Absolutely RNA 96 Microprep Kit

INSTRUCTION MANUAL

Catalog #400793 and #400794
Revision #A

For In Vitro Use Only
400793-12

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Absolutely RNA 96 Microprep Kit

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MATERIALS PROVIDED

Materials provided	Quantity	
	Catalog #400793[a]	Catalog #400794[b]
Lysis buffer	25 ml	125 ml
β-Mercaptoethanol (β-ME) (14.2 M)[c]	0.3 ml	1.0 ml
RNase-free DNase I (lyophilized)[d]	2600 U	4 x 2600 U
DNase reconstitution buffer	0.3 ml	1.5 ml
DNase digestion buffer	11 ml	55 ml
High-salt wash buffer (1.67×)	64 ml	320 ml
Low-salt wash buffer (5×)	2 × 40 ml	2 x 200 ml
Elution buffer (10 mM Tris-HCl, pH 7.5)	12 ml	65 ml
96-well binding plate	2	10
96-well collection plate	4	20
Adhesive plate sealer	12	60
96-well storage mat	2	10

[a] Sufficient reagents are provided to isolate total RNA from two sets of 96 samples of 10 to 5 × 10^5 cells each.

[b] Sufficient reagents are provided to isolate total RNA from ten sets of 96 samples of 10 to 5 × 10^5 cells each.

[c] Once opened, store at 4°C.

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

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

STORAGE CONDITIONS

β-Mercaptoethanol: Once opened, store at 4°C
RNase-free DNase I: Once reconstituted, store at –20°C
All Other Components: Room temperature

ADDITIONAL MATERIALS REQUIRED

Diethylpyrocarbonate (DEPC)
Sulfolane [Sigma (Catalog #T22209)]
Ethanol
Vacuum manifold and tabletop centrifuge (For an alternate centrifugation protocol, see *Appendix*: *Alternate Protocol for Isolation by Centrifugation*)

Revision A

INTRODUCTION

The Absolutely RNA 96 microprep kit allows rapid high-throughput purification of high-quality total RNA from small samples of cultured cells.[1] This simple method of RNA isolation eliminates toxic phenol–chloroform extractions and time-consuming ethanol precipitations used in other RNA purification methods. The method involves the addition of lysis buffer containing the chaotropic salt guanidine thiocyanate, a strong protein denaturant, to lyse cells and prevent RNA degradation by ribonucleases (RNases).[2] Following cell lysis, the sample is transferred to a 96-well binding plate with a silica-based fiber matrix where the RNA binds to the fiber matrix.[3] Treatment with a low-salt wash buffer followed by a DNase digestion step removes contaminating DNA. A series of washes removes the DNase and other proteins. Highly pure RNA is eluted from the fiber matrix with a small volume of low-ionic-strength buffer and captured in a 96-well collection plate. The resulting RNA is suitable for cDNA synthesis, RT-PCR, qualitative and quantitative PCR, and northern blotting.

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., 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 ethanol or methanol.

Sterilizing Labware

Disposable Plasticware

Disposable sterile plasticware is generally free of RNases. If disposable sterile plasticware is unavailable, components such as 1.5-ml 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 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 DEPC, using 0.1% (v/v) DEPC in distilled water. During preparation, mix the 0.1% 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 for cDNA synthesis 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 isolations.

PREPARING THE REAGENTS

80% Sulfolane

Prepare 80% (v/v) sulfolane by diluting 100% sulfolane with RNase-free water.

Preparation of 20 ml of 80% sulfolane is sufficient for processing RNA preparations using 2× 96 well plates (from up to 0.1 ml lysate each). To prepare 20 ml of 80% sulfolane, add 4 ml of RNase-free water to 16 ml of 100% sulfolane.

Note *100% sulfolane is a solid at room temperature. Prior to diluting the sulfolane, melt by incubating in a 37°C waterbath until liquefied (overnight incubation is convenient for this purpose). The 80% sulfolane solution is a liquid at room temperature, and may be stored at room temperature for at least one month.*

If particulate matter is observed, the sulfolane solution may be filtered using a 0.2 µm nylon filter.

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 thoroughly to ensure all the powder goes 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. 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 the quantity of 100% ethanol specified in the following table to the bottle of 1.67× High-Salt Wash Buffer:

Catalog #	Quantity of 100% ethanol	Quantity of 1.67× High-Salt Wash Buffer
400793	43 ml/bottle	64 ml/bottle
400794	214 ml/bottle	320 ml/bottle

After adding the ethanol, mark the container as suggested: *[√] 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 the quantity of 100% ethanol specified in the following table to the bottle of 5× Low-Salt Wash Buffer:

Catalog #	Quantity of 100% ethanol	Quantity of 5× Low-Salt Wash Buffer
400793	160 ml/bottle	40 ml/bottle
400794	800 ml/bottle	200 ml/bottle

After adding the ethanol, mark the container as suggested: *[√] 1× (Ethanol Added)*. Store the 1× Low-Salt Wash Buffer at room temperature.

β-Mercaptoethanol

Once opened, store the β-ME at 4°C.

RNA ISOLATION PROTOCOL

Preparing Adherent Tissue Culture Cells

1. Add 77 µl of β-ME to 11 ml of Lysis Buffer for processing 96 samples (10–5 × 10^5 cells per sample).

   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 plate, tilting the plate to remove any residual medium.

   Note *Cell monolayers can be stored at –80°C for future processing, although addition of Lysis Buffer (the next step) prior to freezing is recommended to minimize RNA degradation.*

3. Add 100 µl of the Lysis Buffer–β-ME mixture to each well of cells.

   Notes *Do not exceed 5 × 10^5 cells per well or the RNA yield may be adversely affected.*

   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 and/or increasing the volume of Lysis Buffer.

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

Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells

1. Add 77 µl of β-ME to 11 ml of Lysis Buffer for processing 96 samples. Do not exceed 5×10^5 cells per sample.

   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 the supernatant.

   Note *Cell pellets can be stored at –80°C for future processing, although addition of Lysis Buffer (the next step) prior to freezing is recommended to minimize RNA degradation.*

4. Add 100 µl of Lysis Buffer–β-ME mixture to each cell pellet (up to 5×10^5 cells).

   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 and/or increasing the volume of Lysis Buffer.*

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

Isolating RNA

1. Add an equal volume (usually 100 µl) of 80% sulfolane to the cell lysate and mix thoroughly by repeated pipetting (3–5 times). Transfer the lysate to the wells of a 96-well binding plate.

   Note *It is very important to use equal volumes of 80% sulfolane and cell lysate. It is also important to vortex until the lysate and sulfolane are thoroughly mixed.*

2. Place the binding plate within the top of the vacuum manifold.

   Note *If some of the 96 wells do not contain samples, seal the tops of the empty wells with tape or cover the entire plate and carefully cut slits over the wells containing samples. Take care not to cross-contaminate the samples when cutting slits in the tape.*

3. Apply vacuum until the lysate-sulfolane mixtures are pulled through the fiber matrix at the bottom of the wells. Release the vacuum.

   Note *Up to this point, the RNA has been protected from RNases by the presence of guanidine thiocyanate.*

4. ***Optional DNase Treatment*** This procedure is recommended if DNA-free total RNA is required. The DNase treatment can be omitted if the removal of DNA is not necessary.

   a. Add 600 µl of 1× Low-Salt Wash Buffer to each well and reapply the vacuum until the wash buffer has been pulled through the fiber matrix. Cover the top of the plate with an adhesive plate sealer and place the plate on top of a 96-well collection plate and spin in a tabletop centrifuge at 1100 × *g* for 10 minutes to dry the fiber matrix.

   b. Prepare the DNase solution by gently mixing 100 µl of reconstituted RNase-free DNase I with 5 ml of DNase Digestion Buffer.

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

   c. Add 50 µl of the DNase solution directly onto the fiber matrix inside each well of the plate. Cover the plate with a fresh plate sealer.

   d. Incubate the plate at room temperature for 15 minutes. During this incubation, discard the fluid in the collection tray of the vacuum manifold.

5. Add 500 µl of 1× High-Salt Wash Buffer to each well of the binding plate. Place the binding plate within the top of the vacuum manifold and apply vacuum until the wash buffer has been pulled through the fiber matrix at the bottom of the wells.

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

6. Release the vacuum. Add 600 µl of 1× Low-Salt Wash Buffer to each well and reapply the vacuum until the wash buffer has been pulled through the fiber matrix.

7. Release the vacuum. Add 600 µl of 1× Low-Salt Wash Buffer to each well and reapply the vacuum until the wash buffer has been pulled through the fiber matrix. Cover the plate with a fresh adhesive plate sealer and place the plate on top of the 96-well collection plate and spin in a tabletop centrifuge at 1100 × *g* for 5 minutes to dry the fiber matrix.

8. Transfer the plate to a fresh 96-well collection plate.

9. Add 30 µl of Elution Buffer directly onto the fiber matrix inside the wells of the plate. Cover the top of the plate with a fresh adhesive plate sealer and incubate the plate for 2 minutes at room temperature. Spin in a tabletop centrifuge at 1100 × *g* for 5 minutes to collect the RNA.

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

   The RNA yield may be increased by performing a second elution.

   The purified RNA is in the Elution Buffer in the collection plate. 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 from >1 × 10^5 cells, 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. When quantifying RNA isolated from <1 × 10^5 cells it will be necessary to use a more sensitive fluorescence based system (i.e. RiboGreen® RNA Quantitation Kit, Molecular Probes, Inc.).

RNA yields may vary depending on cell types used. Yields of 2–8 µg of RNA can be expected from 5 × 10^5 cultured cells, and yields of 80–200 pg can be expected from 10 cultured cells.

TROUBLESHOOTING

Observation	Suggestion
RNA is degraded	Use DEPC-treated or radiation-sterilized plasticware.
RNA yield is poor	Confirm that the cell number is within the recommended range; exceeding 5×10^5 cells will result in a viscous lysate.
	Confirm that 80% sulfolane and cell lysate were combined at a 1:1 ratio prior to loading the 96-well binding plate.
	Incubate the plate for 2 full minutes after adding the Elution Buffer.
	Use 30–100 µl of Elution Buffer.
	Perform the elution twice.
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 with additional Lysis Buffer or use a smaller number of cells, as a highly viscous lysate will cause a large amount of genomic DNA to bind to the fiber matrix.
	Ensure that the plate is centrifuged at 1100 × *g* for 10 minutes before the DNase treatment step.

APPENDIX: ALTERNATE PROTOCOL FOR ISOLATION BY CENTRIFUGATION

This protocol modification provides a means for 96-well RNA isolation without the use of a vacuum manifold. Follow the instructions for *Preparing Adherent Tissue Culture Cells* or *Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells* and then proceed with the following protocol.

Additional Materials Required

800-µl 96-well collection plates (no more than 3.1 cm in height)
Additional plate sealers

Isolating RNA

1. Add an equal volume (usually 100 µl) of 80% sulfolane to the cell lysate and mix thoroughly by repeated pipetting (3–5 times). Transfer the lysate to the wells of a 96-well binding plate.

2. Cover the top of the binding plate with an adhesive plate sealer and place the plate on top of a 96-well collection plate. Spin the plate in a tabletop centrifuge at 1100 × g for 3 minutes or until the lysate-sulfolane mixtures have been pulled through the fiber matrix at the bottom of the wells.

   Note *The RNA is protected up to this point from RNases by the presence of guanidine thiocyanate.*

3. **Optional DNase Treatment** This procedure is recommended if DNA free total RNA is required. The DNase treatment can be omitted if the removal of DNA is not necessary.

   a. Add 600 µl of 1× Low-Salt Wash Buffer to each well and place the binding plate on top of an 800 µl 96-well collection plate. Cover the top of the plate with a fresh plate sealer and spin in a tabletop centrifuge at 1100 × *g* for 2 minutes. Decant the wash and centrifuge for an additional 10 minutes to dry the fiber matrix.

   b. Prepare the DNase solution by gently mixing 100 µl of reconstituted RNase-free DNase I with 5 ml of DNase Digestion Buffer.

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

   c. Add 50 µl of the DNase solution directly onto the fiber matrix inside each well of the plate. Cover the plate with a fresh plate sealer.

   d. Incubate the plate at room temperature for 15 minutes.

4. Add 500 µl of 1× High-Salt Wash Buffer to each well of the binding plate and cover the plate with a fresh plate sealer. Place the binding plate on top of the 800 µl 96-well collection plate and centrifuge for 2 minutes or until the wash buffer has been pulled through the fiber matrix at the bottom of the wells.

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

5. Decant the wash. Add 600 µl of 1× Low-Salt Wash Buffer to each well and cover the plate with a fresh plate sealer. Centrifuge for 2 minutes or until the wash buffer has been pulled through the fiber matrix.

6. Decant the wash. Add 600 µl of 1× Low-Salt Wash Buffer to each well and cover the plate with a fresh plate sealer. Centrifuge for 2 minutes or until the wash buffer has been pulled through the fiber matrix. Decant the wash, cover the plate with a fresh plate sealer, and centrifuge for an additional 5 minutes to dry the fiber matrix.

7. Transfer the plate to a fresh 96-well collection plate.

8. Add 30 µl of Elution Buffer directly onto the fiber matrix inside the wells of the plate and cover the plate with a fresh plate sealer. Incubate the plate for 2 minutes at room temperature. Spin in a tabletop centrifuge at 1100 × g for 5 minutes to collect the RNA.

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

   The RNA yield may be increased by performing a second elution.

   The purified RNA is in the Elution Buffer in the collection plate. 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 from >1 × 10^5 cells, 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. When quantifying RNA isolated from <1 × 10^5 cells it will be necessary to use a more sensitive fluorescence based system (i.e. the RiboGreen RNA Quantitation Kit, Molecular Probes, Inc.).

RNA yields may vary depending on cell types used. Yields of 2–8 µg of RNA can be expected from 5 × 10^5 cultured cells, and yields of 80–200 pg can be expected from 10 cultured cells.

REFERENCES

1. Dolter, K. and Braman, J. (2000) *Strategies* 13(2):56-58.
2. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* 18(24):5294-9.
3. Vogelstein, B. and Gillespie, D. (1979) *Proc Natl Acad Sci U S A* 76(2):615-9.

ENDNOTES

RiboGreen® is a registered trademark of Molecular Probes, Inc.

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.

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Absolutely RNA 96 Microprep Kit

Catalog #400793 and #400794

QUICK-REFERENCE PROTOCOL

Preparing Adherent Tissue Culture Cells

- Add 77 µl of β-ME to 11 ml of Lysis Buffer
- Aspirate the medium from the tissue culture plate
- Add 100 µl of the Lysis Buffer–β-ME mixture to each well of cells

Preparing Tissue Culture Cells Grown in Suspension or Trypsinized Adherent Cells

- Add 77 µl of β-ME to 11 ml of Lysis Buffer for 96 samples containing between 10 cells and 5×10^5 cells each
- Centrifuge the cells at 1000 × g for 5 minutes
- Aspirate the supernatant
- Add 100 µl of Lysis Buffer–β-ME mixture to each cell pellet (up to 5×10^5 cells)

Isolating RNA

- Add 100 µl of 80% sulfolane to the cell lysate and mix thoroughly by repeated pipetting (3–5 times)
- Transfer the lysate to the wells of a 96-well binding plate and seal the empty wells
- Place the binding plate within the top of the vacuum manifold and apply vacuum until the lysate-sulfolane mixtures have been pulled through the fiber matrix at the bottom of the wells, then release the vacuum

Optional DNase Treatment

- Add 600 µl of 1× Low-Salt Wash Buffer to each well and reapply the vacuum until the wash buffer has been pulled through the fiber matrix
- Cover the top of the plate with an adhesive plate sealer and place the plate on top of a 96-well collection plate and spin in a tabletop centrifuge at 1100 x g for 10 minutes to dry the fiber matrix
- Gently mix 100 µl of reconstituted RNase-free DNase I with 5 ml of DNase Digestion Buffer
- Add 50 µl of the DNase solution directly onto the fiber matrix of each well
- Incubate at room temperature for 15 minutes and discard liquid in collection tray of vacuum manifold

- Add 500 µl of 1× High-Salt Wash Buffer to each well and apply vacuum until the wash buffer has been pulled through the fiber matrix at the bottom of the wells, then release the vacuum
- Add 600 µl of 1× Low-Salt Wash Buffer to each well and reapply the vacuum until the wash buffer has been pulled through the fiber matrix, then release the vacuum
- Add 600 µl of 1× Low-Salt Wash Buffer to each well and reapply the vacuum until the wash buffer has been pulled through the fiber matrix
- Cover the plate with a fresh adhesive plate sealer and place the plate on top of the 96-well collection plate and spin in a tabletop centrifuge at 1100 × g for 5 minutes to dry the fiber matrix
- Transfer the plate to a fresh 96-well collection plate
- Add 30 µl of Elution Buffer directly onto the fiber matrix inside the wells and incubate the plate for 2 minutes at room temperature
- Cover the top of the plate with a fresh adhesive plate sealer and spin in a tabletop centrifuge at 1100 × g for 5 minutes to collect the RNA

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