Absolutely RNA miRNA Kit

Instruction Manual

Catalog #400814

Revision D0

Laboratory Reagent.

400814-12

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Absolutely RNA miRNA Kit

CONTENTS

Absolutely RNA miRNA Kit

MATERIALS PROVIDED

Materials Provided	Quantity[a]	Part # on Vial or Package	Storage Conditions
Lysis Buffer	35 ml	400814-13	Room temperature
β-Mercaptoethanol (β-ME) (14.2 M)	210 µl	400814-21	Room temperature[b]
RNase-free DNase I (lyophilized)	2600 U	400711-23	Room temperature[c]
DNase Reconstitution Buffer	0.3 ml	400814-17	Room temperature
DNase Digestion Buffer	2.5 ml	400814-18	Room temperature
High-Salt Wash Buffer (1.67×)	24 ml	400814-14	Room temperature
Low-Salt Wash Buffer (5×)	19 ml	400814-15	Room temperature
Elution Buffer[d]	2.5 ml	400814-16	Room temperature
Prefilter Spin Cups and 2-ml receptacle tubes	50	400814-110	Room temperature
RNA Binding Spin Cups and 2-ml receptacle tubes	50	400800-110	Room temperature
1.5-ml microcentrifuge tubes	50	—	Room temperature

[a] Sufficient reagents are provided to isolate total RNA from 50 samples of up to 25 mg tissue or up to 5×10^6 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

Ethanol
Diethylpyrocarbonate (DEPC)
Homogenizer
Phenol–chloroform [1:1 (v/v)] **(required for tissue samples only)**
Chloroform–isoamyl alcohol [24:1 (v/v)] **(required for tissue samples only)**
Phosphate-buffered saline (PBS; **required for cultured-cell samples only)**
Optional: High-Specificity miRNA QRT-PCR Detection Kit (Catalog #600580)
Optional: Agilent 2100 Bioanalyzer (Part No. G2939AA) and RNA 6000 Nano LabChip kit (Part No. 5067-1511)
Optional: Agilent 2100 Small RNA Assay kit (Part No. 5067-1548)

Revision D0

INTRODUCTION

The Absolutely RNA miRNA Kit provides a rapid method for purification of high-quality, miRNA-containing total RNA from cultured cells or small samples of tissue. The Absolutely RNA miRNA Kit method employs a spin cup with a matrix that binds cellular RNA, with a very low size cutoff, in the presence of ethanol and a chaotropic salt. Both miRNA and mRNA species are quantitatively recovered using this method, allowing gene expression studies of both miRNA and mRNA levels from the same sample using QRT-PCR or microarray analysis.

Extreme purity of RNA is critical in gene expression studies, because contamination with DNA can result in overestimation of total RNA amounts and thus affects the ability to make direct comparisons between samples. In addition, for some assays, contaminating DNA can give rise to amplification or hybridization signal that mimics the signal from the RNA target. The Absolutely RNA miRNA Kit includes a prefilter spin step to remove genomic DNA from tissue culture cell lysates and an additional DNase digestion step to remove residual genomic DNA from tissue samples. Including the DNase digestion step ensures high yields of miRNA-containing total RNA with undetectable levels of DNA.

The simple and effective RNA purification methods for cultured cells or tissue samples are summarized in figure 1. First, cells or tissues are homogenized in the presence of guanidine thiocyanate, one of the strongest protein denaturants, to lyse cells and to prevent RNA degradation by ribonucleases (RNases). Following cell lysis, tissue samples are extracted with phenol to remove proteins. For lysates prepared from cultured cells, phenol extraction is not required. After prefiltration of samples to remove genomic DNA, the filtrate is combined with ethanol and transferred to an RNA-binding spin cup. The following on-column DNase digestion step (required for tissue lysates and optional for cell line lysates) removes any residual DNA. A series of wash steps removes contaminants (including DNase, when this step is performed). Finally, highly pure RNA is eluted in a small volume of low-ionic-strength buffer and captured in a microcentrifuge tube. The resulting total RNA is ideal for real time quantitative RT-PCR and microarray analyses that investigate miRNA or mRNA levels.

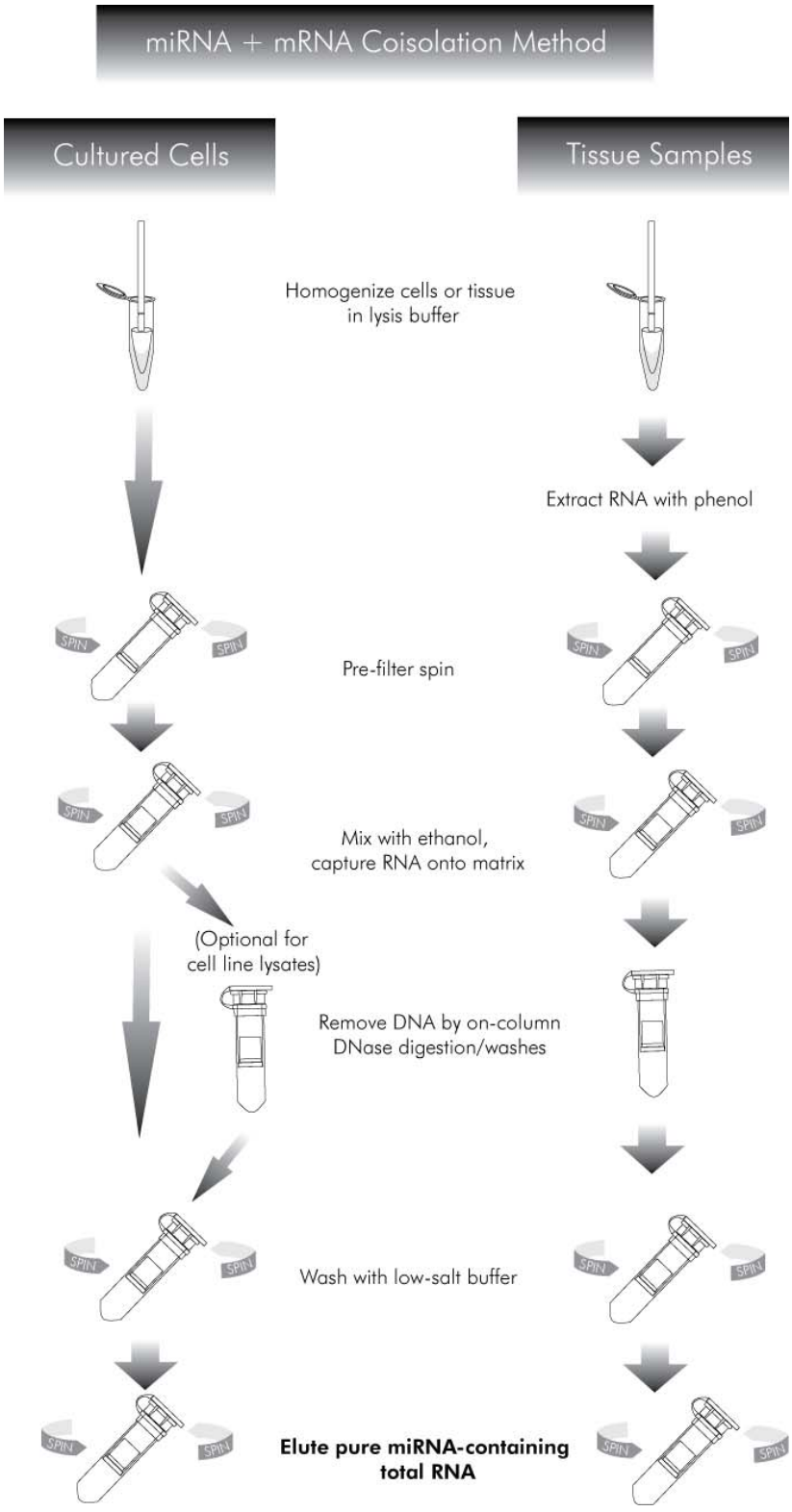

FIGURE 1 Absolutely RNA miRNA Kit method.

PREVENTING SAMPLE CONTAMINATION

Preventing RNase Contamination

Ribonucleases (RNases) 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, 70% methanol or a commercially available RNase decontamination agent.

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 previously used for nucleic acid isolation.

PREPROTOCOL CONSIDERATIONS

Selecting the Appropriate Protocol

Separate protocols are provided for isolation of miRNA-containing total RNA from cultured cells versus tissue samples. It is important to follow the protocol appropriate to your sample type. For example, when isolating RNA from tissues, organic extraction of the lysate is critical prior to column filtration and DNase treatment of the filtrate is required. In contrast, when isolating RNA from cultured cells, organic extraction is omitted and DNase treatment is optional.

When using the cultured cell RNA isolation protocol, the majority of cellular DNA is associated with cellular debris in the lysate and is removed in the prefiltration step. If the cell lysate is highly viscous, and thus requires extensive homogenization, or if your application requires complete absence of cellular DNA, then consider including the DNase digestion step for RNA isolation from cultured cells.

Optimal Sample Sizes and Yield Guidelines

The *RNA Isolation Protocol* is optimized for 1–25 mg of tissue or 1×10^6–5×10^6 tissue culture cells. Yields of 5–15 µg total RNA are typical from 1×10^6 cultured cells. Total RNA yields from individual tissue types vary widely. For each tissue type, the expected total RNA yield is equivalent to yields obtained from similar column-based RNA purification methods. It is important to note, however, that miRNA yield is not directly indicated by total RNA yield, and miRNA composition must be investigated using methods that specifically detect miRNA species. See Verifying *miRNA Recovery* in *Protocols* for suggested methods.

Sample Viscosity Considerations

High viscosity of tissue or cell lysates results in difficult-to-pipette samples and can result in spin cup clogging (and loss of sample) during prefiltration. If your lysate is difficult to pipet, the viscosity may be reduced by dilution with additional Lysis Buffer, or by passing the sample through an 18–20 gauge syringe needle. High viscosity results from preparing the lysate at high cell density. If your cell line or tissue tends to produce highly viscous lysates, reduce the cell number or tissue mass used as starting material.

RNA and RNase Levels in Different Tissues

The amount of total RNA available for isolation varies with tissue type. For example, liver and kidney cells are metabolically active and yield relatively large amounts of RNA while structural cells and fat cells tend to yield less RNA. In addition, there is considerable variation in the abundance and composition of the miRNA component of the total RNA among different cell types and tissue types.

The amount of RNases in different tissue types also varies. The pancreas and spleen are very rich in RNases and extra urgency should be applied to flash-freezing these tissues in liquid nitrogen **immediately** after dissection.

PREPARING THE REAGENTS

β-Mercaptoethanol

Once opened, store the β-ME at 4°C.

Low-Salt Wash Buffer

- Prepare 1× Low-Salt Wash Buffer by adding 76 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, 76 ml).*
- Store the 1× Low-Salt Wash Buffer at room temperature.

High-Salt Wash Buffer

- The High Salt Wash Buffer is required only for isolation protocols that include the DNase digestion step.
- 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, 16 ml).*
- Store the 1× High-Salt Wash Buffer at room temperature.

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 vortex and do not introduce air bubbles into the solution. Store the reconstituted RNase-Free DNase I at –20°C.

Notes *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.*

When isolating RNA from cultured cells, digestion with DNase I is optional.

PROTOCOL: RNA PREPARATION FROM ANIMAL TISSUE

Tissue Lysate Preparation

Note *This protocol is optimized for tissue samples of up to 25 mg.*

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. Use the amount of Lysis Buffer/β-ME mixture indicated in the table below, according to sample size:

Tissue sample	Lysis Buffer	β-ME
<5 mg	200 µl	1.4 µl
5–25 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 25 mg, use the appropriate amount of Lysis Buffer to yield a tissue concentration of 0.04 mg/µl. For samples greater than 25 mg, the resulting lysate should be split between multiple purification columns.

4. Place the tissue sample in a tube containing the Lysis Buffer–β-ME mixture. Homogenize the tissue using the appropriate homogenizer instrument. Fibrous or fatty tissues (such as muscle, skin, kidney, uterus, placenta, lung, brain, breast, esophagus, trachea, and aorta) are most effectively homogenized using a rotating blade homogenizer, such as Polytron homogenizer. Soft tissues (such as liver or spleen) may be homogenized using a mini-homogenizer, such as Kontes Pellet Pestle, or a micro-Dounce homogenizer. Ensure that the instrument is RNase-free.

   Notes *Homogenization results in shearing of genomic DNA and decreases its retention on the Prefilter Spin Cup. Therefore, use the minimum amount of time and the lowest setting required for complete homogenization.*

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

RNA Isolation from Tissue Lysates

Organic Extraction

1. Measure the volume of the homogenate. Phenol-extract the sample once by adding an equal volume of neutral phenol–chloroform [1:1 (v/v)] and vortexing the mixture for 10 seconds. Spin the mixture in a microcentrifuge at maximum speed for 3–4 minutes. Transfer the aqueous (upper) phase to a fresh tube.

2. Measure the volume of aqueous phase collected. Chloroform-extract the sample once by adding an equal volume of chloroform–isoamyl alcohol [24:1 (v/v)] and vortexing the mixture for 10 seconds. Spin the mixture in a microcentrifuge at maximum speed for 2–3 minutes. Transfer the aqueous (upper) phase to a fresh tube.

Prefiltration

Note *Two types of spin cups are provided: Prefilter Spin Cups and RNA Binding Spin Cups. Use care in selecting the correct spin cup for the following steps. The Prefilter Spin Cups contain a thicker layer of filter material and have lids attached directly to the spin cup. The RNA Binding Spin Cups contain a thinner layer of filter material and have lids attached to the receptacle tubes.*

3. Transfer up to 600 µl of the extracted lysate to a Prefilter Spin Cup that is seated in a 2-ml receptacle tube and close the spin cup cap.

4. Spin the tube in a microcentrifuge at maximum speed for 3 minutes.

5. Remove the spin cup from the receptacle tube and discard it. **Retain the filtrate sample in the receptacle tube**. Transfer the sample to a fresh tube.

Loading and Washing the RNA-Binding Spin Cup

6. Add **1.25 volumes** of 100% ethanol to the filtrate and vortex the tube for 15 seconds or until the filtrate and ethanol are mixed thoroughly.

7. Transfer up to 600 µl of the 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 cap, prior to closing it, so that it becomes flexible and the cap can be firmly seated in the tube, to prevent leakage.

8. Spin the spin cup in a microcentrifuge at maximum speed for 1 minute.

9. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube. Load the remaining lysate-ethanol mixture onto the same RNA Binding Spin Cup by repeating steps 7–8.

   Note *The RNA was protected in previous steps from RNases by the presence of guanidine thiocyanate which is removed by the Low-Salt Wash Buffer in the next step.*

10. After discarding the final filtrate, add 600 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 1 minute.

    Note *High RNA purity requires complete removal of organic solvents, including guanidine thiocyanate, in this step. Be sure to rinse the inside of the spin cup thoroughly in all wash steps, including the lip of the spin cup where the cap fits.*

11. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube and dry the matrix by spinning the tube in a microcentrifuge at maximum speed for 2 minutes.

On-Column DNase Treatment and Washes

12. 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. Do not vortex.*

13. Add the 55 µl of DNase solution directly onto the matrix inside the spin cup and cap the spin cup.

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

15. 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 1 minute.

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

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

17. Add 600 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 1 minute.

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

19. Perform a second wash using 600 µl of 1× Low-Salt Wash Buffer by repeating steps 17–18.

20. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube and dry the matrix by spinning the tube in a microcentrifuge at maximum speed for 2 minutes.

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

RNA Elution

22. Add 50 µl of Elution Buffer directly onto the center of the matrix inside the spin cup and cap the spin cup. Incubate the tube for 1 minute at room temperature. Spin the tube in a microcentrifuge at maximum speed for 1 minute.

    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.*

    If preferred, RNA may be eluted with RNase-free H_2O.

    The RNA yield can be increased by using Elution Buffer or RNase-free H_2O warmed to 65°C.

23. To maximize RNA yield, reload the eluate onto the matrix. Incubate the tube for 1 minute at room temperature, then spin the tube in a microcentrifuge at maximum speed for 1 minute.

    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. Aliquot RNA stocks prior to freezing to minimize exposure of the preparation to freeze-thaw cycles.

PROTOCOL: RNA PREPARATION FROM TISSUE CULTURE CELLS

Lysate Preparation from 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 the following section* Lysate Preparation from 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 lysate.*

For a 100-mm dish of confluent cells, it is preferable to trypsinize the cells and use the protocol in the following section, Lysate Preparation from 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 × 10^6–5 × 10^6 tissue culture cells in a 35- or 60-mm tissue culture dish. See Table I for volumes of Lysis Buffer and β-ME to use for other tissue culture well configurations.

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. Wash cells once with phosphate-buffered saline (PBS).

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

TABLE I

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.

5. 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, since high viscosity prevents the sample from passing through the prefiltration column. Sample viscosity can be reduced by, increasing the volume of Lysis Buffer or by passing the lysate through an 18–21 gauge syringe needle.*

6. Proceed with the protocol in *RNA Isolation from Cultured Cell Lysates.*

Lysate Preparation from Cells Grown in Suspension or Trypsinized Adherent Cells

Note *This protocol is optimized for samples of 1 × 10^6–5 × 10^6 tissue culture cells.*

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

3. Aspirate most of the supernatant. Resuspend the cells in 1 ml PBS 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 the supernatant.

   Note *Cell pellets can be flash-frozen and then stored at –80°C or stored in liquid N_2 for future processing. If possible, homogenize cells in Lysis Buffer prior to freezing to minimize RNA degradation.*

5. Add 600 µl 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 *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 is too great, the homogenate may be too viscous to pass through the prefiltration column. Sample viscosity can be reduced by increasing the volume of Lysis Buffer or by passing the lysate through an 18–21 gauge syringe needle.*

6. Proceed with the protocol in *RNA Isolation from Cultured Cell Lysates.*

RNA Isolation from Cultured Cell Lysates

Note *Two types of spin cups are provided: Prefilter Spin Cups and RNA Binding Spin Cups. Use care in selecting the correct spin cup for the following steps. The Prefilter Spin Cups contain a thicker layer of filter material and have lids attached directly to the spin cup. The RNA Binding Spin Cups contain a thinner layer of filter material and have lids attached to the receptacle tubes.*

Prefiltration

1. Transfer up to 600 µl of cell homogenate to a Prefilter Spin Cup that is seated in a 2-ml receptacle tube and close the spin cup cap.

2. Spin the tube in a microcentrifuge at maximum speed for 3 minutes.

3. Remove the spin cup from the receptacle tube and discard it. **Retain the filtrate sample in the receptacle tube**. Transfer the sample to a fresh tube.

Loading and Washing the RNA-Binding Spin Cup

4. Add **1.5 volumes** of 100% ethanol to the filtrate and vortex the tube for 15 seconds or until the filtrate and ethanol are mixed thoroughly.

5. Transfer up to 600 µl of the 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 cap, prior to closing it, so that it becomes flexible and the cap can be firmly seated in the tube, to prevent leakage.

6. Spin the spin cup in a microcentrifuge at maximum speed for 1 minute.

7. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube. Load the remaining lysate-ethanol mixture onto the same RNA Binding Spin Cup by repeating steps 5–6.

   Note *The RNA was protected in previous steps from RNases by the presence of guanidine thiocyanate which is removed by the Low-Salt Wash Buffer in the next step.*

8. After discarding the final filtrate, add 600 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 1 minute.

   Note *High RNA purity requires complete removal of organic solvents, including guanidine thiocyanate, in this step. Be sure to rinse the inside of the spin cup thoroughly in all wash steps, including the lip of the spin cup where the cap fits.*

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

   Note *If performing the optional on-column DNase digestion, proceed to step 10. If omitting DNase digestion, proceed to a second low-salt wash, in step 18.*

On-Column DNase Treatment and Washes (Optional for Cell Lysates)

Note *The majority of cellular DNA is associated with cellular debris in the lysate and is removed in the prefiltration step. If the cell lysate was highly viscous, and thus required extensive homogenization, or if your application requires complete absence of cellular DNA, then consider including the following DNase digestion steps.*

10. Dry the spin cup matrix by spinning the tube in a microcentrifuge at maximum speed for 2 minutes.

11. 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. Do not vortex.*

12. Add the 55 µl of DNase solution directly onto the matrix inside the spin cup and cap the spin cup.

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

14. 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 1 minute.

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

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

16. Add 600 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 1 minute.

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

Final Wash and RNA Elution

Note *Two low-salt washes are required just prior to elution, regardless of protocol options included. If DNase treatment is omitted, the two washes are provided by performing steps 9 and 18 in immediate succession.*

18. Perform a final low salt wash. Add 600 µl of 1× Low-Salt Wash Buffer and cap the spin cup. Spin the tube in a microcentrifuge at maximum speed for 60 seconds.

19. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube and dry the matrix by spinning the tube in a microcentrifuge at maximum speed for 2 minutes.

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

21. Add 50 µl of Elution Buffer directly onto the center of the matrix inside the spin cup and cap the spin cup. Incubate the tube for 1 minute at room temperature. Spin the tube in a microcentrifuge at maximum speed for 1 minute. To maximize RNA yield, reload the eluate onto the matrix, incubate the tube for 1 minute at room temperature. Spin the tube in a microcentrifuge at maximum speed for 1 minute.

    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.*

    If preferred, RNA may be eluted with RNase-free H_2O.

    The RNA yield can be increased by using Elution Buffer or RNase-free H_2O warmed to 65°C.

22. To maximize RNA yield, reload the eluate onto the matrix. Incubate the tube for 1 minute at room temperature, then spin the tube in a microcentrifuge at maximum speed for 1 minute.

    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. Aliquot RNA stocks prior to freezing to minimize exposure of the preparation to freeze-thaw cycles.

ANALYZING RNA QUANTITY AND QUALITY

Spectrophotometric Analysis of the RNA

For Spectrophotometric analysis of the RNA, remove a small sample and dilute it with a buffer of neutral pH (e.g., 10 mM Tris, pH 7.5). Diluting the sample 1:20–1:50 typically yields a solution with a concentration that is appropriate for spectrophotometric analysis.

Measure the optical density (OD) at 230 nm, 260 nm, and 280 nm to quantitate and qualify the RNA (see *Appendix I: Spectrophotometric Analysis of RNA*). Yields will vary, depending on tissue and cell type. Yields of 5–15 µg RNA are typical from 1×10^6 cultured cells. Yields from individual tissue types vary widely. For example, liver and kidney cells are metabolically active and yield relatively large amounts of RNA while structural cells and fat cells tend to yield less RNA.

Analysis of RNA Composition and Quality

Verifying miRNA Recovery

Due to the small size and relative low abundance of miRNAs in cellular RNA preparations, analysis of miRNA content using gel electrophoresis and other traditional molecular biology tools is difficult.

The presence of miRNA in the total RNA preparation may be definitively verified using QRT-PCR experiments that detect one or more standard miRNA targets. Any of the ubiquitous miRNA species, such as the let-7 miRNA family, may be used as the target for QRT-PCR analysis. The High-Specificity miRNA QRT-PCR Detection Kit may be used, in conjunction with an miRNA-specific forward primer of choice, to facilitate the analysis of miRNA recovery in total RNA preparations.

Assessing RNA Quality by RNA Size Distribution

Analyze the quality of the RNA based on the size distribution of recovered RNA molecules by microfluidics analysis on the Agilent 2100 Bioanalyzer. To analyze the total RNA composition of the sample, use the RNA 6000 Nano LabChip kit, and perform analysis using the instrument's Eukaryotic Total RNA Nano series II program.

The Bioanalyzer uses electrophoretic separation on microfabricated chips to determine the quality of total RNA in a quantitative manner. Using the components outlined above, the Bioanalyzer output provides an image of the RNA fragments including the 28S and 18S bands, similar to gel electrophoresis, and calculates the 28S to 18S ratio. The 28S:18S ratio should be >1 for tissue-derived RNA and 1.5–1.8 for cell line-derived RNA. Additionally, the Bioanalyzer calculates the RNA integrity number (RIN) using an algorithm. The instrument software uses the entire electrophoretic trace (all regions and peaks) to calculate the integrity of samples, and reports the RIN using a scale of 1 to 10 (with 1 being the most degraded and 10 being the most intact). As a general guideline, RIN values >7 are expected from total RNA prepared using this kit.

Note *Bioanalyzer analysis using the RNA 6000 Nano LabChip kit is not informative regarding the presence or absence of miRNA in the total RNA sample, since the miRNA species contribute negligible amounts of mass to the total RNA population. See* Verifying miRNA Recovery*, above, for miRNA analysis suggestions.*

The small RNA composition of the sample may also be analyzed semi-quantitatively using the Agilent 2100 Bioanalyzer with the 2100 Small RNA Assay. This system can be used to characterize the sample for the relative abundance of single- and double-stranded nucleic acids in the size range of 10-150 nt. The electropherogram output provides an image of the composition of small nucleic acid molecules in the sample, compared to an RNA ladder. The bands or peaks in the 10 to 40 nt range of the electropherogram include the miRNA portion of the total RNA preparation. However, the Small RNA Assay is not a definitive indicator of miRNA content in the sample, as other small RNA or DNA fragments can contribute to bands or peaks in the 10 to 40 nt range. See *Verifying miRNA Recovery*, above, for miRNA analysis suggestions.

If the Agilent Bioanalyzer method is unavailable, analyze the RNA size distribution in the sample using denaturing agarose gel electrophoresis. (For a protocol, see *Appendix II: Formaldehyde Gel RNA Analysis of RNA*.) When RNA isolated from mammalian sources is viewed on a denaturing agarose gel, the ribosomal bands (28S and 18S) should appear as two bright bands at approximately 4.5 and 1.9 kilobases. The ratio of intensities of the 28S and 18S bands should be 1.5-2.5:1. Lower ratios may indicate that RNA degradation has occurred and that this RNA may not be suitable for gene expression studies. Additional bands, including low molecular weight bands corresponding to the 5S ribosomal RNA and tRNA may also be visible.

TROUBLESHOOTING

Observation	Suggestion
High viscosity of the homogenate makes sample difficult to pipet or causes clogging of the Prefilter Spin Cup	Dilute the homogenate with additional Lysis Buffer.
	Pass the homogenate through an 18–21 gauge needle several times to reduce the viscosity.
RNA is degraded	Use DEPC-treated or radiation-sterilized plasticware.
	Flash-freeze the tissue immediately upon dissection from the animal.
RNA yield is poor	Yield may be reduced by the inability of the lysate to pass through the Prefilter Spin Cup. If your sample is excessively viscous (e.g. difficult to pipette or have a gelatinous appearance) 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.
	For tissue samples, use a rotating blade homogenizer, such as a Polytron homogenizer, in order to effectively grind tissue samples and achieve complete lysis.
	Use buffer or water at neutral pH (pH 7–8) for efficient RNA elution.
	Heat the elution buffer to 65 °C prior to use.
	Increase the volume of Elution Buffer to up to 100 µl. (Note that while yield may be increased, RNA concentration may be reduced.)
	Perform the elution twice, by reloading the eluate onto the RNA Binding Spin Cup to elute residual bound RNA.
	After adding Elution Buffer to the spin cup, incubate the tube at room temperature for 2 minutes before collecting the eluate.
miRNA not detected	Ensure that the correct amount of ethanol was added to the lysate prior to loading the RNA-Binding Spin Cup.
	Verify that appropriate methods are used to evaluate miRNA composition, which varies widely among cell lines and tissue types. Use one or more ubiquitous miRNA species, e.g. members of the let7 miRNA family, as targets for miRNA detection. Use detection methods that are optimized for miRNA, such as the High Specificity miRNA QRT-PCR Detection Kit.
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	During homogenization of cells or tissues, use the minimum amount of time and the lowest setting required for complete homogenization. Excessive homogenization may shear genomic DNA, decreasing the proportion of DNA retained by the Prefilter Spin Cup.
	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*). Do not vortex the DNase solution.
After RT-PCR of RNA prep, PCR products include excessive background bands or smeared bands	Use less RNA in the cDNA synthesis reaction. When 1 µg of total RNA is polyadenylated and converted into cDNA using the High Specificity miRNA QRT-PCR Detection Kit, 1/300 of the cDNA synthesis reaction is enough for rare miRNA to be amplified.

APPENDIX I: SPECTROPHOTOMETRIC ANALYSIS 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 (1:20–1:50). 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 and calculate the concentration using the following formula:

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

   Example *For a 5µl sample diluted to 250 µl (1:50 dilution), which produces an* OD_{260} *reading of 0.10, the concentration is calculated as follows:*

   $0.10 \times 250/5 \times 0.040\ \mu g/\mu l = 0.2\ \mu g/\mu l$

3. Calculate the yield of RNA by multiplying the volume in microliters by the concentration. Using the example above, with an RNA preparation volume of 50 µl, the resulting yield is 10 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.

5. Blank the spectrophotometer at 230 nm with water or buffer. Measure the OD_{230} of the RNA sample and calculate the OD_{260}:OD_{230} ratio as an indicator of RNA purity. An OD_{260}:OD_{230} ratio >1 is expected. OD_{260}:OD_{230} ratios <1 indicate possible contamination with thiocyanate or other organic solvents.

APPENDIX II: FORMALDEHYDE GEL ANALYSIS OF RNA

Preparation of Media and Reagents

10× Loading Dye	10× MOPS Buffer
50% sterile glycerol 1 mM ethylenediaminetetraacetic acid (EDTA) 0.25% bromophenol blue 0.25% xylene cyanol FF	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

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*.

MSDS Information

Material Safety Data Sheets (MSDSs) are provided online at *www.agilent.com*. MSDS documents are not included with product shipments.