TECHNICAL MANUAL

ReliaPrep™ miRNA Cell and Tissue Miniprep System

Instructions for Use of Products
Z6212, Z6211 and Z6210

Revised 12/20
TM469

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ReliaPrep™ miRNA Cell and Tissue Miniprep System

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

1. Description

The purity and integrity of miRNA isolated from cells and tissue samples 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, and next-generation sequencing (NGS). In recent years, RT-PCR and RT-qPCR have emerged as powerful methods to identify and quantitate specific miRNAs from small amounts of total RNA. As the use of amplification as a research tool has grown, the need for methods to rapidly isolate high-quality miRNA, substantially free of genomic DNA contamination, from small amounts of starting material (i.e., tissue samples) has also increased. The ReliaPrep™ miRNA Cell and Tissue Miniprep System is designed to address these needs.

1. Description (continued)

The ReliaPrep™ miRNA Cell and Tissue Miniprep System provides a fast and simple technique for preparing purified and intact total RNA–including miRNA–from tissue samples in as little as 40 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™ miRNA Cell and Tissue Miniprep System	**250 preps**	**Z6212**

For in vitro Research Use Only. Each system contains sufficient reagents for 250 preparations of total RNA (including miRNA) from cultured mammalian cells and tissue samples. Includes:

- 10 packs ReliaPrep™ Minicolumns (50/pack)
- 2 packs Collection Tubes (250/pack)
- 10 packs Elution Tubes (50/pack)
- 150ml Lysis Buffer (LBA)
- 5 vials DNase I (lyophilized)
- 3 × 500µl DNase 10X Buffer
- 206ml RNA Wash Solution (RWA)
- 220ml RNA Dilution Buffer (RDB)
- 4 × 900µl 1-Thioglycerol
- 25ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
ReliaPrep™ miRNA Cell and Tissue Miniprep System	**50 preps**	**Z6211**

For in vitro Research Use Only. Each system contains sufficient reagents for 50 preparations of total RNA (including miRNA) from cultured mammalian cells and tissue samples. Includes:

- 2 packs ReliaPrep™ Minicolumns (50/pack)
- 2 packs Collection Tubes (50/pack)
- 2 packs Elution Tubes (50/pack)
- 30ml Lysis Buffer (LBA)
- 1 vial DNase I (lyophilized)
- 500µl DNase 10X Buffer
- 58.8ml RNA Wash Solution (RWA)
- 44ml RNA Dilution Buffer (RDB)
- 900µl 1-Thioglycerol
- 13ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
ReliaPrep™ miRNA Cell and Tissue Miniprep System	**10 preps**	**Z6210**

For in vitro Research Use Only. Each system contains sufficient reagents for 10 preparations of total RNA (including miRNA) from cultured mammalian cells and tissue samples. Includes:

- 2 packs ReliaPrep™ Minicolumns (10/pack)
- 2 packs Collection Tubes (10/pack)
- 1 pack Elution Tubes (25/pack)
- 6ml Lysis Buffer (LBA)
- 1 vial DNase I (lyophilized)
- 500µl DNase 10X Buffer
- 11.8ml RNA Wash Solution (RWA)
- 9ml RNA Dilution Buffer (RDB)
- 2 × 75µl 1-Thioglycerol
- 1.25ml Nuclease-Free Water

Storage Conditions: Store components at room temperature, 15–30°C. Store the LBA Buffer, with 1-Thioglycerol added, at 2–10°C for up to 30 days. Cap tightly between uses. For information on rehydration of DNase I see Section 4, Preparation of Solutions.

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

Caution: Guanidine thiocyanate (components of LBA Buffer) is harmful and an irritant. 1-Thioglycerol is toxic. Wear gloves and follow standard safety procedures while working with these solutions. When processing animal tissues, follow standard procedures for handling and disposal of hazardous materials.

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3. General Considerations

3.A. Direct Purification of RNA

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™ miRNA Cell and Tissue 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 column. The bound total RNA, including miRNA, is further purified from contaminating salts, proteins and cellular components by simple washing steps. RNase-free DNase I is used to digest contaminating genomic DNA. Finally, total RNA, including miRNA, is eluted from the membrane by the addition of Nuclease-Free Water. This procedure yields an essentially pure fraction of total RNA, including miRNA, after only a single round of purification without organic extractions or precipitations. The procedure is easy to perform with small quantities of cultured cells, and it can be used to process multiple samples.

Processing Capacity

The ReliaPrep™ miRNA Cell and Tissue Miniprep System is optimized for total RNA isolation from a wide range of input cell numbers (1×10^2 to 1×10^6) and tissue masses (0.25–20mg), with a broad spectrum of mRNA and miRNA expression levels.

3.B. Downstream Applications

RNA purified with the ReliaPrep™ miRNA Cell and Tissue Miniprep System is suitable for many molecular biology applications, including RT-qPCR, microarrays and NGS.

For all downstream applications, continue to protect your samples from RNases by wearing gloves and using RNase-free solutions and centrifuge tubes. 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.

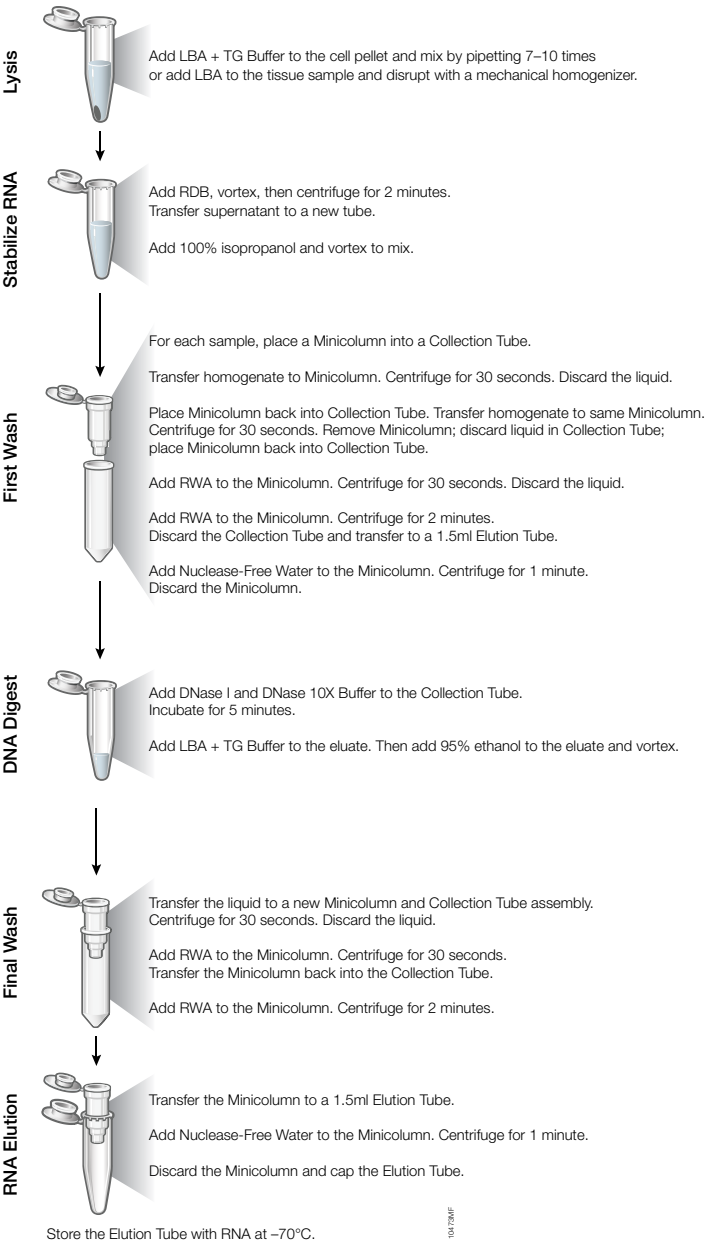

Figure 1. Schematic diagram of the ReliaPrep™ miRNA Cell and Tissue Miniprep System. For protocol details see Sections 5 and 6.

3.C. 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. RNase-Free Elution Tubes are provided with the system.

4. Preparation of Solutions

Before beginning the ReliaPrep™ miRNA Cell and Tissue Miniprep System protocol, three solutions must be prepared.

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

Solution	Preparation Steps	Notes
DNase I	**250** prep size (**Cat.# Z6212**): Add **275µl** of Nuclease-Free Water (provided) as indicated on the vial label or **50** prep size (**Cat.# Z6211**): Add **275µl** of Nuclease-Free Water (provided) as indicated on the vial label or **10** prep size (**Cat.# Z6210**): Add **80µl** 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 5µl 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.**

Solution	Preparation Steps	Notes
LBA + TG Buffer	**250** prep size (**Cat.# Z6212**): Add **3ml** of 1-Thioglycerol to 150ml of LBA Buffer or **50** prep size (**Cat.# Z6211**): Add **650µl** of 1-Thioglycerol to 30ml of LBA Buffer or **10** prep size (**Cat.# Z6210**): Add **120µl** of 1-Thioglycerol to the bottle containing 6ml 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.
RNA Wash Solution	**250** prep size (**Cat.# Z6212**): Add **350ml** of 95% ethanol to the bottle containing 206ml of concentrated RNA Wash Solution (RWA) or **50** prep size (**Cat.# Z6211**): Add **100ml** of 95% ethanol to the bottle containing 58.8ml of concentrated RNA Wash Solution (RWA) or **10** prep size (**Cat.# Z6210**): Add **20ml** of 95% ethanol to the bottle containing 11.8ml 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 15–30°C when tightly capped.

For best results from this system, use fresh tissue samples. If you choose to store harvested cells or tissues, it is best to flash freeze them in a dry ice/ethanol bath, then store at –70°C. The integrity of isolated RNA from stored, harvested tissues is dependent on two key steps: rapid freezing of tissues at time of storage, and direct thawing of the cell pellet into LBA + TG Buffer during purification to ensure rapid lysis and inactivation of nucleases in the sample. After lysis in LBA + TG Buffer samples may be stored at –70°C for up to three months. Due to the chemicals used in the RNA purification procedure and the prevalence of RNases, wear gloves throughout the lysis and purification procedure.

Materials to Be Supplied by the User

- 100% isopropanol, RNase-free
- 95–100% ethanol, RNase-free
- microcentrifuge
- 1.5ml centrifuge tubes

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5. RNA Isolation and Purification from Cell Samples

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

1. To harvest adherent cells, follow the protocol in the Appendix (Section 10.B) prior to cell lysis. For suspension cells proceed directly to Step 2.
2. Collect cells in a sterile centrifuge tube by centrifugation at 300 × *g* for 5 minutes. Remove the medium and wash the cell pellet with ice-cold, sterile 1X PBS (see Section 10.A for the recipe). Centrifuge at 300 × *g* for 5 minutes to collect the cells. Discard the supernatant. Careful removal of all supernatant will aid in efficient preparation of high-quality RNA.
3. Verify that 1-Thioglycerol has been added to the LBA Buffer. Add 200µl LBA + TG Buffer to the washed cell pellet.

   Note: If frozen cell pellets are used as a starting material, add prepared LBA + TG Buffer to the frozen pellets prior to thawing.
4. Disperse the cell pellet and mix well by vortexing and/or pipetting.

   Note: Up to 1×10^6 cells will lyse easily in 200µl of LBA + TG Buffer. The lysis should be followed by pipetting 7–10 times to shear the DNA using a P200 or P1000 pipettor.
5. Add 130µl of RDB to each homogenate and vortex for 10 seconds to mix. Centrifuge for 2 minutes at 12,000 × *g*. Carefully transfer the cleared homogenate to a clean 1.5ml tube, avoiding transfer of any pelleted material.
6. Add 400µl of 100% isopropanol to each cleared homogenate. Mix by vortexing.
7. 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. It is important to label all your tubes and minicolumns to maintain sample identity. Always wear gloves when handling the tubes and minicolumns.
8. Carefully transfer the homogenate to a ReliaPrep™ Minicolumn. Centrifuge at 12,000 × *g* for 30 seconds.
9. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube. Transfer the remaining homogenate liquid onto the same ReliaPrep™ Minicolumn used in Step 8. Centrifuge at 12,000 × g for 30 seconds.
10. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
11. Add 500µl of RWA to each column. Centrifuge at 12,000 × *g* for 30 seconds.
12. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
13. Add 500µl RWA to each column. Centrifuge at 12,000 × *g* for 2 minutes. Transfer the ReliaPrep™ Minicolumn into a 1.5ml Elution Tube.

14. Add 40µl Nuclease-Free Water to each column. Centrifuge at 12,000 × *g* for 1 minute.
15. Transfer 5µl of DNase I and 5µl DNase 10X Buffer to each eluate.
16. Incubate for 5 minutes at room temperature (20–25°C).
17. Add 150µl of LBA + TG Buffer to the DNase treatment tube.
18. Add 300µl of 95% ethanol to the mixture and vortex for 10 seconds to mix. Transfer 500µl of this mixture to ReliaPrep™ Minicolumn #2. Centrifuge at 12,000 × *g* for 30 seconds.
19. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
20. Add 500µl of RWA to each column. Centrifuge at 12,000 × *g* for 30 seconds.
21. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
22. Add 500µl RWA to each column. Centrifuge at 12,000 × *g* for 2 minutes. Transfer the ReliaPrep™ Minicolumn into 1.5ml Elution Tube.
23. Add 15µl Nuclease-Free Water to each column. Centrifuge at 12,000 × *g* for 1 minute.

    Note: If expected yields are greater than 15µg, add an additional 15µl of Nuclease-Free water and repeat the centrifugation step.

Table 2. Recommended 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 1×10^6	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 15µl are used. Elution volumes below 10µl 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, a second elution into a second sterile tube with an additional 15µl of Nuclease-Free Water followed by centrifugation at 12,000 × *g* for 1 minute is recommended. Depending on the number of cells input and RNA expression levels, a second elution may yield as much as 10–20% of additional RNA.

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6. RNA Isolation and Purification from Tissue Samples

Use the following protocol to lyse tissue samples. Use from 0.25mg to a maximum of 20mg per purification. The mass of tissue used may need to be adjusted depending on tissue type, function and RNA expression levels at the time of harvest.

1. Place fresh or flash-frozen tissue samples in a sterile centrifuge tube containing 200µl LBA + TG. Use a mechanical homogenizer (preferred) or mini pestle, disrupt the tissue until a homogeneous homogenate is achieved.
2. The lysis should be followed by pipetting 7-10 times to shear the DNA using a P200 or P1000 pipettor.
3. Add 130µl of RDB to each homogenate and vortex for 10 seconds to mix. Centrifuge at 12,000 × *g* for 2 minutes. Carefully transfer the cleared homogenate to a clean 1.5ml tube avoiding transfer of any pelleted material.
4. Add 400µl of 100% isopropanol to each cleared homogenate. Mix by vortexing.
5. Wear clean gloves and open the packs of tubes and Minicolumns carefully. Remove two ReliaPrep™ Minicolumns (label 1 and 2), two collection Tubes and two Elution tubes for each sample to be processed. Place the Collection Tubes in a microcentrifuge tube rack, and place the ReliaPrep™ Minicolumn into a Collection Tube. Always wear gloves when handling the tubes and minicolumns.
6. Carefully transfer the homogenate to a ReliaPrep™ Minicolumn. Centrifuge at 12,000 × *g* for 30 seconds.
7. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
8. Transfer the remaining homogenate liquid onto the same ReliaPrep™ Minicolumn used in Step 7. Centrifuge at 12,000 × *g* for 30 seconds. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
9. Add 500µl of RWA to each column. Centrifuge at 12,000 × *g* for 30 seconds.
10. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
11. Add 500µl RWA to each column. Centrifuge at 12,000 × *g* for 2 minutes. Transfer the ReliaPrep™ Minicolumn into 1.5ml Elution Tube.
12. Add 40µl Nuclease-Free Water to each column. Centrifuge at 12,000 × *g* for 1 minute.
13. Transfer 5µl of DNase I and 5µl DNase 10X Buffer to each eluate.
14. Incubate for 5 minutes at room temperature (20–25°C).
15. Add 150µl of LBA + TG Buffer to the DNase treatment tube.
16. Add 300µl of 95% ethanol to the mixture and vortex for 10 seconds to mix. Transfer 500µl of this mixture to ReliaPrep™ Minicolumn #2. Centrifuge at 12,000 × *g* for 30 seconds.
17. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.

18. Add 500µl of RWA to each column. Centrifuge at 12,000 × *g* for 30 seconds.
19. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection Tube.
20. Add 500µl RWA to each column. Centrifuge at 12,000 × *g* for 2 minutes. Transfer the ReliaPrep™ Minicolumn into 1.5ml Elution Tube.
21. Add 15µl Nuclease-Free Water to each column. Centrifuge at 12,000 × *g* for 1 minute.

    Note: If expected yields are greater than 15µg, add an additional 15µl of Nuclease-Free water and repeat the centrifugation step.

Table 3. Recommended RNA Elution Volumes per mg of Tissue.

Tissue Input Range	Nuclease-Free Water
0.25–5mg	15µl
>5–10mg	30µl
>10mg	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 elution volumes of less than 15µl are used. Elution volumes below 10µl 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, a second elution into a second sterile tube with an additional 15µl of Nuclease-Free Water followed by centrifugation at 12,000 × *g* for 1 minute is recommended. Depending on the mass of tissue input and RNA expression levels, a second elution may yield as much as 10–20% of additional RNA.

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7. Determination of RNA Yield and Quality

The ReliaPrep™ miRNA Cell and Tissue Miniprep System can be used to isolate intact miRNA from a variety of cell and tissue sources. The yield of total RNA obtained may be determined spectrophotometrically at 260nm, where one absorbance unit (A_{260}) equals 40µg of single-stranded RNA/ml. The purity may also be estimated by spectrophotometry from the relative absorbances at 230, 260 and 280nm (i.e., A_{260}/A_{280} and A_{260}/A_{230}).

RNA isolated with the ReliaPrep™ miRNA Cell and Tissue Miniprep System is substantially free of DNA and contaminating protein and may be used directly for any of the applications listed in Section 3.B. 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 8 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.

8. Troubleshooting

Symptoms	Possible Causes and Comments
Low A_{260}/A_{280} ratios	When processing small numbers of cells, 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_{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 numbers of cells, 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.

Symptoms	Possible Causes and Comments
Low A_{260} (low RNA yield)	Tissue homogenates that have been stored frozen (at –20° 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 numbers of cells, 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 immediately in liquid nitrogen and store at -70°C if they cannot be processed immediately. Samples in LBA + TG 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 Cell 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 RNA Wash Solution. Prepare solutions as instructed in Section 4 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 LBA + TG Buffer for lysis to improve yield and stability if overheating is a problem.

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8. Troubleshooting (continued)

Symptoms	Possible 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 total RNA.
	The sample may contain too much genomic DNA. For cultured cells, do not exceed 20mg tissue per column.
	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. Do not freeze-thaw the DNase more than three times after it has been rehydrated.
	DNase I was not added to the sample. For each isolation to be performed, prepare the DNase incubation mix by combining 5µl DNase I and 5µl 10X buffer to each sample.
	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 Binding Columns	The lysate is too concentrated. If the lysate is difficult to pipet, the tissue lysate is too concentrated. RNA concentration will vary between tissues. If the lysate is too viscous, dilute it with LBA + TG Buffer and split the sample between two columns.
Lysate too viscous; does not pipet easily	The initial lysate is too viscous. Dilute the lysate with LBA + TG Buffer.
	Lysate becomes too viscous while sitting on ice. Briefly mix by drawing up 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}").

9. References

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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.

10. Appendix

10.A. 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.

10.B. Harvesting Procedure for Adherent Cells

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.

Materials to Be Supplied by the User
(Solution compositions are provided in Sections 4 and 10.A.)

- LBA + TG Buffer
- 1X PBS

1. Lysates may be prepared rapidly in the culture plate or flask by the addition of LBA + TG Buffer. Recommended volumes of wash (PBS) and LBA + TG Buffer (lysis) to add per well are shown in Table 4, below.

Table 4. Recommended Wash and Reagent Volumes for Harvesting Adherent Cells.

Plate Type	Wash (PBS/well)	Lysis (LBA + TG Buffer)	RDB	Isopropanol	Total Load Volume
96-well	100µl	100µl	65µl	200µl	365µl
48-well	250µl	100µl	65µl	200µl	365µl
24-well	500µl	100µl	65µl	200µl	365µl
6-well	2.0ml	250µl	160µl	500µl	910µl
T-25 flask	5.0ml	500µl	325µl	1,000µl	1,825µl

2. Remove the culture medium and wash the cells with ice-cold, sterile 1X PBS (Section 10.A). Add LBA + TG Buffer, gently rock the plate or flask to cover adherent cells. Rinse the cells by 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 the viscosity of the lysate. The maximum lysate volume which can be processed with a single Minicolumn is 500µl.

3. Add the appropriate volume of RDB (see Table 4 above) to each homogenate and vortex for 10 seconds to mix. Centrifuge at 12,000 × *g* for 2 minutes. Carefully transfer the cleared homogenate to a clean 1.5ml tube, while avoiding transfer of any pelleted material.

   Note: Cleared lysates from T-25 flasks must be split in half (approximately 400µl each) prior to isopropanol addition. These split volumes can then be loaded onto the same column in two steps.

4. Add the appropriate volume of isopropanol (see Table 4 above) to the sample.

5. Proceed to Step 7 in Section 5, RNA Isolation and Purification Procedure from Cell Samples.

   Note: If you prefer to harvest cells by conventional trypsinization methods, harvest cells and proceed to Step 3 in Section 5.

10.C. Related Products

Product	Size	Cat.#
ReliaPrep™ RNA Tissue Miniprep System	10 preps	Z6110
	50 preps	Z6111
	250 preps	Z6112
ReliaPrep™ gDNA Tissue Miniprep System	100 preps	A2051
	250 preps	A2052
GoTaq® Probe qPCR Master Mix	2ml	A6101
	10ml	A6102
GoTaq® qPCR Master Mix	5ml	A6001
	25ml	A6002

10.D. Summary of Change

The following change was made to the 12/20 revision of this document:

1. Updated protocol instructions in Section 10.B.