Absolutely RNA Microprep Kit

INSTRUCTION MANUAL

Catalog #400805
Revision A

For In Vitro Use Only
400805-12

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Absolutely RNA Microprep Kit

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Absolutely RNA Microprep Kit

MATERIALS PROVIDED

Materials provided	Quantity[a]
Lysis Buffer	35 ml
β-Mercaptoethanol (β-ME) (14.2 M)[b]	300 µl
RNase-free DNase I (lyophilized)[c]	2600 U
DNase Reconstitution Buffer	300 µl
DNase Digestion Buffer	1.5 ml
High-Salt Wash Buffer (1.67×)	24 ml
Low-Salt Wash Buffer (5×)	17 ml
Elution buffer (10 mM Tris-HCl, pH 7.5)	3 ml
RNA-Binding Spin Cups and 2-ml receptacle tubes	50 each
1.5-ml collection tubes	50

[a] Sufficient reagents are provided to isolate total RNA from 50 samples of up to 5×10^5 cells each.

[b] Once opened, store at 4°C.

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

STORAGE CONDITIONS

Upon Receipt: Store all components at room temperature.

β-Mercaptoethanol: Once opened, store at 4°C.
RNase-Free DNase I: Once reconstituted, store at –20°C.
All Other Components: Once prepared, store at 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)]

Revision A

INTRODUCTION

The Absolutely RNA microprep kit allows rapid purification of high-quality total RNA from small samples of cultured cells or cells harvested by laser capture microdissection.

The Absolutely RNA microprep kit is designed for isolation from samples containing a very small number of cells (1 cell to 5×10^5 cells). Treatment of the sample with lysis buffer containing a strong protein denaturant (the chaotropic salt guanidine thiocyanate), results in effective cell lysis and prevents ribonuclease (RNase) degradation of the RNA. Isolation of total RNA is performed in a single micro-spin cup to minimize potential loss of RNA during the procedure. Following cell lysis, the solution is mixed with ethanol and applied to the RNA-binding matrix within the micro-spin cup. The RNA component of the sample binds to the matrix; DNA contamination is efficiently removed following incubation with DNase I directly on the column. The immobilized RNA is washed to remove contaminants, and total RNA is recovered in 30 µl of elution buffer. See Figure 1 for an overview of this procedure.

The purified RNA is suitable for use in a multitude of molecular biology applications, including cDNA synthesis, RT-PCR, qualitative and quantitative RT-PCR, northern blotting, microarray target preparation and differential display.

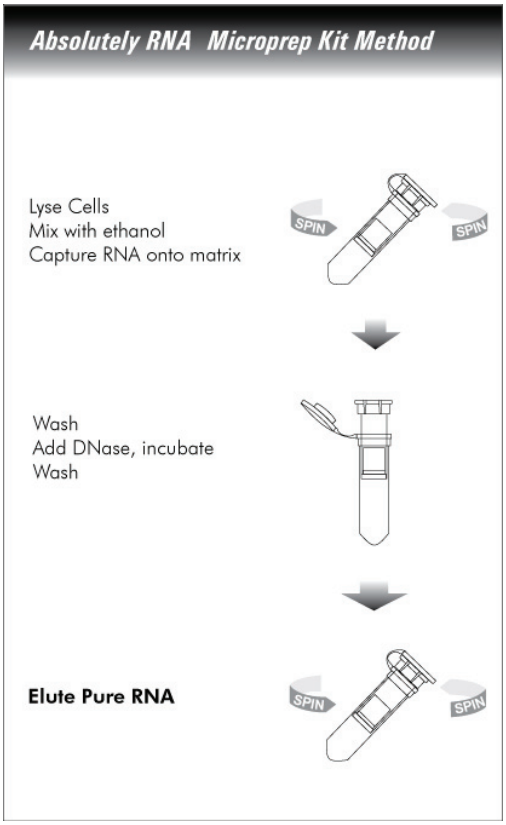

Figure 1 Overview of the Absolutely RNA microprep procedure.

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 thoroughly to ensure that 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 16 ml of 100% ethanol to the bottle of 1.67× High-Salt Wash Buffer.

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

β-Mercaptoethanol

Once opened, store the β-ME at 4°C.

PROTOCOL

RNA Isolation from Cells

1. Add 0.7 µl of β-ME to 100 µl of Lysis Buffer for each sample of up to 5×10^5 cells.

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

   Notes *Do not exceed 5×10^5 cells per sample or the RNA yield may decrease.*

   Prepare a fresh mixture of Lysis Buffer and β-ME before each use.

   Cell pellets can be stored at –80°C for future processing; however, homogenizing the cells in Lysis Buffer (the next step) prior to freezing the pellets is recommended to minimize RNA degradation.

2. Add 100 µl of the Lysis Buffer–β-ME mixture to each cell sample and vortex or pipet the sample repeatedly until homogenized.

   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 vortexing, pipetting, and/or by increasing the volume of Lysis Buffer.*

3. Add an equal volume (usually 100 µl) of 70% ethanol to the cell lysate and mix thoroughly by vortexing for 5 seconds.

   Note *It is important to vortex until the lysate and ethanol are thoroughly mixed.*

4. Transfer this mixture to a RNA-Binding Spin Cup that has been seated within a 2-ml collection tube and snap the cap of the tube onto the top of the spin cup.

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

6. Remove and **retain the spin cup** and discard the filtrate.

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

7. ***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. Replace the spin cup in the collection tube, add 600 µl of 1× Low-Salt Wash Buffer, and cap the tube. 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 collection tube, cap tube, and spin the sample in a microcentrifuge at maximum speed for 2 minutes to dry the matrix.

    c. Prepare the DNase solution by gently mixing 5 µl of reconstituted RNase-free DNase I with 25 µl of DNase Digestion Buffer.

        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 tube.

    e. Place the samples in a 37°C incubator for 15 minutes.

8. Add 500 µl of 1× High-Salt Wash Buffer to the spin cup, cap the tube, and spin the sample in a microcentrifuge at maximum speed for 30–60 seconds.

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

9. Remove and **retain the spin cup,** discard the filtrate, and replace the spin cup in the collection tube. Add 600 µl of 1× Low-Salt Wash Buffer. Cap the tube and spin the sample in a microcentrifuge at maximum speed for 30–60 seconds.

10. Remove and **retain the spin cup,** discard the filtrate, and replace the spin cup in the collection tube. Add 300 µl of 1× Low-Salt Wash Buffer. Cap the tube and spin the sample in a microcentrifuge at maximum speed for 2 minutes to dry the matrix.

11. Transfer the spin cup to a 1.5-ml collection tube.

12. Add 30 µl of Elution Buffer directly onto the matrix inside the spin cup. Snap the cap of the collection tube onto the spin cup and incubate the sample at room temperature for 2 minutes. Spin the sample in a microcentrifuge for 1 minute. This elution step may be repeated to increase the yield of total RNA.

    Note *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 may be increased by using Elution Buffer warmed to 60°C.

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

Quantifying the RNA

To quantify RNA isolated from at least 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. RNA yields may vary with the cell type. 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.

To quantify RNA isolated from less than 1×10^5 cells, it is necessary to use a more sensitive fluorescence-based system (e.g., RiboGreen® RNA quantitation kit, Molecular Probes, Inc.).

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.
	Incubate the spin cup for 2 full minutes after adding the Elution Buffer.
	Perform the elution twice and/or increase the volume of Elution Buffer. Any volume between 30 and 100 µl can be used.
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, ensuring that the surface of the matrix is completely covered. It is possible to use as little as 10 µl of Elution Buffer as long as the matrix is completely covered. The use of <30 µl of Elution Buffer, however, will lower the RNA yield from a large number of cells.
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 matrix.
	Ensure that the spin cup is centrifuged at maximum speed for 2 full minutes before DNase treatment.

APPENDIX I: 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 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 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.

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. This protocol is written for reactions of 60 µl or less. The volumes may be scaled up for larger reaction volumes.

1. Add 1.4 µl of β-ME to 200 µl of Lysis Buffer.
2. Adjust the reaction volume to 60 µl with water or buffer (e.g., 10 mM Tris, pH 7).
3. Mix the Lysis Buffer–β-ME mixture and 140 µl of 100% ethanol into the reaction.
4. Transfer the mixture to a RNA-Binding Spin Cup that is seated in a 2-ml receptacle tube and cap the tube.
5. Spin the tube in a microcentrifuge for 30–60 seconds.
6. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.
7. Add 500 µl of 1× High-Salt Wash Buffer and cap the tube. Spin the tube in a microcentrifuge for 30–60 seconds.
8. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.
9. Add 500 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin the tube in a microcentrifuge for 30–60 seconds.
10. Remove and **retain the spin cup** and discard the filtrate. Replace the spin cup in the receptacle tube.
11. Add 300 µl of 1× Low-Salt Wash Buffer and cap the tube. Spin the tube in a microcentrifuge for 2 minutes.
12. Transfer the spin cup to a 1.5-ml microcentrifuge tube and discard the 2-ml receptacle tube.
13. Add 30 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. This elution step may be repeated to increase the yield of RNA.

APPENDIX III: ISOLATING RNA FROM CELLS HARVESTED BY LASER CAPTURE MICRODISSECTION

This protocol can be used with CapSure transfer film carriers [Arcturus Engineering, Inc. (*www.arctur.com*)] to isolate RNA from cells harvested by laser capture microdissection.

1. Add 0.7 µl of β-ME to 100 µl of Lysis Buffer for each sample.

2. For each sample, add 100 µl of the Lysis Buffer–β-ME mixture to an Eppendorf standard 0.5-ml microcentrifuge tube [Brinkmann Instruments, Inc. #22 36 430-8 (*www.brinkmann.com*)].

3. Snap a CapSure transfer film carrier containing laser-captured cells onto each microcentrifuge tube.

4. Invert and vortex each tube for 15–30 seconds to lyse the cells that were captured on the CapSure transfer film carrier.

5. Proceed with the protocol in *RNA Isolation from Cells*, beginning with the addition of 70% ethanol at step 3.

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 Microprep Kit

QUICK-REFERENCE PROTOCOL

- Add 0.7 µl of β-ME to 100 µl of Lysis Buffer for each sample of ≤5 × 10^5 cells
- Add 100 µl of Lysis Buffer–β-ME mixture to each cell sample and vortex or pipet repeatedly until homogenized
- Add an equal volume of 70% ethanol to the cell lysate and mix thoroughly by vortexing for 5 seconds
- Transfer this 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

Optional 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 5 µl of reconstituted RNase-free DNase I with 25 µl of DNase Digestion Buffer
- Add the DNase solution directly onto the matrix of the spin cup
- Incubate at 37°C for 15 minutes

- Add 500 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 collection tube
- Add 30 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

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.