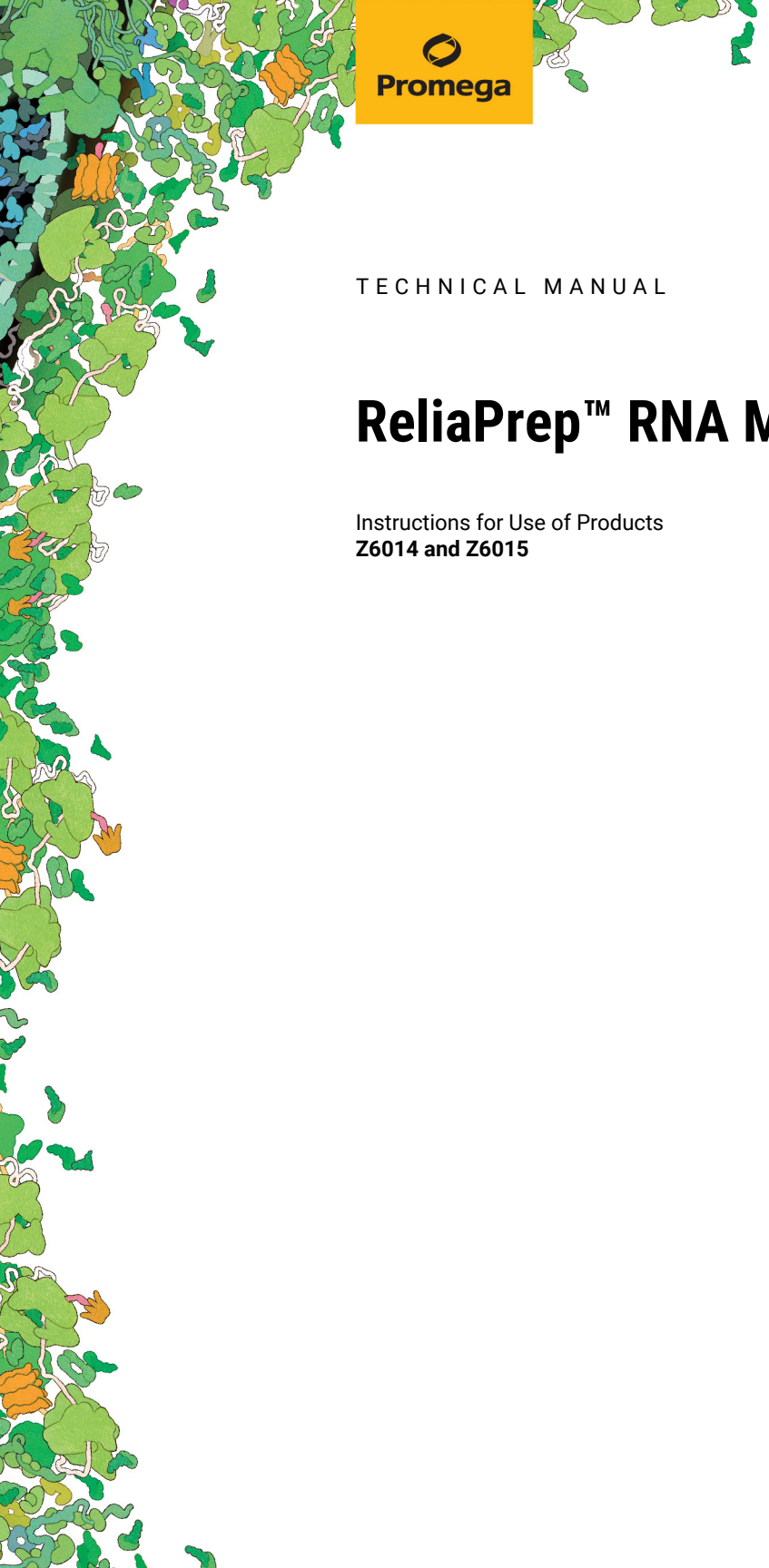

TECHNICAL MANUAL

ReliaPrep™ RNA Miniprep System

Instructions for Use of Products
Z6014 and Z6015

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TM774

ReliaPrep™ RNA Miniprep System

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The purity and integrity of RNA isolated from mammalian samples, whether cultured cells or animal tissue, are critical for its effective use in applications such as reverse transcription PCR (RT-PCR), reverse transcription quantitative PCR (RT-qPCR), RNase protection assays, Northern blot analysis, oligo(dT) selection of poly(A)+ RNA, in vitro translation, microarray analysis and sequencing. In recent years, RT-PCR and RT-qPCR have emerged as powerful methods to identify and quantitate specific mRNAs from small amounts of total RNA and mRNA. As the use of amplification as a research tool has grown, the need for methods to rapidly isolate high-quality RNA, substantially free of genomic DNA contamination, from small amounts of starting material (i.e., cultured cells or animal tissue) has also increased. The ReliaPrep™ RNA Miniprep System has been designed to address these needs.

The ReliaPrep™ RNA Miniprep System provides a fast and simple technique for preparing purified and intact total RNA from both cultured cells and animal tissues in as little as 30 minutes, depending on the number of samples to be processed. The system also incorporates a DNase treatment step that is designed to substantially reduce genomic DNA contamination, which can interfere with amplification-based methodologies. Purification is achieved without the use of phenol:chloroform extractions or ethanol precipitations.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ReliaPrep™ RNA Miniprep System	**250 preps**	**Z6015**

For in vitro Research Use Only. Each system contains sufficient reagents for 250 preparations of total RNA from cultured mammalian cells or animal tissue. Includes:

- 5 packs ReliaPrep™ Minicolumns (50/pack)
- 2 packs Collection Tubes (250/pack)
- 5 packs Elution Tubes (50/pack)
- 150ml BL Buffer
- 150ml LBA Buffer (LBA)
- 220ml RNA Dilution Buffer (RDB)
- 24ml Column Wash Solution (CWE)
- 3 vials DNase I (lyophilized)
- 2 × 750µl $MnCl_2$, 0.09M
- 11ml Yellow Core Buffer
- 206ml RNA Wash Solution (RWA)
- 4 × 900µl 1-Thioglycerol
- 13ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
ReliaPrep™ RNA Miniprep System	**50 preps**	**Z6014**

For in vitro Research Use Only. Each system contains sufficient reagents for 50 preparations of total RNA from cultured mammalian cells or animal tissue. Includes:

- 1 pack ReliaPrep™ Minicolumns (50/pack)
- 2 packs Collection Tubes (50/pack)
- 1 pack Elution Tubes (50/pack)
- 32.5ml BL Buffer
- 30ml LBA Buffer (LBA)
- 44ml RNA Dilution Buffer (RDB)
- 5ml Column Wash Solution (CWE)
- 1 vial DNase I (lyophilized)
- 250µl $MnCl_2$, 0.09M
- 2.5ml Yellow Core Buffer
- 35ml RNA Wash Solution (RWA)
- 2 × 900µl 1-Thioglycerol
- 13ml Nuclease-Free Water

Note: The Yellow Core Buffer is colored yellow to allow users to visualize whether the membrane is completely covered by the DNase mixture in the DNase step. The dye has no effect on the quality or downstream performance of the RNA.

2.A. Component and Kit Storage Conditions

Storage Conditions: Upon receipt, remove and store the 1-Thioglycerol at +2°C to +10°C. Store the BL Buffer and LBA Buffer, with 1-Thioglycerol added, at +2°C to +10°C for up to 30 days. Cap tightly between uses. For information on rehydration of DNase I see Section 4.A, Preparing Solutions. Store all other components at +15°C to +30°C.

Do not combine or replace components of the ReliaPrep™ RNA Miniprep System with components from any Wizard® *Plus* or Wizard® *Plus* SV DNA Purification System.

Caution: Guanidine thiocyanate and guanidine hydrochloride are harmful and irritants that are components of the BL Buffer, Lysis Buffer (LBA) and Column Wash Solution (CWE). 1-Thioglycerol is toxic. Wear gloves and follow standard safety procedures while working with these solutions. When processing human cells or tissue, follow standard procedures for handling and disposal of hazardous materials.

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3. General Considerations

3.A. Considerations for RNA Purification

The successful isolation of intact RNA requires four essential steps: Effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and removal of contaminating DNA and proteins. The most important step is the immediate inactivation of endogenous RNases that are released from membrane-bound organelles upon cell disruption (1).

The ReliaPrep™ RNA Miniprep System combines the disruptive and protective properties of guanidine thiocyanate (GTC) and 1-Thioglycerol to inactivate the ribonucleases present in cell extracts and tissue homogenates. GTC disrupts nucleoprotein complexes, allowing the RNA to be released into solution and isolated free of protein. Nucleic acids in lysates are bound to the ReliaPrep™ Minicolumns by centrifugation. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts, thus favoring adsorption of nucleic acids to the minicolumn. RNase-Free DNase I is applied directly to the membrane to digest contaminating genomic DNA. The bound total RNA is further purified from contaminating salts, proteins and cellular components by simple washing steps. Finally, the total RNA is eluted from the membrane by the addition of Nuclease-Free Water. This procedure yields an essentially pure fraction of total RNA after only a single round of purification without organic extractions or precipitations. The procedure is easy to perform with small quantities of cultured cells or animal tissues, and it can be used to process multiple samples.

Processing Capacity

The ReliaPrep™ RNA Miniprep System was developed and optimized for total RNA isolation from a wide range of input cell numbers, 1×10^2 to 5×10^6 cultured cells or a wide range of input tissue mass, 0.25–20mg, with a broad spectrum of RNA expression levels.

Downstream Applications

RNA purified with the ReliaPrep™ RNA Miniprep System is suitable for many molecular biology applications, including RT-PCR, microarrays and Northern blot hybridizations.

For all downstream applications, continue to protect your samples from RNases by wearing gloves and using solutions and centrifuge tubes that are RNase-free. The use of a ribonuclease inhibitor such as Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511) can help ensure protection from nucleases that may be introduced into purified RNA during downstream processing.

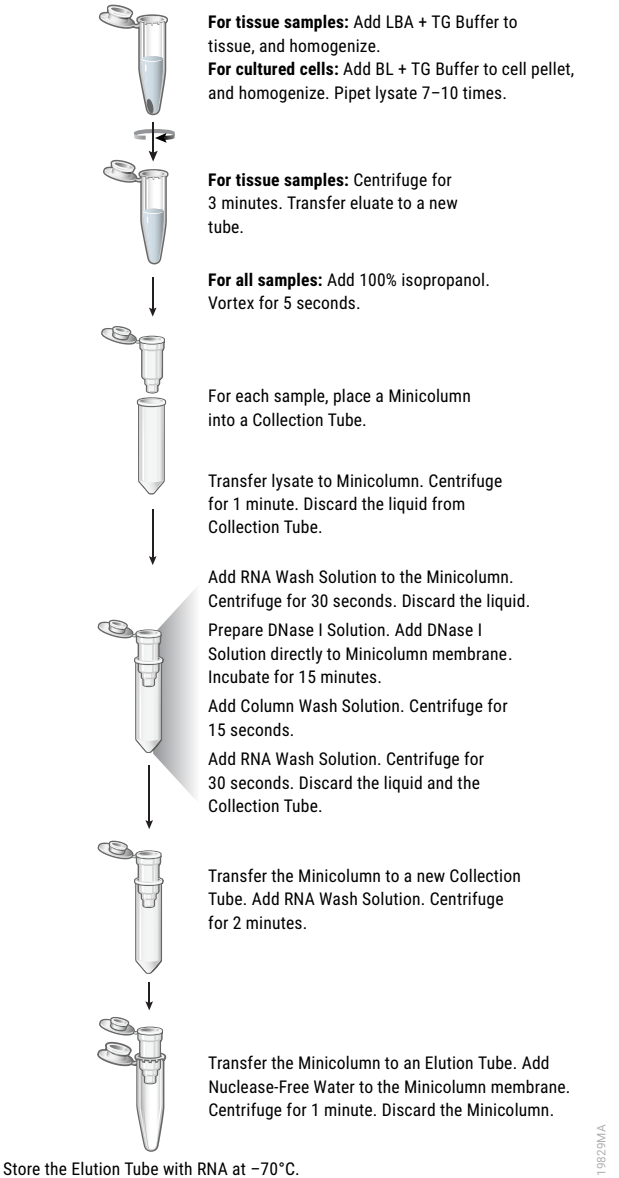

Figure 1. Schematic diagram of the ReliaPrep™ RNA Miniprep System. For protocol details, see Section 4.

3.B. Materials to be Supplied by the User

(Solution compositions are provided in Section 8.B.)

- 100% isopropanol, RNase-free
- 95% ethanol, RNase-free
- microcentrifuge
- PBS buffer, 1X
- trypsin-EDTA solution, 1X (for tissue samples only)
- small homogenizer (e.g., BioSpec Tissue-Tearor or Omni Micro homogenizer; for tissue samples only)

4. RNA Isolation and Purification Protocols

For best results from this system, use fresh cell or tissue samples. If you choose to store harvested cells as cell pellets, it is best to flash freeze the pellets in a dry ice/ethanol bath and then store at –70°C. RNA integrity depends on the rapid freezing of cells and direct thawing of the cell pellet into BL + TG Buffer to ensure rapid lysis and inactivation of nucleases in the sample. After lysis in BL + TG Buffer samples may be stored at –20°C to –70°C for up to 3 months.

If you choose to store harvested tissues, flash freeze the pellets in liquid nitrogen and then store at –70°C. RNA integrity depends on the rapid freezing of tissue and direct thawing into LBA + TG Buffer to ensure rapid lysis and inactivation of nucleases in the sample. After lysis in LBA + TG Buffer samples may be stored at –20°C to –70°C for up to 3 months.

Note: Three protocols are provided below, one protocol for cell samples and two protocols for tissue samples. The first protocol (Section 4.B) is used with cell cultures and cell pellets. The second (Section 4.C) addresses non-fibrous tissues (e.g., liver, spleen, brain). The third protocol (Section 4.D) is for use with fibrous tissues (e.g., lung, heart) and requires additional purification steps to yield best results.

Due to the toxicity of the chemicals used in the RNA purification procedure and the prevalence of RNases, wear gloves throughout the lysis and purification procedure.

4.A. Preparing Solutions

Before beginning the ReliaPrep™ RNA Miniprep System protocol, four solutions must be prepared.

Note: Throughout this document, BL Buffer, LBA Buffer, RNA Wash Solution (RWA) and Column Wash Solution (CWE) refer to the solutions supplied with the ReliaPrep™ RNA Miniprep System. Once prepared as described below, these solutions are referred to as BL + TG Buffer or LBA + TG Buffer, RNA Wash Solution and Column Wash Solution.

Solution	Preparation Steps	Notes
DNase I	**250** prep size (**Cat.# Z6015**): Add **275µl** of Nuclease-Free Water (provided) as indicated on the vial label or **50** prep size (**Cat.# Z6014**): Add **275µl** of Nuclease-Free Water (provided) as indicated on the vial label	**Gently** mix by swirling the vial of solution. **Do not vortex.** We recommend dispensing the rehydrated DNase into working aliquots (e.g., into 5–10 equal aliquots) using sterile RNase-free microcentrifuge tubes. A total of 3µl of rehydrated DNase I is required per RNA purification. Store rehydrated DNase I at –20°C. **Do not vortex the DNase I solution.** **Do not freeze-thaw aliquots of rehydrated DNase I more than three times.**
BL + TG Buffer (for cell samples)	**250** prep size (**Cat.# Z6015**): Add **1,500µl** of 1-Thioglycerol to 150ml of BL Buffer or **50** prep size (**Cat.# Z6014**): Add **325µl** of 1-Thioglycerol to 32.5ml of BL Buffer	**After adding 1-Thioglycerol (TG), mark on the bottle that you have performed this step.** Store the BL + TG Buffer at 2–10°C for up to 30 days. Cap tightly between uses. **1-Thioglycerol is very viscous and must be pipetted carefully.**
LBA + TG Buffer (for tissue samples)	**250** prep size (**Cat.# Z6015**): Add **3,000µl** of 1-Thioglycerol to 150ml of LBA Buffer or **50** prep size (**Cat.# Z6014**): Add **650µl** of 1-Thioglycerol to 30ml of LBA Buffer	**After adding 1-Thioglycerol (TG), mark on the bottle that you have performed this step.** Store the LBA + TG Buffer at 2–10°C for up to 30 days. Cap tightly between uses. **1-Thioglycerol is very viscous and must be pipetted carefully.**

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4.A. Preparing Solutions (continued)

Solution	Preparation Steps	Notes
RNA Wash Solution	**250** prep size (**Cat.# Z6015**): Add **350ml** of 95% ethanol to the bottle containing 206ml of concentrated RNA Wash Solution (RWA) or **50** prep size (**Cat.# Z6014**): Add **60ml** of 95% ethanol to the bottle containing 35ml of concentrated RNA Wash Solution (RWA)	**After adding ethanol to RNA Wash Solution (RWA), mark on the bottle label that this step has been performed.** The reagent is stable at +15°C to +30°C when tightly capped.
Column Wash Solution	**250** prep size (**Cat.# Z6015**): Add **36ml** of 95% ethanol to the bottle containing 24ml of concentrated Column Wash Solution (CWE) or **50** prep size (**Cat.# Z6014**): Add **7.5ml** of 95% ethanol to the bottle containing 5ml of concentrated Column Wash Solution (CWE)	**After adding ethanol to the Column Wash Solution (CWE), mark on the bottle label that this step has been performed.** The reagent is stable at +15°C to +30°C when tightly capped**.**

4.B. Preparing Cell Lysates

Use the following protocol to lyse cultured cells from adherent or suspension cultures. Use from 1×10^2 cells to a maximum of 5×10^6 cells per purification. The number of cells used may need to be adjusted, depending on cell type, function and RNA expression levels when collected.

To prepare cell lysates from adherent cell cultures, follow the protocol bellow beginning at Step 1. For adherent cells grown in multiwell plates, a yield of up to 1×10^6 cells per well is possible, depending on the cell type and well size. To prepare cell lysates from suspension cell cultures, proceed directly to Step 4.

Preparing Cell Lysates from Adherent Cell Cultures

1. Lysates can be prepared rapidly in the culture plate or flask by adding BL + TG Buffer. Recommended volumes of wash (PBS) and BL + TG Buffer (lysis) to add per well are shown in Table 1.

   Note: If you prefer to harvest cells by conventional trypsinization methods, harvest cells and proceed to Step 5.

   Table 1. Recommended Wash and Lysis Volumes for Harvesting Adherent Cells.

Plate Type	Wash (PBS/well)	Lysis (BL + TG Buffer)
96-well	100µl	100µl
48-well	250µl	100µl
24-well	500µl	100µl
6-well	2.0ml	250µl
T-25 flask	5.0ml	500µl

2. Remove the culture medium and wash the cells with ice-cold, sterile 1X PBS (Section 8.B). Add BL + TG Buffer and gently rock the plate or flask to cover adherent cells. Rinse the cells by repeatedly pipetting the lysate over the well surface 7–10 times. Collect the lysate, and transfer it to a new microcentrifuge tube.

   Note: For 96-well to 6-well plates, scraping is not required for individual wells. For T-25 flasks, scraping after the lysis step may increase yields due to lysate viscosity. The maximum lysate volume that can be processed with a single minicolumn is 500µl.

3. Proceed to Step 8.

4.B. Preparing Cell Lysates (continued)

Preparing Cell Lysates from Suspension Cell Cultures

4. **Collect cells in a sterile centrifuge tube** by centrifugation at 300 × *g* for 5 minutes. **Wash the cell pellet with ice-cold, sterile 1X PBS** (see Section 8.B for the recipe). **Centrifuge at 300 × *g* for 5 minutes** to collect the cells. **Discard the supernatant.** Carefully removing all supernatant will aid in efficient preparation of high-quality RNA.

5. Verify that 1-Thioglycerol has been added to the BL Buffer. Add BL + TG Buffer to the washed cell pellet in accordance with Table 1 below.

   Note: If frozen cell pellets are used as a starting material, add prepared BL + TG Buffer to the frozen pellets prior to thawing.

   Table 2. Recommended Amounts of Cells and Solutions for Suspension Cell Lysis.

Cell Input Range	BL + TG Buffer	100% Isopropanol
1×10^2 to 5×10^5	100µl	35µl
$>5 \times 10^5$ to 2×10^6	250µl	85µl
$>2 \times 10^6$ to 5×10^6	500µl	170µl

6. Disperse the cell pellet and mix well by vortexing and/or pipetting.

   Note: Up to 2×10^6 cells will lyse easily in 250µl of BL + TG Buffer. The lysis should be followed by pipetting 7–10 times to shear the DNA using a P200 or P1000 pipettor. For more than 2×10^6 cells, best results will be achieved by passing the lysate through a 20-gauge needle to shear the genomic DNA. Repeat 4–5 times. Expel the lysate into a 1.5ml tube.

   The maximum volume of lysate that can be efficiently processed is 500µl per minicolumn. If a lysate contains more RNA than the capacity of the minicolumn allows, some RNA will be lost during the wash steps.

7. Proceed to Step 8.

Purifying RNA from Prepared Cell Lysates

8. Add isopropanol as recommended in Table 2. Mix by vortexing for 5 seconds.

9. Wear clean gloves and open the packs of tubes and minicolumns carefully. Remove one ReliaPrep™ Minicolumn, two Collection Tubes and one Elution Tube for each sample to be processed. Place the Collection Tubes in a microcentrifuge tube rack, and place the ReliaPrep™ Minicolumn into a Collection Tube. Label all your tubes and minicolumns to maintain sample identity. Always wear gloves when handling the tubes and minicolumns.

10. Transfer the lysate to a ReliaPrep™ Minicolumn and centrifuge at 12,000–14,000 × *g* for 30 seconds at 20–25°C.

11. Remove the ReliaPrep™ Minicolumn and **discard the liquid in the Collection Tube**. Place the ReliaPrep™ Minicolumn back into the Collection Tube. Verify that the RNA Wash Solution has been diluted with ethanol as described in Section 4.A. **Add 500µl of RNA Wash Solution** to the ReliaPrep™ Minicolumn. **Centrifuge at 12,000–14,000 × *g* for 30 seconds**.

12. Empty the Collection Tube as before, and place it in the microcentrifuge rack. In a sterile tube, prepare the DNase I incubation mix by combining (in this order) the following amount of each reagent per sample:
    24µl of Yellow Core Buffer
    3µl of 0.09M $MnCl_2$
    3µl of DNase I enzyme
    Mix by gentle pipetting; do not vortex. Prepare only the amount of DNase I incubation mix needed. Store the DNase I mix on ice while it is thawed. Apply 30µl of this freshly prepared DNase I incubation mix directly to the membrane inside the minicolumn. Make sure that the solution is in direct contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to visualize.

    Note: Do not mix the Yellow Core Buffer and 0.09M $MnCl_2$ prior to Step 12. The Yellow Core Buffer and 0.09M $MnCl_2$ should be stored separately and mixed immediately prior to each set of RNA preparations.

13. **Incubate for 15 minutes at room temperature** (+20°C to +25°C). After this incubation, **add 200µl of Column Wash Solution** (as prepared in Section 4.A; verify that ethanol has been added) to the ReliaPrep™ Minicolumn. **Centrifuge at 12,000–14,000 × *g* for 15 seconds**. There is no need to empty the Collection Tube before the next step.

14. **Add 500µl of RNA Wash Solution** (with ethanol added) and **centrifuge at 12,000–14,000 × *g* for 30 seconds**. Empty wash solutions and discard the Collection Tube.

15. Place the ReliaPrep™ Minicolumn into a new Collection Tube. Add 300µl of RNA Wash Solution (with ethanol added). Centrifuge at high speed for 2 minutes.

16. For each sample, remove one capped 1.5ml Elution Tube. Transfer the ReliaPrep™ Minicolumn from the Collection Tube to the Elution Tube, and add Nuclease-Free Water to the membrane according to the **Elution volume listed in Table 3**. Be sure to completely cover the surface of the membrane with the water. Place the ReliaPrep™ Minicolumn in the centrifuge with the lids of the Elution Tubes facing out. Centrifuge at 12,000–14,000 × *g* for 1 minute. Remove the minicolumn and discard. Cap the Elution Tube containing the purified RNA and store at –70°C.

    Table 3. Recommended RNA Elution Volumes per Number of Cells.

Cell Input Range	Nuclease-Free Water
1×10^2 to 5×10^5	15µl
$>5 \times 10^5$ to 2×10^6	30µl
$>2 \times 10^6$ to 5×10^6	50µl

Note: If more concentrated RNA is required the elution volume can be decreased. While the concentration of the RNA may increase, the total yield of RNA obtained may decrease, especially when using elution volumes of less than 10µl. Elution volumes below 7µl are not recommended. Alternatively, RNA can be concentrated by vacuum-drying and resuspending in a smaller volume of water. If maximum recovery of RNA is essential, we recommend a second elution into a second sterile tube with an additional 15µl of Nuclease-Free Water followed by centrifugation at 12,000–14,000 × *g* for 1 minute. Depending on the number of input cells and RNA expression levels, a second elution may yield as much as 10–20% of additional RNA.

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4.C. Purifying RNA from Non-Fibrous Tissues

Use the following protocol to lyse tissue samples. Use 0.25–20mg of tissue per purification.

1. Verify that 1-Thioglycerol has been added to the LBA Buffer. Add LBA + TG Buffer to the tissue sample in accordance with Table 4 below.

   Table 4. Recommended Amounts of Tissue and Solutions for Lysis.

Tissue Input Range	LBA + TG Buffer	100% Isopropanol
≤5mg	250µl	85µl
>5mg (maximum 20mg)	500µl	170µl

2. Up to 20mg of tissue will lyse easily in combination with disruption using a tissue homogenizer.

   Note: The lysis should be followed by pipetting 7–10 times to shear the DNA using a P200 or P1000 pipettor. Clear homogenates by centrifugation for 3 minutes at 14,000 × *g* and then transfer supernatant to a clean tube.

   The maximum volume of lysate that can be efficiently processed is 500µl per column. If a lysate contains more RNA than the capacity of the column allows, some RNA will be lost during the wash steps.

3. Add isopropanol as recommended in Table 4. Mix by vortexing for 5 seconds.

4. Wear clean gloves and open the packs of tubes and minicolumns carefully. Remove one ReliaPrep™ Minicolumn, two Collection Tubes and one Elution Tube for each sample to be processed. Place the Collection Tubes in a microcentrifuge tube rack, and place the ReliaPrep™ Minicolumn into a Collection Tube. Label all your tubes and minicolumns to maintain sample identity. Always wear gloves when handling the tubes and minicolumns.

5. Transfer the lysate to a ReliaPrep™ Minicolumn and centrifuge at 12,000–14,000 × *g* for 1 minute at 20–25°C. If the lysate does not completely clear the column, repeat the centrifugation for an additional 1 minute.

6. Remove the ReliaPrep™ Minicolumn and **discard the liquid in the Collection Tube**. Place the ReliaPrep™ Minicolumn back into the Collection Tube. Verify that the RNA Wash Solution has been diluted with ethanol as described in Section 4.A. **Add 500µl of RNA Wash Solution** to the ReliaPrep™ Minicolumn. **Centrifuge at 12,000–14,000 × *g* for 30 seconds**.

7. Empty the Collection Tube as before and place it in the microcentrifuge rack. In a sterile tube, prepare the DNase I incubation mix by combining (in this order) the following amount of each reagent per sample:
   24µl of Yellow Core Buffer
   3µl of 0.09M $MnCl_2$
   3µl of DNase I enzyme
   Mix by gentle pipetting; do not vortex. Prepare only the amount of DNase I incubation mix needed. Store the DNase I mix on ice while it is thawed. Apply 30µl of this freshly prepared DNase I incubation mix directly to the membrane inside the column. Make sure that the solution is in direct contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to see.

Note: Do not mix the Yellow Core Buffer and 0.09M $MnCl_2$ prior to Step 7. The Yellow Core Buffer and 0.09M $MnCl_2$ should be stored separately and mixed immediately prior to each set of RNA preparations.

8. **Incubate for 15 minutes at room temperature** (+20°C to +25°C). After this incubation, **add 200µl of Column Wash Solution** (as prepared in Section 4.A; verify that ethanol has been added) to the ReliaPrep™ Minicolumn. **Centrifuge at 12,000–14,000 × *g* for 15 seconds**. There is no need to empty the Collection Tube before the next step.

9. **Add 500µl of RNA Wash Solution** (with ethanol added) and **centrifuge at 12,000–14,000 × *g* for 30 seconds**. Empty wash solutions, and discard the Collection Tube.

10. Place the ReliaPrep™ Minicolumn into a new Collection Tube. Add 300µl of RNA Wash Solution (with ethanol added). Centrifuge at high speed for 2 minutes.

11. For each sample, remove one capped 1.5ml Elution Tube. Transfer the ReliaPrep™ Minicolumn from the Collection Tube to the Elution Tube, and add Nuclease-Free Water to the membrane according to the **elution volume listed in Table 5**. Be sure to completely cover the surface of the membrane with the water. Place the ReliaPrep™ Minicolumn in the centrifuge with the lids of the Elution Tubes facing out. Centrifuge at 12,000–14,000 × *g* for 1 minute. Remove the column and discard. Cap the Elution Tube containing the purified RNA and store at –70°C.

Table 5. Recommended RNA Elution Volumes per Mass of Tissue.

Tissue Input Range	Nuclease-Free Water
5mg or less	15µl
>5mg	30µl

Note: If more concentrated RNA is required, the elution volume can be decreased. While the concentration of the RNA may increase, the total yield of RNA obtained may decrease, especially when elution volumes of less than 10µl are used. Elution volumes below 7µl are not recommended. Alternatively, RNA can be concentrated by vacuum-drying and resuspending in a smaller volume of water. If expected yields of >30µg of RNA are essential, we recommend a second elution into a second sterile tube with an additional 15µl of Nuclease-Free Water followed by centrifugation at 12,000–14,000 × *g* for 1 minute. Depending on the tissue mass and RNA expression levels, a second elution may yield as much as 30–40% of additional RNA.

4.D. Purifying RNA from Fibrous Tissues

Use the following protocol to process fibrous tissues. Use 0.25–20mg of tissue per purification.

1. Verify that 1-Thioglycerol has been added to the LBA buffer. Add LBA + TG Buffer to the tissue sample in accordance with Table 6 below.

   Table 6. Recommended Amounts of Tissue and Solutions for Lysis.

Tissue Input Range	LBA + TG Buffer	RDB Addition (µl)	Total Volume (µl)	100% Isopropanol
≤5mg	250µl	250µl	500µl	170µl
>5mg (maximum 20mg)	500µl	500µl	1,000µl	340µl

   Note: Up to 20mg of tissue will lyse easily in LBA + TG in combination with the use of a tissue homogenizer. The lysis should be followed by pipetting 7–10 times to shear the DNA using a P200 or P1000 pipettor.

2. Add an equal volume of RNA Dilution Buffer (RDB) and mix by vortexing for 10 seconds. Incubate 1 minute at room temperature. A visible precipitate may appear. Clear homogenates by centrifugation at room temperature for 3 minutes at 10,000 *× g* to pellet insoluble debris. Transfer the cleared lysates to clean tubes, taking care to avoid any pelleted debris.

   The maximum volume of lysate that can be efficiently processed is 500µl per column. If a lysate contains more RNA than the capacity of the column (approximately 80–85µg), some RNA may be lost during the wash steps.

3. Add isopropanol as recommended in Table 6.

4. Wear clean gloves and open the packs of tubes and minicolumns carefully. Remove one ReliaPrep™ Minicolumn, two Collection Tubes and one Elution Tube for each sample to be processed. Place the Collection Tubes in a microcentrifuge tube rack, and place the ReliaPrep™ Minicolumn into a Collection Tube. Be sure to label all your tubes and minicolumns to maintain sample identity. Always wear gloves when handling the tubes and minicolumns.

5. Transfer up to 700µl of lysate to a ReliaPrep™ Minicolumn and centrifuge at 12,000–14,000 *× g* for 1 minute at 20–25°C.

   Note: If 500µl LBA + TG volume was used for homogenizing, transfer the lysate in two 670µl aliquots, centrifuging after each transfer.

6. Remove the ReliaPrep™ Minicolumn, and **discard the liquid in the Collection Tube**. Place the ReliaPrep™ Minicolumn back into the Collection Tube. Verify that the RNA Wash Solution has been diluted with ethanol as described in Section 4.A. **Add 500µl of RNA Wash Solution** to the ReliaPrep™ Minicolumn. **Centrifuge at 12,000–14,000 × *g* for 30 seconds**.

7. Empty the Collection Tube as before and place it in the microcentrifuge rack. In a sterile tube, prepare the DNase I incubation mix by combining (in this order) the following amount of each reagent per sample:
   24µl of Yellow Core Buffer
   3µl of 0.09M $MnCl_2$
   3µl of DNase I enzyme
   Mix by gentle pipetting; do not vortex. Prepare only the amount of DNase I incubation mix needed. Store the DNase I mix on ice while it is thawed. Apply 30µl of this freshly prepared DNase I incubation mix directly to the membrane inside the column. Make sure that the solution is in direct contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to see.

   Note: Do not mix the Yellow Core Buffer and 0.09M $MnCl_2$ prior to Step 7. The Yellow Core Buffer and 0.09M $MnCl_2$ should be stored separately and mixed immediately prior to each set of RNA preparations.

8. **Incubate for 15 minutes at room temperature** (20–25°C). After this incubation, **add 200µl of Column Wash Solution** (as prepared in Section 4.A; verify that ethanol has been added) to the ReliaPrep™ Minicolumn. **Centrifuge at 12,000–14,000 × *g* for 15 seconds**. There is no need to empty the Collection Tube before the next step.

9. **Add 500µl of RNA Wash Solution** (with ethanol added) and **centrifuge at 12,000–14,000 × *g* for 30 seconds**. Empty wash solutions, and discard the Collection Tube.

10. Place the ReliaPrep™ Minicolumn into a new Collection Tube. Add 300µl of RNA Wash Solution (with ethanol added). Centrifuge at high speed for 2 minutes.

11. For each sample, remove one capped 1.5ml Elution Tube. Transfer the ReliaPrep™ Minicolumn from the Collection Tube to the Elution Tube, and add Nuclease-Free Water to the membrane according to the **elution volume listed in Table 7**. Be sure to completely cover the surface of the membrane with the water. Place the ReliaPrep™ Minicolumn in the centrifuge with the lids of the Elution Tubes facing out. Centrifuge at 12,000–14,000 × *g* for 1 minute. Remove the column and discard. Cap the Elution Tube containing the purified RNA and store at –70°C.

Table 7. Recommended RNA Elution Volumes per Mass of Tissue.

Tissue Input Range	Nuclease-Free Water
>5mg	15µl
>5 to 20mg	30µl

Note: If more concentrated RNA is required, the elution volume can be decreased. While the concentration of the RNA may increase, the total yield of RNA obtained may decrease, especially when elution volumes of less than 10µl are used. Elution volumes below 7µl are not recommended. Alternatively, RNA can be concentrated by vacuum-drying and resuspending in a smaller volume of water. If expected yields of >30µg of RNA are essential, we recommend a second elution into a second sterile tube with an additional 15µl of Nuclease-Free Water followed by centrifugation at 12,000–14,000 × *g* for 1 minute. Depending on the tissue mass and RNA expression levels, a second elution may yield as much as 30–40% of additional RNA.

5. Determining RNA Yield and Quality

Yield and Purity

The ReliaPrep™ RNA Miniprep System can be used to isolate intact RNA from a variety of cell or tissue sources. The yield of total RNA obtained may be determined spectrophotometrically at 260nm, where 1 absorbance unit (A_{260}) equals 40µg of single-stranded RNA/ml. The purity may also be estimated by spectrophotometry from the relative absorbances at 230nm, 260nm and 280nm (i.e., A_{260}/A_{280} and A_{260}/A_{230}). If the number of cells estimated as starting material is less than 5×10^4 or the mass of tissue estimated as starting material is less than 0.5mg, spectrophotometric analysis will not yield accurate results due to the lack of sensitivity of this method for low concentrations of nucleic acid. Alternatively, RNA yields from cell inputs as low as 100 cells or tissue inputs as low as 0.25mg can be determined using the QuantiFluor® RNA System (Cat.# E3310).

RNA isolated with the ReliaPrep™ RNA Miniprep System is substantially free of DNA and contaminating protein and may be used directly for any of the applications listed in Section 3.A. Pure RNA will exhibit an A_{260}/A_{280} ratio of 2.0. However, it should be noted that, due to the variations between individual starting materials and in performing the procedure, the expected range of A_{260}/A_{280} ratios for RNA will be 1.7–2.1. If the RNA ratio is less than 1.7, refer to Section 6 for possible causes and suggestions on improving the purity of the RNA. Using this protocol, the RNA will usually exhibit an A_{260}/A_{230} ratio of 1.8–2.2. A low A_{260}/A_{230} ratio may indicate guanidine contamination that can interfere with downstream processing. If sufficient quantities of RNA are available, determine the integrity of the purified RNA by denaturing agarose gel electrophoresis. Several methods are suitable for this purpose using either formaldehyde (2,3) or glyoxal (4) as the denaturing agent. The ratio of 28S to 18S eukaryotic ribosomal RNAs should be approximately 2:1 by ethidium bromide staining, indicating that no gross degradation of RNA has occurred. In RNA samples that have been degraded, this ratio will be reversed since the 28S ribosomal RNA characteristically is degraded to an 18S-like species. Refer to Sections 3 and 6 for suggestions on avoiding RNA degradation.

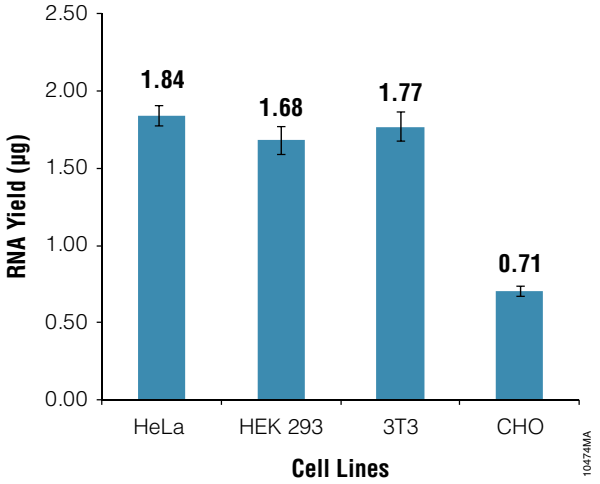

Figure 2. Average total RNA yield obtained from four cultured cell lines using the ReliaPrep™ RNA Miniprep System. One hundred-thousand cells were used for each purification, with 4 replicates processed per cell line. RNA yield was calculated from the concentration obtained at 260nm using a NanoDrop® 1000 instrument and the elution volume.

5. Determining RNA Yield and Quality (continued)

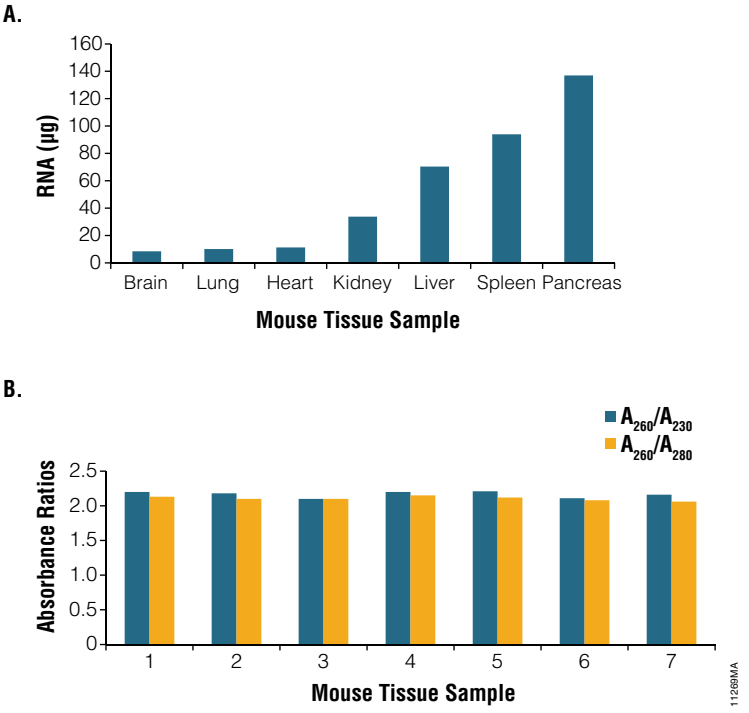

Figure 3. Average yield and typical purity of RNA isolated from 10mg mouse tissue samples using the ReliaPrep™ RNA Miniprep System. RNA from ten milligrams of various mouse tissues, fibrous and non-fibrous, was purified using the appropriate protocols, with 3 replicates processed per tissue type. **Panel A.** RNA yield was calculated by spectrophotometric analysis where 1 O.D. 260 equals 40µg/ml RNA. **Panel B.** Purity was determined by analysis of $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios indicative of the lack of protein contamination and guanidine contamination, respectively. In both cases, a ratio greater than 1.8 is indicative of high-purity RNA.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
Low A_{260}/A_{280} ratios	When processing small cell numbers or tissue amounts, the expected yield of RNA may be below the level of detection; spectroscopic methods may not be accurate when expected yields of RNA drop below approximately 2ng/µl for cells or 15ng/µl for tissue. As the signal approaches the limit of detection for the instrument, background noise may lead to inaccurate readings.
	Low A_{260}/A_{280} ratios are typically due to protein contamination. Several methods may be used for removing contaminating protein from RNA solutions. The most expedient method is to perform a phenol:chloroform extraction. This organic extraction should yield higher A_{260}/A_{280} ratios. However, loss of RNA (up to 40%) should be expected.
Low A_{260}/A_{230} ratios	When processing small cell numbers or tissue amounts, the expected yield of RNA may be below the level of detection; spectroscopic methods may not be accurate when expected yields of RNA drop below approximately 2ng/µl. As the signal approaches the limit of detection for the instrument, background noise may lead to inaccurate readings.
	Low A_{260}/A_{230} ratios are typically due to guanidine thiocyanate contamination. Precipitate the RNA by adding NaCl to a final concentration of 0.1M. Add 2.5 volumes of ethanol. Incubate for 30 minutes at –20°C. Collect the RNA by centrifugation at 10,000 × *g* for 15 minutes at 4°C. Resuspend the RNA in Nuclease-Free Water. When processing small samples, further concentration of the RNA may be challenging as precipitation of dilute samples may be inefficient.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low A_{260} (low RNA yield)	Cell or tissue lysates that have been stored frozen (at –20°C or –70°C) may have decreased amounts of total RNA. For optimal performance, purify the RNA as soon as the lysate is prepared.
	RNA yield is below the level of detection for spectroscopic quantitation methods. When processing small cell numbers or tissue amounts, the expected yield of RNA may be below the level of detection, particularly when processing less than 100,000 cells. Spectroscopic methods may not be accurate when expected yields of RNA drop below approximately 2ng/µl (or 30ng of total RNA). Accurate quantitation may be possible only with fluorescent dye-based methods or by qPCR.
	Sample RNA may be of poor quality. Samples that were not lysed or frozen immediately upon isolation may have decreased amounts of RNA with reduced integrity. Freeze cells or tissue immediately in liquid nitrogen and store at –70°C if they cannot be processed immediately. Samples in BL + TG Buffer and LBA Buffer should be stored at –20°C to –70°C.
	The binding capacity of the membrane in the minicolumn was exceeded. If the lysate contains more RNA than the capacity of the minicolumn, the excess RNA will be washed away during the wash steps. When maximum recovery is essential, divide the lysate and perform multiple purifications. Pool the resulting RNA solutions, and determine the total yield obtained.
	The ReliaPrep™ RNA Miniprep System uses a multiple-step procedure that requires the correct reagents to be used in the correct order. This ensures that the RNA remains bound to the membrane during the purification process.
	Ethanol may not have been added to the Column Wash Solution or to the RNA Wash Solution. Prepare solutions as instructed in Section 4.A before beginning the procedure.
	Lysate was allowed to overheat during processing. Work as quickly as possible. Lysates can be placed on ice during sample preparation. Use ice-cold BL + TG Buffer or LBA +TG Buffer for lysis to improve yield and stability if overheating is a problem.

Symptoms	Causes and Comments
Genomic DNA contamination seen when performing PCR	Reaction may contain too much sample. Reduce total RNA input to 50–100ng in control PCR. Generally, the RNA-specific product is seen from a rare message in RT-PCR using 50ng of total RNA.
	The sample may contain too much genomic DNA. For cultured cells, do not exceed 5×10^6 cells per minicolumn.
	When the suggested input amounts are used in the system, most purified RNA samples do not show genomic DNA contamination in RT-PCR. However, dense cultures may contain too much DNA to eliminate. If DNA contamination is a problem in a sample, we recommend performing a post-RNA isolation DNase treatment using RQ1 RNase-Free DNase (Cat.# M6101) followed by phenol:chloroform extraction. For more information, see reference 3.
Genomic DNA contamination	The DNase I enzyme may be inactive. Resuspend and store the lyophilized DNase according to the directions in Section 4.A. Do not freeze-thaw the DNase more than three times after it has been rehydrated.
	$MnCl_2$ or DNase I was not added to the Yellow Core Buffer. For each isolation to be performed, prepare the DNase incubation mix by combining **24µl of Yellow Core Buffer, 3µl of 0.09M $MnCl_2$ and 3µl of DNase I enzyme** in a sterile tube just before use. Prepare the DNase incubation mix fresh for each set of RNA isolations. Do not vortex.
	The DNase I Solution is not in full contact with the membrane during digestion. Visually inspect to ensure that the DNase I solution completely covers the membranes during the DNA digestion. The solution is yellow to make this easier.
	The DNase step was omitted or not performed correctly. The DNase step should be performed to eliminate the possibility of host DNA contaminating the system.
Clogged minicolumns	The lysate is too concentrated. If the lysate is difficult to pipet, the cell lysate is too concentrated. RNA concentration will vary between cell lines. If the lysate is too viscous, dilute it with BL + TG Buffer for cell lysates or LBA + TG Buffer for tissue lysates before adding RNA Wash Buffer. Use no more than 500µl of lysate per minicolumn.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Lysate too viscous; does not pipet easily	The initial lysate is too viscous. Dilute the lysate with BL + TG Buffer for cell lysates or LBA + TG Buffer for tissue lysates.
	Lysate becomes too viscous while sitting on ice. Briefly mix by passing the lysate through a 20-gauge needle to shear genomic DNA.
RNA degradation	RNase was introduced during handling. Use DEPC-treated glassware and solutions and disposable plasticware when manipulating and storing RNA. Wear gloves at all times. RNases introduced after elution will degrade RNA.
	RNA was degraded during sample prep. Work quickly during sample preparation (see comments under "Low A_{260}").

7. References

1. Chirgwin, J.M. *et al*. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–9.
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3. Ausubel, F.M. *et al*. eds. (1993) In: *Current Protocols in Molecular Biology.* Greene Publishing Associates and John Wiley and Sons, New York, NY.
4. McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**, 4835–8.

8. Appendix

8.A. Creating a Ribonuclease-Free Environment

Ribonucleases are extremely difficult to inactivate (1). Take care to avoid inadvertently introducing RNase activity into your RNA during or after the isolation procedure. This is especially important if the starting material has been difficult to obtain or is irreplaceable. The following notes may help you to prevent accidental RNase contamination of your sample.

1. Two of the most common sources of RNase contamination are the user's hands and bacteria or molds that may be present on airborne dust particles (1). To prevent contamination from these sources, use sterile technique when handling the reagents supplied with the kit. Wear gloves at all times.
2. Whenever possible, sterile disposable plasticware should be used for handling RNA. These materials are generally RNase-free and thus do not require pretreatment to inactivate RNase. Autoclaved Elution Tubes are provided with the system.

8.B. Composition of Buffers and Solutions

PBS buffer, 10X (per liter)

11.5g	Na_2HPO_4
2g	KH_2PO_4
80g	NaCl
2g	KCl

Dissolve in 1 liter of sterile, deionized water. The pH of 1X PBS will be 7.4.

trypsin-EDTA solution, 1X

0.05%	trypsin (w/v)
0.53mM	EDTA

Dissolve in 1X PBS.

RNA Wash Solution (RWA) (concentrated)

162.8mM	potassium acetate
27.1mM	Tris-HCl (pH 7.5 at 25°C)

After dilution with ethanol the final concentration (approximate) is 60mM potassium acetate, 10mM Tris-HCl (pH 7.5 at 25°C) and 60% ethanol.

Yellow Core Buffer

0.0225M	Tris (pH 7.5)
1.125M	NaCl
0.0025%	yellow dye (w/v)