above the level of the remaining fluid. Place the bottom of the tube on a pad of Kimwipes, and carefully withdraw the fluid with an automatic pipettor. Invert the tube and allow any remaining fluid to drain into the pad of Kimwipes. Return the tube to an upright position. Check that the pellet of RNA has not fallen out of the tube.

   Depending on the amount of RNA present, the pellet may be invisible in the tube, but it can be seen easily enough if it falls onto the pad of Kimwipes. Should this occur, use sterile forceps to return the pellet of RNA to the tube.

8. Fill the bottom of the tube with 70% ethanol at room temperature. Invert the tube and drain off the ethanol, again checking that the pellet of RNA is not dislodged.

   Washing with ethanol removes CsCl from the pellet of RNA, making it easier to dissolve.

9. Allow the pellet of RNA to dry at room temperature. Dissolve the pellet of RNA in the appropriate volume of TE (pH 7.6) containing 0.1% SDS by drawing the fluid repeatedly into an automatic pipettor fitted with a disposable tip.

Volume of homogenate	Volume of TE (pH 7.6)/ 0.1% SDS
3.3 ml	100 μl
10.0 ml	300 μl
26.0 ml	1.0 ml

   If the RNA is difficult to dissolve, freeze and thaw the sample twice and then heat it to 45°C. This usually breaks up the pellets and allows them to dissolve rapidly. Alternatively, the pellets can be homogenized in the TE/SDS solution in a Con-Torque tissue grinder (made by Eberbach and available from Fisher) or a Polytron homogenizer (Brinkmann) (RNAase-free).

   Transfer the RNA solution to a microfuge tube. Rinse the bottom of the ultracentrifuge tube with 25 μl of TE (pH 7.6) and add to the microfuge tube.

10. To the solution of RNA in the microfuge tube, add 150 μl of TE (pH 7.6), 30 μl of 3 M sodium acetate (pH 5.2), and 0.9 ml of ice-cold ethanol. Mix well, and store for at least 30 minutes at 0°C.

11. Collect the precipitate of RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Discard the supernatant.

12. Wash the pellet with 70% ethanol, recentrifuge briefly, and allow the pellet of nucleic acid to dry in the air.

13. Redissolve the RNA in a small volume of water. Add 3 volumes of ethanol, and store the preparation at −70°C until it is needed.

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i. The RNA extracted from cultured cells and from most tissues is pure enough to be used for northern hybridization and nuclease-S1 analysis without further treatment. However, RNA extracted from certain tissues (e.g., spleen) may be contaminated with protein, which should be removed by extraction with organic solvents. The solution of RNA (step 9) is extracted once with phenol:chloroform and once with chloroform alone. The RNA is then recovered from the aqueous phase by precipitation with ethanol as described in step 10. RNA isolated from liver is often contaminated with glycogen, which can be removed by precipitating the RNA with salt. Add 3 volumes of 4 M sodium acetate (pH 7.0), mix well, and store at 0°C overnight. Recover the RNA by centrifugation at 8000*g* for 15 minutes at 4°C.

ii. RNA isolated from cells transfected with DNA or from cells infected with DNA viruses may be contaminated with template DNA, which must be removed before the RNA can be used for translation, northern hybridization, or synthesis of cDNA (see steps 10–17, page 7.14). RNA prepared from uninfected or untransfected cells generally contains only a small quantity of cellular DNA that generally does not compromise experiments with the RNA.

EXTRACTION OF RNA WITH GUANIDINE HCl AND ORGANIC SOLVENTS

This is a modification of methods described by Strohman et al. (1977) and MacDonald et al. (1987). Cells and tissues are prepared as described on pages 7.6–7.7.

1. *Either*

   a. To a fragment of tissue or a cell pellet, add 10 volumes of guanidine HCl homogenization buffer I.

   or

   b. To each confluent monolayer of cells, add 1 ml of guanidine HCl homogenization buffer I. Allow the solution to spread across the plate until all of the cells are covered. Scrape the viscous lysate to the edge of the plate with a policeman, and transfer it to a tube for homogenization.

Guanidine HCl homogenization buffer I

8 M guanidine HCl (M_r = 95.6)
0.1 M sodium acetate (pH 5.2)
5 mM dithiothreitol
0.5% sodium lauryl sarcosinate

Add 191 g of guanidine HCl to 8.35 ml of 3 M sodium acetate (pH 5.2) and 6.25 ml of 0.2 M dithiothreitol. Add H_2O to 237.5 ml and mix well. Add 12.5 ml of 10% sodium lauryl sarcosinate and mix by vortexing.

2. Homogenize the lysates with a Con-Torque tissue grinder (made by Eberbach and available from Fisher) or a Polytron homogenizer (Brinkmann) (RNAase-free) for 1 minute at room temperature.

3. Centrifuge the homogenate at 5000*g* for 10 minutes at room temperature.

   RNA may be purified from the supernatant by centrifugation through a cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) as described on page 7.20 or by the steps below, which involve differential precipitation of DNA and RNA.

4. Transfer the supernatant to a fresh tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2). Mix well. Add 0.5 volume of ice-cold ethanol and mix thoroughly. Store the solution for at least 2 hours at 0°C.

5. Recover the nucleic acids by centrifugation at 5000*g* for 10 minutes at 0°C. Discard the supernatant, and allow the pellet of nucleic acids to dry at room temperature.

6. Dissolve the pellet in a small volume of guanidine HCl homogenization buffer II. Use 10–15 ml of buffer for every gram of original cells or tissue.

   The pellet is often difficult to dissolve, and it may be necessary to use a homogenizer to assist in dissolution.

   Guanidine HCl homogenization buffer II

   8 M guanidine HCl (M_r = 95.6)
   0.1 M sodium acetate (pH 7.0)
   1 mM dithiothreitol
   20 mM EDTA (pH 8.0)

   Add 191 g of guanidine HCl to 8.35 ml of 3 M sodium acetate (pH 7.0), 1.25 ml of 0.2 M dithiothreitol, and 10 ml of 0.5 M EDTA (pH 8.0). Add H_2O to 250 ml and mix well.

7. Add 0.5 volume of ice-cold ethanol. Immediately mix the solution. Store the solution for at least 2 hours at −20°C.

8. Recover the nucleic acids by centrifugation at 5000*g* for 10 minutes. Discard the supernatant, and allow the pellet of nucleic acids to dry at room temperature.

9. Repeat steps 7 and 8 twice (i.e., a total of three precipitations with ethanol).

10. Dissolve the pellet in a minimal volume of 0.02 M EDTA (pH 8.0) (~5 ml for every gram of original cells or tissue) as follows:

    a. Add approximately one half of the EDTA solution, vortex for 1–2 minutes, and centrifuge at 3000*g* for 2 minutes. Remove and save the supernatant.

    b. Add the second half of the EDTA solution and vortex for 1–2 minutes. The pellet of nucleic acids should dissolve completely.

    c. Pool the EDTA solutions.

11. Add an equal volume of chloroform:1-butanol (4:1) and vortex.

12. Centrifuge at 5000*g* for 10 minutes at room temperature. Transfer the aqueous (upper) phase to a fresh tube. Repeat the extraction with organic solvents.

13. Transfer the aqueous phase to a fresh tube, and add 3 volumes of 4 M sodium acetate (pH 7.0). Store the tube for at least 1 hour at −20°C.

14. Centrifuge at 5000*g* for 20 minutes at 0°C. Under these conditions, the cellular DNA remains soluble, while the RNA is precipitated.

15. Remove the supernatant, and wash the pellet once with 3 M sodium acetate (pH 7.0) at 4°C. Centrifuge at 5000*g* for 20 minutes at 0°C.

16. Remove as much as possible of the supernatant, and dissolve the pellet in a minimal volume of 0.2% SDS, 0.05 M EDTA (pH 8.0) (~1 ml per gram of cells or tissue).

    If the SDS precipitates, add 0.1 N NaOH dropwise while agitating the solution until the pH reaches ~7.5.

17. Add 2 volumes of ice-cold ethanol. Store for at least 2 hours at 0°C. Recover the RNA by centrifugation at 5000*g* for 10 minutes at 4°C.

18. Wash the pellet with 70% ethanol, recentrifuge briefly, and allow the pellet of nucleic acid to dry in the air.

19. Redissolve the RNA in a small volume of water. Add 3 volumes of ethanol, and store the preparation at −70°C until it is needed.

    To recover the RNA, remove an aliquot, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Note

RNA isolated from cells transfected with DNA or from cells infected with DNA viruses may be contaminated with template DNA which must be removed before the RNA can be used for translation, northern hybridization, or synthesis of cDNA (see steps 10–17, page 7.14). RNA prepared from uninfected or untransfected cells generally contains only a small quantity of cellular DNA that generally does not compromise experiments with the RNA.

Selection of Poly(A)$^+$ RNA

In contrast to rRNAs and tRNAs, the vast majority of mRNAs of mammalian cells carry tracts of poly(A) at their 3′ termini. mRNAs can therefore be separated from the bulk of cellular RNA by affinity chromatography on oligo(dT)-cellulose (Edmonds et al. 1971; Aviv and Leder 1972). This is an essential step when preparing mRNA that is to be used as a template for the construction of cDNA libraries. Poly(A)$^+$ RNA usually yields better results than total RNA when analyzed by northern hybridization or in nuclease-S1 assays. Oligo(dT)-cellulose can be prepared as described by Gilham (1964) or obtained commercially.

1. Suspend 0.5–1.0 g of oligo(dT)-cellulose in 0.1 N NaOH.

2. Pour a column of oligo(dT)-cellulose (0.5–1.0 ml packed volume) in a sterile Dispocolumn (Bio-Rad) (or a pasteur pipette, plugged with sterile glass wool that has been treated with diethyl pyrocarbonate [DEPC] and autoclaved [see page 7.3]). Wash the column with 3 column volumes of sterile water.

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

   Up to 10 mg of total RNA can be loaded onto 1 ml of packed oligo(dT)-cellulose. If smaller quantities of total RNA are used, the amount of oligo(dT)-cellulose should be reduced accordingly to avoid loss of poly(A)$^+$ RNA both on the column and during the subsequent steps.

3. Wash the column with sterile 1 × column-loading buffer until the pH of the effluent is less than 8.0.

   1 × Column-loading buffer

   20 mM Tris · Cl (pH 7.6)
   0.5 M NaCl
   1 mM EDTA (pH 8.0)
   0.1% sodium lauryl sarcosinate

   To prepare sterile column-loading buffer, mix appropriate amounts of RNAase-free stock solutions of Tris · Cl (pH 7.6), NaCl, and EDTA (pH 8.0) (see page 7.4). Autoclave the mixture for 15 minutes at 15 lb/sq. in. on liquid cycle. Allow the solution to cool to approximately 65°C, and then add sodium lauryl sarcosinate from a 10% stock solution that has been heated to 65°C for 30 minutes. Alternatively, 0.05 M sodium citrate can be substituted for Tris · Cl, and the sodium citrate/NaCl/EDTA mixture and sodium lauryl sarcosinate can then be treated with DEPC (see page 7.4).

4. Dissolve the RNA in sterile water, and heat the solution to 65°C for 5 minutes. Cool the solution to room temperature quickly, and add an

equal amount of 2 × column-loading buffer. Apply the solution to the column, and immediately begin to collect the eluate in a sterile tube. When all of the RNA solution has entered the column, add 1 column volume of 1 × column-loading buffer and continue to collect the eluate.

Heating the RNA disrupts regions of secondary structure that might involve the poly(A)$^+$ tail.

5. When all of the solution has eluted, heat the collected eluate to 65°C for 5 minutes and reapply the eluate to the top of the column. Again collect the material flowing through the column.

6. Wash the column with 5–10 column volumes of 1 × column-loading buffer, collecting 1-ml fractions. Read the absorbance at 260 nm of each fraction collected from the column. Initially, the OD_{260} will be very high as the nonpolyadenylated RNA passes through the column. The later fractions should have very little or no absorbance at 260 nm.

In some protocols, the column is now washed with 5 column volumes of 1 × column-loading buffer containing 0.1 M NaCl. However, very little or no nonpolyadenylated RNA elutes from the column during this wash, which can therefore be omitted.

7. Elute the poly(A)$^+$ RNA from the oligo(dT)-cellulose with 2–3 column volumes of sterile, RNAase-free elution buffer. Collect fractions equivalent in size to 1/3 to 1/2 of the column volume.

Elution buffer

10 mM Tris · Cl (pH 7.6)
1 mM EDTA (pH 8.0)
0.05% SDS

The stock solutions of Tris · Cl and EDTA used to make elution buffer should be freshly autoclaved (15 minutes at 15 lb/sq. in. on liquid cycle) and then diluted with the appropriate amount of sterile water. The SDS should then be added from a concentrated stock solution (10% or 20%) made in sterile water. Do not attempt to sterilize elution buffer by autoclaving since it froths excessively.

8. Measure the absorbance of the solution at 260 nm, using cuvettes that have been soaked for 1 hour in concentrated HCl:methanol (1:1) and then washed extensively in water that has been treated with DEPC and autoclaved (see page 7.4).

Pool the fractions containing RNA that has eluted from the column.

The material obtained after a single round of chromatography on oligo(dT)-cellulose usually contains approximately equal quantities of polyadenylated and nonpolyadenylated species of RNA. To purify poly(A)$^+$ RNA further, heat the RNA to 65°C for 3 minutes and then cool it quickly to room temperature. Adjust the concentration of NaCl in the eluted RNA to 0.5 M, and carry out a second round of chromatography on the same column of oligo(dT)-cellulose.

9. To the poly(A)$^+$ RNA eluted from the oligo(dT)-cellulose column, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Mix well. Add 2.5 volumes of ice-cold ethanol, mix, and store for at least 30 minutes on ice.

10. Recover the poly(A)$^+$ RNA by centrifugation at 10,000*g* for 15 minutes at 4°C. Carefully discard the supernatant, and wash the pellet (which is often invisible) with 70% ethanol. Recentrifuge briefly, and allow the pellet of nucleic acid to dry in the air.

11. Redissolve the RNA in a small volume of water. Measure the absorbance of the solution at 260 nm, using cuvettes that have been soaked for 1 hour in concentrated HCl:methanol (1:1) and then washed extensively in water that has been treated with DEPC and autoclaved (see page 7.4).

12. Transfer the solution of poly(A)$^+$ RNA from the cuvettes to a polypropylene tube, add 3 volumes of ethanol, mix well, and store the preparation at −70°C until it is needed.

    To recover the RNA, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Notes

i. A solution whose $OD_{260} = 1$ contains approximately 40 μg of RNA per milliliter.

ii. The yield of poly(A)$^+$ RNA from 10^7 cultured mammalian cells should be 1–5 μg. Usually between 1% and 2% of the total RNA applied to the column is recovered as poly(A)$^+$ RNA.

iii. Columns of oligo(dT)-cellulose can be stored at 4°C and reused many times. Between uses, regenerate the column by sequential washing with NaOH, water, and column-loading buffer as described in steps 1, 2, and 3 above.

iv. The sodium salt of lauryl sarcosine is relatively insoluble and may therefore impede the flow of the column if the room temperature is less than 18°C. This can be avoided by using LiCl instead of NaCl in the column-loading buffer.

v. When many RNA samples are to be processed, it may be more efficient to use batch affinity chromatography on oligo(dT)-cellulose. Begin at step 4 and, after dissolving the RNA in sterile water and adding 2× column-loading buffer, add 0.3 g (dry weight) of oligo(dT)-cellulose for each 0.5 mg of RNA. Shake the suspension gently for 15 minutes at room temperature. Centrifuge the suspension at 1500*g* for 4 minutes at 15°C, discard the supernatant, and wash the oligo(dT)-cellulose four or five times with 5 ml of sterile 1× column-loading buffer at room temperature. Elute the

poly(A)$^+$ RNA with four 1-ml washes of sterile elution buffer, and continue with step 8 above.

Alternatively, paper filters in which poly(U) residues have been embedded (e.g., Amersham Hybond-mAP) may be used (Wreschner and Herzberg 1984). Total cellular RNA is spotted onto the filters, which are then washed in DEPC-treated 0.1 M NaCl and 70% ethanol. Poly(A)$^+$ RNA is eluted by heating the filters to 70°C in water for 5 minutes. These filters bind up to 20 μg of poly(A)$^+$ RNA per square centimeter and are extremely useful when isolating small amounts of poly(A)$^+$ RNA from many samples simultaneously. Instructions for using these filters are provided by the commercial manufacturers.

vi. Some protocols call for the use of poly(U)-Sephadex rather than oligo(dT)-cellulose. Although both resins give excellent results, oligo(dT)-cellulose is preferred for its durability. However, poly(U)-Sephadex has a faster flow rate, which is a convenience when large volumes are to be passed through the column.

FRACTIONATION OF RNA BY SIZE IN THE PRESENCE OF METHYLMERCURIC HYDROXIDE

Two methods are used to isolate mRNA molecules of particular size: electrophoresis through agarose gels and sedimentation through sucrose gradients. Both methods have been used to estimate the sizes of mRNAs that code for particular proteins and to enrich populations of mRNA to be used for cDNA cloning for species of interest. Electrophoresis through agarose gels gives better separation of different-size molecules of RNA, but the recovery of RNA from sucrose gradients is much more efficient. Before fractionation, the RNA is treated with methylmercuric hydroxide, a reagent that reacts primarily with the imino bonds of uridine and guanosine in RNA (Gruenwedel and Davidson 1966). Because these bonds may be involved in Watson-Crick base pairing, methylmercuric hydroxide is an effective denaturing agent that disrupts all secondary structure in RNA.

The electrophoretic mobility of RNA in the presence of methylmercuric hydroxide is an inverse function of the $\log_{10}$ of its molecular weight (Bailey and Davidson 1976). Methylmercuric hydroxide can be displaced from NH groups in purines and pyrimidines and inactivated by a number of sulfhydryl compounds. For example:

$$CH_3HgOH + RSH \rightarrow CH_3HgSR + H_2O.$$

These compounds can therefore be used to reverse the binding of methylmercuric hydroxide to RNA, which can then be translated in a cell-free protein-synthesizing system or used as a template for the synthesis of cDNA. Because methylmercuric hydroxide reacts with free radicals that are used for polymerization of acrylamide, it cannot be incorporated into polyacrylamide gels. It is therefore used only in agarose gels or in sucrose density gradients.

Methylmercuric hydroxide is extremely toxic. It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly. A thoughtful discussion that places the hazards of using alkylmercurials in reasonable perspective and describes simple and sensible precautions may be found in Junghans (1983).

Note: Although fractionation of RNA by electrophoresis in the presence of methylmercuric hydroxide has been used extensively, the hazards associated with the use of methylmercury usually prompt investigators to use an alternative protocol (see pages 7.40 and 7.43).

Electrophoresis of RNA through Agarose Gels Containing Methylmercuric Hydroxide

Caution: Methylmercuric hydroxide is extremely toxic. It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly.

1. Prepare the gel (1% agarose for RNA molecules 1 kb or larger in size; 1.4% agarose for smaller species of RNA). Dissolve the agarose in 1 × methylmercury gel-running buffer, and allow the solution to cool to 55°C before adding methylmercuric hydroxide to a final concentration of 5 mM.

   1 × Methylmercury gel-running buffer

   50 mM boric acid
   5 mM $Na_2B_4O_7 \cdot 10\ H_2O$
   10 mM Na_2SO_4

   If necessary, adjust the pH to 8.1.

   Buffers that contain nitrogen bases, EDTA, or chloride ions should not be used because these compounds form complexes with methylmercuric hydroxide.

   Methylmercuric hydroxide dissociates from nucleic acids when the pH is < 7.0.

2. Mix equal volumes of 2 × gel-loading solution and the solution of RNA (up to 10 μg may be loaded per standard 0.6-cm slot).
   2 × gel-loading solution is prepared as follows:

1 M methylmercuric hydroxide	25 μl
4 × methylmercury gel-running buffer	500 μl
100% glycerol	200 μl
H_2O	275 μl
bromophenol blue	0.2% w/v

3. Load the samples and run the gel at 5–6 V/cm for 12–16 hours.

   Methylmercuric hydroxide is added to the gel but not to the methylmercury gel-running buffer. The compound is uncharged and does not migrate rapidly out of the gel. However, if the gel is submerged in buffer during electrophoresis, the methylmercury ions may diffuse from the gel. To avoid this problem, adjust the level of the buffer so that it maintains full electrical contact with the ends of the gel but does not spill onto the surface of the gel. After the samples have been loaded and the RNA has entered the gel, cover the gel with Saran Wrap to prevent drying during electrophoresis.

4. After electrophoresis, RNA may be stained by incubating the gel for 30–45 minutes in 0.1 M ammonium acetate containing 0.5 μg/ml ethidium bromide. The ammonium ions convert methylmercury to a charged, nonvolatile complex and enhances binding of ethidium bromide to RNA.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain

this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

For a description of alternative methods of staining RNA, see page 7.51.

Note

Although RNA may be transferred from gels containing methylmercuric compounds to nitrocellulose filters or charged nylon membranes, this procedure is not recommended. It is less hazardous and equally effective to transfer RNA that has been fractionated through gels containing glyoxal/DMSO or formaldehyde (see pages 7.40–7.42 or 7.43–7.45, respectively).

RECOVERY OF RNA FROM AGAROSE GELS CONTAINING METHYLMERCURIC HYDROXIDE

This is a modification of the method described by Lemischka et al. (1981).

Caution: Methylmercuric hydroxide is extremely toxic. It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly.

1. Prepare and run the gel as described above, except use low-melting-temperature agarose (Wieslander 1979).

2. After electrophoresis, soak the gel in 0.1 M dithiothreitol for 30–40 minutes.

   Dithiothreitol forms an insoluble complex with methylmercury; other sulfhydryl reagents, such as mercaptoethanol, form soluble complexes that retain volatility.

3. Cut the gel into slices approximately 3 mm in width. Stained tracks containing fragments of DNA of known size, 18S and 28S human rRNAs, or 9S rabbit β-globin mRNA may be used as molecular-weight standards. The sizes of these RNAs are 6333, 2366, and 710 nucleotides, respectively. Alternatively, mixtures of RNAs of known sizes can be purchased from BRL.

   Dithiothreitol absorbs ultraviolet light, making detection of small amounts of RNA difficult. This problem can be avoided by using as markers radioactive RNAs generated by transcription in vitro of cloned DNAs with bacteriophage-encoded DNA-dependent RNA polymerases (see Chapter 10).

4. Transfer each gel slice to a polypropylene tube, and add at least 4 volumes of 0.5 M ammonium acetate preheated to 65°C. Be sure to use a large volume of ammonium acetate so that the gel slice dissolves completely. Otherwise, agarose will be carried over into the aqueous phase during subsequent extraction with phenol and chloroform.

5. Heat the samples at 65°C until the agarose is completely dissolved. Vortex the samples well.

6. Extract the samples with phenol equilibrated with Tris · Cl (pH 7.6) (see Appendix B) at room temperature. Separate the phases by centrifugation at 2000*g* for 10 minutes at 4°C. During extraction with organic solvents, agarose becomes a powder and forms a layer at the interface during centrifugation.

7. Reextract the aqueous phase at least twice more with chloroform. Repeated extractions with chloroform may be required to remove the agarose completely.

8. Transfer the aqueous phase to a fresh tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2). Mix well. Add 3 volumes of ethanol, and store the solution for at least 1 hour at −70°C.

9. Recover the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C. Carefully discard the supernatant, and wash the pellet (which is usually invisible) with 70% ethanol. Recentrifuge briefly, and allow the pellet of RNA to dry in the air.

10. Redissolve the RNA in a small volume (5–10 μl) of water, add 3 volumes of ethanol, mix well, and store the preparation at −70°C until it is needed.

    To recover the RNA, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Note

RNA fractionated in this way can be translated in in vitro protein-synthesizing systems and is an efficient template for cDNA synthesis with reverse transcriptase.

Fractionation of RNA by Centrifugation through Sucrose Gradients Containing Methylmercuric Hydroxide

This is a modification of the procedure described by Schweinfest et al. (1982).

Caution: Methylmercuric hydroxide is extremely toxic. It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly.

1. Prepare one or more sucrose gradients (10–30% w/v) containing 10 mM methylmercuric hydroxide in 9/16″ × 3 1/2″ ultracentrifuge tubes (Beckman SW41 or equivalent). Up to 100 μg of RNA can be loaded on a single gradient.

   The sucrose should be dissolved in sterile water, and the solutions should then be treated overnight with 0.1% diethyl pyrocarbonate (DEPC) at 37°C and heated to 100°C for 15 minutes. After the sucrose solutions have cooled to room temperature, sterile solutions of 1 M Tris · Cl (pH 7.4) and 0.5 M EDTA (pH 7.4) should be added to give final concentrations of 10 mM Tris · Cl (pH 7.4) and 1 mM EDTA (pH 7.4). Finally, methylmercuric hydroxide is added to a final concentration of 10 mM.

   The apparatus used to pour the sucrose gradients and the centrifuge tubes should be treated overnight with 0.1% DEPC (or for 1 hour with 3% H_2O_2) and then rinsed thoroughly with sterile, DEPC-treated water (see page 7.4).

   Caution: DEPC is suspected to be a carcinogen and should be handled with care.

2. Dissolve the RNA (up to 100 μg) in a small volume (<100 μl/100 μg) of sterile, RNAase-free TE (pH 7.4) (see page 7.4). Add methylmercuric hydroxide to a final concentration of 20 mM, and immediately load the RNA solution onto the gradients (<100 μg/gradient).

3. Centrifuge the gradients at 34,000 rpm for 15 hours at 4°C in a Beckman SW41 rotor (or its equivalent).

4. Collect 0.3-ml fractions through a hypodermic needle inserted into the bottom of the centrifuge tube. Dilute each of the fractions with an equal volume of sterile, DEPC-treated water containing 5 mM β-mercaptoethanol. Add 60 μl of DEPC-treated 3 M sodium acetate (pH 5.2) to each fraction. Mix the samples well. Add 1.5 ml of ice-cold ethanol and mix well. Store the samples for at least 30 minutes at 0°C.

5. Centrifuge the samples at 12,000*g* for 15 minutes at 0°C. Remove and discard the supernatants (which should be treated as toxic waste). Add 1 ml of 70% ethanol, vortex briefly, and recentrifuge the samples.

6. Discard the supernatants, and allow the pellets of RNA (which are usually invisible) to dry at room temperature.

7. Redissolve the RNAs in a small volume (5–10 μl) of water, add 3 volumes of ethanol, mix well, and store the preparation at −70°C until it is needed.
   To recover the RNAs, add 3 M sodium acetate (pH 5.2) to a final

concentration of 0.3 M, mix well, and centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.

Note

RNA fractionated in this way can be translated in in vitro protein-synthesizing systems and is an efficient template for cDNA synthesis with reverse transcriptase.

Analysis of RNA

A number of methods have been developed to quantitate, measure the size of, and map the 5′ and 3′ termini of specific mRNA molecules in preparations of cellular RNA. These include:

- *Northern hybridization (RNA blotting),* in which the size and amount of specific mRNA molecules in preparations of total or poly(A)$^+$ RNA are determined (Alwine et al. 1977, 1979). The RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to activated cellulose (Alwine et al. 1977; Seed 1982b), nitrocellulose (Goldberg 1980; Thomas 1980; Seed 1982a), or glass or nylon membranes (Bresser and Gillespie 1983) (see below). The RNA of interest is then located by hybridization with radiolabeled DNA or RNA followed by autoradiography.
- *Dot and slot hybridization,* in which an excess of radiolabeled probe is hybridized to RNA that has been immobilized on a solid support (Kafatos et al. 1979; Thomas 1980; White and Bancroft 1982). Densitometric tracings of the resulting autoradiographs can allow comparative estimates of the amount of the target sequence in various preparations of RNA.
- *Mapping RNA using nuclease S1 or ribonuclease,* in which the precise positions of the 5′ and 3′ termini of the mRNA and the locations of splice junctions can be rigorously determined (Berk and Sharp 1977; Weaver and Weissmann 1979). Labeled or unlabeled RNA or DNA probes derived from various segments of the genomic DNA are hybridized to mRNA, often under conditions favoring the formation of DNA:RNA hybrids (Casey and Davidson 1977). The products of the hybridization are then digested with nuclease S1 or RNAase under conditions favoring digestion of single-stranded nucleic acids only. Analysis of the digestion products by gel electrophoresis yields important quantitative and qualitative information about the mRNA structure.
- *Primer extension,* in which a small radiolabeled fragment of DNA is hybridized to the mRNA and used as a primer for reverse transcriptase. The resulting product should extend to the extreme 5′ terminus of the mRNA, and thus the size of the product reflects the number of nucleotides from the position of the label to the 5′ terminus of the mRNA.
- *Solution hybridization,* in which the absolute concentration of the sequence of interest is calculated from the rate of hybridization of a small amount of a specific radioactive probe with a known quantity of purified cellular RNA (see, e.g., Roop et al. 1978; Durnam and Palmiter 1983). Alternatively, an excess of a radiolabeled probe is incubated with a known amount of RNA. The concentration of the RNA of interest can then be estimated from the amount of radioactivity that becomes resistant to nuclease S1 (see, e.g., Favaloro et al. 1980; Beach and Palmiter 1981; Williams et al. 1986).

- *Filter hybridization,* in which purified cellular RNA is end-labeled with ^{32}P and hybridized to a large excess of the homologous DNA that has been immobilized on a solid support (Williams et al. 1986).

Below we describe northern hybridization. Dot and slot hybridization of both crude and purified preparations of RNA are described beginning on page 7.53; nuclease-S1 and RNAase analysis of specific hybrids, beginning on pages 7.58 and 7.71, respectively; and analysis of mRNA by primer extension, beginning on page 7.79.

NORTHERN HYBRIDIZATION

Initially, northern hybridization was carried out exclusively with RNA immobilized on diazotized cellulose (diazobenzyloxymethyl [DBM]-cellulose) (Alwine et al. 1977) (see Chapter 9, pages 9.35–9.36). Subsequently, *o*-aminophenylthioether (APT)-cellulose was developed, which is easier to prepare and more stable than DBM-cellulose (Seed 1982a,b). However, the use of these activated celluloses for immobilization and hybridization of RNA was largely obviated when it was shown that RNA denatured by glyoxal and dimethyl sulfoxide (DMSO) (McMaster and Carmichael 1977), methylmercuric hydroxide (Thomas 1980, 1983), or formaldehyde (Rave et al. 1979; Goldberg 1980; Seed 1982a) binds tightly to nitrocellulose and hybridizes to radioactive probes with high efficiency (White and Bancroft 1982). The sensitivity with which RNA bound to nitrocellulose can be detected by hybridization is such that species of mRNA comprising no more than 0.001% of the mRNA can be rapidly identified and easily quantitated. Large molecules of denatured RNA (>9 kb) transfer from the gel with high efficiency, so that fragmentation of the RNA is generally unnecessary.

The attachment of denatured RNA to nitrocellulose is presumed to be noncovalent but is essentially irreversible. It is therefore possible to hybridize sequentially RNA immobilized on nitrocellulose to a series of radioactive probes without significant loss of the bound nucleic acid. Unfortunately, nitrocellulose filters are not usually durable enough to withstand more than two rounds of hybridization and washing. This difficulty can be solved by transferring the RNA to positively charged nylon membranes, which stand up well to many rounds of hybridization without loss of hybridization signal. However, the background hybridization to many types of nylon membranes is considerably higher than it is to nitrocellulose filters. We therefore recommend that nylon membranes be used only when it is known that the RNA will be hybridized to many probes sequentially. Each manufacturer provides specific instructions for the transfer of nucleic acids to their particular type of charged nylon membrane. These instructions should be followed exactly, since they presumably have been shown to yield the best results. Practical information about the use of nylon membranes can also be found in Chapter 9, pages 9.42–9.43, and in Reed and Mann (1985).

A wide variety of probes may be used to detect RNA transferred to nitrocellulose filters or nylon membranes, including double-stranded DNA labeled by nick translation, single-stranded DNA prepared by primer extension of an oligonucleotide annealed to a recombinant M13 bacteriophage, radiolabeled synthetic oligonucleotides, and RNA synthesized in vitro with prokaryotic DNA-dependent RNA polymerases (e.g., bacteriophage SP6, T7, or T3 RNA polymerase). Methods to synthesize and use these probes are discussed in Chapters 10 and 11.

Below we describe methods for electrophoresis of denatured RNA through agarose gels, for the transfer of the fractionated RNA to nitrocellulose filters and nylon membranes, and for hybridization of the immobilized RNA to radiolabeled probes. The protocol presented for northern hybridization also works well with most types of positively charged nylon membranes but may not be optimal for any particular brand.

Electrophoresis of RNA after Denaturation with Glyoxal and Dimethyl Sulfoxide

This method is adapted from that of McMaster and Carmichael (1977). Gels containing glyoxal/dimethyl sulfoxide (DMSO) are more difficult to run than those containing formaldehyde (see page 7.43): They must be run more slowly and also require that the electrophoresis buffer be circulated in order to avoid creating unacceptable H^+ gradients during electrophoresis. Although the resolving powers of the two gel systems are approximately equal (Miller 1987), the bands of RNA detected by northern hybridization are usually sharper when the RNA has been fractionated through gels containing glyoxal/DMSO.

1. In sterile microfuge tubes, mix:

6 M glyoxal	5.4 μl
DMSO	16.0 μl
0.1 M sodium phosphate (pH 7.0)	3.0 μl
RNA (up to 10 μg)	5.4 μl

Glyoxal is usually obtained as a 40% solution (6 M). Because it readily oxidizes in air, the glyoxal solution must be deionized before use by passage through a mixed-bed resin (Bio-Rad AG 501-X8) until its pH is greater than 5.0. It is then stored at −20°C in small aliquots in tightly capped tubes. Each aliquot should be used only once and then discarded. 0.1 M sodium phosphate (pH 7.0) is prepared by mixing 3.9 ml of 1 M NaH_2PO_4 with 6.1 ml of 1 M Na_2HPO_4 and 90 ml of H_2O. The sodium phosphate solution should be treated with diethyl pyrocarbonate (DEPC) and then sterilized by autoclaving (see page 7.4).

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

Up to 10 μg of RNA may be analyzed in each lane of the gel. Abundant mRNAs (0.1% or more of the mRNA population) can usually be detected by northern analysis of 10–20 μg of total cellular RNA. For detection of rare RNAs, between 0.5 and 3.0 μg of poly(A)$^+$ RNA should be applied to each lane of the gel.

2. Close the tops of the microfuge tubes, and incubate the RNA solutions for 60 minutes at 50°C. Chill the samples in ice water, and then centrifuge them for 5 seconds to deposit all of the fluid in the bottoms of the microfuge tubes.

3. While incubating the RNAs at 50°C, pour a horizontal agarose gel. For RNAs up to 1 kb in length, use 1.4% agarose; for larger RNAs, use 1% agarose. The gels are poured and run in 10 mM sodium phosphate (pH 7.0). After dissolving the agarose in the sodium phosphate, cool the solution to 70°C and add solid sodium iodoacetate to a final concentration of 10 mM (to inactivate RNAases). Cool the solution to 50°C, and then pour the gel and allow it to set for at least 30 minutes before loading the RNA.

Electrophoresis tanks used for electrophoresis of RNA should be cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H_2O_2. After 10 minutes at room temperature, the tank should be rinsed thoroughly with water that has been treated with DEPC (see page 7.4).

Because glyoxal reacts with ethidium bromide, the gels are poured and run in the absence of the dye.

4. Cool the RNA samples to 0°C, add 4 μl of sterile, DEPC-treated glyoxal/DMSO gel-loading buffer, and immediately load the samples into the wells of the gel. As molecular-weight markers, use glyoxylated RNAs of known size, for example, 18S and 28S rRNAs or 9S rabbit β-globin mRNA. The sizes of these RNAs are 6333, 2366, and 710 nucleotides, respectively. Alternatively, mixtures of RNAs of known size can be purchased from BRL. The markers are usually loaded into the outside lanes of the gel so that they can be cut from the gel after electrophoresis and stained with ethidium bromide. If possible, leave an empty lane between the markers and the samples that are to be transferred to a nitrocellulose filter or nylon membrane.

Glyoxal/DMSO gel-loading buffer

50% glycerol
10 mM sodium phosphate (pH 7.0)
0.25% bromophenol blue
0.25% xylene cyanol FF

5. Run the gel submerged in 10 mM sodium phosphate at 3–4 V/cm. Constant recirculation of the sodium phosphate (Figure 7.1) is required to maintain the pH within acceptable limits (glyoxal dissociates from RNA at pH >8.0). Alternatively, the sodium phosphate may be changed every 30 minutes during the run.

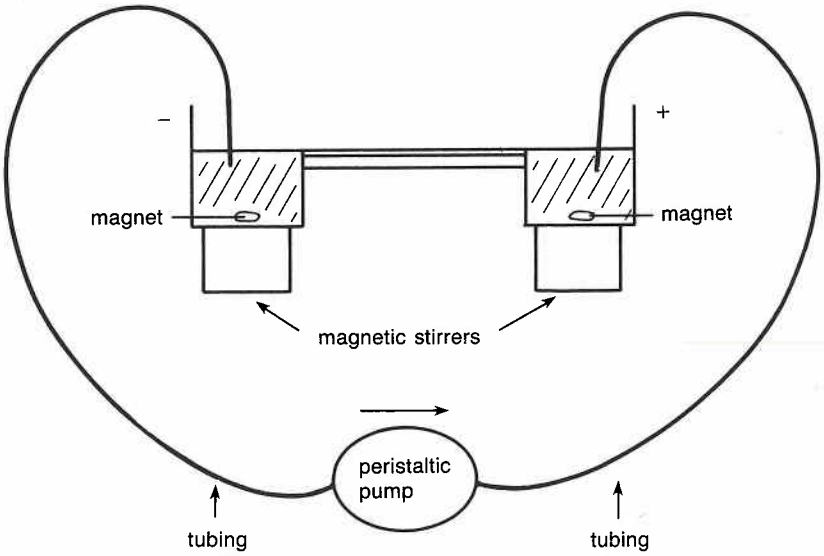


FIGURE 7.1
Recirculation of buffer during electrophoresis of RNA. Either of two methods are used to maintain the pH of the electrophoresis buffer within acceptable limits: (1) The electrophoresis apparatus is placed on a pair of magnetic stirrers so that the buffer in each chamber can be mixed by means of Teflon-covered magnets. (2) A peristaltic pump is used to circulate buffer slowly from one chamber of the apparatus to the other.

6. At the end of the run (when the bromophenol blue has migrated approximately 8 cm), the lanes containing the markers may be cut from the gel and stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30–45 minutes. Align a transparent ruler with the gel, and photograph the gel and ruler by ultraviolet illumination. Use the photograph to measure the distance from the loading well to each of the bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated. Use the resulting curve to calculate the sizes of the RNA species detected by hybridization after transfer from the gel to a solid support.

   Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

   Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

For a description of alternative methods of staining RNA, see page 7.51.

7. Transfer the RNA from the gel to a nitrocellulose filter as described on pages 7.46–7.48 or to a nylon membrane as described on pages 7.49–7.50.

Electrophoresis of RNA through Gels Containing Formaldehyde

This method is adapted from those of Lehrach et al. (1977), Goldberg (1980), and Seed (1982a).

Caution: Formaldehyde vapors are toxic. Solutions containing formaldehyde should be prepared in a chemical hood, and electrophoresis tanks containing formaldehyde solutions should be kept covered whenever possible.

1. Prepare 5 × formaldehyde gel-running buffer.

5 × Formaldehyde gel-running buffer

0.1 M MOPS (pH 7.0)
40 mM sodium acetate
5 mM EDTA (pH 8.0)

Dissolve 20.6 g of 3-(*N*-morpholino)propanesulfonic acid (MOPS) in 800 ml of diethyl pyrocarbonate (DEPC)-treated 50 mM sodium acetate (see page 7.4 for DEPC treatment of solutions). Adjust the pH to 7.0 with 2 N NaOH. Add 10 ml of DEPC-treated 0.5 M EDTA (pH 8.0). Adjust the volume of the solution to 1 liter with DEPC-treated water. Sterilize the solution by filtration through a 0.2-micron Millipore filter, and store it at room temperature protected from light. The buffer yellows with age if it is exposed to light or is autoclaved. Straw-colored buffer works well, but darker buffer does not.

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

2. Prepare the gel by melting the appropriate amount of agarose in water, cooling it to 60°C, and adding 5 × formaldehyde gel-running buffer and formaldehyde to give final concentrations of 1 × and 2.2 M, respectively. (One part of a stock 12.3 M formaldehyde solution should be diluted with 3.5 parts of agarose in water and 1.1 parts of 5 × formaldehyde gel-running buffer.) Cast the gel in a chemical hood, and allow the gel to set for at least 30 minutes at room temperature.

The fractionation properties of gels containing formaldehyde and different concentrations of agarose have been measured by Lehrach et al. (1977) and Miller (1987).

Formaldehyde (M_r = 30.03) is usually obtained as a 37% solution (12.3 M) in water. Check that the pH of the concentrated solution is greater than 4.0.

3. Prepare the samples by mixing the following in a sterile microfuge tube:

RNA (up to 30 μg)	4.5 μl
5 × formaldehyde gel-running buffer	2.0 μl
formaldehyde	3.5 μl
formamide	10.0 μl

Incubate the samples for 15 minutes at 65°C, and then chill them on ice. Centrifuge the samples for 5 seconds to deposit all of the fluid in the bottom of the microfuge tubes.

Up to 30 μg of RNA may be analyzed in each lane of the gel. Abundant mRNAs (0.1% or more of the mRNA population) can usually be detected by northern analysis of 10–20 μg of total cellular RNA. For detection of rare RNAs, between 0.5 and 3.0 μg of poly(A)$^+$ RNA should be applied to each lane of the gel.

Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

Some workers add a small amount of ethidium bromide (1 μl of a 1 mg/ml solution in DEPC-treated water) to the samples before electrophoresis (Fourney et al. 1988). Although this solves the problem of staining RNA in the gel after electrophoresis, it can reduce the efficiency of northern hybridization, especially when the samples contain small amounts of poly(A)$^+$ RNA.

4. Add 2 μl of sterile, DEPC-treated formaldehyde gel-loading buffer.

Formaldehyde gel-loading buffer

50% glycerol
1 mM EDTA (pH 8.0)
0.25% bromophenol blue
0.25% xylene cyanol FF

5. Before loading the samples, prerun the gel for 5 minutes at 5 V/cm. Immediately load the samples into the lanes of the gel. As molecular-weight markers, use RNAs of known size, for example, 18S and 28S rRNAs or 9S rabbit β-globin mRNA. The sizes of these RNAs are 6333, 2366, and 710 nucleotides, respectively. Alternatively, mixtures of RNAs of known size can be purchased from BRL. The markers are usually loaded into the outside lanes of the gel so that they can be cut from the gel after electrophoresis and stained with ethidium bromide. If possible, leave an empty lane between the markers and the samples that are to be transferred to a nitrocellulose filter or nylon membrane.

DNA and RNA migrate at different rates through agarose gels containing formaldehyde: RNA migrates faster than DNA of equivalent size (Wicks 1986). Although DNA markers are preferable because they run as sharp bands, they cannot readily be used to measure the absolute size of unknown RNAs.

6. Run the gel submerged in 1 × formaldehyde gel-running buffer at 3–4 V/cm. Constant recirculation of the buffer is not necessary, but after 1–2 hours the buffer from each reservoir should be collected, mixed, and returned to the gel apparatus.

7. At the end of the run (when the bromophenol blue has migrated approximately 8 cm), the lanes containing the markers may be cut from the gel

and stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30–45 minutes. Align a transparent ruler with the gel, and photograph the gel and ruler by ultraviolet illumination. Use the photograph to measure the distance from the loading well to each of the bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated. Use the resulting curve to calculate the sizes of the RNA species detected by hybridization after transfer from the gel to a solid support.

Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

Ultraviolet radiation is dangerous, especially to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

For a description of alternative methods of staining RNA, see page 7.51.

Gels containing formaldehyde are less rigid than nondenaturing agarose gels, and care must be exercised in handling them.

8. Transfer the RNA from the gel to a nitrocellulose filter as described on the following pages or to a nylon membrane as described on pages 7.49–7.50.

Transfer of Denatured RNA to Nitrocellulose Filters

Glyoxylated RNA may be transferred immediately after electrophoresis from agarose gels to nitrocellulose filters by capillary elution, vacuum transfer, or electroblotting (see Chapter 9, pages 9.34–9.37, for a discussion of the relative merits of these techniques). Capillary elution is carried out as described below; vacuum transfer and electroblotting should be performed according to the instructions of the manufacturer of the apparatus that is used.

Although further treatment of agarose gels before transfer is unnecessary (Thomas 1980) and may be detrimental (Thomas 1983), gels containing formaldehyde must be rinsed in several changes of diethyl pyrocarbonate (DEPC)-treated water (see page 7.4) to remove the formaldehyde. However, if the gel contains more than 1% agarose or is more than 0.5 cm thick or if the RNA to be analyzed is greater than 2.5 kb in length, soak the gel for 20 minutes in 0.05 N NaOH. This treatment partially hydrolyzes the RNA and improves the efficiency of transfer. Then rinse the gel in RNAase-free water and soak it for 45 minutes in 20 × SSC. The gel is then placed in contact with the nitrocellulose filter or nylon membrane and the RNA is transferred to the solid support (nitrocellulose or charged nylon) in an ascending flow of buffer (Figure 7.2).

Cautions: DEPC is suspected to be a carcinogen and should be handled with care.

Formaldehyde is toxic and should be handled with care in a chemical hood.

Note: Gels containing formaldehyde are less rigid than nondenaturing agarose gels, and care must be exercised in handling them.

1. Transfer the gel to a glass baking dish, and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.

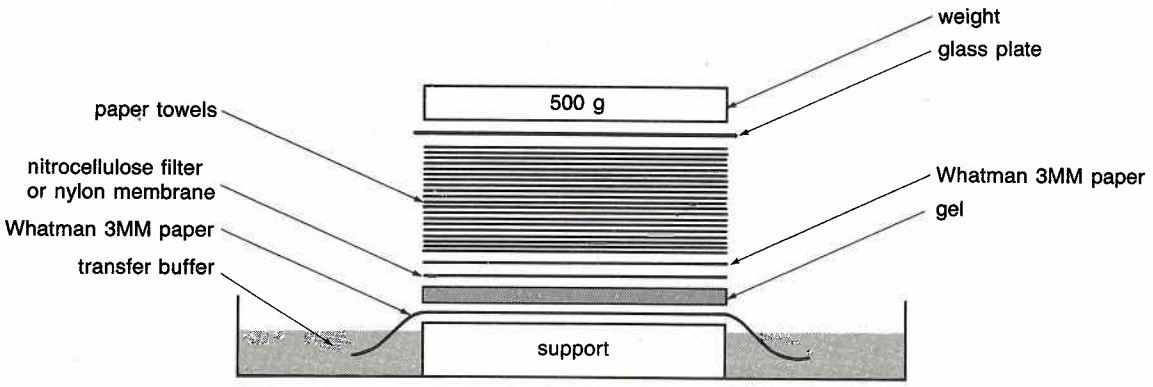


FIGURE 7.2
Capillary transfer of nucleic acids from agarose gels to solid supports. Buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The nucleic acid is eluted from the gel by the moving stream of buffer and is deposited on a nitrocellulose filter or nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

2. Place a piece of Whatman 3MM paper on a piece of Plexiglas or a stack of glass plates to form a support that is longer and wider than the gel. Place the support inside a large baking dish. Fill the dish with 20 × SSC until the level of the liquid reaches almost to the top of the support. When the 3MM paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod.

3. Using a fresh scalpel or a paper cutter, cut a piece of nitrocellulose filter (Schleicher and Schuell BA85 or equivalent) about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the filter. A nitrocellulose filter that has been touched by greasy hands will not wet!

4. Float the nitrocellulose filter on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the filter in 20 × SSC for at least 5 minutes. Using a clean scalpel blade, cut a corner from the nitrocellulose filter to match the corner cut from the gel.

   The rate at which different batches of nitrocellulose filters wet varies enormously. If the filter is not saturated after floating for several minutes on water, it should be replaced with a new filter, since the transfer of RNA to an unevenly wetted filter is unreliable. The original filter should not be discarded but should be autoclaved for 5 minutes between pieces of 3MM paper saturated with 2 × SSC. This usually results in complete wetting of the filter. The autoclaved filter, sandwiched between the autoclaved 3MM papers saturated with 2 × SSC, may be stored at 4°C in a sealed plastic bag until it is needed.

5. Place the gel on the support in an inverted position so that it is centered on the wet 3MM paper. Make sure that there are no air bubbles between the 3MM paper and the gel.

6. Surround, but do not cover, the gel with Saran Wrap or Parafilm. This serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on the top of the gel. If these towels are not precisely stacked, they tend to droop over the edge of the gel and may touch the support. This type of short-circuiting is a major reason for inefficient transfer of RNA from the gel to the filter.

7. Place the wet nitrocellulose filter on top of the gel so that the cut corners are aligned. One edge of the filter should just extend over the edge of the line of slots at the top of the gel. Do not move the filter once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the filter and the gel.

8. Wet two pieces of 3MM paper (cut to exactly the same size as the gel) in 2 × SSC and place them on top of the wet nitrocellulose filter. Smooth out any air bubbles with a glass rod.

9. Cut a stack of paper towels (5–8 cm high) just smaller than the 3MM papers. Place the towels on the 3MM papers. Put a glass plate on top of the stack and weigh it down with a 500-g weight (see Figure 7.2). The

objective is to set up a flow of liquid from the reservoir through the gel and the nitrocellulose filter, so that RNA molecules are eluted from the gel and are deposited on the nitrocellulose filter.

10. Allow transfer of RNA to proceed for 6–18 hours. As the paper towels become wet, they should be replaced.

11. Remove the paper towels and the 3MM papers above the gel. Turn over the gel and the nitrocellulose filter and lay them, gel side up, on a dry sheet of 3MM paper. Mark the positions of the gel slots on the filter with a very-soft-lead pencil or a ballpoint pen.

12. Peel the gel from the filter and discard it. Soak the filter in 6 × SSC for 5 minutes at room temperature. This removes any pieces of agarose sticking to the filter.

Remove the filter from the 6 × SSC and allow excess fluid to drain away. Place the filter flat on a paper towel to dry for at least 30 minutes at room temperature.

To assess the efficiency of transfer of RNA, the gel may be stained for 45 minutes in a solution of ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) and examined by ultraviolet illumination.

Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

13. Place the dried filter between two pieces of 3MM paper, and bake the filter for 30 minutes to 2 hours at 80°C in a vacuum oven.

The filter will become extremely brittle and may become yellow if baked too long.

If the filter is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

14. *For filters containing glyoxylated RNA only:* Before hybridization, remove glyoxal from the RNA by washing the filter with 20 mM Tris · Cl (pH 8.0) at 65°C.

Transfer of Denatured RNA to Nylon Membranes

The methods used to transfer RNA from gels to nylon membranes are similar to those used for transfer to nitrocellulose filters and include vacuum transfer, electroblotting, or capillary elution (see Chapter 9, pages 9.34–9.37, for a discussion of the relative merits of these techniques). Vacuum transfer and electroblotting should be carried out according to the instructions of the manufacturer of the apparatus that is used. Capillary elution is carried out essentially as described on pages 7.46–7.48. Gels containing formaldehyde must be rinsed in several changes of DEPC-treated water to remove the formaldehyde before transfer. Because charged nylon membranes retain nucleic acids in alkaline solution (Reed and Mann 1985), glyoxylated RNA can be eluted from agarose gels in 7.5 mN NaOH (Vrati et al. 1987). This partially hydrolyzes the RNA and thereby increases the speed and efficiency of transfer of large (>2.3 kb) RNAs. In addition, alkali removes glyoxal adducts from the mRNA (Thomas 1983), eliminating the need for postfixation stripping (step 14, page 7.48). Finally, because RNA transferred in alkaline transfer buffers becomes irreversibly fixed to the charged nylon membrane, there is no need to bake the membrane or to expose it to ultraviolet irradiation before hybridization.

The sole disadvantage of nylon membranes is a tendency to give increased levels of background hybridization, especially with RNA probes. The level of background hybridization is almost always significantly higher when the membrane has been exposed to high concentrations of alkali for extended periods of time. In many cases, this problem can be overcome by using increased amounts of blocking agents in the prehybridization and hybridization steps.

Alkaline transfer of glyoxylated RNA to charged nylon membranes is carried out essentially as described on pages 7.46–7.48, except that the transfer buffer is 7.5 mN NaOH. After transfer is completed (4.5–6.0 hours), the membranes should be rinsed briefly in 2 × SSC, 0.1% SDS and allowed to dry at room temperature.

When transfer is carried out with a neutral transfer buffer, nylon membranes must be treated to immobilize the nucleic acid after transfer is complete. RNA becomes fixed to the nylon membrane if it is thoroughly dried or if it is exposed to low doses of ultraviolet irradiation. To fix the RNA to the membrane either place the dried membrane between two pieces of 3MM paper and bake the membrane for 30 minutes to 2 hours at 80°C in a vacuum or conventional oven, or expose the side of the membrane carrying the RNA to a source of ultraviolet irradiation (254 nm). The latter method, although a nuisance to set up, is preferred because it greatly enhances the hybridization signal obtained with some brands of positively charged nylon membranes (Khandjian 1987). However, for maximum effect, it is important to make sure that the membrane is not overirradiated. The aim is to form cross-links between a small fraction of the bases in the RNA and the positively charged amine groups on the surface of the membrane (Church and Gilbert 1984). Overirradiation results in the covalent attachment of a higher proportion of thymines, with a consequent decrease in hybridization signal. Most manufacturers advise that damp nylon membranes should be exposed to a total of 1.5 J/sq. cm. and that dry membranes should be exposed to 0.15 J/sq. cm.

However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal. In addition, the system should be recalibrated routinely.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

If the membrane is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

Staining RNA Before and After Transfer to Nitrocellulose Filters

Prolonged staining with ethidium bromide is not recommended before RNA is transferred from agarose gels to nitrocellulose filters because saturation of the nucleic acid with the dye appears to reduce the efficiency of transfer. However, brief staining with ethidium bromide does not detectably inhibit transfer and has the advantage of allowing RNA to be detected both in the gel and on the filter.

METHOD 1

1. After electrophoresis is completed, gels containing glyoxal/DMSO should be immersed in 10 mM sodium phosphate (pH 7.0) containing ethidium bromide (0.5 μg/ml). Gels containing formaldehyde should be washed in RNAase-free water and immersed in 20× SSC before staining in 20× SSC containing ethidium bromide (0.5 μg/ml).

2. After staining for 5–10 minutes at room temperature, examine and photograph the gel in ultraviolet light as described in Chapter 6, page 6.19.

3. Transfer the RNA from the gel to a nitrocellulose filter as described on pages 7.46–7.48. After transfer, the stained RNA is usually visible when the filter is examined by ordinary illumination.

Note

This method works only when each lane of the gel contains considerable quantities of RNA (e.g., 5 μg or more of mRNA).

METHOD 2

RNA may also be stained on the nitrocellulose filter as follows: A lane may be cut from the filter after it has been baked or the entire filter may be stained after hybridization and exposure to X-ray film (A. Efstratiadis, unpubl.).

1. Soak the dried filter in 5% acetic acid for 15 minutes at room temperature.

2. Transfer the filter to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 5–10 minutes at room temperature.

3. Rinse the filter in water for 5–10 minutes. RNAs used as molecular-weight standards should appear as sharp bands. Total poly(A)$^+$ mRNA appears as a smear composed of many individual species whose sizes range between <500 bases and >5 kb. The median size of mRNA is approximately 2 kb.

Note

Staining with ethidium bromide is not recommended when RNA is to be transferred from gels to nylon membranes. RNA may be stained with methylene blue after transfer from agarose/formaldehyde gels to certain types of nylon membrane. For details of this method, see Herrin and Schmidt (1988).

Hybridization and Autoradiography

The conditions for prehybridization, hybridization, and washing of RNA immobilized on filters are essentially the same as those used for DNA. These are described in detail in Chapter 9, pages 9.47–9.55. In brief:

1. Prehybridize the filter for 1–2 hours in

   either (at 42°C)
   50% formamide
   5 × SSPE
   2 × Denhardt's reagent
   0.1% SDS

   or (at 68°C)
   6 × SSC
   2 × Denhardt's reagent
   0.1% SDS

2. Add the denatured radiolabeled probe directly to the prehybridization fluid. To detect low-abundance mRNAs, use at least 0.1 μg of probe whose specific activity exceeds 2×10^8 cpm/μg (see Chapter 10). Continue incubation for 16–24 hours at the appropriate temperature.

   It is important to remember that RNA is complementary to only one of the two DNA strands. Therefore, if a single-stranded probe is utilized, it must be complementary to the RNA strand.

3. Wash the filter for 20 minutes at room temperature in 1 × SSC, 0.1% SDS, followed by three washes of 20 minutes each at 68°C in 0.2 × SSC, 0.1% SDS.

4. Establish an autoradiograph by exposing the filter for 24–48 hours to X-ray film (Kodak XAR-2 or equivalent) at −70°C with an intensifying screen (see Appendix E).

DOT AND SLOT HYBRIDIZATION OF RNA

Dot hybridizations were originally performed by spotting a small sample of the RNA preparation onto dry nitrocellulose, which was then dried, hybridized with a specific ^{32}P-labeled DNA or RNA probe, and exposed to X-ray film (Kafatos et al. 1979). Although accurate quantitation was not feasible because of the large and variable size of the spots, it was nevertheless possible in many cases to obtain a good idea of the intensity of specific gene expression in specific tissues or cultured cells. Recently, this technique has been improved in two ways:

1. Filtration manifolds have been designed to accept a large number of samples and to deposit the nucleic acids onto the nitrocellulose in a fixed pattern that allows the results to be quantitated by scanning densitometry (see, e.g., Brown et al. 1983; Chapman et al. 1983). The filtration manifold consists of a Lucite block containing a number of slots into which the samples are applied. The manifold fits onto a suction platform containing a sheet of nitrocellulose onto which the samples are deposited. These manifolds are available commercially (e.g., Minifold II, Schleicher and Schuell).
2. A method involving slot blots has been developed (White and Bancroft 1982) to measure the concentration of a specific RNA in unfractionated cytoplasm prepared from freshly harvested or frozen cultured cells or animal tissues. This improvement eliminates the tedious task of purifying RNA from large numbers of samples.

Slot Hybridization of RNA

1. Wet a piece of nitrocellulose (0.45-micron pore size) briefly in water and soak it in 20 × SSC for 1 hour at room temperature. Meanwhile, clean the manifold carefully with 0.1 N NaOH and then rinse it well with sterile water.

2. Place two sheets of heavy, absorbent paper, previously wetted with 20 × SSC, on the top of the vacuum unit of the apparatus. Place the wet nitrocellulose on the bottom of the sample wells cut into the upper section of the manifold. Smooth away any air bubbles trapped between the upper section of the manifold and the nitrocellulose. Clamp the two parts of the manifold together, and connect the vacuum unit to a vacuum line.

3. Fill all of the slots with 10 × SSC, and apply gentle suction until all of the fluid has passed through the nitrocellulose filter. Turn off the vacuum and refill the slots with 10 × SSC.

4. Mix the RNA (dissolved in 10 μl of H_2O) with:

100% formamide	20 μl
formaldehyde (37%)	7 μl
20 × SSC	2 μl

   Incubate the mixture for 15 minutes at 68°C, and then cool the samples on ice.

   Caution: Formaldehyde is toxic and should be handled with care in a chemical hood.

   Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

   Formaldehyde (M_r = 30.03) is usually obtained as a 37% solution (12.3 M) in water. Check that the pH of the concentrated solution is greater than 4.0.

   The volume of the samples containing the denatured RNA is not critical. However, the final solution should consist of:
   50% formamide
   7% formaldehyde
   1 × SSC

   Up to 20 μg of RNA may be applied to each slot of the manifold (John et al. 1984).

   The RNA may be denatured with methylmercuric hydroxide (see page 7.30) or glyoxal (see page 7.40) instead of formaldehyde.

5. Add 2 volumes of 20 × SSC to each of the samples.

6. Apply gentle suction to the manifold until the 10 × SSC in the slots has passed through the nitrocellulose filter. Turn off the suction.

7. Load the samples into the slots, and then apply gentle suction. After all of the samples have passed through the filter, rinse each of the slots twice with 1 ml of 10× SSC.

8. After the second rinse has passed through the filter, continue suction for 5 minutes to dry the nitrocellulose filter.

9. Remove the nitrocellulose filter from the manifold, and allow it to dry completely at room temperature. Bake the filter for 2 hours at 80°C in a vacuum oven.

10. Hybridize the filter to a radiolabeled probe as described on page 7.52. By using single-stranded probes (either DNA or RNA) radiolabeled to a specific activity of $> 5 \times 10^8$ cpm/μg, it is possible to detect mRNAs that are present at approximately 5 copies per cell if 20 μg of total cellular RNA is applied to a single slot.

    To calibrate the system, add known amounts of unlabeled RNA synthesized in vitro by bacteriophage SP6 or T7 RNA polymerase from a cloned copy of the DNA probe.

Slot Hybridization of Cytoplasmic RNA

This procedure is a slight modification of that described by White and Bancroft (1982). The method relies on the use of small amounts of Nonidet P-40 (NP-40) to break open the cytoplasmic membrane but not the nuclear membrane.

1. Collect 10^6–10^7 cells by centrifugation at 1000*g* for 5 minutes. Resuspend the pellet in 1 ml of phosphate-buffered saline (PBS) lacking calcium and magnesium ions (see Appendix B). Transfer the cell suspension to a sterile microfuge tube, and centrifuge it at 12,000*g* for 15 seconds at 4°C in a microfuge.

2. Discard the supernatant, and resuspend the cells by brief vortexing in 45 μl of ice-cold TE (pH 7.2). Add 5 μl of a 5% solution of NP-40. Vortex briefly. Store the lysate for 5 minutes at 0°C.

   Vanadyl-ribonucleoside complexes (see pages 7.4–7.5) may be added at this stage to a final concentration of 0.1 M. This is usually necessary only when cells rich in RNAases (e.g., macrophages or pancreatic cells) are processed. Vanadyl-ribonucleoside complexes are preferred to protein inhibitors of RNAase because they do not clog the nitrocellulose filter.

3. Add another 5 μl of 5% NP-40. Vortex the cell lysate briefly.

   The amount of NP-40 required to achieve dissolution of the cytoplasmic membranes of the cell without lysis of the nuclei varies from cell line to cell line. When carrying out this procedure for the first time with a new cell line, monitor the appearance of the cells during steps 1–3 by phase-contrast microscopy. If necessary, adjust the concentration of NP-40 to obtain the maximum yield of free nuclei and the minimum number of unlysed cells.

4. Remove the nuclei by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge.

5. Transfer the supernatant to a fresh tube, taking care not to disturb the soft pellet.

6. Add 30 μl of 20× SSC to the supernatant. Vortex the solution briefly. Add 20 μl of 37% formaldehyde, vortex again, and close the top of the tube.

   Caution: Formaldehyde is toxic and should be handled with care in a chemical hood.

   Formaldehyde (M_r = 30.03) is usually obtained as a 37% solution (12.3 M) in water. Check that the pH of the concentrated solution is greater than 4.0.

7. Incubate the tube for 15 minutes at 60°C.

   The samples may be stored at −70°C at this stage, if necessary.

8. Dilute 5–10 μl of the sample with 200 μl of 20× SSC, and load it onto a nitrocellulose filter in a manifold as described on page 7.55 beginning at step 7. (For preparation of the manifold, see page 7.54, steps 1–3 and 6.)

If the filter clogs during loading, remove the insoluble material by centrifugation of the sample at 12,000*g* for 1 minute (after step 7). Be aware that this procedure can result in loss of RNA from the sample.

9. Hybridize the filter to a radiolabeled probe as described on page 7.52. By using single-stranded probes (either DNA or RNA) radiolabeled to a specific activity of $>5 \times 10^8$ cpm/μg, it is possible to detect mRNAs that are present at approximately 5 copies per cell if 20 μg of total cellular RNA is applied to a single slot.

To calibrate the system, add known amounts of unlabeled RNA synthesized in vitro by bacteriophage SP6 or T7 RNA polymerase from a cloned copy of the DNA probe.

MAPPING RNA WITH NUCLEASE S1

Nuclease S1 is used

- To map the location of 5′ and 3′ termini of mRNA on DNA templates
- To locate the 5′ and 3′ splice junctions in relation to sites of cleavage with restriction enzymes in cloned genes or double-stranded cDNA
- To quantitate the amount of specific classes of mRNA in RNAs extracted from tissues or cultured cells

The procedure described originally (Berk and Sharp 1977) was based on the observation of Casey and Davidson (1977) that hybridization conditions can often be established to minimize the formation of DNA:DNA hybrids while promoting the formation of DNA:RNA hybrids. When double-stranded DNA is denatured and incubated with mRNA under these conditions, those segments of DNA (exons) that are transcribed into mRNA form hybrids and the remainder of the DNA remains single-stranded. DNA that has not formed duplexes is hydrolyzed with nuclease S1, whereas DNA that has hybridized to RNA is protected from digestion. The size of the protected fragment of DNA is then determined by electrophoresis under native or denaturing conditions through agarose or acrylamide gels. By using DNA fragments derived from different segments of the gene of interest, it is possible to map the termini of the mRNA and locate the positions of introns within the gene (Berk and Sharp 1977; Favaloro et al. 1980) (Figure 7.3). In this type of experiment, the double-stranded DNA need not be radiolabeled: The fragments of DNA that survive digestion with nuclease S1 can be detected efficiently by Southern hybridization using uniformly radiolabeled probes. Alternatively, double-stranded fragments of DNA that are radiolabeled at only one end by phosphorylation or by filling of recessed 3′ termini (see Chapter 10) may be used directly in the annealing reaction. This kind of asymmetric labeling allows the polarity of the mRNA with respect to the double-stranded DNA to be established directly (Weaver and Weissmann 1979) (Figure 7.4).

Although it is simple to isolate double-stranded fragments of DNA that can be used as probes, it is not always easy or possible to establish conditions that suppress formation of DNA:DNA hybrids and yet allow annealing of DNA to RNA to proceed efficiently. This problem can be avoided if single-stranded probes (either DNA or RNA) are used in the hybridization mixture. Annealing can then be carried out under standard conditions because a complementary strand is not present to compete with the hybridization of the mRNA to the probe. Until recently, such single-stranded probes could be obtained only by physical separation of the complementary strands of a fragment of DNA (usually by gel electrophoresis [Hayward 1972]). This method was laborious, did not work with every fragment of DNA, and hardly ever produced probes that were completely strand-specific. However, during the last few years, new methods have become available to prepare single-stranded probes of defined length and polarity from virtually any restriction fragment. These probes are now the reagents of choice for the analysis of RNA. Techniques to generate these probes are described in Chapter 10.

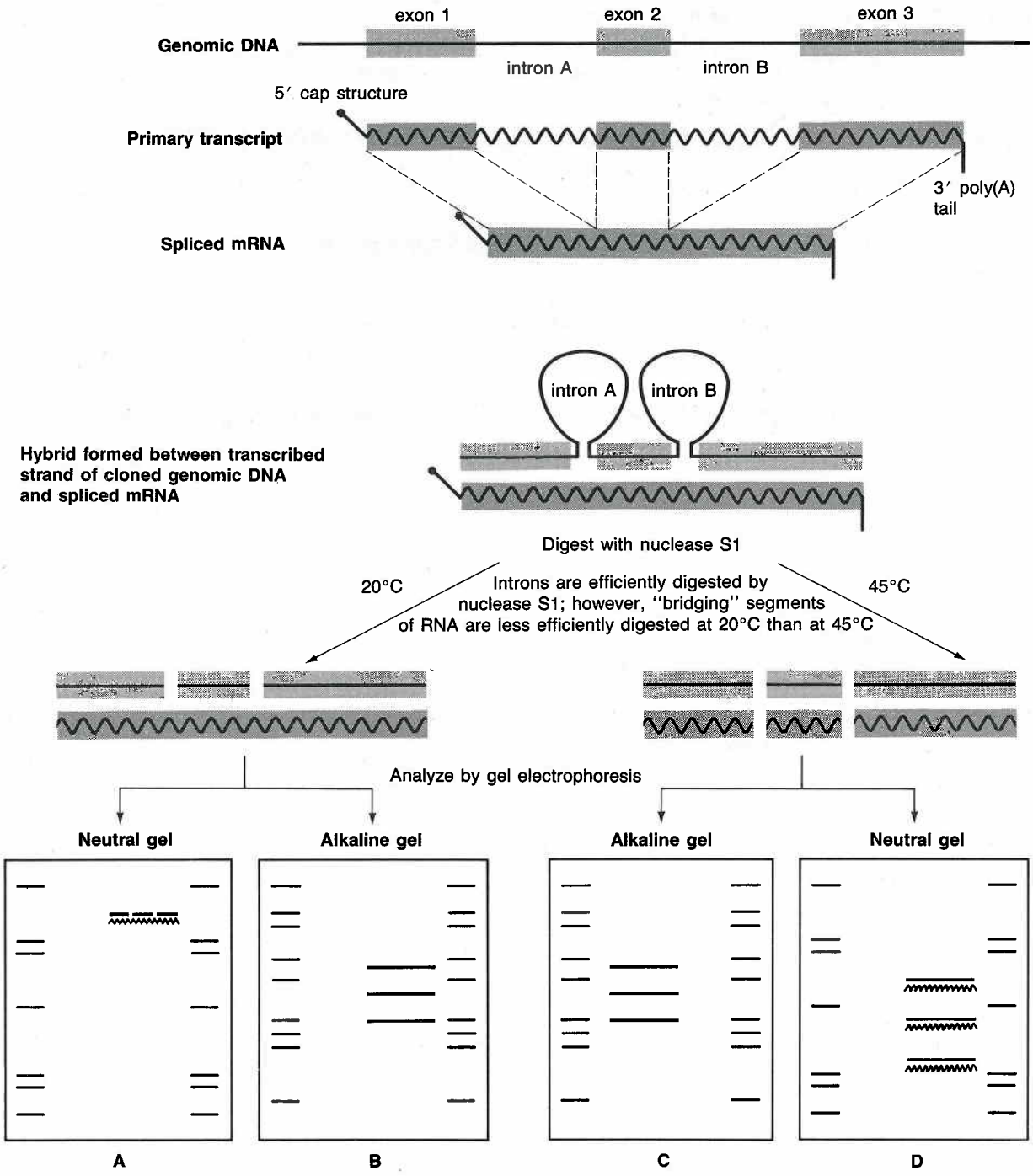


FIGURE 7.3
Nuclease-S1 analysis of RNA. Hybrids formed between the transcribed strand of genomic DNA and mRNA contain single-stranded loops of DNA (introns). Digestion of these hybrids with nuclease S1 at 20°C generates molecules whose RNA moieties are intact but whose DNAs contain gaps at the sites of the introns. These molecules migrate as a single band when analyzed by gel electrophoresis under nondenaturing conditions (gel A). In alkaline gels (gel B), however, the RNA is hydrolyzed and the individual fragments of DNA separate according to their sizes. When digestion is carried out at 45°C, both the DNA and RNA strands of the parental hybrid are cleaved to yield a series of smaller DNA:RNA hybrids that can be separated by electrophoresis under nondenaturing conditions (gel D). The DNA moieties in these hybrids (gel C) are the same size as those detected in gel B.

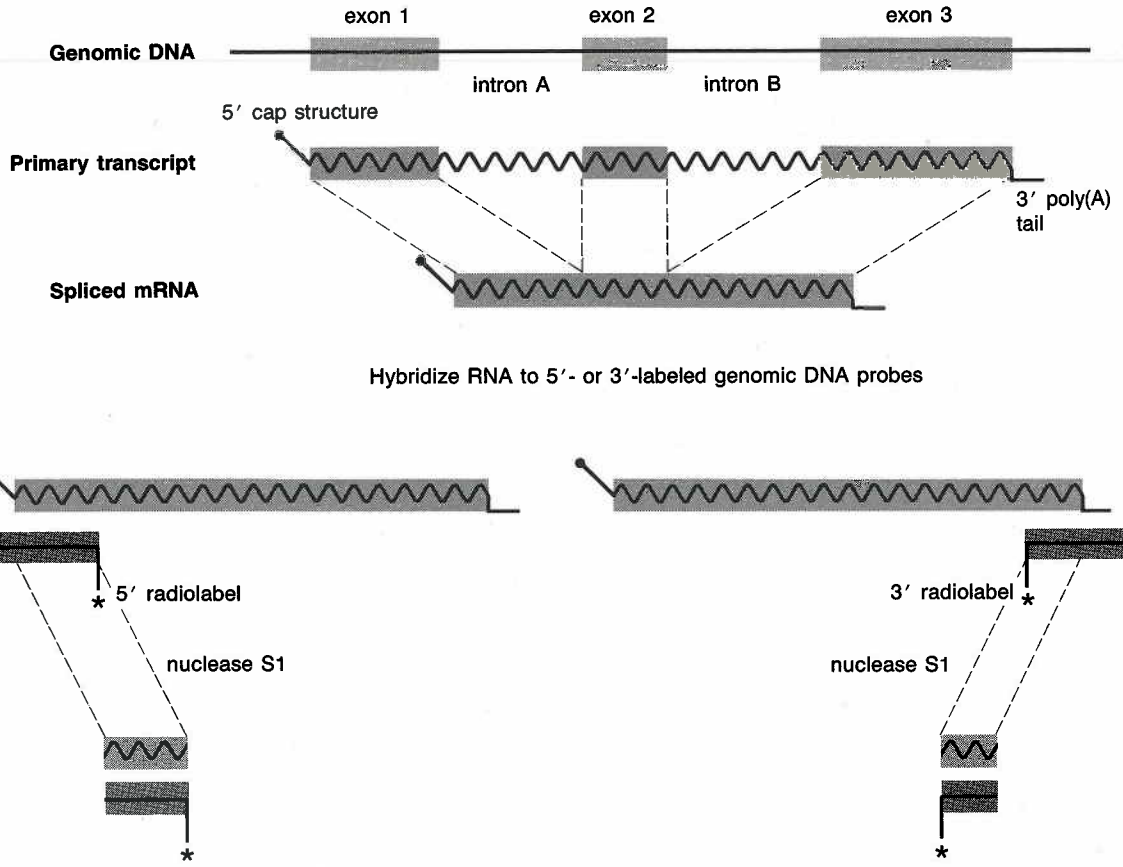


FIGURE 7.4 (See facing page for legend.)

The first method given below describes the use of double-stranded DNA probes to map RNA with nuclease S1. The second, and more modern, method uses radiolabeled single-stranded probes. Whichever type of probe is used, it is important to realize that nuclease-S1 analysis of the structure of eukaryotic RNAs is not free of artifacts. For example, small mismatches in DNA:RNA heteroduplexes are relatively resistant to the action of the nuclease (Berk and Sharp 1977). Conversely, regions of perfect heteroduplex that are rich in rU:dA sequences are susceptible to cleavage (Miller and Sollner-Webb 1981). Third, a single molecule of DNA frequently can be protected from the action of the nuclease by simultaneous hybridization to two different RNA molecules (Lopata et al. 1985). Fourth, nuclease S1 cleaves the segment of DNA opposite a looped-out region of RNA comparatively inefficiently (Sisodia et al. 1987). Although many of these problems can be solved by using a range of concentrations of nuclease S1, by performing the digestion at different temperatures, or by using a combination of nucleases (e.g., RNAase H and nuclease S1) (Sisodia et al. 1987), it is important to realize that cleavage by nuclease S1 of a DNA:RNA hybrid does not necessarily reflect a divergence in sequence between the two nucleic acids and that resistance to digestion is not necessarily synonymous with identity. Mapping with nuclease S1 should therefore be regarded as a useful, but not infallible, guide to the structure of RNAs.

FIGURE 7.4
Mapping the 5′ and 3′ termini of mRNAs. Hybrids formed between mRNA and DNA probes radiolabeled at either their 5′ or their 3′ termini are digested with nuclease S1. By measuring the size of the nuclease-resistant fragments of DNA, it is possible to estimate the distance between the radiolabel and the 5′ and 3′ termini of the mRNA. A similar strategy can be used to map the position of 3′ and 5′ splice sites.

1. Mix in a sterile microfuge tube:

DNA	0.1–1.0 μg
RNA	0.5–250 μg

The amount of DNA included in the reaction depends on its molecular weight. For fragments approximately 5 kb in length, 1 μg of DNA is required; for smaller fragments, proportionately less DNA should be used.

The amount of RNA required depends on the concentration of the sequences of interest. Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of DNA and RNA that yield acceptable results. To detect mRNA sequences present in low concentrations, up to 250 μg of RNA may be used in a 50-μl hybridization reaction. For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reaction can be scaled down to 10 μl.

All reactions should contain the same amount of RNA to ensure that digestion with nuclease S1 is carried out under standard conditions. If necessary, adjust the amount of RNA in the hybridization reactions by adding carrier RNA.

Carrier RNA can be prepared by dissolving commercially available yeast tRNA at a concentration of 10 mg/ml in sterile TE (pH 7.6) containing 0.1 M NaCl. The solution is then extracted twice with phenol (equilibrated in Tris · Cl [pH 7.6] [see Appendix B]) and twice with chloroform, and the RNA is recovered by precipitation with 2.5 volumes of ethanol at room temperature. The precipitated RNA is redissolved at a concentration of 10 mg/ml in sterile TE (pH 7.6), divided into small aliquots, and stored at −20°C.

2. Precipitate the mixed DNA and RNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. Store the solution at 0°C for at least 30 minutes. Recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Discard the supernatant, wash the pellet with 70% ethanol, and recentrifuge. Carefully remove all of the ethanol, and store the pellet of nucleic acids at room temperature until the last visible traces of ethanol have evaporated. Do not allow the pellet to become desiccated, otherwise it will be difficult to dissolve.

3. Redissolve the pellet of nucleic acids in 30 μl of hybridization buffer. Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

It is often difficult to obtain complete dissolution in hybridization buffer of the pellet of nucleic acids that has been precipitated with ethanol. This problem is exacerbated if the pellet is dried in a desiccator. Sometimes the pellet can be redissolved by a combination of vigorous pipetting and heating to 60°C. If difficulties persist, or if equivalent signals are not obtained from duplicate samples of RNA, the following procedure is recommended:

a. After step 2, dissolve the pellet in 40–50 μl of water. Evaporate the sample in a rotary evaporator until it is *just* dry.
b. Add 30 μl of hybridization buffer. The hydrated pellet goes into solution quickly and easily and gives extremely reproducible results.

Hybridization buffer

40 mM PIPES (pH 6.4)
1 mM EDTA (pH 8.0)
0.4 M NaCl
80% formamide

PIPES: Use the disodium salt of PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

4. Close the lid of the tube tightly, and incubate the hybridization reaction in a water bath set at 85°C for 10 minutes to denature the nucleic acids.

5. Rapidly transfer the tube to a water bath set at the desired hybridization temperature. Do not allow the tube to cool below the hybridization temperature during transfer. The hybridization temperature, which depends on the G + C content of the DNA, is chosen so as to minimize the formation of DNA:DNA hybrids while allowing DNA:RNA hybrids to form. Figure 7.5 shows the approximate hybridization temperatures for DNAs of different G + C content (Dean 1987). It is advisable to carry out a series of preliminary experiments to find out the optimal hybridization conditions for the RNA being used.

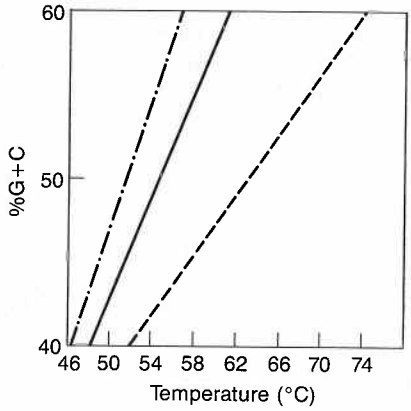


FIGURE 7.5
Optimization of yields of DNA:RNA hybrids. The graph (solid line) shows the temperature calculated to produce maximal yields of DNA:RNA hybrids when denatured DNA is annealed in the presence of RNA complementary to only one strand of the DNA. The broken lines show the calculated T_m for DNA:DNA (- · · · -) and DNA:RNA (---) hybrids. (Redrawn, with permission, from Dean 1987.)

6. Hybridize the DNA and RNA for 12–16 hours at the appropriate temperature. Then open the lid of the hybridization tube, taking care to keep the body of the tube submerged in the water. Rapidly add 300 μl of ice-cold nuclease-S1 mapping buffer, and immediately remove the tube from the water bath. Rapidly mix the contents of the tube by vortexing, and then transfer the tube to a water bath set at the temperature appropriate for digestion with nuclease S1 (see note below). Incubate for 1–2 hours, depending on the degree of digestion desired.

Nuclease-S1 mapping buffer

0.28 M NaCl
0.05 M sodium acetate (pH 4.5)
4.5 mM $ZnSO_4$
20 μg/ml single-stranded DNA (carrier DNA)
100–1000 units/ml nuclease S1

It is necessary to titrate the nuclease S1 each time a new probe or RNA is used.

Single-stranded DNA may be prepared by dissolving fragmented salmon sperm DNA in water at a concentration of 1 mg/ml. Add NaCl to a final concentration of 0.1 M, and extract the DNA solution with phenol:chloroform until no interface is visible. Recover the DNA from the aqueous phase by ethanol precipitation, and redissolve the DNA in a small volume of water. Adjust the concentration of the DNA solution to 1 mg/ml. Immerse the tube containing the DNA solution in a boiling-water bath for 20 minutes. Chill the DNA solution in ice water. Store the denatured DNA at 4°C.

A variety of temperatures and nuclease-S1 concentrations have been used to analyze DNA:RNA hybrids. At 20°C, nuclease S1 at a concentration of 100–1000 units/ml will degrade loops of DNA but will not efficiently digest segments of RNA molecules that bridge loops of DNA (see Figure 7.3). This can be an advantage when mapping intron/exon borders in segments of genomic DNA. The partially digested molecule shown in Figure 7.3 will migrate through agarose gels at neutral pH at approximately the same rate as double-stranded DNA; in alkaline conditions, the RNA "bridge" will be hydrolyzed, liberating two smaller pieces of single-stranded DNA. From the sizes of these fragments, it is often possible to assign locations to intron/exon junctions in the original segment of genomic DNA (Berk and Sharp 1977). For digestion of the single-stranded regions of DNA:RNA hybrids, higher temperatures (37°C–45°C) or increased quantities of nuclease S1 are generally required. However, it is rarely possible to establish conditions that will lead either to complete resistance to digestion or to complete digestion of structures of the type shown in Figure 7.3. The changing ratios of the various digestion products obtained under different conditions can often provide useful clues about the distribution of intron/exon borders in genomic DNA.

7. Chill the reaction to 0°C. Add 80 μl of nuclease-S1 stop mixture and mix.

Nuclease-S1 stop mixture

4 M ammonium acetate
50 mM EDTA (pH 8.0)
50 μg/ml tRNA (carrier RNA)

For preparation of carrier RNA, see notes to step 1, page 7.62.

8. Extract the reaction once with phenol:chloroform. After centrifugation at 12,000*g* for 2 minutes at room temperature in a microfuge, transfer the aqueous supernatant to a fresh tube. Add 2 volumes of ethanol, mix, and store the tube at −20°C for 1 hour.

9. Recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully remove all of the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

10. Dissolve the pellet in 40 μl of TE (pH 7.4).

11. Add 10 μl of loading buffer (50% glycerol and 0.2% bromocresol green), and mix well.

12. Analyze the nuclease-S1-resistant hybrids by electrophoresis through an alkaline and/or a neutral agarose gel (see Chapter 6). As molecular-weight markers, use end-labeled fragments of DNA of known size.

13. Following electrophoresis, transfer the DNA from either type of gel to a nitrocellulose filter or nylon membrane as described on pages 7.46–7.50. (Alkaline gels do not require soaking in denaturation solution.)

14. Hybridize the filters to an appropriate ^{32}P-labeled DNA probe as described on page 7.52.

Mapping of RNA with Nuclease S1 and Single-stranded DNA Probes

Single-stranded DNA probes are prepared by one of two methods:

- Separation of the strands of a fragment of double-stranded DNA
- De novo synthesis of a strand from a single-stranded template

Strand-separated probes are prepared by using restriction enzymes, singly or in combination, to generate a DNA fragment of suitable size (usually 100–500 nucleotides) with a 5′ extension at one end and a 3′ extension at the other. One strand of the fragment will therefore be up to 8 nucleotides longer than the other. This difference in size is sufficient to allow separation of the two strands by electrophoresis through a polyacrylamide gel under denaturing conditions. Alternatively, if no convenient restriction sites are available, it is sometimes possible to purify the desired strand by agarose or polyacrylamide gel electrophoresis of denatured DNA under neutral conditions (Hayward 1972) (see Chapter 6). Either prior to or after electrophoresis, the 5′ terminus of the strand of interest is dephosphorylated with alkaline phosphatase and radiolabeled by addition of ^{32}P catalyzed by bacteriophage T4 polynucleotide kinase.

De novo synthesis can be used to produce either end-labeled or uniformly labeled probes in vitro. In both cases, an oligonucleotide is used to prime the synthesis of a probe that is complementary to a single-stranded DNA template (usually derived from a recombinant bacteriophage M13). End-labeled probes are prepared by phosphorylating the 5′ terminus of the oligonucleotide primer; uniformly labeled probes are prepared by incorporating radiolabeled dNTPs into the growing strand. In both cases, the newly synthesized strand of DNA can be separated from the unlabeled template by digestion with a restriction enzyme that recognizes a unique site in the newly formed double-stranded DNA. The radiolabeled probe can then be separated from the linearized single-stranded DNA by electrophoresis through a polyacrylamide gel under denaturing conditions.

Detailed descriptions of methods used to prepare and isolate radiolabeled single-stranded DNA probes are given in Chapter 10.

1. Precipitate 0.5–150 μg of RNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. After storage at 0°C for 30 minutes, recover the RNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Rinse the pellet with 70% ethanol and recentrifuge. Carefully remove all of the ethanol, and store the pellet of nucleic acid at room temperature until the last visible traces of ethanol have evaporated.

   The amount of RNA required depends on the concentration of the sequences of interest and on the specific activity of the radiolabeled DNA. Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of DNA and RNA that yield acceptable results. With DNAs that have been radiolabeled to high specific activity ($>10^9$ cpm/μg), 10 μg of total RNA is usually sufficient to allow detection of mRNA species that are present at the level of 1–5 copies/cell. To detect sequences present in lower concentrations (e.g., in RNA extracted from heterogeneous populations of cells), up to 150 μg of RNA may be used in a 30-μl hybridization reaction. For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reactions can be scaled down to 10 μl.

   All reactions should contain the same amount of RNA to ensure that digestion with nuclease S1 is carried out under standard conditions. If necessary, adjust the amount of RNA in the hybridization reactions by adding carrier RNA. (For preparation of carrier RNA, see notes to step 1, page 7.62.)

2. Redissolve the RNA in 30 μl of hybridization buffer. Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

   Hybridization buffer

   40 mM PIPES (pH 6.4)
   1 mM EDTA (pH 8.0)
   0.4 M NaCl
   80% formamide

   PIPES: Use the disodium salt of PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

   Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

   It is often difficult to obtain complete dissolution in hybridization buffer of RNA that has been precipitated with ethanol. This problem is exacerbated if the pellet of RNA is dried in a desiccator. Sometimes the RNA can be redissolved by a combination of vigorous pipetting and heating to 60°C. If difficulties persist, or if equivalent signals are not obtained from duplicate samples of RNA, the following procedure is recommended:

a. After step 1, dissolve the RNA in 40–50 μl of water. Evaporate the sample in a rotary evaporator until it is *just* dry.
b. Add 30 μl of hybridization buffer. The hydrated RNA goes into solution quickly and easily and gives extremely reproducible results.

3. Add radiolabeled single-stranded DNA probe (~ 0.01 pmole—corresponding to ~ 1.6 ng of a fragment 400 nucleotides in length). The specific activity of the probe should be $> 5 \times 10^7$ cpm/μg, and it should be added in a volume of < 1 μl (see Chapter 10).

When using this method to measure the concentration of specific RNA sequences, it is essential to use an excess of radiolabeled probe. This is usually determined empirically by nuclease-S1 digestion of a series of hybridization mixtures containing different ratios of RNA:DNA. If necessary, the system can be calibrated by setting up a series of control reactions containing a constant amount of radiolabeled single-stranded DNA and increasing amounts of RNA transcribed in vitro from an appropriate double-stranded DNA template (see Chapter 10).

Single-stranded probes uniformly labeled to high specific activity should be used within a few days to avoid problems caused by radiochemical degradation. End-labeled probes can be stored for up to 1 week.

4. Incubate the hybridization reaction at 85°C for 10 minutes to denature the nucleic acids, and then quickly transfer the reaction to a 30°C water bath. Incubate the hybridization reaction for 12–16 hours.

5. Add to each reaction 300 μl of ice-cold nuclease-S1 mapping buffer. Mix well, and incubate for 1–2 hours at 12°C–45°C, depending on the expected structure of the DNA:RNA hybrid and the desired degree of digestion (see note below).

Nuclease-S1 mapping buffer

0.28 M NaCl
0.05 M sodium acetate (pH 4.5)
4.5 mM $ZnSO_4$
20 μg/ml single-stranded DNA (carrier DNA)
100–1000 units/ml nuclease S1

It is necessary to titrate the nuclease S1 each time a new probe or RNA is used.

Single-stranded DNA may be prepared by dissolving fragmented salmon sperm DNA at a concentration of 1 mg/ml in water. Add NaCl to a final concentration of 0.1 M, and extract the DNA solution with phenol:chloroform until no interface is visible. Recover the DNA from the aqueous phase by ethanol precipitation, and redissolve the DNA in a small volume of water. Adjust the concentration of the DNA solution to 1 mg/ml. Immerse the tube containing the DNA solution in a boiling-water bath for 20 minutes. Chill the DNA solution in ice water. Store the denatured DNA at 4°C.

A variety of temperatures and nuclease-S1 concentrations have been used to analyze DNA:RNA hybrids. At 20°C, nuclease S1 at a concentration of 100–1000 units/ml will degrade loops of DNA but will not efficiently digest segments of RNA molecules that bridge loops of DNA (see Figure 7.3). This can be an advantage when mapping intron/exon borders in segments of genomic DNA. The partially digested molecule shown in Figure 7.3 will migrate through agarose gels at neutral pH at approximate-

ly the same rate as double-stranded DNA; in alkaline conditions, the RNA "br will be hydrolyzed, liberating two smaller pieces of single-stranded DNA. From sizes of these fragments, it is often possible to assign locations to intron/e junctions in the original segment of genomic DNA (Berk and Sharp 1977). digestion of the single-stranded regions of DNA:RNA hybrids, higher temperatu (37°C–45°C) or increased quantities of nuclease S1 are generally required. Howev it is rarely possible to establish conditions that will lead either to complete resi tance to digestion or to complete digestion of structures of the type shown in Figu 7.3. The changing ratios of the various digestion products obtained under differen conditions can often provide useful clues about the distribution of intron/exo borders in genomic DNA.

6. Chill the reaction to 0°C. Add 80 μl of nuclease-S1 stop mixture and mix.

Nuclease-S1 stop mixture

4 M ammonium acetate
50 mM EDTA (pH 8.0)
50 μg/ml tRNA (carrier RNA)

For preparation of carrier RNA, see notes to step 1, page 7.62.

7. Recover the nucleic acids from the nuclease-S1 digest by precipitation with 2 volumes of ethanol. After storage for 60 minutes at −20°C, centrifuge the tube at 12,000*g* for 15 minutes at 4°C. Carefully wash the pellet with 70% ethanol and recentrifuge. Remove the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

8. Dissolve the pellet in 4 μl of TE (pH 7.4).

9. Add 6 μl of formamide loading buffer and mix well.

Formamide loading buffer

80% formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized as described in step 2, page 7.67.

10. Heat the nucleic acids for 5 minutes at 95°C, and then immediately transfer the tube to an ice bath.

11. Analyze the radiolabeled DNA by electrophoresis through a polyacrylamide/7 M urea gel (see Chapter 13) cast according to the expected sizes

of the radioactive fragments. As molecular-weight markers, use end-labeled fragments of DNA of known size.

% Polyacrylamide/urea gel	Size of band (nucleotides)
4	>250
6	60–250
8	40–120
10	20–60
12	10–50

Tracking dyes migrate through denaturing polyacrylamide gels at the following approximate positions equivalent to nucleotide migration:

% Polyacrylamide/urea gel	Xylene cyanol FF (nucleotides)	Bromophenol blue (nucleotides)
4	155	30
6	110	25
8	75	20
10	55	10

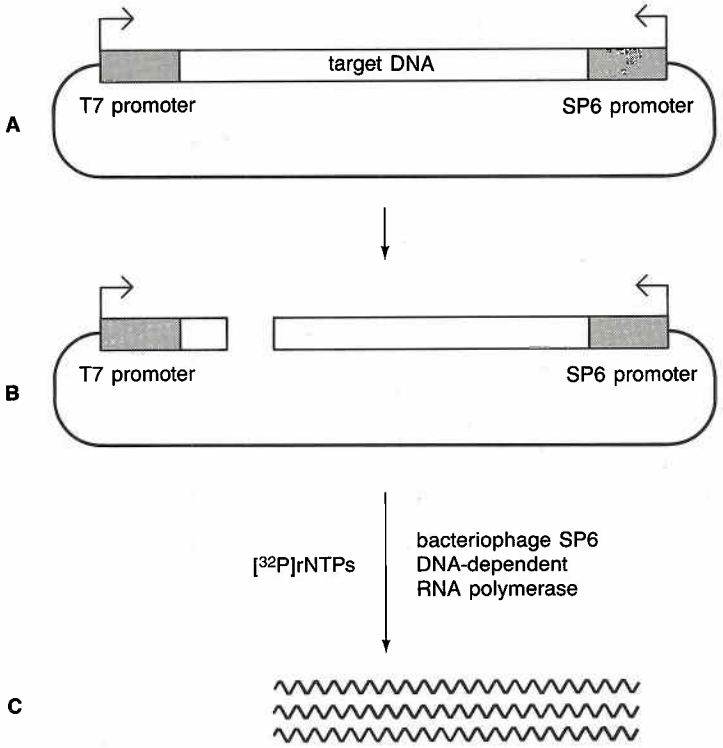
12. Establish an autoradiographic image of the gel as described in Appendix E.

MAPPING OF RNA WITH RIBONUCLEASE AND RADIOLABELED RNA PROBES

Radiolabeled RNA probes can be used in hybridization experiments in the same way as single-stranded DNA probes to detect and quantitate specific RNAs and to study gene and mRNA structure (Lynn et al. 1983; Zinn et al. 1983; Cox et al. 1984). The primary differences between the two procedures are the methods used to synthesize the probes and the use of RNAase to digest regions of the probe that do not form hybrids with the corresponding gene or mRNA (Zinn et al. 1983).

The strategy for mapping mRNA with radiolabeled RNA probes generated in vitro is illustrated in Figure 7.6. A segment of DNA containing all or part of the gene of interest is inserted into a polycloning site immediately downstream from a bacteriophage T7 or SP6 promoter, in an orientation that leads to the production of antisense RNA (Zinn et al. 1983; Melton et al. 1984). The recombinant plasmid is digested with a restriction enzyme that cleaves at a convenient site within the gene or at a site in the plasmid downstream from it. The linearized plasmid is then transcribed in the presence of ^{32}P-labeled rNTPs with the appropriate bacteriophage DNA-dependent RNA polymerase to produce an RNA that extends from the initiation site of the promoter to the end of the DNA fragment. An excess of this ^{32}P-labeled single-stranded RNA is hybridized in solution with the RNA being tested so that all complementary sequences are driven into ^{32}P-labeled RNA:RNA hybrids. The amount of radiolabeled RNA required to force hybridization to completion is usually determined empirically in preliminary experiments. After unhybridized material has been removed by digestion with RNAase, the radiolabeled RNA:RNA hybrid is then detected and quantitated by polyacrylamide gel electrophoresis under denaturing conditions, followed by autoradiography.

Mapping of RNA with RNAase is extremely sensitive: Reconstruction experiments have shown that as little as 0.1 pg of mRNA can be detected by exposing the autoradiograph for several days (Melton et al. 1984). The sensitivity of this method is therefore approximately 20-fold greater than that attainable with double-stranded DNA probes or end-labeled single-stranded DNA probes and approximately equal to that attainable with uniformly labeled single-stranded DNA probes. Furthermore, the digestion of RNA:RNA hybrids with RNAase appears to suffer from fewer artifacts than digestion of RNA:DNA hybrids with nuclease S1. For these reasons and because of the relative ease with which radiolabeled RNA probes can be synthesized, it is not surprising that RNAase digestion of RNA:RNA hybrids has become a standard method to quantitate mRNA molecules, to map their termini, and to determine the positions of introns within the corresponding gene.



Clone target DNA into a plasmid downstream from a prokaryotic promoter (bacteriophage SP6 or T7)

Linearize recombinant plasmid with restriction enzyme that cleaves at distal end of target DNA

Transcribe linear DNA in vitro with appropriate DNA-dependent RNA polymerase and remove template DNA by digestion with pancreatic DNAase I

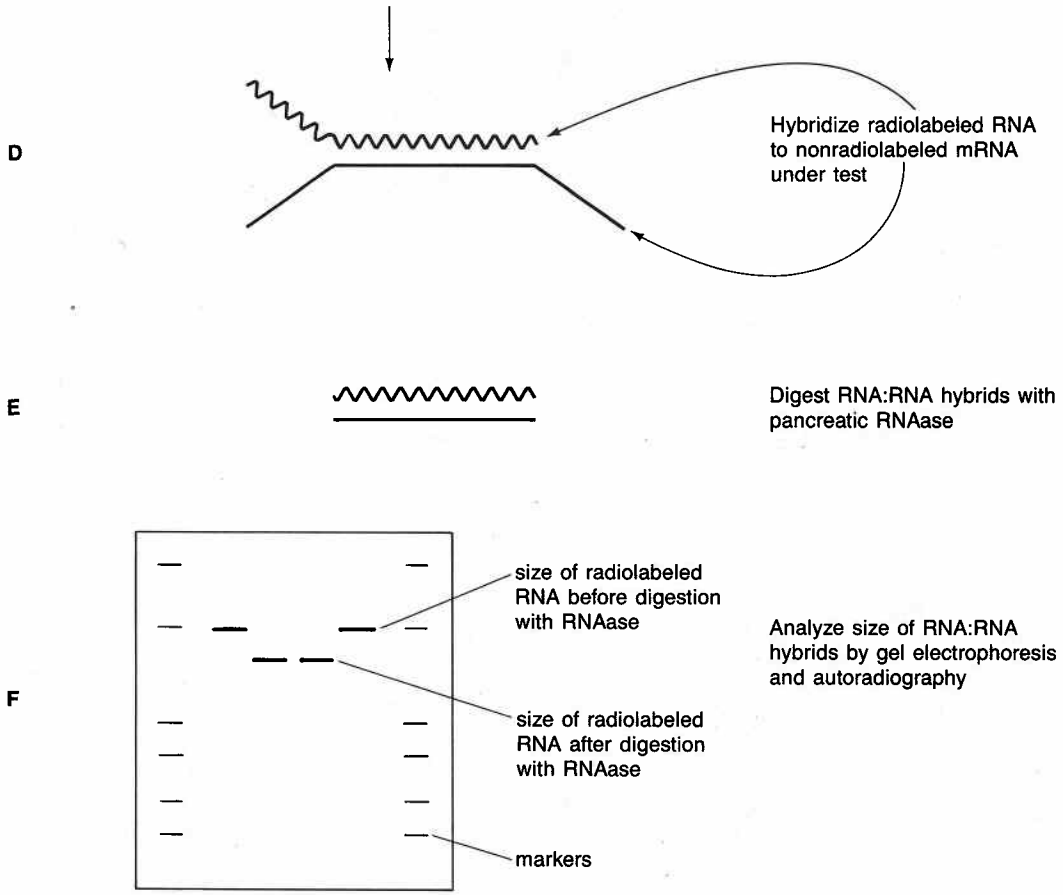


FIGURE 7.6
Mapping of mRNAs with radiolabeled RNA probes. Radiolabeled RNA, synthesized in vitro from a cloned copy of the template DNA (steps A–C), is hybridized to unlabeled test mRNA (step D). After removal of the unhybridized tails (step E), the size of the radiolabeled RNA that is resistant to RNAase is determined by gel electrophoresis (step F). By using probes complementary to appropriate segments of the template DNA, it is possible to map the positions of the 5′ and 3′ termini of mRNAs and the positions of 5′ and 3′ splice sites.

1. Synthesize a uniformly labeled, strand-specific RNA probe as described in Chapter 10. The probe should be used within a few days to avoid problems caused by radiochemical damage to the RNA.

2. Precipitate 0.5–150 μg of the RNA that is to be analyzed by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. Store the solution at 0°C for 30 minutes, and then recover the RNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol and recentrifuge. Carefully remove all of the ethanol, and store the pellet at room temperature until the last visible traces of ethanol have evaporated.

   The amount of unlabeled RNA required depends on the concentration of the sequences of interest and the specific activity of the radiolabeled complementary RNA. Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of RNAs that yield acceptable results. With RNA probes that have been radiolabeled to high specific activity ($>10^9$ cpm/μg), 10 μg of total RNA is usually sufficient to allow detection of mRNA species that are present at the level of 1–5 copies/cell. To detect sequences present in lower amounts (e.g., in RNA extracted from heterogeneous populations of cells), up to 150 μg of RNA may be used in a 30-μl hybridization reaction. For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reactions can be scaled down to 10 μl.

   When comparing different preparations of RNA, make sure that all of the reactions contain the same amount of RNA. In this way, digestion with RNAase is carried out under standard conditions. If necessary, adjust the amount of RNA in the hybridization reactions by adding carrier RNA. (For preparation of carrier RNA, see notes to step 1, page 7.62.)

3. Redissolve the RNA to be analyzed in 30 μl of hybridization buffer containing a small molar excess of radiolabeled probe. Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

Hybridization buffer

40 mM PIPES (pH 6.4)
1 mM EDTA (pH 8.0)
0.4 M NaCl
80% formamide

PIPES: Use the disodium salt of PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

It is often difficult to obtain complete dissolution in hybridization buffer of RNA that has been precipitated with ethanol. The problem is exacerbated if the pellet is dried in a desiccator. Sometimes the RNA can be redissolved by a combination of vigorous pipetting and heating to 60°C. If difficulties persist, or if equivalent signals are not obtained from duplicate samples of RNA, the following procedure is recommended:

a. After step 2, dissolve the RNA in 40–50 μl of water. Evaporate the sample in a rotary evaporator until it is *just* dry.
b. Add 30 μl of hybridization buffer. The hydrated RNA goes into solution quickly and easily and gives extremely reproducible results.

Usually, 1×10^5 to 5×10^5 cpm of probe are used per hybridization. A single in vitro transcription reaction with bacteriophage SP6 or bacteriophage T7 DNA-dependent RNA polymerase generates enough probe (~2 pmoles) for more than 200 hybridization reactions. However, because background increases as more probe is added to the hybridization reaction, add only enough probe to achieve a small molar excess. This is usually determined empirically by RNAase digestion of a series of hybridization mixtures containing different ratios of probe RNA to target RNA. If necessary, the system can be calibrated by setting up a series of control reactions containing a constant amount of radiolabeled RNA and increasing amounts of unlabeled RNA transcribed in vitro from the opposite strand of an appropriate double-stranded DNA template (see Chapter 10).

4. Incubate the hybridization mixture at 85°C for 10 minutes to denature the RNAs. Quickly transfer the hybridization mixture to a water bath set at the annealing temperature. Incubate the mixture for 8–12 hours.

The optimal temperature for annealing varies from RNA to RNA, presumably depending on such factors as G + C content and propensity to form secondary structures. In most cases, satisfactory results are obtained when the RNA is annealed at 45°C–50°C. Optimal conditions for the hybridization of specific probes can be established by reconstruction experiments, in which the radiolabeled probe is hybridized at temperatures ranging between 25°C and 65°C to unlabeled RNA transcribed in vitro from the opposite strand of an appropriate double-stranded DNA template (see Chapter 10).

5. Cool the hybridization mixture to room temperature, and add 300 μl of RNAase digestion mixture.

RNAase digestion mixture

300 mM NaCl
10 mM Tris · Cl (pH 7.4)
5 mM EDTA (pH 7.5)
2 μg/ml RNAase T1
40 μg/ml RNAase A

Digest the hybridization reaction for 60 minutes at 30°C.

Concentrated stock solutions of RNAases are usually made up in 10 mM Tris · Cl (pH 7.5), 15 mM NaCl and stored at −20°C (RNAase A, 10 mg/ml [see Appendix B]; RNAase T1, 100 μg/ml).

The time and temperature of the digestion with RNAase should be determined empirically if the signal-to-noise ratio is unacceptable.

6. Add 20 μl of 10% SDS and 10 μl of a 10 mg/ml solution of proteinase K. Incubate the reaction for 30 minutes at 37°C.

The solution of proteinase K should be freshly prepared. Solutions that have been frozen or thawed several times do not work well and sometimes lead to the degradation of RNA.

7. Add 400 μl of phenol:chloroform, vortex for 30 seconds, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge.

8. Transfer the upper, aqueous phase to a fresh tube, carefully avoiding the interface between the organic and aqueous phases.

9. Add 20 μg of tRNA and 750 μl of ice-cold ethanol. Mix well by vortexing, and store the solution at −20°C for 30 minutes.

10. Recover the RNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully remove the ethanol and wash the pellet with 500 μl of 70% ethanol. Recentrifuge.

11. Carefully remove all of the ethanol, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

12. Resuspend the precipitate in 10 μl of formamide loading buffer.

Formamide loading buffer

80% formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized as described in step 3, page 7.74.

13. Heat the nucleic acids for 5 minutes at 95°C, and then immediately transfer the tube to an ice bath.

14. Analyze the radiolabeled RNA by electrophoresis through a polyacrylamide/7 M urea gel (see Chapter 13) cast according to the expected size of the radioactive fragments (see step 11, page 7.70). As molecular-weight markers, use end-labeled fragments of DNA of known size.

The relative mobility of RNA and DNA through polyacrylamide/7 M urea gels varies according to the conditions used for electrophoresis. In general, the faster the gel is run, the less the difference in mobility between RNA and DNA molecules of identical size. Under the conditions normally used (40–45 V/cm), RNA runs approximately 5%–10% slower than DNA of the same size. Thus, a 90-nucleotide RNA will migrate at approximately the same rate as a 100-nucleotide DNA. If absolute measurement of the size of the RNA is required, a series of radioactive probes of defined length can be generated by in vitro transcription of a double-stranded DNA template after digestion with restriction enzymes that cleave at sites progressively more distal to a bacteriophage promoter or by end-labeling of commercially available RNA markers.

15. Establish an autoradiographic image of the gel as described in Appendix E.

Notes

i. It is important to remove the template DNA completely from the transcription reaction by digestion with RNAase-free pancreatic DNAase I (see Chapter 10). Trace amounts of partially degraded template DNA in the hybridization mixture will lead to the formation of DNA:RNA hybrids that will be detected on the autoradiograph. This problem can be minimized by titrating the amount of probe needed for each test sample to give a strong signal without a large amount of background.

ii. A control hybridization reaction containing 20 μg of tRNA instead of the RNA being tested should always be carried out to check for spurious protection of the probe from digestion by RNAases. RNAase-resistant fragments can be formed if the probe contains self-complementary sequences or if it is contaminated with a small quantity of RNA transcribed from the opposite strand of the DNA template. These rare complementary transcripts are thought to be generated by transcription initiated by bacteriophage T7 and SP6 RNA polymerases at the ends of the template (Schenborn and Mierendorf 1985). Significant quantities of these extraneous transcripts are synthesized in vitro only when the linearized DNA templates carry protruding 3′ termini. This problem can therefore be minimized by using a restriction enzyme that generates 5′ protruding termini to linearize the plasmid or by modifying the termini with bacteriophage T4 DNA polymerase before the transcription reaction. If all other procedures fail, it is possible to purify a probe of the desired length by agarose or polyacrylamide gel electrophoresis.

iii. Problems occasionally arise because of degradation of the probe before the hybridization reaction or, more rarely, because of the synthesis of transcripts that are less than full-length. In both cases, many smaller fragments are observed in addition to a protected RNA fragment of the expected size. Check that none of the reaction buffers or enzymes is contaminated with RNAase, and analyze an aliquot of the probe by electrophoresis through a polyacrylamide gel under denaturing conditions. In a typical reaction with bacteriophage SP6 or T7 RNA polymerase, more than 90% of the transcripts will appear as a single band of the expected size. Contamination with RNAase will result in the appearance of a smear of smaller fragments of RNA in the gel. This problem can be circumvented by making fresh buffers and checking them for RNAase contamination using the gel electrophoresis assay. Transcripts that are less than full-length may also be generated if the conditions for transcription are not optimal (see Melton et al. 1984; Krieg and Melton 1987) or if there are signals for termination of transcription in the template. Once again, this problem can be solved by purifying the transcripts of the correct length by agarose or polyacrylamide gel electrophoresis.

iv. Certain batches of ^{32}P-labeled UTP contain inhibitors that can cause

premature termination of nascent transcripts. This problem can usually be circumvented by using another radiolabeled nucleotide.

ANALYSIS OF RNA BY PRIMER EXTENSION

Primer extension is used to map and quantitate the 5′ termini of RNA and to detect precursors and processing intermediates of mRNA. The test RNA is hybridized with an excess of a single-stranded DNA primer (a synthetic oligonucleotide or a restriction fragment) radiolabeled at its 5′ terminus. Reverse transcriptase is then used to extend this primer to produce cDNA complementary to the RNA template. The length of the resulting end-labeled cDNA, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions, reflects the distance between the end-labeled nucleotide of the primer and the 5′ terminus of the RNA. The yield of cDNA is approximately proportional to the concentration of the target sequences in the mRNA preparation.

If desired, the end-labeled cDNA can be extracted from the gel and sequenced by the Maxam-Gilbert method. It is also possible to sequence the cDNA directly by including dideoxyribonucleoside triphosphates in the primer-extension reaction, but this should be done only after it has been established that a single species of cDNA is synthesized during the reaction.

Either single-stranded or denatured double-stranded DNAs can be used as primers. In the latter case, the conditions for hybridization are adjusted to suppress the formation of DNA:DNA hybrids in favor of DNA:RNA hybrids. Because it is not usually possible to prevent completely the formation of DNA:DNA hybrids, it is advisable always to include controls that contain no RNA in the hybridization reaction. Fragments of double-stranded DNA used as primers are usually 75–150 nucleotides in length and are radiolabeled at both ends by phosphorylation or by incorporation of radiolabeled dNTPs at a recessed 3′ terminus.

Single-stranded DNA primers, which are preferred for experiments of this type, are synthetic oligonucleotides 30–40 nucleotides in length. Primers of this type have two main advantages: They eliminate the formation of DNA:DNA hybrids and they can be precisely designed to hybridize to specific sequences of the mRNA. In this way, it is possible to avoid potential problems caused by secondary structure in the mRNA and to maximize resolution of the cDNA products from the primer by gel electrophoresis.

It is important to use primers whose target sequences are located within 100 nucleotides of the 5′ terminus of the mRNA. Primers that hybridize to more-distal sites frequently generate heterogeneous extension products because of the tendency of reverse transcriptase to stop or pause in regions of high secondary structure in the template RNA.

1. Radiolabel the fragment of DNA to be used as a primer. Double-stranded DNA may be labeled either by phosphorylation or by filling of a recessed 3′ terminus (see Chapter 10). Oligonucleotides are labeled by phosphorylation (see Chapter 11).

   The length of the primer is not critical and can range from 75 to 150 or more nucleotides. However, the shorter the primer, the greater the difference in size between it and the extended product.

2. Mix 10^4–10^5 cpm of the DNA primer with 0.5–150 μg of the RNA that is to be analyzed. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. Store the solution at −20°C for 30 minutes, and then recover the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol and recentrifuge. Carefully remove all of the ethanol, and store the pellet at room temperature until the last visible traces of ethanol have evaporated.

   The amount of unlabeled RNA required depends on the concentration of the sequences of interest. Before embarking on large-scale experiments, it is advisable to carry out preliminary experiments to establish the range of concentrations of RNAs that yield acceptable results. Usually, 10 μg of total RNA is sufficient to allow mapping of the 5′ termini of mRNA species that are present at the level of 1–5 copies/cell. To analyze RNAs present in lower amounts (e.g., in RNA extracted from heterogeneous populations of cells), up to 150 μg of RNA may be used in a 30-μl hybridization reaction. For ease of manipulation in subsequent steps, it is advisable to keep the hybridization volume to 30 μl or less. If reagents are in short supply, the hybridization reactions can be scaled down to 10 μl.

   To minimize problems caused by contamination with RNAase, treat all of the reagents and tubes as described on pages 7.3–7.4.

3. Redissolve the precipitated nucleic acids in 30 μl of hybridization buffer. Pipette the solution up and down many times to make sure that the pellet is completely dissolved.

Hybridization buffer

40 mM PIPES (pH 6.4)
1 mM EDTA (pH 8.0)
0.4 M NaCl
80% formamide

PIPES: Use the disodium salt of PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) to prepare the buffer, and adjust the pH with 1 N HCl.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

It is often difficult to obtain complete dissolution in hybridization buffer of the pellet of nucleic acids that has been precipitated with ethanol. This problem is exacerbated if the pellet is dried in a desiccator. Sometimes the pellet can be redissolved by a combination of vigorous pipetting and heating to 60°C. If difficulties persist, or if variable signals are obtained from duplicate samples of RNA, the following procedure is recommended:

a. After step 2, dissolve the pellet in 40–50 μl of water. Evaporate the sample in a rotary evaporator until it is *just* dry.
b. Add 30 μl of hybridization buffer. The hydrated pellet goes into solution quickly and easily and gives extremely reproducible results.

Usually, 10^4–10^5 cpm of primer are used per hybridization. However, because the number of artifactual bands increases as more primer is added to the hybridization reaction, it is advisable to carry out a series of pilot reactions that contain a constant amount of RNA and different amounts of primer.

4. Incubate the hybridization mixture at 85°C for 10 minutes to denature the nucleic acids. Quickly transfer the hybridization mixture to a water bath set at the annealing temperature. Incubate the mixture for 8–12 hours.

The optimal temperature for annealing varies from RNA to RNA, presumably depending on such factors as G + C content, its propensity to form secondary structures, and the length of the primer. Optimal conditions should be established in a series of pilot experiments. With double-stranded DNA primers, the aim is to minimize formation of DNA:DNA hybrids and to maximize annealing of the primer to its target RNA. In most cases, satisfactory results are obtained at 40°C–50°C. With oligonucleotide primers, the aim is to minimize formation of mismatched hybrids. Primers 30–40 nucleotides in length usually yield satisfactory results when annealed to RNA at 30°C.

5. Add 170 μl of water and 400 μl of ethanol. Mix well, and store at 0°C for 1 hour. Collect the precipitate of nucleic acids by centrifugation at 12,000*g* for 15 minutes at 0°C in a microfuge. Carefully discard the supernatant and wash the pellet with 500 μl of 70% ethanol. Recentrifuge. Carefully discard the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

6. Redissolve the primer:RNA hybrids in 20 μl of reverse transcriptase buffer.

Reverse transcriptase buffer

50 mM Tris · Cl (pH 7.6)
60 mM KCl
10 mM $MgCl_2$
1 mM of each dNTP
1 mM dithiothreitol
1 unit/μl placental RNAase inhibitor
50 μg/ml actinomycin D

Add 50 units of murine reverse transcriptase, and mix well by gentle vortexing. Try to avoid making bubbles. This is sometimes difficult because reverse transcriptase is supplied in a buffer containing Triton X-100. Bubbles may be removed by brief centrifugation in a microfuge.

Incubate the reaction for 2 hours at 37°C.

Once initiated by a primer, synthesis of a cDNA molecule usually proceeds to the 5′ terminus of the RNA template. However, regions of the template that are rich in secondary structure may cause reverse transcriptase to pause or stop, resulting in cDNAs that are less than full-length. This artifact can be minimized by performing the reaction in the presence of higher (5 mM) concentrations of dNTPs and using a primer that is complementary to sequences of the mRNA that lie within 50–100 nucleotides of its 5′ terminus. However, to distinguish between artifacts of reverse transcription and true heterogeneity of 5′ termini, it is often necessary to carry out nuclease-S1 analysis with probes labeled at their 5′ termini.

Actinomycin D inhibits synthesis of double-stranded DNA by reverse transcriptase without significantly affecting the yield of first-strand DNA. It therefore prevents the synthesis of "hairpin" molecules that are formed when the first strand of DNA serves as a primer/template. The efficiency of this self-priming is believed to vary widely from mRNA to mRNA but is generally quite low. However, since actinomycin D does not affect the efficiency with which mRNA is copied into DNA, its routine use is recommended to reduce the possibility of obtaining artifactual products of the primer-extension reaction. Stock solutions of actinomycin D are usually prepared in ethanol at a concentration of 5 mg/ml, stored at −20°C in the dark, and diluted into the reaction mixture immediately before use.

Caution: Actinomycin D is a teratogen and a carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench. Solutions of actinomycin D are light-sensitive.

7. Add 1 μl of 0.5 M EDTA (pH 8.0) and 1 μl of DNAase-free pancreatic RNAase (5 μg/ml) (see Appendix B). Incubate the reaction for 30 minutes at 37°C.

8. Add 150 μl of TE (pH 7.6), containing 0.1 M NaCl, and 200 μl of phenol:chloroform. Vortex for 30 seconds, and centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge.

9. Transfer the upper, aqueous phase to a fresh tube, carefully avoiding the interface between the organic and aqueous phases.

10. Add 500 μl of ethanol. Mix well, and store at 0°C for 1 hour.

11. Recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully remove the ethanol and wash the pellet with 500 μl of 70% ethanol. Recentrifuge.

12. Carefully remove all of the ethanol, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.

13. Dissolve the pellet in 4 μl of TE (pH 7.4), add 6 μl of formamide loading buffer, and mix well.

Formamide loading buffer

80% formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized as described in step 3, page 7.80.

14. Heat the mixture for 5 minutes at 95°C, and immediately transfer the tube to an ice bath.

15. Analyze the radiolabeled DNA by electrophoresis through a polyacrylamide/7 M urea gel (see Chapter 13) cast according to the expected sizes of the radioactive fragments (see step 11, page 7.70). As molecular-weight markers, use end-labeled fragments of DNA of known size.

16. Establish an autoradiographic image of the gel as described in Appendix E.

References

Alwine, J.C., D.J. Kemp, and G.R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci.* **74:** 5350.

Alwine, J.C., D.J. Kemp, B.A. Parker, J. Reiser, J. Renart, G.R. Stark, and G.M. Wahl. 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. *Methods Enzymol.* **68:** 220.

Aviv, H. and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci.* **69:** 1408.

Bailey, J.M. and N. Davidson. 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* **70:** 75.

Beach, L.R. and R.D. Palmiter. 1981. Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proc. Natl. Acad. Sci.* **78:** 2110.

Berger, S.L. and C.S. Birkenmeier. 1979. Inhibition of intractable nucleases with ribonucleoside–vanadyl complexes: Isolation of messenger ribonucleic acid from resting lymphocytes. *Biochemistry* **18:** 5143.

Berk, A.J. and P.A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12:** 721.

Birnboim, H.C. 1988. Rapid extraction of high molecular weight RNA from cultured cells and granulocytes for Northern analysis. *Nucleic Acids Res.* **16:** 1487.

Blackburn, P., G. Wilson, and S. Moore. 1977. Ribonuclease inhibitor from human placenta. Purification and properties. *J. Biol. Chem.* **252:** 5904.

Bresser, J. and D. Gillespie. 1983. Quantitative binding of covalently closed circular DNA to nitrocellulose in NaI. *Anal. Biochem.* **129:** 357.

Brown, P.C., T.D. Tlsty, and R.T. Schimke. 1983. Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. *Mol. Cell. Biol.* **3:** 1097.

Casey, J. and N. Davidson. 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. *Nucleic Acids Res.* **4:** 1539.

Chapman, A.B., M.A. Costello, F. Lee, and G.M. Ringold. 1983. Amplification and hormone-regulated expression of a mouse mammary tumor virus-*Eco gpt* fusion plasmid in mouse 3T6 cells. *Mol. Cell. Biol.* **3:** 1421.

Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18:** 5294.

Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci.* **81:** 1991.

Cox, K.H., D.V. DeLeon, L.M. Angerer, and R.C. Angerer. 1984. Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. Biol.* **101:** 485.

Cox, R.A. 1968. The use of guanidinium chloride in the isolation of nucleic acids. *Methods Enzymol.* **12B:** 120.

Dean, M. 1987. Determining the hybridization temperature for S1 nuclease mapping. *Nucleic Acids Res.* **15:** 6754.

de Martynoff, G., E. Pays, and G. Vassart. 1980. The synthesis of a full length DNA complementary to thyroglobulin 33 S messenger RNA. *Biochem. Biophys. Res. Commun.* **93:** 645.

Durnam, D.M. and R.D. Palmiter. 1983. A practical approach for quantitating specific mRNAs by solution hybridization. *Anal. Biochem.* **131:** 385.

Edmonds, M., M.H. Vaughan, Jr., and H. Nakazato. 1971. Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: Possible evidence for a precursor relationship. *Proc. Natl. Acad. Sci.* **68:** 1336.

Favaloro, J., R. Treisman, and R. Kamen. 1980. Transcription maps of polyoma virus-specific RNA: Analysis

by two-dimensional nuclease S1 gel mapping. *Methods Enzymol.* **65:** 718.
Fedorcsak, I. and L. Ehrenberg. 1966. Effects of diethyl pyrocarbonate and methyl methanesulfonate on nucleic acids and nucleases. *Acta Chem. Scand.* **20:** 107.
Fourney, R.M., J. Miyakoshi, R.S. Day III, and M.C. Paterson. 1988. Northern blotting: Efficient RNA staining and transfer. *Bethesda Res. Lab. Focus* **10(1):** 5.
Gilham, P.T. 1964. Synthesis of polynucleotide celluloses and their use in fractionation of polynucleotides. *J. Am. Chem. Soc.* **86:** 4982.
Glišin, V., R. Crkvenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13:** 2633.
Goldberg, D.A. 1980. Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. *Proc. Natl. Acad. Sci.* **77:** 5794.
Gruenwedel, D.W. and N. Davidson. 1966. Complexing and denaturation of DNA by methylmercuric hydroxide. I. Spectrophotometric studies. *J. Mol. Biol.* **21:** 129.
Hayward, G.S. 1972. Gel electrophoretic separation of the complementary strands of bacteriophage DNA. *Virology* **49:** 342.
Herrin, D.L. and G.W. Schmidt. 1988. Rapid, reversible staining of Northern blots prior to hybridization. *BioTechniques* **6:** 196.
John, M.E., M.C. John, P. Ashley, R.J. MacDonald, E.R. Simpson, and M.R. Waterman. 1984. Identification and characterization of cDNA clones specific for cholesterol side-chain cleavage cytochrome P-450. *Proc. Natl. Acad. Sci.* **81:** 5628.
Junghans, R.P. 1983. A review of the toxicity of methylmercury compounds with application to occupational exposures associated with laboratory uses. *Environ. Res.* **31:** 1.
Kafatos, F.C., C.W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res.* **7:** 1541.
Khandjian, E.W. 1987. Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Bio/Technology* **5:** 165.
Krieg, P.A. and D.A. Melton. 1987. *In vitro* RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* **155:** 397.
Kumar, A. and U. Lindberg. 1972. Characterization of messenger ribonucleoprotein and messenger RNA from KB cells. *Proc. Natl. Acad. Sci.* **69:** 681.
Kurachi, K., E.W. Davie, D.J. Strydom, J.F. Riordan, and B.L. Vallee. 1985. Sequence of the cDNA and gene for angiogenin, a human angiogenesis factor. *Biochemistry* **24:** 5494.
Lehrach, H., D. Diamond, J.M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16:** 4743.
Lemischka, I.R., S. Farmer, V.R. Racaniello, and P.A. Sharp. 1981. Nucleotide sequence and evolution of a mammalian α-tubulin messenger RNA. *J. Mol. Biol.* **151:** 101.
Lopata, M.A., B. Sollner-Webb, and D.W. Cleveland. 1985. Surprising S1-resistant trimolecular hybrids: Potential complication in interpretation of S1 mapping analyses. *Mol. Cell. Biol.* **5:** 2842.
Lynn, D.A., L.M. Angerer, A.M. Bruskin, W.H. Klein, and R.C. Angerer. 1983. Localization of a family of mRNAs in a single cell type and its precursors in sea urchin embryos. *Proc. Natl. Acad. Sci.* **80:** 2656.
MacDonald, R.J., G.H. Swift, A.E. Przybyla, and J.M. Chirgwin. 1987. Isolation of RNA using guanidinium salts. *Methods Enzymol.* **152:** 219.
McMaster, G.K. and G.G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci.* **74:** 4835.
Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12:** 7035.
Miller, K. 1987. Gel electrophoresis of RNA. *Bethesda Res. Lab. Focus* **9:** 14.
Miller, K.G. and B. Sollner-Webb. 1981. Transcription of mouse rRNA genes by

RNA polymerase I: In vitro and in vivo initiation and processing sites. *Cell* **27:** 165.

Rave, N., R. Crkvenjakov, and H. Boedtker. 1979. Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. *Nucleic Acids Res.* **6:** 3559.

Reed, K.C. and D.A. Mann. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13:** 7207.

Roop, D.R., J.L. Nordstrom, S.Y. Tsai, M.-J. Tsai, and B.W. O'Malley. 1978. Transcription of structural and intervening sequences in the ovalbumin gene and identification of potential ovalbumin mRNA precursors. *Cell* **15:** 671.

Scheele, G. and P. Blackburn. 1979. Role of mammalian RNase inhibitor in cell-free protein synthesis. *Proc. Natl. Acad. Sci.* **76:** 4898.

Schenborn, E.T. and R.C. Mierendorf, Jr. 1985. A novel transcription property of SP6 and T7 RNA polymerases: Dependence on template structure. *Nucleic Acids Res.* **13:** 6223.

Schweinfest, C.W., R.W. Kwiatkowski, and R.P. Dottin. 1982. Molecular cloning of a DNA sequence complementary to creatine kinase M mRNA from chickens. *Proc. Natl. Acad. Sci.* **79:** 4997.

Seed, B. 1982a. Attachment of nucleic acids to nitrocellulose and diazonium-substituted supports. In *Genetic engineering: Principles and methods* (ed. J.K. Setlow and A. Hollaender), vol. 4, p. 91. Plenum Publishing, New York.

———. 1982b. Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. *Nucleic Acids Res.* **10:** 1799.

Sela, M., C.B. Anfinsen, and W.F. Harrington. 1957. The correlation of ribonuclease activity with specific aspects of tertiary structure. *Biochim. Biophys. Acta* **26:** 502.

Sisodia, S.S., D.W. Cleveland, and B. Sollner-Webb. 1987. A combination of RNase H and S1 nuclease circumvents an artifact inherent to conventional S1 analysis of RNA splicing. *Nucleic Acids Res.* **15:** 1995.

Stallcup, M.R. and L.D. Washington. 1983. Region-specific initiation of mouse mammary tumor virus RNA synthesis by endogenous RNA polymerase II in preparations of cell nuclei. *J. Biol. Chem.* **258:** 2802.

Strohman, R.C., P.S. Moss, J. Micou-Eastwood, D. Spector, A. Przybyla, and B. Paterson. 1977. Messenger RNA for myosin polypeptides: Isolation from single myogenic cell cultures. *Cell* **10:** 265.

Strydom, D.J., J.W. Fett, R.R. Lobb, E.M. Alderman, J.L. Bethune, J.F. Riordan, and B.L. Vallee. 1985. Amino acid sequence of human tumor derived angiogenin. *Biochemistry* **24:** 5486.

Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci.* **77:** 5201.

———. 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. *Methods Enzymol.* **100:** 255.

Ullrich, A., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W.J. Rutter, and H.M. Goodman. 1977. Rat insulin genes: Construction of plasmids containing the coding sequences. *Science* **196:** 1313.

Vrati, S., D.A. Mann, and K.C. Reed. 1987. Alkaline Northern blots: Transfer of RNA from agarose gels to Zeta-Probe membrane in dilute NaOH. *Mol. Biol. Rep. (Bio-Rad Laboratories)* **1 (3):** 1.

Weaver, R.F. and C. Weissmann. 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: The 5′ termini of 15 S β-globin mRNA precursor and mature 10 S β-globin mRNA have identical map coordinates. *Nucleic Acids Res.* **7:** 1175.

White, B.A. and F.C. Bancroft. 1982. Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. *J. Biol. Chem.* **257:** 8569.

Wicks, R.J. 1986. RNA molecular weight determination by agarose gel electrophoresis using formaldehyde as denaturant: Comparison of RNA and DNA molecular weight markers. *Int. J. Biochem.* **18:** 277.

Wieslander, L. 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal. Biochem.* **98:** 305.

Williams, D.L., T.C. Newman, G.S. Shelness, and D.A. Gordon. 1986. Measurement of apolipoprotein mRNA by DNA-excess solution hybridization with single-stranded probes. *Methods Enzymol.* **128:** 671.

Wreschner, D.H. and M. Herzberg. 1984. A new blotting medium for the simple isolation and identification of highly resolved messenger RNA. *Nucleic Acids Res.* **12:** 1349.

Zinn, K., D. DiMaio, and T. Maniatis. 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. *Cell* **34:** 865.