Absolutely RNA Miniprep Kit

INSTRUCTION MANUAL

Catalog #400800
Revision A

For In Vitro Use Only
400800-12

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Absolutely RNA Miniprep Kit

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Absolutely RNA Miniprep Kit

MATERIALS PROVIDED

Material provided	Quantity[a]	Storage conditions
Lysis Buffer	35 ml	Room temperature
β-Mercaptoethanol (β-ME) (14.2 M)	0.3 ml	Room temperature[b]
RNase-free DNase I (lyophilized)	2600 U	Room temperature[c]
DNase Reconstitution Buffer	0.3 ml	Room temperature
DNase Digestion Buffer	2 × 1.5 ml	Room temperature
High-Salt Wash Buffer (1.67×)	24 ml	Room temperature
Low-Salt Wash Buffer (5×)	17 ml	Room temperature
Elution Buffer[d]	12 ml	Room temperature
Prefilter Spin Cups (blue) and 2-ml receptacle tubes	50	Room temperature
RNA Binding Spin Cups and 2-ml receptacle tubes	50	Room temperature
1.5-ml microcentrifuge tubes	50	Room temperature

[a] Sufficient reagents are provided to isolate total RNA from 50 samples of 40 mg of tissue or 1×10^7 cells.

[b] Once opened, store at 4°C.

[c] Once reconstituted, store at –20°C.

[d] 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA.

STORAGE CONDITIONS

All Components: Room Temperature

Caution *Guanidine thiocyanate in the lysis buffer and high-salt wash buffer is an irritant.*

ADDITIONAL MATERIALS REQUIRED

Diethylpyrocarbonate (DEPC)
Ethanol [100% and 70% (v/v)]
Homogenizer

Revision A

INTRODUCTION

The Absolutely RNA miniprep kit provides a rapid method for purification of high-quality total RNA from small samples of tissue or cultured cells. Extreme purity of RNA is critical because contaminating DNA present in the RNA sample can give rise to amplification products that mimic the amplification product expected from the RNA target. The Absolutely RNA miniprep kit eliminates these problems by providing high yields of total RNA with undetectable levels of DNA from animal tissues and cultured cells. This simple and effective method of RNA purification eliminates toxic phenol–chloroform extractions and time-consuming ethanol precipitations used in other RNA purification methods.

The Absolutely RNA method employs a spin cup with a matrix that binds RNA in the presence of a chaotropic salt while a series of washes removes contaminants. The lysis buffer contains guanidine thiocyanate, one of the strongest protein denaturants, to lyse the cells and to prevent RNA degradation by ribonucleases (RNases). Following cell lysis, the sample is prefiltered in a spin cup to remove particles and to reduce the amount of DNA. The filtrate is then transferred to an RNA-binding spin. Treatment with a low-salt wash buffer and digestion with DNase removes the remaining DNA. A series of washes removes the DNase and other proteins. Highly pure RNA is eluted from the spin cup matrix with a small volume of low-ionic-strength buffer and captured in a microcentrifuge tube. The highly pure RNA is ideal for conventional RT-PCR and real time quantitative RT-PCR and is suitable for cDNA synthesis, RT-PCR, northern blotting, RNase protection assay, and primer extension analysis.

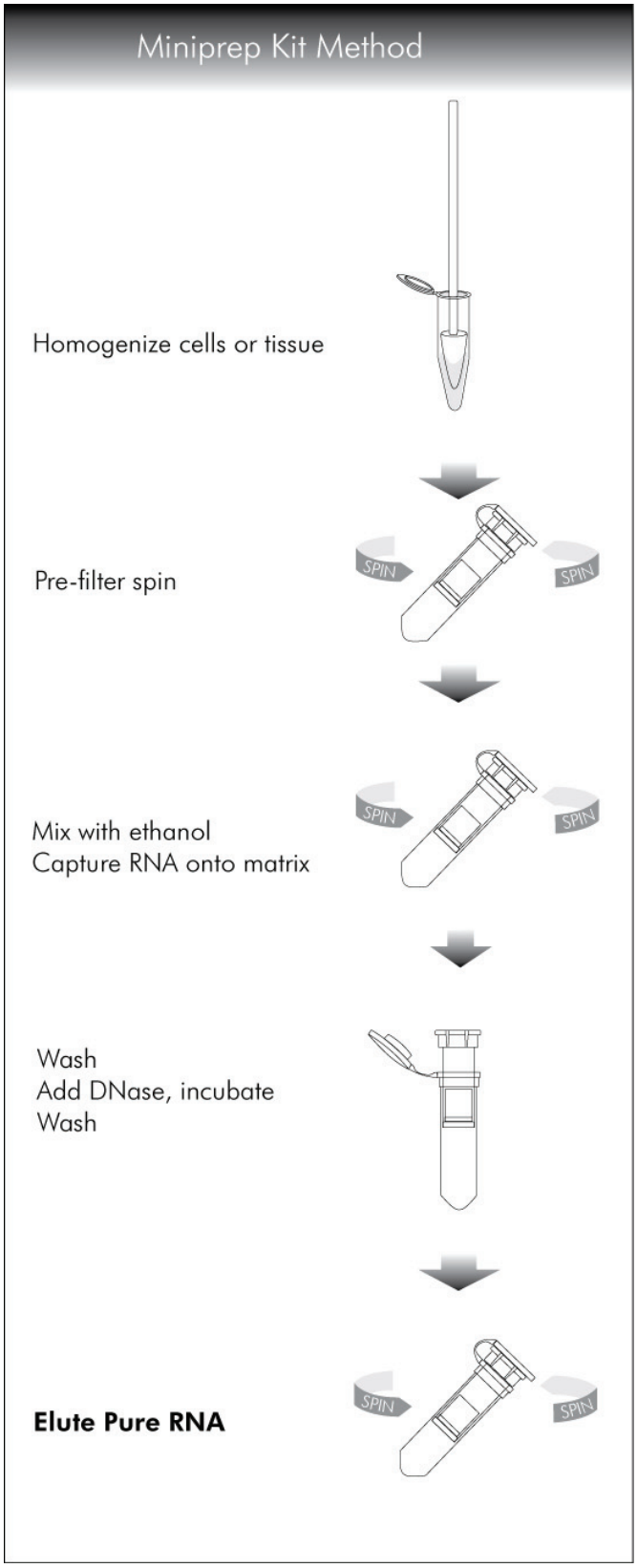

FIGURE 1 Absolutely RNA miniprep kit method.

PREVENTING SAMPLE CONTAMINATION

Preventing RNase Contamination

Ribonucleases are very stable enzymes that hydrolyze RNA. RNase A can be temporarily denatured under extreme conditions, but it readily renatures. RNase A can therefore survive autoclaving and other standard methods of protein inactivation. The following precautions can prevent RNase contamination:

- **Wear gloves at all times** during the procedures and while handling materials and equipment**,** as RNases are present in the oils of the skin.

- Exercise care to ensure that all equipment (e.g., the homogenizer, centrifuge tubes, etc.) is as free as possible from contaminating RNases. Avoid using equipment or areas that have been exposed to RNases. Use sterile tubes and micropipet tips only.

- Micropipettor bores can be a source of RNase contamination, since material accidentally drawn into the pipet or produced by gasket abrasion can fall into RNA solutions during pipetting. Clean micropipettors according to the manufacturer's recommendations. We recommend rinsing both the interior and exterior of the micropipet shaft with 70% ethanol or 70% methanol.

Sterilizing Labware

Disposable Plasticware

Disposable sterile plasticware is generally free of RNases. If disposable sterile plasticware is unavailable, components such as microcentrifuge tubes can be sterilized and treated with diethylpyrocarbonate (DEPC), which chemically modifies and inactivates enzymes, according to the following protocol:

Caution *DEPC is toxic and extremely reactive. Always use DEPC in a fume hood.* ***Read and follow the manufacturer's safety instructions.***

1. Add DEPC to deionized water to a final DEPC concentration of 0.1% (v/v) and mix thoroughly.

2. Place the plasticware to be treated into a separate autoclavable container. Carefully pour the DEPC-treated water into the container until the plasticware is submerged.

3. Leave the container and the beaker used to prepare DEPC-treated water in a fume hood overnight.

4. For disposal, pour the DEPC-treated water from the plasticware into another container with a lid. Autoclave the bottle of waste DEPC-treated water and the container with the plasticware for at least 30 minutes. Aluminum foil may be used to cover the container, but it should be handled with gloves and cut from an area untouched by ungloved hands.

Nondisposable Plasticware

Remove RNases from nondisposable plasticware with a chloroform rinse. Before using the plasticware, allow the chloroform to evaporate in a hood or rinse the plasticware with DEPC-treated water.

Electrophoresis Gel Boxes

To inactivate RNases on electrophoresis gel boxes, treat the gel boxes with 3% (v/v) hydrogen peroxide for 10–15 minutes and then rinse them with RNase-free water.

Glassware or Metal

To inactivate RNases on glassware or metal, bake the glassware or metal for a minimum of 8 hours at 180°C.

Treating Solutions with DEPC

Treat water and solutions (except those containing Tris base) with 0.1% (v/v) DEPC in distilled water. During preparation, mix the 0.1% DEPC solution thoroughly, allow it to incubate overnight at room temperature, and then autoclave it prior to use. If a solution contains Tris base, prepare the solution with autoclaved DEPC-treated water.

Preventing Nucleic Acid Contamination

If the isolated RNA will be used to synthesize cDNA for cDNA library construction or PCR amplification, it is important to remove any residual nucleic acids from equipment that was used for previous nucleic acid isolation.

PREPARING THE REAGENTS

RNase-Free DNase I

Reconstitute the lyophilized RNase-Free DNase I by adding 290 µl of DNase Reconstitution Buffer to the vial. Mix the contents gently but thoroughly to ensure all the powder (including powder on the cap) dissolves into solution. Do not introduce air bubbles into the solution. Store the reconstituted RNase-Free DNase I at –20°C.

Note *DNase Reconstitution Buffer is easily added to the vial of DNase with a syringe and needle or by carefully removing the cap and adding buffer with a pipettor. Gentle mixing is necessary because the DNase I is very sensitive to denaturation.*

High-Salt Wash Buffer

- Prepare 1× High-Salt Wash Buffer by adding 16 ml of 100% ethanol to the bottle of 1.67× High-Salt Wash Buffer.
- After adding the ethanol, mark the container as follows: *[√] 1× (Ethanol Added).*
- Store the 1× High-Salt Wash Buffer at room temperature.

Low-Salt Wash Buffer

- Prepare 1× Low-Salt Wash Buffer by adding 68 ml of 100% ethanol to the bottle of 5× Low-Salt Wash Buffer.
- After adding the ethanol, mark the container as follows: *[√] 1× (Ethanol Added).*
- Store the 1× Low-Salt Wash Buffer at room temperature.

70% Ethanol

Prepare 70% (v/v) ethanol by diluting 95% or 100% ethanol with RNase-free water. To prepare 100 ml of 70% ethanol, for example, add sufficient water to 70 ml of 100% ethanol or 74 ml of 95% ethanol to make a final volume of 100 ml.

β-Mercaptoethanol

Once opened, store the β-ME at 4°C.

PREPROTOCOL CONSIDERATIONS

RNA and RNase Levels in Different Tissues

The amount of RNA available for isolation varies with tissue type (see Table I). Liver and kidney cells are metabolically active and produce relatively large amounts of RNA. Structural cells and fat cells tend to have less RNA. The amount of RNases in different tissue types also varies. The pancreas and spleen are very rich in RNases and should be flash-frozen in liquid nitrogen as quickly as possible after dissection.

Sample Size and the RNA Isolation Protocol

The *RNA Isolation Protocol* is optimized for 20–40 mg of tissue or 1×10^6–1×10^7 tissue culture cells. RNA yields will vary, depending on tissue and cell type. Anticipated yields from various tissues are shown below (see Table I). Yields of 5–25 µg RNA can be expected from 1×10^6 cultured cells.

The binding capacity of the spin cup can accommodate approximately 200µg of RNA. To optimize the protocol for smaller samples (5–20 mg of tissue or 1×10^4–1×10^6 tissue culture cells), modify the protocol as indicated in *Appendix I: Protocol Modifications for Small Samples*. RNA can be isolated from small samples without using the protocol modifications, but the RNA yield may be lower.

Table I

Anticipated RNA Yield

Source	Average yield/mg
Rodent liver	3 µg
Rodent brain	0.7 µg
Rodent kidney	2 µg
Rodent spleen	3 µg
Rodent testis	2 µg

RNA ISOLATION PROTOCOLS

Preparing Animal Tissue

Note *This protocol is optimized for tissue samples of 20–40 mg. For samples of 5–20 mg, see* Preprotocol Considerations: Sample Size and the RNA Isolation Protocol *and* Appendix I: Protocol Modifications for Small Samples.

1. Flash-freeze the tissue in liquid nitrogen immediately upon dissection from the organism to minimize RNA degradation.

   Note *Flash-frozen tissue can be stored at –80°C.*

2. Once flash-frozen, fracture the tissue, quickly weigh a sample, and return the tissue to liquid nitrogen. Keep the sample frozen in liquid nitrogen or on dry ice until ready for cell lysis.

3. Add 7 µl of β-ME to the Lysis Buffer for each milliliter of Lysis Buffer required:

Tissue sample	Lysis Buffer	β-ME
5–20 mg	100–250 µl	0.7–1.75 µl
20–25 mg	350 µl	2.5 µl
25–40 mg	600 µl	4.2 µl

   Caution *The Lysis Buffer contains the irritant guanidine thiocyanate.*

   Notes *The Lysis Buffer–β-ME mixture must be prepared fresh for each use.*

   If the sample is greater than 40 mg, use the appropriate amount of Lysis Buffer to yield a concentration of 0.07 mg/µl.

4. Place the tissue sample in a tube containing the Lysis Buffer–β-ME mixture. Homogenize the tissue using a rotating blade homogenizer (such as Polytron® homogenizer), a mini-homogenizer (such as Kontes Pellet Pestle), or a micro-Dounce homogenizer. Ensure that the instrument is RNase-free.

   Note *If frozen promptly, the homogenate can be stored at –80°C for future use.*

5. Proceed with the protocol in *Isolating RNA.*

Preparing Adherent Tissue Culture Cells

Note *Adherent cells can either be lysed in the tissue culture dish using this protocol or treated with trypsin to detach them from the dish and then lysed using the protocol in* Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells. *Efficient collection of the cell lysate from cells lysed in the dish may be difficult, due to the viscosity of the solution.*

For a 100-mm dish of confluent cells, it is preferable to trypsinize the cells and use the protocol in Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells. *Confluent cells in a 100-mm dish are difficult to lyse over such a large area, and the lysate has a high viscosity.*

This protocol is optimized for samples of 1 × 10^6–1 × 10^7 tissue culture cells in a 35- or 60-mm tissue culture dish. For samples of 1 × 10^4–1 × 10^6 cells, see Preprotocol Considerations: Sample Size and the RNA Isolation Protocol *and* Appendix I: Protocol Modifications for Small Samples. *See Table II for volumes of Lysis Buffer and β-ME to use.*

1. Add 4.2 µl of β-ME to 600 µl of Lysis Buffer.

   Caution *The Lysis Buffer contains the irritant guanidine thiocyanate.*

   Note *The Lysis Buffer–β-ME mixture must be prepared fresh for each use.*

2. Aspirate the medium from the tissue culture dish, tilting the dish to remove any residual medium.

3. Add the Lysis Buffer–β-ME mixture to the dish of cells and spread evenly over the surface of the dish.

TABLE II

Plasticware	Lysis Buffer[a]	β-ME[a]
60-mm dish	600 µl/dish	4.2 µl
35-mm dish	350–600 µl/dish	2.5–4.2 µl
6-well plate	350–600 µl/well	2.5–4.2 µl
12-well plate	350 µl/well	2.5 µl
24-well plate	200 µl/well	1.4 µl
96-well plate	100 µl/well	0.7 µl

[a] Volumes of reagents are independent of the cell density.

4. Mix and collect the cell lysate by repeated pipetting and transfer the lysate to a microcentrifuge tube. Vortex the tube to homogenize the lysate.

   Note *Ensure that the viscosity of the lysate is low. High viscosity causes a decrease in RNA yield and an increase in DNA contamination. The viscosity can be reduced by additional pipetting, vortexing, increasing the volume of Lysis Buffer and/or passing the lysate through an 18–21 gauge syringe needle.*

5. Proceed with the protocol in *Isolating RNA*.

Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells

Note *This protocol is optimized for samples of 1 × 10^6–1 × 10^7 tissue culture cells. For samples of 1 × 10^4–1 × 10^6 cells, see* Preprotocol Considerations: Sample Size and the RNA Isolation Protocol *and* Appendix I: Protocol Modifications for Small Samples.

1. Add 7 µl of β-ME to the Lysis Buffer for each milliliter of Lysis Buffer required:

Cell number	Lysis Buffer	β-ME
1 × 10^4–1 × 10^5	100 µl	0.7 µl
1 × 10^5–1 × 10^6	200 µl	1.4 µl
1 × 10^6–5 × 10^6	350 µl	2.5 µl
5 × 10^6–1 × 10^7	600 µl	4.2 µl

Caution *The Lysis Buffer contains the irritant guanidine thiocyanate.*

Note *The Lysis Buffer–β-ME mixture must be prepared fresh for each use.*

2. Centrifuge the cells at 1000 × *g* for 5 minutes.

3. Aspirate most of the supernatant. Resuspend the cells in the residual supernatant and transfer the cell suspension to a microcentrifuge tube.

4 Collect the cells into a loose pellet by spinning the tube in a microcentrifuge at low speed (~3000 rpm) for 5 minutes. Discard all the supernatant.

Note *Cell pellets can be stored at –80°C for future processing, although homogenization in Lysis Buffer prior to freezing is recommended to minimize RNA degradation.*

5. Add the appropriate amount of Lysis Buffer–β-ME mixture to the cell pellet and homogenize the sample by vortexing or repeated pipetting. Ensure that the viscosity of the lysate is low.

Note *Although lysis volumes are given for a specific number of cells, individual cell mass can vary significantly. Generally, fibroblasts and carcinoma cell lines have a greater cell mass than cells that grow in suspension. If the cell mass in the homogenate is too large, the high viscosity causes a decrease in RNA yield and an increase in DNA contamination. If vortexing or repeated pipetting has not decreased the viscosity, the viscosity can be reduced by increasing the volume of Lysis Buffer and/or passing the lysate through an 18–21 gauge syringe needle.*

6. Proceed with the protocol in *Isolating RNA*.

Isolating RNA

Note *Two types of spin cups are provided: Prefilter Spin Cups (blue) and RNA Binding Spin Cups.*

1. Transfer up to 700 µl of homogenate to a Prefilter Spin Cup that is seated in a 2-ml receptacle tube and snap the cap of the receptacle tube onto the spin cup.

2. Spin the tube in a microcentrifuge at maximum speed for 5 minutes.

3. Remove the spin cup from the receptacle tube and discard it. **Retain the filtrate**. Vigorously flex the hinge of the spin cup, prior to closing it, so that it becomes flexible and the cap can be firmly seated in the tube, to reduce the chance of leakage during vortexing.

4. Add an equal volume of 70% ethanol to the filtrate and vortex the tube for 5 seconds or until the filtrate and ethanol are mixed thoroughly.

5. Transfer up to 700 µl of this mixture to an RNA Binding Spin Cup that is seated in a fresh 2-ml receptacle tube and cap the spin cup. Vigorously flex the hinge of the spin cup, prior to closing it, so that it becomes flexible and the cap can be firmly seated in the tube, to reduce the chance of leakage during vortexing.

6. Spin the mixture in a microcentrifuge at maximum speed for 30–60 seconds.

7. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube. For samples homogenized in >350 µl of Lysis Buffer, repeat steps 5–7 with the remaining mixture.

   Note *The RNA was protected in previous steps from RNases by the presence of guanidine thiocyanate.*

8. ***DNase Treatment*** This procedure is recommended for RT-PCR applications.

    a. Add 600 µl of 1× Low-Salt Wash Buffer and snap the cap of the receptacle tube onto the spin cup. Spin the sample in a microcentrifuge at maximum speed for 30–60 seconds.

    b. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 2 minutes.

    c. Prepare the DNase solution by gently mixing 50 µl of DNase Digestion Buffer with 5 µl of reconstituted RNase-Free DNase I.

        Note *Gentle mixing is necessary because the DNase I is very sensitive to denaturation.*

    d. Add the DNase solution directly onto the matrix inside the spin cup and cap the spin cup.

    e. Incubate the sample at 37°C for 15 minutes in an air incubator.

9. Add 600 µl of 1× High-Salt Wash Buffer to the spin cup and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 30–60 seconds.

    Caution *The High-Salt Wash Buffer contains the irritant guanidine thiocyanate.*

10. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.

11. Add 600 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 30–60 seconds.

12. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.

13. Add 300 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 2 minutes to dry the matrix.

14. Transfer the spin cup to a 1.5-ml microcentrifuge tube and discard the 2-ml receptacle tube.

15. Add 30–100 µl of Elution Buffer directly onto the center of the matrix inside the spin cup and cap the spin cup. Incubate the tube for 2 minutes at room temperature. Spin the tube in a microcentrifuge at maximum speed for 1 minute. Repeat this elution step to maximize the yield of RNA.

    Notes *The Elution Buffer must be added directly onto the matrix of the spin cup to ensure that the Elution Buffer permeates the entire matrix.*

    The RNA yield can be increased by using Elution Buffer warmed to 60°C.

    The purified RNA is in the Elution Buffer in the microcentrifuge tube. The RNA can be stored at –20°C for up to one month or at –80°C for long-term storage.

Quantitating the RNA

To quantitate the RNA, remove a small sample and dilute it with a buffer of neutral pH (e.g., 10 mM Tris, pH 7.5). Measure the optical density (OD) at 260 nm and 280 nm to quantitate and qualify the RNA (see *Appendix III: Spectrophotometric Quantification of RNA*). Yields may vary, depending on tissue and cell type. Yields of 5–25 µg RNA can be expected from 1×10^6 cultured cells. Anticipated yields from homogenized tissue are shown in Table I (see *Preprotocol Considerations*).

TROUBLESHOOTING

Observation	Suggestion
Prefilter Spin Cup clogs	Centrifuge the homogenate for an additional 5–10 minutes
	Dilute the homogenate with additional Lysis Buffer
RNA is degraded	Use DEPC-treated or radiation-sterilized plasticware
	Flash-freeze the tissue immediately upon dissection from the animal
RNA yield is poor	Pass the homogenate through an 18–21 gauge needle several times to reduce the viscosity
	Dilute the homogenate with additional Lysis Buffer
	Use a smaller amount of tissue or cells
	Use a rotating blade homogenizer, such as a Polytron homogenizer, that will grind the tissue samples thoroughly
	Use buffer or water at neutral pH (pH7–8) for efficient RNA elution
	Use 30–100 µl of Elution Buffer
	Perform the elution twice
	Incubate the tube for 2 full minutes after adding the Elution Buffer
	For small samples (5–20 mg of tissue or 1×10^4–1×10^6 tissue culture cells), see *Appendix I: Protocol Modifications for Small Samples*
	Increase the volume of Lysis Buffer used for homogenization
Final RNA concentration is too low for use in subsequent applications	Concentrate the RNA under vacuum without heat
	Use a smaller volume of Elution Buffer
DNA contamination	Dilute the homogenate to reduce the viscosity of the lysate before proceeding
	DNase is sensitive to denaturation. Ensure that the DNase is mixed **gently** during resuspension and that the DNase has been properly resuspended and stored (see *Preparing the Reagents*)
Gel of electrophoresed PCR products has excessive background bands or the bands are smeared	Use less RNA in the cDNA synthesis reaction. When 1 µg of total RNA is converted into cDNA in a 50-µl reaction, 1/5 of the cDNA synthesis reaction is enough for extremely rare messages to be amplified

APPENDIX I: PROTOCOL MODIFICATIONS FOR SMALL SAMPLES

These modifications of the *RNA Isolation Protocol* optimize the protocol for small samples: 5–20 mg of tissue or 1×10^4–1×10^6 tissue culture cells.

Extremely Small Samples

For the highest RNA yield from samples of 5–10 mg of tissue or 1×10^4–5×10^5 tissue culture cells, modify the *RNA Isolation Protocol* as follows: Decrease the volume of Lysis Buffer–β-ME mixture used for homogenization to 100–250 µl and omit the prefiltration steps (steps 1–3 in *Isolating RNA*). (Omitting the prefiltration steps may result in a small amount of detectable DNA contamination.)

Small Samples

For the highest RNA yield from samples of 10–20 mg of tissue or 5×10^5–1×10^6 tissue culture cells, modify the *RNA Isolation Protocol* as follows:

Preparing Small Samples

Decrease the volume of Lysis Buffer–β-ME mixture used for homogenization (add 7 µl of β-ME to the Lysis Buffer for each milliliter of Lysis Buffer required):

Sample size	Lysis buffer	β-ME
5–20 mg of tissue	100–250 µl	0.7–1.75 µl
1×10^4–1×10^5 tissue culture cells	100 µl	0.7 µl
1×10^5–1×10^6 tissue culture cells	200 µl	1.4 µl

Isolating RNA from Small Samples

Begin the protocol in *Isolating RNA* as follows:

1. Transfer the homogenate to a Prefilter Spin Cup that is seated in a 2-ml receptacle tube.
2. Spin the tube in a microcentrifuge for **15 seconds** or for the minimum time required to pull the homogenate through the filter.
3. Add 100 µl of Lysis Buffer to the Prefilter Spin Cup.
4. Spin the tube in a microcentrifuge for 2 minutes.
5. Remove the spin cup from the receptacle tube and discard it. **Retain the filtrate.**
6. Proceed with the protocol in *Isolating RNA*, beginning with the addition of 70% ethanol at step 4.

APPENDIX II: PURIFYING RNA FOLLOWING AN ENZYMATIC REACTION

This alternate protocol can be used to purify RNA following an enzymatic reaction, such as DNase digestion or in vitro transcription. This method is most efficient for RNA molecules of at least 100 nucleotides in length.

1. Add 1.75 µl of β-ME to 250 µl of Lysis Buffer.

2. Mix the Lysis Buffer–β-ME mixture and 250 µl of 70% ethanol into the reaction.

3. Transfer the mixture to an RNA Binding Spin Cup that is seated in a 2-ml receptacle tube and cap the tube.

4. Spin the tube in a microcentrifuge for 30–60 seconds.

5. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.

6. Add 500 µl of 1× High-Salt Wash Buffer and cap the tube. Spin the tube in a microcentrifuge for 30–60 seconds.

7. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.

8. Add 500 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin the tube in a microcentrifuge for 30–60 seconds.

9. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.

10. Add 300 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin the tube in a microcentrifuge for 2 minutes.

11. Transfer the spin cup to a 1.5-ml microcentrifuge tube and discard the 2-ml receptacle tube.

12. Add 30–100 µl of Elution Buffer directly to the matrix inside the spin cup. Incubate the sample at room temperature for 2 minutes. Spin the tube in a microcentrifuge for 30–60 seconds. Repeat this step for a maximum yield of RNA.

APPENDIX III: SPECTROPHOTOMETRIC QUANTIFICATION OF RNA

Note *Accurate spectrophotometric measurement requires an* $OD_{260} \geq 0.05$.

1. Blank the spectrophotometer at 260 nm with an appropriate buffer (e.g., 10 mM Tris, pH 7.5) near neutral pH.

2. Prepare an appropriate dilution of the RNA sample (1:50–1:100). Place a piece of laboratory film (e.g., Parafilm® laboratory film) over the top of the cuvette and mix the sample well. The conversion factor for RNA is 0.040 µg/µl per OD_{260} unit. Take the spectrophotometric reading. For a reading of 0.10, calculate the concentration as follows:

   Concentration = A_{260} × dilution factor × conversion factor

   Example $0.10 \times 500/5 \times 0.040\ \mu g/\mu l = 0.4\ \mu g/\mu l$

3. Calculate the yield of RNA by multiplying the volume in microliters by the concentration. For example, in the sample above a volume of 100 µl results in a yield of 40 µg.

4. Blank the spectrophotometer at 280 nm with water or buffer. Measure the OD of the RNA sample at 280 nm. The ratio of the 260 nm measurement to the 280 nm measurement indicates purity. Ratios of 1.8 to 2.1 are very pure. Lower ratios indicate possible protein contamination, or low pH in the solution used as a diluent for the spectrophotometric readings.

APPENDIX IV: FORMALDEHYDE GEL PROTOCOL

Preprotocol Considerations

Caution *Formaldehyde is a suspected carcinogen and must be used and disposed of in accordance with federal, state and local regulations. Always use formaldehyde in a fume hood.*

The secondary structure of mRNA present in the total RNA must be denatured if the molecules are to migrate at their true molecular weight. The percentage of agarose used affects resolution and transfer. High agarose concentrations improve resolution but decrease the rate and efficiency of RNA transfer to membranes. Agarose concentrations of 0.8–1.2% are recommended.

Protocol

1. Dry the RNA samples under vacuum without heat. 1–5 µg of RNA works well for most applications. More than 15 µg of RNA may cause the lanes to become distorted with ribosomal RNA.

   Note *The RNA can be dried completely without severe resuspension problems, since the loading buffer contains 50% formamide.*

2. For 100 ml of a 1% agarose gel, melt 1 g of agarose in 88 ml of RNase-free water.

3. Add 10 ml of 10× MOPS buffer[§] to the agarose solution. Allow the gel solution in the flask to cool to approximately 60°C while preparing an electrophoresis gel apparatus. Place the gel apparatus on a level space inside a fume hood. Add 2.7 ml of 37% formaldehyde to the cooled agarose. Swirl to mix and quickly pour the agarose into the gel apparatus. If the RNA on the gel will be transferred to a membrane, the gel should only be thick enough to handle easily (0.5–0.75 cm). Allow the gel to solidify in the fume hood.

[§] See *Preparation of Media and Reagents*.

4. While the gel is solidifying, prepare 10 µl of sample loading buffer for each sample. Prepare the loading buffer by mixing the components listed below (no more than 12 hours before use):

   For 100 µl total volume of loading buffer use:

   10 µl 10× MOPS buffer
   11.5 µl RNase-free water
   50 µl of deionized formamide
   17.5 µl 37% formaldehyde solution
   10 µl 10× loading dye[§]
   1 µl 10 mg/ml ethidium bromide

   Note *This solution is not stable.* ***Do not*** *use after 12 hours.*

5. Cover the solidified gel with 1× MOPS buffer. Carefully pull the comb out and connect the electrophoresis apparatus to a power supply.

6. Resuspend the vacuum-dried RNA in 5–10 µl of loading buffer. Heat the resuspended RNA at 65°C for 10–15 minutes, chill on ice for 1–2 minutes, centrifuge to collect condensation and immediately load onto the gel.

7. Electrophorese the gel until the bromphenol blue has run one-half to three-quarters the length of the gel (depending on the resolution desired). Ethidium bromide in the loading dye will migrate to the negative electrode, and the bromphenol blue and xylene cyanol will travel to the positive electrode with the RNA sample.

   Note *Formaldehyde gels are more fragile than other agarose gels. Use caution when moving the gel. Wear UV-protective safety glasses or a full safety mask to prevent UV damage to the face and skin.*

8. Examine the gel with UV illumination.

Expected Results

The majority of eukaryotic mRNA falls within the size range of 400–2000 bases. If a size marker is unavailable, the upper and lower ribosomal RNA bands can be used to help size the RNA. The large 28S band is ~5 kb, and the smaller 18S band is ~2 kb. These numbers are only approximate, since ribosomal RNA sizes vary between species.

[§] See *Preparation of Media and Reagents*.

PREPARATION OF MEDIA AND REAGENTS

10× Loading Dye

50% sterile glycerol
1 mM ethylenediaminetetraacetic acid (EDTA)
0.25% bromophenol blue
0.25% xylene cyanol FF

10× DNase I Buffer

500 mM Tris-HCl (pH 7.5)
100 mM $MgCl_2$
0.5 mg/ml nuclease-free bovine serum albumin (BSA)

10× MOPS Buffer

0.2 M MOPS (3-[N-morpholino] propanesulfonic acid)
0.05 M sodium acetate
0.01 M ethylenediaminetetraacetic acid (EDTA)
Bring to a final pH of 7.0 with NaOH
Do not autoclave

ENDNOTES

Parafilm® is a registered trademark of the American National Can Company.
Polytron® is a registered trademark of Kinematica AG.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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Absolutely RNA Miniprep Kit

QUICK-REFERENCE PROTOCOL

Preparing Animal Tissue

- Dissect the tissue from the organism and immediately flash-freeze in liquid nitrogen
- Once flash-frozen, fracture the tissue, quickly weigh a sample, and return the tissue to liquid nitrogen; store the sample in liquid nitrogen or on dry ice
- Prepare the Lysis Buffer–β-ME mixture:

Tissue sample	Lysis Buffer	β-ME
5–20 mg	100–250 µl	0.7–1.75 µl
20–25 mg	350 µl	2.5 µl
25–40 mg	600 µl	4.2 µl

- Homogenize the tissue in the Lysis Buffer–β-ME mixture

Preparing Adherent Tissue Culture Cells

- Prepare 100–600 µl of a Lysis Buffer–β-ME mixture (7 µl of β-ME/ml of Lysis Buffer)
- Aspirate the medium from the tissue culture dish
- Add the Lysis Buffer–β-ME mixture to the dish and spread evenly over the surface
- Transfer the lysate to a microcentrifuge tube
- Vortex the tube to homogenize the lysate

Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells

- Prepare the Lysis Buffer–β-ME mixture:

Cell number	Lysis Buffer	β-ME
1×10^4–1×10^5	100 µl	0.7 µl
1×10^5–1×10^6	200 µl	1.4 µl
1×10^6–5×10^6	350 µl	2.5 µl
5×10^6–1×10^7	600 µl	4.2 µl

- Centrifuge the cells at 1000 × *g* for 5 minutes.

- Aspirate most of the supernatant. Resuspend the cells in the remaining supernatant and transfer the cells to a microcentrifuge tube. Spin in a microcentrifuge at low speed (~3000 rpm) for 5 minutes. Remove all the supernatant

- Add the Lysis Buffer–β-ME mixture and vortex or repeatedly pipet to homogenize

Isolating RNA

- Transfer up to 700 µl of homogenate to a seated Prefilter Spin Cup (blue)

- Spin in a microcentrifuge at maximum speed for 5 minutes

- Discard the spin cup and **retain the filtrate**

- Add an equal volume of 70% ethanol to the filtrate and vortex the tube for 5 seconds or until the filtrate and ethanol are mixed thoroughly

- Transfer up to 700 µl of the mixture to a seated RNA Binding Spin Cup; spin in a microcentrifuge at maximum speed for 30–60 seconds; **retain the spin cup** and discard the filtrate; repeat with remaining sample, if necessary

DNase Treatment

- Add 600 µl of 1× Low-Salt Wash Buffer and spin in a microcentrifuge at maximum speed for 30–60 seconds
- **Retain the spin cup** and discard the filtrate; replace the spin cup and spin the tube in a microcentrifuge at maximum speed for 2 minutes
- Gently mix 50 µl of DNase Digestion Buffer with 5 µl of reconstituted RNase-Free DNase I
- Add the DNase solution directly onto the matrix
- Incubate at 37°C for 15 minutes

- Add 600 µl of 1× High-Salt Wash Buffer and spin in a microcentrifuge at maximum speed for 30–60 seconds

- **Retain the spin cup** and discard the filtrate

- Add 600 µl of 1× Low-Salt Wash Buffer and spin in a microcentrifuge at maximum speed for 30–60 seconds

- **Retain the spin cup** and discard the filtrate

- Add 300 µl of 1× Low-Salt Wash Buffer and spin in a microcentrifuge at maximum speed for 2 minutes to dry the matrix

- Transfer the spin cup to a 1.5-ml microcentrifuge tube

- Add 30–100 µl of Elution Buffer directly onto the matrix and incubate for 2 minutes at room temperature; spin in a microcentrifuge at maximum speed for 1 minute; repeat

The purified RNA is in the Elution Buffer in the microcentrifuge tube. Store at –80°C for the long term or at –20°C for the short term.