

Preface

RNA MOLECULES PARTICIPATE IN AND REGULATE a vast array of cellular processes, and the scientific community is now well into a new era in which some aspect of RNA biology—as a tool, a therapeutic, a diagnostic, or a central player in a fundamental biological process—is becoming increasingly important. Nevertheless, beginning RNA research can be daunting, and without a thorough understanding of the challenges and complexities inherent in handling this fragile nucleic acid, forays into the RNA world can be quite frustrating. For example, the introduction of many kits designed to perform everything from purification to high-throughput sequencing has been both a boon and a bane to RNA science. Although these kits have made RNA analysis accessible to nearly every laboratory, they give many researchers a false sense of security—they believe that whatever comes out of the final tube is exactly what they expect. Unfortunately, unbeknownst to the investigator, this is often not the case.

We have written this manual with all RNA scientists—novices and more advanced researchers—in mind. Chapters 1 through 3 are specifically targeted to those new to the area or those needing some “refreshing” of fundamental principles and techniques. We have emphasized “common sense” approaches and highlight the fact that it is essential to monitor the yield and quality of any RNA preparation after each step. Anyone contemplating RNA research should be comfortable with the material in these chapters before moving on to the more complex procedures in the chapters that follow.

Chapters 4 through 6 are written for those who wish to investigate RNA-related topics in more depth. Here, we describe a variety of techniques, some quite complex, designed primarily to explore specific RNA–protein interactions and elucidate the mechanisms of RNA-processing reactions. Chapter 7 describes current methodologies for using RNA interference as a tool for determining gene function, and Chapter 8 presents methods for performing increasingly popular genome-wide investigations. Finally, the Appendix includes a few methods that we have found to be useful but did not fit into any particular area, as well as recipes for commonly used reagents and some general reference information.

Almost all of the methods described in this manual are routinely used in one or more of our laboratories. In the few cases where we do not have direct experience, we have enlisted the expertise of trusted colleagues. Although we have attempted to be comprehensive, we realize that this is an impossible task. New approaches, especially in the area of genome-wide analyses, appear with high frequency. Moreover, no one method is ideally suited to any particular question. We fully realize that there are alternative approaches available for nearly every method that we describe. In addition, we acknowledge that many specialized techniques, such as circular permutation and direct approaches to RNA analysis (e.g., fingerprinting and thin-layer chromatography), have not been described at all because we believe that these methods would be of limited appeal to most RNA researchers.

It is often difficult to identify with any precision the source of particular methods because as they are adopted by different laboratories, they “evolve.” Accordingly, we have not attempted to provide extensive referencing for the protocols; only a few representative publications are cited. We apologize in advance to all of those authors who have published methods or improvements thereon who have not been cited in this manual.

Finally, we note that many “standard” molecular biology techniques, such as protein gels, western blotting, cloning, etc., are not covered in this manual. Such material is covered in great detail in the latest edition of *Molecular Cloning: A Laboratory Manual*, and readers requiring this information are strongly encouraged to consult that manual in conjunction with this one.

In *RNA: A Laboratory Manual*, we have attempted to describe a broad scope of current techniques used in RNA research. We hope that this manual will enable any researcher to approach a wide variety of problems from the most fundamental to the highly sophisticated with confidence and high expectation of success.

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Purification of RNA from Natural Sources

CHAPTER 1 OUTLINED A VARIETY of fundamental and practical considerations applicable to all experiments dealing with RNA. Chapter 2 presents detailed protocols for several basic techniques. It is impossible to envision every experimental scenario, but the procedures described in this chapter should have general utility. It is important to remember to adjust the scale of each approach to the scale of your particular applications.

In general, all RNA purification approaches involve three steps: solubilization, deproteinization (i.e., removing protein and sometimes DNA from RNA, also called extraction), and recovery of RNA free from other contaminants. Some protocols are designed to remove DNA during extraction, whereas in others contaminating DNA is removed after recovery of protein-free nucleic acids. All purification methods are designed to minimize loss of RNA due to endogenous (or exogenous) nucleases and to maximize recovery. In many cases, it is advantageous to enrich for specific classes of RNAs such as messenger RNAs (mRNAs) or small RNAs either before or after purification. Solubilization is achieved by dissolving biological material in either ionic detergents (e.g., sodium dodecyl sulfate [SDS]) or chaotropic salts (e.g., guanidinium isothiocyanate). For soft tissues (e.g., liver, blood) and tissue culture cells, solubilization is accomplished simply by adding a solution of detergent or chaotropic salt. In other cases, e.g., bone, kidney, most plant cells, and other cells with rigid cell walls, it is necessary to disrupt the material enzymatically or mechanically before solubilization.

In this chapter, we first discuss widely applicable general methods for solubilization and extraction of RNA; we then discuss general methods for recovering extracted RNA. We show how these methods can be used to purify RNA from tissue culture cells, yeast, and bacteria, and subsequently outline a variety of methods designed to either enrich or deplete specific types of RNA once total RNA is purified. The strategies for RNA purification are outlined in Figure 2-1.

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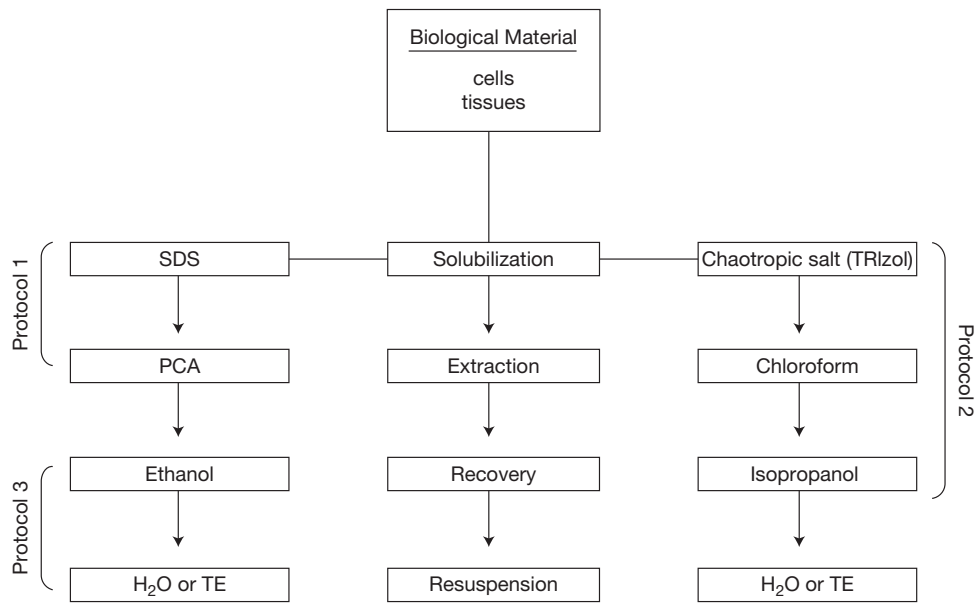


FIGURE 2-1. Flow chart outlining purification strategies.

WWW RESOURCES

http://www.ambion.com/techlib/append/rna_yields.html RNA yields from tissues and cells, technical resource appendix, Applied Biosystems.

<http://www1.qiagen.com/HB/RNeasyMini/> 2006. *RNeasy Mini Handbook*, 4th ed. QIAGEN.

Protocol 1

SDS Solubilization and Phenol Extraction

This method is of wide utility and is routinely used to deproteinize RNAs in biological material that has been solubilized in SDS. SDS (at least 0.25%) is an ionic detergent that dissolves membranes, disrupts protein–nucleic acid interactions, and inactivates ribonucleases. Once solubilized, addition of phenol (or phenol:chloroform:isoamyl alcohol [PCA] 25:24:1) completely denatures the protein, and it becomes insoluble in aqueous solution. Throughout the rest of the book, this method is referred to as PCA extraction. PCA extraction is the method of choice for preparing cytoplasmic RNA from tissue culture cells or in any other situation (e.g., enzyme reactions) where solubilization in SDS is easily achievable.

Note that SEVAG is 24:1 chloroform:isoamyl alcohol, which is often mixed 1:1 with TE-saturated neutralized phenol to give PCA (Sevag et al. 1938).

MATERIALS

The recipe for the item marked with <R> is at the end of the protocol or in the Common Recipes Appendix.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

Reagents

Biological material or sample containing RNA and protein (e.g., analytical reactions)

Chloroform <!>

Isoamyl alcohol <!>

Phenol (purchased), saturated and equilibrated <!>

Proteinase K (Roche) (10 mg/mL in 20 mM Tris, pH 7.5) <!>

SDS extraction buffer (10x) <R>

Sodium acetate (NaOAc) (3 M, pH 5.2, RNase-free)

Equipment

Centrifuge (large scale, Beckman J6B or equivalent) or microcentrifuge (small scale)

Freezer (–80°C) (optional; see Step 2)

Incubator or water bath (42°C) (optional; see Step 2)

Pipettes according to scale, e.g., Pipetman tips or baked 5-mL and 10-mL glass pipettes

Tubes (1.5-mL microcentrifuge or 12-mL polypropylene [Sarstedt] with lids)

Vortex mixer

OVERVIEW

Before beginning this protocol, prepare the PCA 25:24:1 solution, 10x SDS extraction buffer, 3 M NaOAc, and proteinase K. In addition, it is necessary to have the RNA-containing sample prepared and ready for solubilization and extraction. Such samples can be cytoplasmic extracts from tissue culture cells or “soft” tissues (see Protocol 5), analytical RNA-processing reactions, or enzyme reactions. It is never advisable to use SDS solubilization and phenol extraction to prepare RNA from material that contains large amounts of DNA (e.g., whole mammalian cells or mammalian cell nuclei). Such

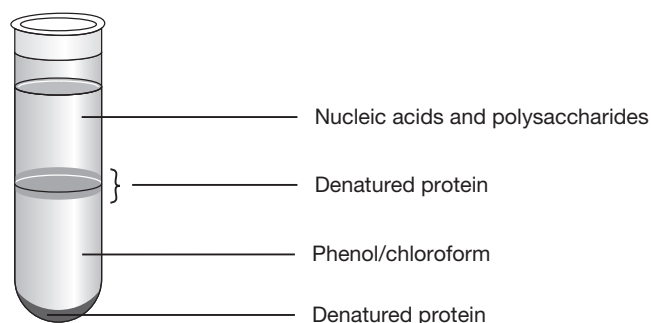


FIGURE 2-2. Phases formed after centrifugation in phenol extraction procedure.

samples become highly viscous when exposed to SDS. In these situations, we recommend using TRIzol (see Protocol 2).

In general, the aqueous RNA- and protein-containing solution will be brought up to 0.3 M NaOH, 0.5% SDS, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.5, and emulsified with the organic mixture. The SDS disrupts protein–nucleic acid interactions and, together with the chloroform and phenol, denatures the proteins so that they become insoluble in the aqueous solution. The charged nucleic acids will remain in the salty aqueous phase, whereas the proteins, with their aromatic and aliphatic side chains, will partition to the organic phase. Because phenol:chloroform is immiscible with water, centrifugation results in the formation of two phases: a lower organic phase that contains denatured proteins and an upper aqueous phase that contains nucleic acid (Fig. 2-2). The isoamyl alcohol facilitates the separation of the two phases. Removing the aqueous phase recovers the RNA from the protein, and a second organic extraction with chloroform removes the considerable amount (~6% by volume) of phenol that dissolves in the aqueous phase with the RNA and that can contaminate the RNA. When protein concentration is high (i.e., when cytoplasmic preparations are extracted), some denatured protein can form an insoluble gel at the organic–aqueous interface. Because this gel can trap some nucleic acid, it is recommended that samples containing high concentrations of proteins be predigested with proteinase K (this is one of the few enzymes that is active in SDS). Treatment with proteinase K greatly reduces or eliminates the interface. When appropriately performed, PCA extraction results in a quantitative yield of RNA from SDS-solubilized material. The procedure is outlined in the flow chart in Figure 2-3.

METHOD

For specific methods for processing of biological materials (e.g., cells and tissues) see Protocols 5, 7, and 8.

1. Starting with a solution of the RNA and protein to be processed, estimate the volume to be extracted (i.e., the total volume of the aqueous RNA solution should be $\geq 200 \mu\text{L}$; if not, increase to $200 \mu\text{L}$ with RNase-free water). Note this starting volume and whether the solution has a large amount of protein (as in a cell extract) or a small amount ($\leq 100 \mu\text{g/mL}$, as in an enzyme reaction or for RNA that has been partially purified already).
2. Add a 1/9 volume 10x SDS buffer to the solution of RNA and protein, e.g., cytoplasmic extract (see Protocol 5), to be extracted. (We recommend $200 \mu\text{L}$ as the minimum volume to be extracted.) Vortex and keep at room temperature for immediate extraction; otherwise, freeze at -80°C until extraction. If frozen, thaw quickly and proceed to next steps.
3. In all cases, except those with minimal protein concentration (e.g., enzyme reactions such as removing DNase or ligase), add proteinase K to a concentration of $100 \mu\text{g/mL}$ and incubate the

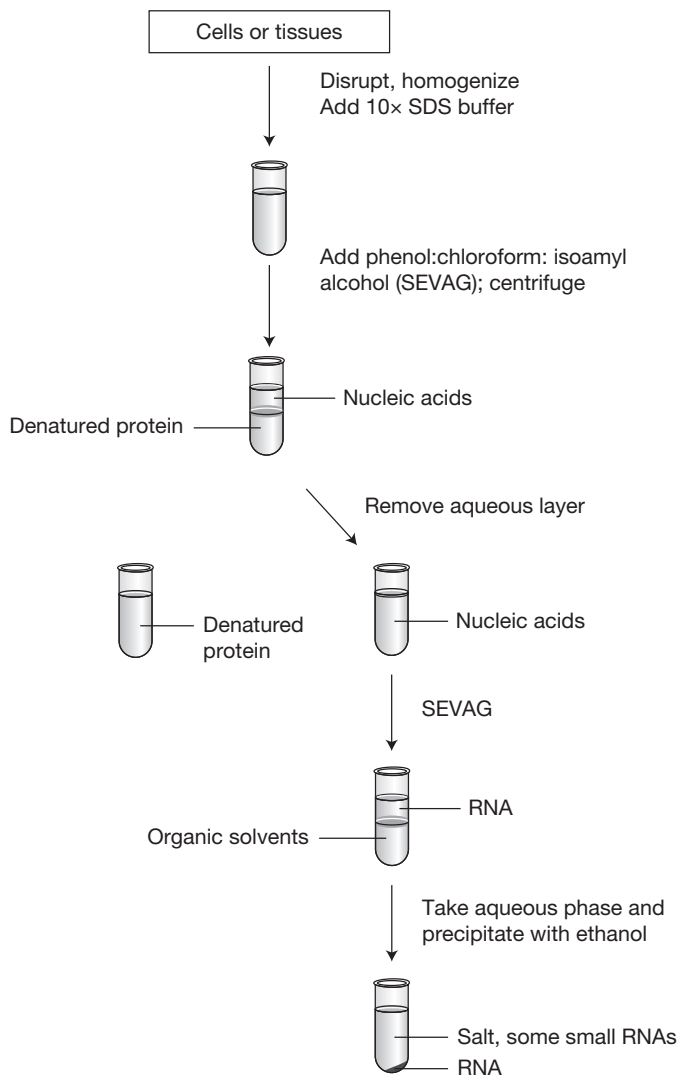


FIGURE 2-3. Flow chart for phenol extraction procedure.

mixture for 20 min at 42°C. If the protein concentration is negligible, skip this step and move to Step 4.

When in doubt regarding protein concentration, use proteinase K.

4. Make the solution 0.3 M NaOAc, pH 5.2. For every volume of solution to be extracted, add 2 volumes of PCA and vortex vigorously for 1 min. Centrifuge (maximum speed in a microcentrifuge for 2 min or at 2000 rpm in a J6B or clinical centrifuge for 5 min at room temperature) to separate the phases. The organic phase will be at the bottom (see Fig. 2-2). If protein is present, it will appear as a white precipitate at the interface between the phases.
5. Remove the aqueous (top) phase, which contains the RNA, to a fresh tube. Be careful not to disturb the interface and if necessary, leave a drop of the aqueous phase behind to avoid contamination of the RNA sample with phenol.
6. Add 2 volumes of chloroform:isoamyl alcohol without phenol to the aqueous phase. Vortex vigorously to remove residual phenol from the aqueous phase.
7. Centrifuge as previously in Step 4. Remove the aqueous phase for subsequent ethanol precipitation (see Protocol 3). We recommend that samples be ethanol precipitated immediately.

8. Resuspend RNA in H₂O, quantitate, and check quality on an agarose gel (see Protocol 13).

TIPS AND TROUBLESHOOTING

1. SDS and potassium salts (e.g., KCl or KOAc) are not compatible; this combination forms an insoluble potassium dodecyl sulfate precipitate. Accordingly, never use potassium salts ≥ 20 mM for phenol/SDS extraction.
2. SDS and chloroform can form a white emulsion that prevents phase separation. This occurs when the salt concentration is too low; ensure that the salt concentration in the aqueous solution to be extracted is at least 0.3 M.
3. After PCA extraction, the aqueous phase should be clear. If not, dilute it with an equal volume of 1× SDS buffer and repeat the extractions.

SOLUTION AND REAGENT RECIPE

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!.>.

SDS Buffer (10×)

Reagent	Quantity (for 500 mL)	Final concentration (10×)
SDS (10%) <!.>	250 mL	5%
EDTA (500 mM, pH 8.0) <!.>	10 mL	10 mM
Tris-HCl (1 M, pH 7.5) <!.>	100 mL	200 mM
H ₂ O	140 mL	

Store indefinitely at room temperature.

READING LIST

Sevag MG, Lackman DB, Smolens J. 1938. The isolation of the components of streptococcal nucleoproteins in serologically active form. *J Biol Chem* **124**: 425–436.

Protocol 13

Determining the Yield and Quality of Purified RNA

After any RNA purification by any method, it is necessary to ascertain both the amount (yield) and the integrity (quality) of the RNA obtained. This is essential for any subsequent analysis and critical for any comparative analyses.

METHODS FOR DETERMINING YIELD

Before attempting to use any of the following procedures, it is important to have a “ballpark” idea of what you expect the yield to be (see Table 2-1).

Determining Yield by Spectrophotometry or Fluorometry

The easiest way to determine the quantity of RNA in a sample is to measure the absorbance at 260 nm (A_{260}) using a spectrophotometer. Because the bases in RNA absorb UV light in the 250–265-nm range, one can use this property to quantitatively measure the concentration of an RNA solution, using an average absorbance for the four nucleotide bases. A solution of RNA at 40 $\mu\text{g}/\text{mL}$ will have an absorbance of ~ 1 . Accordingly, if 50 μL of the same solution is diluted in 1 mL of H_2O and read in a 1-mL cuvette, the absorbance will be 0.05; this is the absolute minimum that we recommend for accurate OD readings. The advent of nanospectrophotometers, such as the Thermo-Fisher NanoDrop and GE Healthcare NanoVue, has greatly increased the ease, sensitivity, and accuracy of determining the concentration of low-volume (microliters) samples by UV absorbance (see below). With this equipment, one can measure the A_{260} without dilution and with minimal waste of the sample. If you do not have access to these spectrophotometers, we recommend that OD be used as a measure of concentration, only when you have a significant amount of RNA present in a concentrated solution, e.g., at least 1.0 $\mu\text{g}/\mu\text{L}$. We have often observed students interpret A_{260} readings of 0.003 or less, but measurements as low as this are essentially meaningless.

Although measuring A_{260} is generally a reliable way to quantitate RNA concentrations, this method can be confounded if the RNA is contaminated with DNA, protein, or phenol, all of which absorb some UV light at 260 nm. A diagnostic for protein contamination is absorbance at 280 nm. Phenol and TRIzol Reagent both absorb at 270 nm and 230 nm. The chaotropic agents guanidine-HCl and guanidinium isothiocyanate, commonly used for RNA purification, absorb at ~ 230 nm and ~ 260 nm, respectively. For purified DNA, the $A_{260}:A_{280}$ ratio should be ~ 1.8 , and for purified RNA, this ratio should be ~ 2.0 , because the unpaired bases in RNA absorb more UV light than the base-paired bases in duplex DNA. However, these ratios are “rules of thumb” and assume an average base composition of the RNA sample, because different bases each have different $A_{260}:A_{280}$ ratios. If the $A_{260}:A_{280}$ ratio is < 2.0 , protein contamination is probable and reextraction with phenol is recommended. If the A_{260} is high, phenol contamination is probable and another round of ethanol precipitation and resuspension is recommended. Contamination with DNA is harder to detect because RNA and DNA essentially have identical absorbance spectra. Therefore, if the A_{260} of the sample is higher than expected, and protein and phenol contamination have been ruled out, the sample is likely contaminated with DNA. In this case, DNase treatment followed by phenol extraction and ethanol precipitation is recommended (see Protocols 1 and 3). In addition, it is possible to use the $A_{260}:A_{230}$ ratio as an indicator of nucleic acid purity, with ratios commonly in the range of 2.0–2.2. Note that absorbance is pH dependent, so for accurate readings, keep the pH constant and near 7.5.

UV Absorbance Determination of RNA Concentrations Using a Nanospectrophotometer

The development of small-volume (0.5–2 μL) UV-visible spectrophotometers can now be used to sensitively measure RNA or DNA concentrations and fluorochrome (e.g., Cy3/Cy5) dye coupling to allylamine-modified cDNA, or for protein concentration determination. These instruments (e.g., the Thermo-Fisher NanoDrop or GE Healthcare NanoVue nanospectrophotometers) use fiber optic technology and surface tension to hold a 1- μL sample in place; thus, they eliminate the need for traditional sample holding by cuvettes. The dynamic range of these instruments is high, ranging from 2 ng/ μL to 3700 ng/ μL . The following discussion provides practical information about the use of this equipment and interpretation of results. Step-by-step instructions for operation are supplied in the manufacturers' instrument manuals.

Procedure

This procedure is simple. Place 1.0–1.5 μL of the RNA sample onto the sample pedestal. The UV absorbance of the sample is then read either at a fixed wavelength or in a UV-visible scan. Typically, pure RNA (or DNA) samples are read at A_{260} and A_{280} . It is possible also to use these instruments to scan a full UV and visible wavelength absorbance spectrum, from 220 nm to 750 nm. The UV-visible-wavelength scanning procedure is more useful in microarray studies when one wants to quantify fluorochrome dye coupling to cDNA (see Chapter 8, Protocol 1). Figure 2-6 shows some results obtained using a nanospectrophotometer and how these results are interpreted.

TIPS AND TROUBLESHOOTING

1. As with the UV absorbance methods described previously, any compounds (e.g., free nucleotides, phenol, or other organic compounds such as guanidinium isothiocyanate) that absorb UV light near 260 nm will interfere. Often, a simple ethanol precipitation can be used to remove the contaminating substance.
2. When adding a sample to the pedestal, ensure that the sample is placed over the “eye” (the eye is the little metal circle with the tiny hole in the NanoDrop).
3. Bubbles are incompatible with accurate readings. Although the instructions may claim that only 1 μL is needed, in practice a larger volume (1.3–1.5 μL) will produce more reliable readings because the droplet will have better optical characteristics. Small-volume droplets can give incomplete coverage across the pedestal.
4. It is possible to saturate the spectrophotometer with high concentration solutions of RNA or DNA. This will cause an underestimate of the true concentration. Try to obtain readings using solutions at ≤ 2.5 $\mu\text{g}/\mu\text{L}$. For samples that give higher readings (2500 ng/ μL), it is a good idea to dilute 1:10 and read the dilution to get the most accurate reading.

Fluorescent Dye Binding for RNA and DNA Quantitation

As an alternative to spectrophotometry, RNA can be quantitated using fluorescent dye binding. This is a sensitive assay for detecting and determining the quantity of RNA (and contaminating DNA) present in a purified RNA sample or in crude extracts or chromatographic fractions. It is ~1000 times more sensitive than using UV absorbance and can detect RNA at 1 ng/mL. In addition, this method can be used for quantitation of *in vitro*-transcribed RNA samples or for determining RNA concentrations before northern blotting, RNase protection, RT-PCR, or cDNA library preparation. It is similar in concept to fluorescent dye binding to DNA (e.g., SYBR Green) used in quantitative PCR (qPCR) (see Chapter 4, Protocol 19). The RiboGreen RNA reagent, a proprietary fluorescent

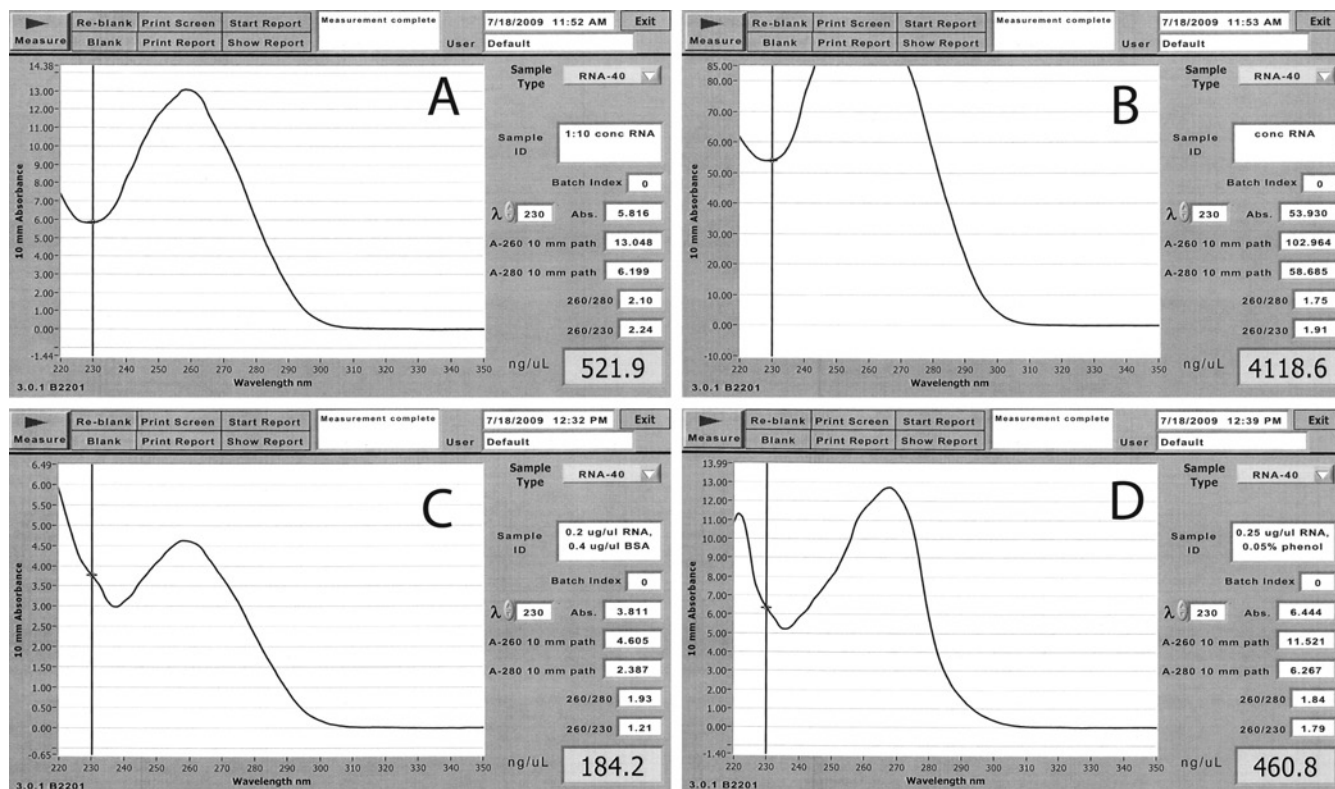


FIGURE 2-6. Using the microspectrophotometer (e.g., Nanodrop), according to the manufacturer's instructions, purified RNA was evaluated. (A) Purified yeast RNA was read at a concentration estimated at 0.5 μ g/ μ L. (B) Purified yeast RNA was read at a concentration to be 5 μ g/mL. Note that the detector has been saturated at this concentration and is underestimating the RNA content by 20%. (C) Mixture of yeast RNA and bovine serum albumin (BSA). Note the increased absorption at 220 nm due to the presence of peptide bonds; however, the increase in 280 is less noticeable, owing to the fact that BSA has relatively few tryptophans (3/607) or tyrosines (21/607). In this case, the peptide bond absorption at 220 is a better indicator (BSA has 606 peptide bonds/mole), as shown by the 260:230 ratio (230 is used because RNA has a local minimum at 230, whereas the peptide bond contributes at this wavelength; see A). The 260:230 ratio in the BSA-contaminated sample is 1.21, whereas it is 2.24 for the purified RNA. The 260:280 ratio has dropped from 2.1 for pure RNA to 1.9 in this sample. Thus, although this RNA passes the >1.8 test for 260:280, it should be rejected or reextracted on the basis of its 260:230 ratio. (D) Mixture of 250 ng/ μ L purified yeast RNA and 0.05% v/v of water-saturated phenol in water (monophasic). Note that absorption is at 270 $>$ 260 and that the RNA amount is overestimated. Phenol has a second peak near 224 that is obvious in this spectrum, and the RNA minimum at 230 has shifted up toward 240, which is a phenol minimum. Recognizing the presence of phenol in the spectrum by noting these features is more valuable than simply evaluating the 260:280 and 260:230 ratios, but it can be seen that these have dropped in comparison to the purified sample.

dye, preferentially binds to RNA, but it can also detect DNA (see the following Tips and Troubleshooting section). This method is useful when making RNA from nuclear fractions that might be contaminated with DNA and for assaying very small quantities of RNA prepared from limited quantities of starting material. Step-by-step instructions for using this reagent are supplied in the manufacturer's user manual.

TIPS AND TROUBLESHOOTING

1. If you suspect that the RNA sample of interest is contaminated with DNA (perhaps cellular genomic DNA in a total RNA preparation), you can DNase-I treat the sample (Protocol 11) or use PicoGreen, which detects double-stranded DNA only.

2. The assay remains linear in the presence of several compounds that commonly contaminate nucleic acid preparations, although the signal intensity may be affected. Thus, to serve as an effective control, treat the RNA solution used to prepare the standard curve the same way as the experimental samples; it should contain similar levels of such compounds.
3. There can be some interference with the fluorescence assay by salts, organic solvents, detergents, proteins, or other compounds. If these are present in the sample, control for this by placing them in the standard curve RNA dilutions.

Determining Yield by Gel Electrophoresis

For samples of total or cytoplasmic RNA (with rRNA), a simple and straightforward way to determine yield is to separate a small aliquot of the RNA on an agarose gel and stain with ethidium bromide or SYBR Gold (see Protocol 12). Bands of rRNA (28S and 18S) are visualized, and their intensity is compared to that of a preparation of known quantity. In our laboratories, we keep a “stock” of high-quality cytoplasmic RNA prepared from tissue culture cells as a reference. This stock can be diluted as appropriate to obtain the equivalent amount of RNA that you expect from the sample. Because SYBR Gold is 10-fold more sensitive than ethidium bromide, it should be used for small amounts of RNA.

An advantage of this technique is that it measures both the quantity and the quality of RNA; i.e., sharp and distinct rRNA bands without a pronounced haze below them is a good sign that the preparation is not significantly degraded. The only disadvantage of this approach is sensitivity; at least 500 ng of RNA must be available to sacrifice for this assessment. To assess quality, sufficient quantity must be loaded to detect degraded material.

Use of Rapid Capillary Electrophoresis on an Agilent Bioanalyzer for RNA Sample Quality Control

A bioanalyzer provides a convenient and more sensitive way to assess RNA quality. The Agilent 2100 Bioanalyzer basically performs small-volume electrophoresis, similar to capillary DNA sequencing but in a “chip” format that allows analysis of 12 samples at once. The bioanalyzer can be used for analysis of the quality of total RNA preparations by visualizing the rRNA bands and intact mRNA, and to detect and quantify RT-PCR products (using DNA chips; see Klinck et al. 2008; Venables et al. 2008, 2009 in the Reading List at the end of this protocol). Agilent RNA kits, which are designed for use with the Agilent 2100 Bioanalyzer only, contain chips and reagents designed for analysis of RNAs. Each RNA chip contains an interconnected set of microchannels (capillaries) that is used for separation of nucleic acid fragments based on their size as they are driven through the microchannels electrophoretically. The following discussion provides practical information about the use of this equipment and interpretation of results obtained running RNA samples on a bioanalyzer. Step-by-step instructions for operation of this equipment are supplied in the manufacturer’s user manuals.

A computer runs the machine, analyzes the traces, and presents the data. Of interest is the trace that estimates the amount of each rRNA that is present and their ratios. Because the larger rRNA is more susceptible to degradation because of its greater length, the ratio of 28S to 18S rRNA is a convenient measure of the integrity of the sample. This information is displayed by the software. The amount of RNA in the sample is determined by comparison to a fixed known amount of the RNA ladder RNAs. Thus, it is possible to obtain both concentration and integrity of the sample. Because the data are simple to archive and compare, many laboratories that handle and compare many samples believe that this approach is superior to gel analysis, primarily because of its data archiving and comparison ability.

Expected Results of Bioanalyzer Analysis of RNA Quality

The quality of purified total RNA can be analyzed using a bioanalyzer, such as the Agilent 2100. In Figure 2-7, this bioanalyzer was used to show the presence of 18S and 28S rRNA (mammalian), 18S

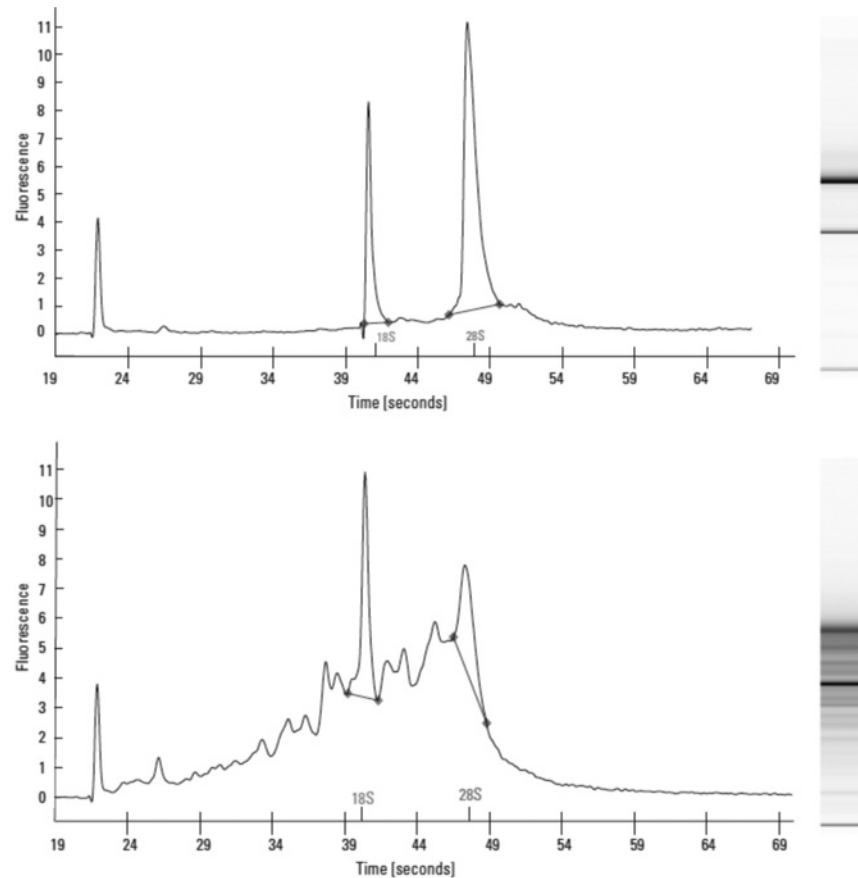


FIGURE 2-7. Analysis of intact and degraded total RNA. Electropherograms and gel-like images of intact total RNA (*top*) and partially degraded total RNA (*bottom*). Partially degraded RNA shows a decrease in the 18S/28S ribosomal band ratio and an increase in fragmentation products. The RNA ladder markers give discrete peaks following electrophoresis and the instrument software can generate a “virtual gel” pattern (*right*). (Reprinted, with permission, from <http://www.chem.agilent.com> [publication 5988-7650EN. ©Agilent Technologies 2002].)

and 25S/26S rRNA (yeast), 16S and 23S rRNA (bacteria), and small RNA species in total RNA. Typically, for total RNA samples, two major rRNA peaks are observed (because these account for >90% of the total RNA in cells: for eukaryotic total RNA, the two rRNA peaks correspond to the 18S and 28S rRNAs, for yeast total RNA the two large rRNA peaks correspond to the 18S and 25S/26S rRNAs, for bacterial total RNA, the two large rRNA peaks correspond to the 16S and 23S rRNAs) (see Fig. 2-7).

TIPS AND TROUBLESHOOTING

1. Sample preparation for the RNA Nano Chip:
 - i. Ideally, sample concentrations should be 100–200 ng/μL; RNA concentrations as low as 50 ng/μL can be used.
 - ii. Each sample well must contain a total of 6 μL (1 μL for the RNA Pico Chip).
 - iii. The nano marker must be placed in every sample well and the ladder well.
 - iv. Add water or nano marker to unused wells to bring the volume up to 6 μL.

- v. Use the chip within 5 min of preparation to prevent evaporation. Cover the chip with plastic wrap or parafilm if it will be left standing for any length of time.
 - vi. RNA samples may be denatured to remove secondary structure. Denature for 2 min at 70°C before placing the samples in the wells of the chip.
 - vii. Genomic DNA can produce stray bands or clog the capillaries in the chip. To check for genomic DNA contamination, treat the samples with DNase I. Run a DNase-I-treated sample next to an untreated sample.
2. It is important not to leave the used chip in the bioanalyzer after the run is complete because this will dry out the electrodes and make them difficult to clean.
 3. A critical part of the assay is preparing the chip. This involves loading the capillaries with the “gel” matrix material and the dyes that will stain the RNA so that the detector in the machine can measure it. The chip is loaded with a syringe system that is fairly easy to use. Fill each well (make sure that there are no bubbles!), add the sample, vortex, and load into the machine according to the instructions.
 4. Because this protocol uses small-volume electrophoresis, the samples must be in a low-ionic-strength solution (preferably in RNase-free water or 10 mM TE buffer).
 5. Accurate pipetting is very important for reproducible results. Make sure to use properly calibrated pipettes and to place the tip into the center and bottom of each well in the chip when dispensing. To avoid bubbles, do not push past the first resistance point on the pipette. You may pipette up and down gently to mix samples in the wells of the chip.
 6. Protect the gel-dye mix from light by covering the tube with foil. Return the reagents to the cold room when you are finished.
 7. When using the priming station, press down slowly and steadily on the plunger when priming. After releasing, the plunger should come up to at least 0.7 mL in 1–2 sec. If this does not occur, check that the gasket is clean and retry. If it still does not prime well, change the gasket (see Agilent 2100 instrument manual).

Determining Yield by Quantitative or Semiquantitative PCR

When it is not possible to quantitate the amount of RNA in a preparation by any of the methods previously described (e.g., when the amount of expected RNA is very small), reverse transcription followed by qPCR or semi-qPCR is recommended (see Chapter 3, Protocols 18 and 19). These techniques are extremely sensitive and provide information with regard to both the quantity and quality of RNA. In addition, these approaches allow a determination of whether the sample is significantly contaminated with DNA. We recommend assaying a housekeeping mRNA (e.g., actin, glyceraldehyde 3-phosphate dehydrogenase [GAPDH], tubulin, etc.). Again, as with gel electrophoresis, we recommend using a “stock” RNA preparation (diluted as appropriate) as a reference. Parallel assessment with and without reverse transcription will show any DNA contamination (i.e., amplification without reverse transcription indicates the presence of DNA, unless the PCR primer pairs are in different exons separated by a large intron; in this case, the genomic DNA contamination product will be too large to show up). For RNA preparations in which large RNAs have been removed, it is necessary to assay a small RNA (e.g., a constitutively expressed miRNA) by RT-PCR.

All of the techniques described above are useful methods for assessing the quantity of RNA that has been prepared; gel electrophoresis and qPCR or semi-qPCR also provide information regarding quality. Common sense indicates that if the yield is far less ($\geq 30\%$) than expected, something has gone wrong with the preparation and the RNA is suspect. Rather than proceeding with a suspect preparation, start over from the beginning.

NORTHERN BLOTTING TO ASSESS THE QUALITY OF RNA PREPARATIONS

As discussed previously, gel electrophoresis, visualization of RNA bands, and qPCR or semi-qPCR (in all cases compared to a reference) are valuable methods for assessing quantity as well as quality of RNA. Nevertheless, each of these methods can be potentially misleading. PCR will amplify fragments of RNA, and rRNA visualization is a somewhat crude method for assessing integrity of an RNA preparation. Moreover, spectrophotometry tells nothing about the integrity of the RNA preparation. By far, the best method for assessing the quality of an RNA preparation is northern blotting (see Chapter 3, Protocols 7–10). Because this technique visualizes the entire RNA, it is diagnostic for any degradation. Therefore, if you are working with specific RNAs, we recommend assaying each preparation by northern blotting. Again, this is best done by comparing the quality (i.e., sharp band) and quantity of the signal obtained to those obtained from a reference northern blot performed on RNA of known quantity and quality.

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