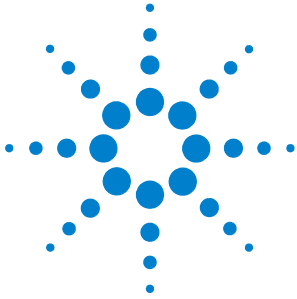
Agilent Total RNA Isolation Mini Kit (10)



Protocol

First edition
January 2005

New protocols: Fibrous tissue, Bacterial, and Yeast RNA isolations

Store Kit and all reagents at room temperature.

Research Use Only

Agilent Technologies

Notices

Manual Part Number

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Acknowledgements

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Contents

5 Troubleshooting

1 Introduction

Product Description

The Agilent Total RNA Isolation Mini Kit provides a phenol-free, spin column technique for the preparation of concentrated, highly purified, intact total cellular RNA. This kit can be used for isolation of total RNA from mammalian tissues and cultured cells. Recoveries of total cellular RNA are comparable to or greater than those obtained by typical silica or glass fiber methodologies. Typical results are shown in Table 1.

An important advantage of this rapid and convenient method is the removal of contaminating genomic DNA (gDNA) from the isolated total cellular RNA. Virtually all gDNA is removed in the initial stage of the protocol by centrifuging the cell or tissue lysate through a unique mini prefiltration column. Typically, this method results in a 100- to 1000-fold lower level of gDNA, relative to typical silica or glass fiber isolation methods. For the majority of applications, including microarray gene expression analysis, no supplemental DNase treatment step is necessary when using total RNA purified by this method. For applications requiring concentrated RNA samples, RNA can be eluted from the isolation column with as low as 10 µL of water. Total RNA is recovered with high yield, and without the addition of buffers or solvents that may interfere with subsequent enzymatic, chemical, or physical manipulations.

The intact and highly-purified RNA prepared by this method is ideal for cDNA synthesis and labeling, RT-PCR and quantitative RT-PCR, Microarray Gene Expression experiments, Northern Blotting, RNase protection assays and other applications.

Table 1 Typical yields of total cellular RNA *

Sample	RNA yield
Mouse or rat tissues	**µg/mg tissue**
Brain	0.8
Heart	2
Kidney	2.7
Liver	4.5
Pancreas	15
Skeletal muscle	1
Spleen	4.7
Thymus	3.2
Cultured cells	**µg per 10^6 cells**
293HEK	10
HeLa	15
NIH3T3	15
Bacteria	**µg per 10^8cells**
E. coli	9.2
B. subtilis	7.2
Yeast	**µg per 10^7cells**
S. cerevisiae	17.5

* Determined by optical absorbance at 260 nm, in 10 mM Tris/1 mM EDTA, pH 8 (40 µg/mL RNA per unit A_{260}). Variation is typically 15% relative standard deviation. Recoveries for tissues are typical for a 10–15 mg sample. Significantly lower RNA yields may indicate a systematic problem with the experimental conditions.

Kit Components

This kit includes the components listed in Table 2.

Table 2 Kit contents

Amount	Component
25 mL	Lysis solution
8 mL	Wash solution
25 mL	Nuclease-free water
10 each	Mini isolation columns (blue) paired with 2 mL collection tubes
10 each	Mini prefiltration columns (natural) paired with 2 mL collection tubes
10 each	1.5 mL RNase-free final collection tubes

Additional Materials Required

In order to perform the protocols, you will also need the materials listed in Table 3.

Table 3 Additional materials required

Materials needed
1 X PBS, sterile (for cultured cells)
100% Ethanol
14.3 M β-Mercaptoethanol (neat) (β-ME)
Microcentrifuge
Nuclease-free water
Tissue homogenizer such as a conventional rotor-stator homogenizer
Lysozyme (for bacteria)
Proteinase K (for fibrous tissues)
Lyticase/Zymolase (for yeast)
D-Sorbitol (for yeast)
0.5 M EDTA, pH 7.5 (for yeast)

Reagent Storage

Store all reagents at room temperature.

Ordering Information

Re-order part number 5185-6000, Agilent Total RNA Isolation Mini Kit (50).

Related Products

Agilent Mini Prefiltration Columns and Collection Tubes (50), part number 5188-2736

2 Preparation

Reagents

Before beginning either protocol, prepare the reagents listed below.

70% Ethanol solution

Prepare 50 mL of 70% ethanol solution by combining 35 mL of ACS 100%, molecular biology or equivalent grade ethanol and 15 mL of RNase-free water. Mix well.

Wash solution

To prepare the wash solution, add 32 mL of ACS 100%, molecular biology or equivalent grade ethanol to the provided bottle labeled **Wash Solution**. Mix well.

Lysis solution

Prepare by adding 250 µL of β-mercaptoethanol (β-ME) to the bottle labeled **Lysis Solution**. To prepare smaller amounts, add 10 µL of β-ME per 1 mL of lysis solution.

Store the lysis solution at 4 °C after the addition of β-ME.

WARNING **Lysis solution contains guanidine isothiocyanate, which may be harmful if inhaled, swallowed, or absorbed through the skin. Guanidine isothiocyanate causes skin, eye, and respiratory irritation. Do not mix with bleach or acids. Wear gloves when using the lysis solution and follow your institution's policies for chemical hygiene and disposal.**

RNase Free Technique

Since RNases are extremely stable enzymes, it is very difficult to inactivate them. A policy of avoidance is central to successful work with RNA. The following general principles will help you avoid RNase contamination of your RNA sample:

- Wear clean laboratory gloves at all times during the RNA isolation procedure and whenever handling lab equipment and any materials involved in the method.
- Clean the lab bench, pipettors, homogenizer, and other equipment with RNase decontamination solutions. Rinse well with nuclease-free water.
- Use only RNase-free filtered pipette tips and microcentrifuge tubes.
- If possible, use sterile disposable plasticware. Otherwise, decontaminate glassware by baking it overnight at 180 °C.
- Clean and decontaminate (for example, by treatment with 3% hydrogen peroxide) any electrophoresis gel boxes, then rinse with RNase-free water.

3
RNA Isolation Protocols

This chapter describes the protocols available for the Total RNA Isolation Mini Kit.

Agilent Total RNA Isolation Protocol for Animal Tissues

This method is designed for mini scale RNA isolation from animal tissues, and is optimized for 2.5–30 mg of tissue samples. Typical yields of RNA for a variety of mouse tissues and immortalized cell lines are shown in Table 1.

NOTE **Columns included**
This kit includes two types of color-coded columns: a mini prefiltration column (natural) and a mini isolation column (blue). Be sure to use the correct column specified by the protocol.

NOTE All steps are to be done at room temperature.

WARNING **To avoid injury, wear appropriate personal protective equipment when using liquid nitrogen.**

1 **Collect** the tissue sample then process it immediately or flash freeze it in liquid nitrogen. Store flash-frozen tissue at –70 °C.

   Alternatively, tissues may be preserved in RNA*later*™ (Cat # 7020, Ambion, Austin, TX) according to manufacturer's directions.

2 **Weigh** the sample (samples stored in RNA*later* may be thawed), then place tissue in a tube containing prepared Lysis Solution (to which β-ME has been added). Use 20 µL of Lysis Solution per milligram of tissue to be homogenized. Tube must be large enough to accommodate probe of homogenizer. The homogenizer probe should reach the bottom of tube for best homogenization. Samples smaller than 5 mg must be homogenized in a minimum of 100 µL Lysis solution.

3 **Immediately** and vigorously homogenize using a conventional rotor-stator homogenizer with a stainless steel probe for 30 seconds at 15,000 rpm. This is 50% of speed for an Omni International TH homogenizer for (OMNI International, Warrenton, VA). To reduce foaming, move the probe from side to side rather than up and down. Larger volumes (more than 10 mL) may require slightly longer homogenization times.

   If you will not process the homogenate immediately, store it at –70 °C. To process frozen homogenate, thaw it at 37 °C for 15–20 minutes and vortex vigorously.

4 **Centrifuge** up to 600 µL of homogenate (equivalent to 30 mg of tissue) through a mini prefiltration column (natural) for **3 minutes** at full speed (for a typical microcentrifuge, approximately 16,000 × g). This step ensures complete homogenization of the tissue and removes cellular contaminants. Mini prefiltration columns **cannot** be reused. If processing more than 600 µL, use a new prefiltration column.

NOTE

Guidance for Column Loading
For lowest levels of gDNA contamination in larger samples, Agilent recommends processing 15 mg equivalents of tissue with each mini prefiltration column. Clarified homogenate can then be pooled for processing on the isolation column. The maximum capacity of the prefiltration column and the mini isolation column is 15 mg for spleen and 10 mg for pancreas.

5 **Add 70% Ethanol** to the clarified homogenate, using a volume equal to the amount of homogenate initially added to the prefiltration column. **Mix** until the solution appears homogenous. Incubate mixture for 5 minutes at room temperature.

6 **Add** the ethanol/lysis mixture (up to 700 µL) to the mini isolation column (blue), then **centrifuge** for **30 seconds** at full speed. Discard the flow-through, and replace the RNA-loaded column in the 2 mL collection tube. If the homogenate/ethanol mixture volume exceeds 700 µL, add aliquots successively onto the mini isolation column, then centrifuge and discard the flow-through as described above. Centrifugation time may be increased to 1 minute to ensure complete flow-through of viscous samples.

7 **Add** 500 µL of prepared Wash Solution (to which ethanol has been added) to the mini isolation column, then **centrifuge** for **30 seconds** at full speed. Discard the flow-through, then replace the mini isolation column in the same collection tube. **Repeat** step 7 one more time for a total of two washes with Wash Solution.

8 **Centrifuge** the mini isolation column for **2 minutes** at full speed to completely remove trace amounts of Wash Solution.

9 **Transfer** the mini isolation column into a new 1.5 mL RNase-free final collection tube. Add 10–50 µL of nuclease-free water to the top center of membrane (without touching membrane). **Incubate 1 minute**, then **centrifuge** for **1 minute** at full speed.

NOTE

If more concentrated RNA samples are desired for downstream application, the elution volume may be as 10 µL. However, if the final RNA concentration exceeds 3 µg/µL, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 10 µL nuclease-free water for elution and determine the concentration by $A_{260\ nm}$. If the concentration is greater than 3 µg/µL, residual RNA may be remaining on the membrane. Apply an additional 10 µL to isolation column, spin 1 minute 16,000 x g and determine concentration of the second elution. Pool with initial elution if significant RNA is present in second elution.

Agilent Total RNA Isolation Protocol for Cultured Cells

This method is designed for mini-scale RNA isolation from cultured cells, and is optimized for $1 \times 10^5 - 5 \times 10^6$ cells per column. Typical yields of RNA for a variety of mouse tissues and immortalized cell lines are shown in Table 1.

For more than 5×10^6 cells, use multiple mini prefiltration and mini isolation columns.

NOTE **Columns included**
This kit includes two types of color-coded columns: a mini prefiltration column (natural) and a mini isolation column (blue). Be sure to use the correct column specified by the protocol.

NOTE All steps are to be done at room temperature.

NOTE **Volume of Lysis Solution**
Cells should be lysed in a minimum volume of 100 µL not to exceed 1×10^7 cells per mL. The capacity of the prefiltration column is 600 µL.

1 Collect the cells

For suspension cells: Spin the cells at low speed (~300 × g for 5 minutes) and remove the supernatant from the tube.

For adherent cells: Aspirate the culture medium and gently wash with PBS. Lyse directly in the culture vessel by scraping or vigorously pipetting with an appropriate volume (see Note) of prepared Lysis Solution (to which β-ME has been added). Adherent cells can also be trypsinized and pelleted prior to lysis.

Cell pellets which have not been lysed with Lysis Solution may be frozen at –70 °C. Cell pellets may also be preserved in RNA*later* according to manufacturer's directions.

2 **Prepare the lysate**

For suspension cells and adherent cells which have not been lysed in culture vessel: Add an appropriate volume of Lysis Solution (see Note) and **vortex** for **1 minute**.

For cells preserved in RNA*later*: Cells may be collected according to manufacturer's directions. Add an appropriate volume of Lysis Solution (see Note) and **vortex** for **1 minute**.

If you will not process the lysate immediately, store it at –70 °C. To process frozen lysate, thaw it at 37 °C for 15–20 minutes and vigorously vortex.

3 **Centrifuge** up to 600 µL of the cell homogenate through a mini prefiltration column (**natural**) for **3 minutes** at full speed (for a typical microcentrifuge, approximately 16,000 × g). This step ensures complete homogenization of the cells and removes cellular contaminants. Mini prefiltration columns **cannot** be reused. If processing more than 600 µL, use an additional new prefiltration column.

4 **Mix an equal volume of 70% Ethanol** with the clarified homogenate. Use volume equal to the amount of homogenate added to the prefiltration column. **Mix** until the solution appears homogenous. **Incubate** mixture for **5 minutes** at room temperature.

5 **Add the ethanol/lysis mixture** (up to 700 µL) to the mini isolation column (**blue**), then **centrifuge** for **30 seconds** at full speed. Discard the flow-through, and replace the RNA-loaded column in the 2 mL collection tube. If the homogenate/ethanol mixture volume exceeds 700 µL, add aliquots successively onto the mini isolation column, then centrifuge and discard the flow-through as described above. Centrifugation time may be increased to 1 minute to ensure complete flow-through of viscous samples.

6 **Add** 500 µL of prepared Wash Solution (to which ethanol has been added) to the mini isolation column, then **centrifuge** for **30 seconds** at full speed. Discard the flow-through, then replace the mini isolation column in the same collection tube. **Repeat** step 6 one more time for a total of two washes with Wash Solution.

7 **Centrifuge** the mini isolation column for **2 minutes** at full speed to remove trace amounts of Wash Solution.

8 **Transfer** the mini isolation column into a new 1.5 mL RNase-free final collection tube. **Add** 10–50 µL of nuclease-free water to the top center of membrane (without touching membrane). **Incubate 1 minute**, then **centrifuge** for **1 minute** at full speed.

NOTE If more concentrated RNA samples are desired for downstream application, the elution volume may be as 10 µL. However, if the final RNA concentration exceeds 3 µg/µL, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 10 µL nuclease-free water for elution and determine the concentration by $A_{260\ nm}$. If the concentration is greater than 3 µg/µL, residual RNA may be remaining on the membrane. Apply an additional 10 µL to isolation column, spin 1 minute 16,000 x g and determine concentration of the second elution. Pool with initial elution if significant RNA is present in second elution.

Agilent Total RNA Isolation Protocol for Fibrous Tissues

This method is designed for mini-scale RNA isolation from fibrous animal tissues, such as heart and skeletal muscle, and has been optimized for 10 mg of tissue. Typical yields are shown in Table 1.

NOTE **Columns included**
This kit includes two types of color-coded columns: a mini prefiltration column (natural) and a mini isolation column (blue). Be sure to use the correct column specified by the protocol.

NOTE All steps are to be done at room temperature, unless otherwise indicated.

NOTE In order to perform RNA isolations from fibrous tissues, you will also need:

100% Ethanol
Proteinase K, 20 mg/mL

Before you begin, set a water bath or heat block to 55 °C

1 **Collect** the tissue sample, then process it immediately or flash freeze it in liquid nitrogen. Store flash-frozen tissue at –70 °C. Alternatively, tissues may be preserved in RNAlater (Cat # 7020, Ambion, Austin, TX) according to manufacturer's directions.

2 **Weigh** the sample (samples stored in RNAlater may be thawed), then place tissue in a tube containing prepared Lysis Solution (to which ß-ME has been added). Use 20 µL of Lysis Solution per

milligram of tissue to be homogenized. Tube must be large enough to accommodate probe of homogenizer. The homogenizer probe should reach the bottom of tube for best homogenization. Samples smaller than 5 mg must be homogenized in a minimum of 100 µL Lysis solution.

3 **Immediately** and vigorously homogenize using a conventional rotor-stator homogenizer with a stainless steel probe for **1 minute** at 15,000 rpm. This is 50% of speed for an Omni International TH homogenizer (OMNI International, Warrenton, VA). To reduce foaming, move the probe from side to side rather than up and down. Larger volumes (more than 10 mL) may require slightly longer homogenization times.

   If you will not process the homogenate immediately, **store** it at –70 °C. To process frozen homogenate, **thaw** it at 37 °C for **15–20 minutes** and vortex vigorously.

4 **Centrifuge** up to 200 µL of homogenate to a mini-prefiltration column (natural) for **3 minutes** at full speed (for a typical micro-centrifuge, approx 16,000 x g). This step ensures complete homogenization of the tissue and removes cellular contaminants. Mini prefiltration columns **cannot** be reused. If processing more than 200 µL, use a new prefiltration column.

5 **Discard** column and **add** 390 µL of nuclease-free water to the filtrate. **Mix** well and **add** 10 µL of 20 mg/mL Proteinase K. **Mix** and **incubate 15 minutes** at 55 °C.

6 **Add** 600 µL of 100% ethanol, **mix** well and **incubate 5 minutes** at room temperature.

7 **Add** the ethanol/lysis mixture (up to 600 µL) onto the mini isolation column (blue) and **centrifuge** for **30 seconds** at full speed. **Discard** flow through and **replace** the RNA-loaded column in the 2 mL collection tube. **Add** the remaining ethanol/lysis mixture onto the mini isolation column, then **centrifuge,** and **discard** flow-through as described above. Centrifugation times may be increased to 1 minute to ensure complete flow-through of viscous samples.

8 **Add** 500 µL of prepared Wash Solution (to which ethanol has been added) to the mini isolation column, then **centrifuge** for **30 seconds** at full speed. **Discard** the flow-through, then **replace** the mini isolation column in the same collection tube. **Repeat** step 8 one more time for a total of two washes with Wash Solution.

9 **Centrifuge** the mini isolation column for **2 minutes** at full speed to completely remove trace amounts of Wash Solution.

10 **Transfer** the mini isolation column into a new 1.5 mL RNase-free final collection tube. **Add** 10–50 µL of nuclease-free water to the top center of membrane (without touching membrane). **Incubate 1 minute**, then **centrifuge** for **1 minute** at full speed.

NOTE

If more concentrated RNA samples are desired for downstream application, the elution volume may be as 10 µL. However, if the final RNA concentration exceeds 3 µg/µL, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 10 µL nuclease-free water for elution and determine the concentration by $A_{260\ nm}$. If the concentration is greater than 3 µg/µL, residual RNA may be remaining on the membrane. Apply an additional 10 µL to isolation column, spin 1 minute 16,000 x g and determine concentration of the second elution. Pool with initial elution if significant RNA is present in the second elution.

Agilent Total RNA Isolation Protocol for Bacteria

This method is designed for mini-scale RNA isolations from bacteria and was developed using 5 x 10^8 cells. Typical yields of bacteria are shown in Table 1. For different bacterial strains, the number of cells can be optimized considering RNA content. The capacity of the Isolation Column is approximately 100 µg total RNA.

NOTE **Columns included**
This kit includes two types of color-coded columns: a mini prefiltration column (natural) and a mini isolation column (blue). Be sure to use the correct column specified by the protocol.

NOTE All steps are to be done at room temperature, unless indicated otherwise.

NOTE In order to perform Bacterial RNA isolations, you will also need:

100% Ethanol
Lysozyme
TE, pH 8.0

Bacterial cultures are to be grown overnight at 30 °C or for a few hours (typically 3 hours) at 37 °C. Growth conditions are dependent on the particular strain used. Avoid isolating RNA from saturated cultures. $OD_{600\ nm}$ readings can approximate culture density (a reading of 1 at 600 nm is approximately 10^9 bacteria). Serial dilutions of culture should be plated to confirm culture density at a particular absorbance reading.

Lysozyme from lyophilized powder is made in TE pH 8.0 just prior to use. Lysozyme should be 400 µg/mL for isolations from gram-negative strains and 3 mg/mL for gram-positive strains. Using frozen aliquots of lysozyme may result in reduced yields.

All steps are to be done at room temperature, unless indicated otherwise. Use RNase Free Technique.

1 **Collect the bacteria**. Centrifuge cultures at 4 °C between 3,000–6,000 x g. The growth medium must be thoroughly removed from the cell pellet by draining or aspirating. Additionally, an absorbent material can be used to remove final traces of medium from the centrifuge tube or bottle. The cell pellet can be frozen at –80 °C.

2 **Resuspend** pellet in 100 µL of Lysozyme prepared in TE pH 8.0. Gram-negative cells should be digested for 5–20 minutes and gram-positive cells should be digested for 20–40 minutes.

3 **Add** 200 µL of prepared Lysis Solution to digested cells and **mix** well by pipetting. **Add** this mixture to a Prefiltration Column and **centrifuge** for **3 minutes** at full speed (for a typical microcentrifuge, approximately 16,000 x g).

4 **Add** 300 µL 100% Ethanol to the flow-through from the Prefiltration Column. **Mix** well by pipetting and **incubate** for **5 minutes**.

5 **Add** the ethanol/lysis mixture to an Isolation Column, **centrifuge 30 seconds** at full speed. Discard the flow-through, and replace the RNA-loaded column in the 2-mL collection tube.

6 **Add** 500 µL of prepared Wash Solution (to which ethanol has been added) to the mini isolation column, then **centrifuge** for **30 seconds** at full speed. **Discard** the flow-through, then **replace** the mini isolation column in the same collection tube. **Repeat** step 6 one more time for a total of two washes with Wash Solution.

7 **Centrifuge** Isolation Column for **2 minutes** at 16,000 x g to remove final traces of Wash Solution.

8 **Transfer** the mini isolation column into a new 1.5 mL RNase-free final collection tube. **Add** 10–50 µL of nuclease-free water to the top center of membrane (without touching membrane). **Incubate 1 minute**, then **centrifuge** for **1 minute** at full speed.

NOTE If more concentrated RNA samples are desired for downstream application, the elution volume may be as 10 µL. However, if the final RNA concentration exceeds 3 µg/µL, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 10 µL nuclease-free water for elution and determine the concentration by $A_{260\ nm}$. If the concentration is greater than 3 µg/µL, residual RNA may be remaining on the membrane. Apply an additional 10 µL to isolation column, spin 1 minute 16,000 x g and determine concentration of the second elution. Pool with initial elution if significant RNA is present in second elution.

Agilent Total RNA Isolation Protocol for Yeast

This method is designed for mini-scale RNA isolations from yeast and was developed using 5 x 10^8 cells. Typical yields of *S. cerevisiae* are shown in Table 1. For different yeast strains, the number of cells can be optimized considering RNA content. The capacity of the Isolation Column is approximately 100 µg total RNA.

NOTE

Columns included
This kit includes two types of color-coded columns: a mini prefiltration column (natural) and a mini isolation column (blue). Be sure to use the correct column specified by the protocol.

NOTE

All steps are to be done at room temperature, unless indicated otherwise.

NOTE

In order to perform yeast RNA isolations, you will also need:

100% Ethanol
ß-mereaptoethanol
Lyticase/Zymolase
D-sorbitol
0.5 M EDTA, pH 7.5

Yeast cultures are typically grown overnight at 30 °C. Growth conditions are dependent on the particular strain used. Avoid isolating RNA from saturated cultures. $OD_{600\ nm}$ readings can approximate culture density (a reading of 1 at 600 nm is approximately 10^7 yeast cells). Serial dilutions of culture should be plated to confirm culture density at a particular absorbance reading.

Before you begin, set a shaking waterbath or incubator at 30 °C and a refrigerated centrifuge at 4 °C.

Prepare 100 mL of Sorbitol/EDTA buffer (1 M Sorbitol, 0.1 M EDTA, pH 7.5) in nuclease-free water and sterile-filter. Each yeast RNA isolation requires 2 mL of buffer.

Lyticase should be dissolved in cold nuclease-free water to a final concentration of 500 units/mL and can be stored at 4 °C up to 1 week.

All steps are to be done at room temperature, unless indicated otherwise. Use RNase Free Technique.

1 **Collect the yeast**. Centrifuge cultures at 1000 x g at 4 °C.The growth medium must be thoroughly removed from the cell pellet by draining or aspirating. Additionally, an absorbent material can be used to remove final traces of medium from the centrifuge tube or bottle. Set centrifuge to 25 °C.

2 **Resuspend** pellet in 2 mL of Sorbitol/EDTA buffer per 5 x 10^7 cells. Add 2 µL of ß-mE and 500 µL Lyticase per 5 x 10^7 cells. **Mix** gently by inversion.

3 **Incubate** for **15 minutes** at 30 °C, shaking at 100 rpm.

NOTE Handle spheroplasts gently to avoid lysis until step 6.

4 **Centrifuge** spheroplasts for 5 minutes at 300 x g at room temperature.

5 **Carefully remove** supernatant by aspirating. An absorbant material can be used to remove final traces of supernatant from tube.

6 **Add** 350 µL of prepared Lysis Solution per 5 x 10^7 spheroplasts. Homogenize with a rotor-stator homogenizor with a stainless steel probe for 30 seconds at 15000 rpm. To reduce foaming, move the probe side to side rather than up and down. Alternatively, the lysate may be vortexed vigorously for 1 minute, although this may result in reduced yields.

7 **Centrifuge** 350 µL of lysate through a mini-prefiltration column (natural) for **3 minutes** at full speed (for a typical microcentrifuge, approximately 16,000 x g). This step ensures complete homogenization and removes cellular contaminants. Mini-prefiltration columns **cannot** be reused. Use an additional prefiltration column if processing more than 350 µL .

8 **Mix an equal volume of 100% Ethanol** with the clarified lysate. Use a volume equal to the volume of homogenate added to the mini-prefiltration column in step 7. **Mix** until the solution appears homogenous. **Incubate** mixture for **20 minutes** at room temperature.

9 **Add** the ethanol/lysis mixture to an Isolation Column, **centrifuge 30 seconds** at full speed. Discard the flow-through, and replace the RNA-loaded column in the 2-mL collection tube. Centrifugation time may be increased to 1 minute to ensure complete flow-through of sample.

10 **Add** 500 µL of prepared Wash Solution (to which ethanol has been added) to the mini isolation column, then **centrifuge** for **30 seconds** at full speed. **Discard** the flow-through, then **replace** the mini isolation column in the same collection tube. **Repeat** step 10 one more time for a total of two washes with Wash Solution.

11 **Centrifuge** Isolation Column for **2 minutes** at 16,000 x g to remove final traces of Wash Solution.

12 **Transfer** the mini isolation column into a new 1.5 mL RNase-free final collection tube. **Add** 10–50 µL of nuclease-free water to the top center of membrane (without touching membrane). **Incubate 1 minute**, then **centrifuge** for **1 minute** at full speed.

NOTE

If more concentrated RNA samples are desired for downstream application, the elution volume may be as 10 µL. However, if the final RNA concentration exceeds 3 µg/µL, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 10 µL nuclease-free water for elution and determine the concentration by $A_{260\ nm}$. If the concentration is greater than 3 µg/µL, residual RNA may be remaining on the membrane. Apply an additional 10 µL to isolation column, spin 1 minute 16,000 x g and determine concentration of the second elution. Pool with initial elution if significant RNA is present in second elution.

4 Quality Analysis and Quantitation of Total RNA Samples

This section provides guidance for determining the quality and yield of total RNA collected with this kit using three methods: UV absorption, the Agilent 2100 Bioanalyzer, and gel electrophoresis.

UV Absorbance

The concentration of total RNA can be determined by measuring the optical absorbance at 260 nm (A_{260}) with a spectrophotometer. Dilute the samples in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) for accurate spectrophotometry. A_{260}/A_{280} ratio is influenced by the pH of the sample. Reliable measurements can be made in buffered solutions: 0.5 xTE, pH 7.0. Samples with A_{260} less than 0.10 are subject to increasing experimental variance. A reading of 1 unit at A_{260} is equivalent to 40 µg RNA per mL.

The ratio of A_{260} to A_{280} provides a measure of RNA purity, with respect to protein and other contaminants that absorb strongly in the near UV. A_{260} to A_{280} ratio values between 1.8 and 2.1 indicate high purity total RNA, as should be obtained in the use of this kit. Values below 1.8 indicate a systematic problem, and the RNA should be used with caution.

For a detailed protocol regarding spectrophotometry of RNA, see "Molecular Cloning. A Laboratory Manual", Volume 3, Section A8.20 (Sambrook & Russel, 2001).

Agilent 2100 Bioanalyzer

We recommend using the Agilent 2100 bioanalyzer for assessing RNA quality and integrity of total RNA samples. The RNA Integrity Number (RIN) software algorithm automatically assigns an integrity number to eukaryotic RNA samples analyzed on RNA 6000 Nano and Pico assays. The parameters used to determine integrity cover the entire electrophoretic trace of the RNA sample, rather than sole determination of the ratio of 28S to 18S ribosomal RNA. RIN allows researchers to compare reproducibility of RNA isolations and subsequent downstream experiments.

Additional Information

- Efficient Method for Isolation of High Quality Concentrated Cellular RNA with Extremely Low Levels of Genomic DNA Contamination. Agilent Technologies, publication 5989-0322EN www.agilent.com/chem
- Efficient Removal of Transfected Plasmid DNA from Total RNA Prepared with the Agilent Total RNA Isolation Mini Kit, Agilent Technologies, publication 5989-1597EN www.agilent.com/chem
- High-Purity Bacterial RNA Isolated with the Agilent Total RNA Isolation Mini Kit, Agilent Technologies, publication 5988-2281EN www.agilent.com/chem
- Quantitation Comparison of Total RNA Using the Agilent 2100 Bioanalyzer, Ribogreen Analysis, and UV Spectraphotometry. Agilent Technologies, publication 5988-7650EN www.agilent.com/chem
- RNA Integrity Number (RIN) - Standardization of RNA Quality Control, Agilent Technologies, publication 5989-1165EN www.agilent.com/chem.
- The Total RNA Story. Agilent Technologies, publication 5988-2281EN www.agilent.com/chem

5 Troubleshooting

Table 4 below lists several common symptoms that can result from processing techniques, and suggests appropriate fixes.

Table 4 Common problems and suggested solutions

Symptom	Suggestion
Prefiltration column clogged	Reduce viscosity with one of the following procedures: • Dilute homogenate with additional lysis solution. • Homogenize the lysate more thoroughly. • Use less of the starting material. • Centrifuge the lysate before prefiltering.
RNA yield is low	• Ensure that the disruption and homogenization is sufficient. Grind the tissue samples thoroughly. • Purify RNA as soon as the lysate is prepared. If using lysates stored at –70 °C, thaw at 37 °C for **15–20 minutes** before use and vortex vigorously. • Check the sample integrity. Ensure that the tissue is frozen in liquid nitrogen **immediately** after separation from the organism. • Ensure that the columns (both mini prefiltration and mini isolation) are not overloaded with the sample. • Use specialized protocol for difficult sample type (bacterial, fibrous tissue).
RNA is degraded	• Closely follow the RNase free technique for cleaning equipment (see "RNase Free Technique" on page 10). • Ensure that the tissue is not thawed before immersion in lysis solution. • Check the sample integrity. Ensure that the tissue is homogenized **immediately** after harvest or is **immediately** frozen in liquid nitrogen. • Purify RNA as soon as the lysate is prepared. • Use RNAlater for samples that will be stored.
A_{260}/A_{280} Ratio is low	The A_{260}/A_{280} ratio is influenced by the pH of the sample. Reliable measurements can be made in buffered solutions, such as 1 × TE, pH 8.0.
High genomic DNA	Dilute sample and use additional prefiltration column.

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