



Promega

Technical Manual

ReliaPrep™ RNA Cell Miniprep System

INSTRUCTIONS FOR USE OF PRODUCTS Z6010, Z6011 AND Z6012.



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ReliaPrep™ RNA Cell Miniprep System

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1. Description

The purity and integrity of RNA isolated from cultured cells 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) has also increased. The ReliaPrep™ RNA Cell Miniprep System has been designed to address these needs.

1. Description (continued)

The ReliaPrep™ RNA Cell Miniprep System provides a fast and simple technique for preparing purified and intact total RNA from cultured cells 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 Cell Miniprep System	250 preps	Z6012

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

- 5 packs ReliaPrep™ Minicolumns (50/pack)
- 2 packs Collection Tubes (250/pack)
- 5 packs Elution Tubes (50/pack)
- 150ml BL Buffer
- 24ml Column Wash Solution (CWE)
- 3 vials DNase I (lyophilized)
- 2 × 750µl MnCl₂, 0.09M
- 11ml Yellow Core Buffer
- 206ml RNA Wash Solution (RWA)
- 2 × 900µl 1-Thioglycerol
- 13ml Nuclease-Free Water

Product	Size	Cat.#
ReliaPrep™ RNA Cell Miniprep System	50 preps	Z6011

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

- 1 pack ReliaPrep™ Minicolumns (50/pack)
- 2 packs Collection Tubes (50/pack)
- 1 pack Elution Tubes (50/pack)
- 32.5ml BL Buffer
- 5ml Column Wash Solution (CWE)
- 1 vial DNase I (lyophilized)
- 250µl MnCl₂, 0.09M
- 2.5ml Yellow Core Buffer
- 35ml RNA Wash Solution (RWA)
- 900µl 1-Thioglycerol
- 13ml Nuclease-Free Water

2. Product Components and Storage Conditions (continued)

Product	Size	Cat.#
ReliaPrep™ RNA Cell Miniprep System	10 preps	Z6010

For in vitro Research Use Only. Each system contains sufficient reagents for 10 preparations of total RNA from cultured mammalian cells. Includes:

- 1 pack ReliaPrep™ Minicolumns (10/pack)
- 2 packs Collection Tubes (10/pack)
- 1 pack Elution Tubes (25/pack)
- 2 × 3.25ml BL Buffer
- 1ml Column Wash Solution (CWE)
- 1 vial DNase I (lyophilized)
- 250µl MnCl₂, 0.09M
- 300µl Yellow Core Buffer
- 11.8ml RNA Wash Solution (RWA)
- 75µl 1-Thioglycerol
- 1.25ml 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.

Storage Conditions: Upon receipt, remove and store the 1-Thioglycerol at 2°–10°C. Store the BL 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.A, Preparation of Solutions. Store all other components at 15°–30°C.

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

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

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™ RNA Cell Miniprep System combines the disruptive and protective properties of guanidine thiocyanate (GTC) and 1-Thioglycerol to inactivate the ribonucleases present in cell extracts. 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. 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, and it can be used to process multiple samples.

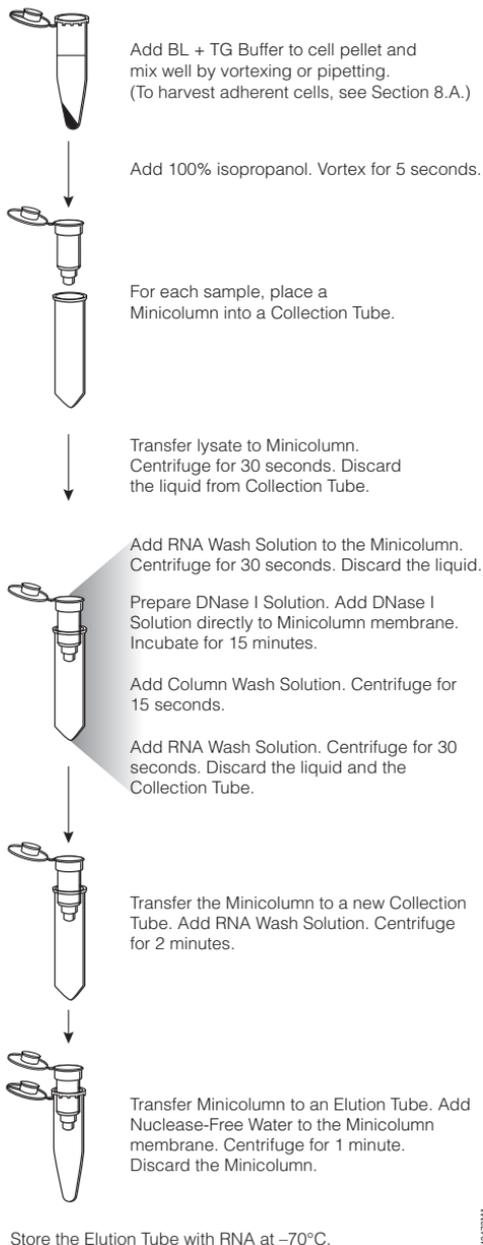
Processing Capacity

The ReliaPrep™ RNA Cell 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, with a broad spectrum of RNA expression levels.

3.B. Downstream Applications

RNA purified with the ReliaPrep™ RNA Cell 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.



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Figure 1. Schematic diagram of the ReliaPrep™ RNA Cell Miniprep System. For protocol details see Sections 4 and 8.A.

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. Autoclaved Elution Tubes are provided with the system.

4. RNA Isolation and Purification Procedure

For best results from this system, use fresh cell 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, 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 three months. 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.

Materials to Be Supplied by the User

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

- 100% isopropanol, RNase-free
- 95% ethanol, RNase-free
- microcentrifuge

4.A. Preparation of Solutions

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

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

4.A. Preparation of Solutions (continued)

Solution	Preparation Steps	Notes
DNase I	<p>250 prep size (Cat.# Z6012): Add 275µl of Nuclease-Free Water (provided) as indicated on the vial label or 50 prep size (Cat.# Z6011): Add 275µl of Nuclease-Free Water (provided) as indicated on the vial label or 10 prep size (Cat.# Z6010): Add 80µl of Nuclease-Free Water (provided) as indicated on the vial label.</p>	<p>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.</p> <p> Do not vortex the DNase I solution.</p> <p> Do not freeze-thaw aliquots of rehydrated DNase I more than three times.</p>
BL + TG Buffer	<p>250 prep size (Cat.# Z6012): Add 1,500µl of 1-Thioglycerol to 150ml of BL Buffer or 50 prep size (Cat.# Z6011): Add 325µl of 1-Thioglycerol to 32.5ml of BL Buffer or 10 prep size (Cat.# Z6010): Add 32.5µl of 1-Thioglycerol to the bottle containing 3.25ml of BL Buffer.</p>	<p>After adding 1-Thioglycerol (TG), mark on the bottle that you have performed this step. Store the BL + TG Buffer at 2°C–10°C for up to 30 days. Cap tightly between uses.</p>

4.A. Preparation of Solutions (continued)

Solution	Preparation Steps	Notes
RNA Wash Solution	<p>250 prep size (Cat.# Z6012): Add 350ml of 95% ethanol to the bottle containing 206ml of concentrated RNA Wash Solution (RWA)</p> <p>or</p> <p>50 prep size (Cat.# Z6011): Add 60ml of 95% ethanol to the bottle containing 35ml of concentrated RNA Wash Solution (RWA)</p> <p>or</p> <p>10 prep size (Cat.# Z6010): Add 20ml of 95% ethanol to the bottle containing 11.8ml of concentrated RNA Wash Solution (RWA).</p>	<p>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.</p>
Column Wash Solution	<p>250 prep size (Cat.# Z6012): Add 36ml of 95% ethanol to the bottle containing 24ml of concentrated Column Wash Solution (CWE)</p> <p>or</p> <p>50 prep size (Cat.# Z6011): Add 7.5ml of 95% ethanol to the bottle containing 5ml of concentrated Column Wash Solution (CWE)</p> <p>or</p> <p>10 prep size (Cat.# Z6010): Add 1.5ml of 95% ethanol to the bottle containing 1ml of concentrated Column Wash Solution (CWE).</p>	<p>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.</p>

4.B. Preparation of Cell Lysates

Use the following protocol to lyse cultured cells from suspension or adherent 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 at the time of harvest.

1. To harvest adherent cells, follow the protocol in the Appendix (Section 8.A) prior to cell lysis. For suspension cells proceed directly to Step 2.
2. **Collect cells in a sterile centrifuge tube** by centrifugation at $300 \times g$ for 5 minutes. **Wash the cell pellet with ice-cold, sterile 1X PBS** (see Section 8.B for the recipe). **Centrifuge at $300 \times g$ 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 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 1. Recommended Amounts of Cells and Solutions for Cell Lysis.

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

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

Note: Up to 2×10^6 cells will lyse easily in 250 μ l 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 500 μ l per column. If a lysate contains more RNA than the capacity of the column allows, some RNA will be lost during the wash steps.

5. Add Isopropanol as recommended in Table 1. Mix by vortexing for 5 seconds.

4.B. Preparation of Cell Lysates (continued)

6. 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.
 7. Transfer the lysate to a ReliaPrep™ Minicolumn and centrifuge at 12,000–14,000 × g for 30 seconds at 20°–25°C.
 8. 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**.
 9. 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 0.09M MnCl₂
 - 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 visualize.
-  **Note:** Do not mix the Yellow Core Buffer and 0.09M MnCl₂ prior to Step 9. The Yellow Core Buffer and 0.09M MnCl₂ should be stored separately and mixed immediately prior to each set of RNA preparations.
10. **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.
 11. **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.
 12. 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.

4.B. Preparation of Cell Lysates (continued)

13. 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 2**. 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 2. 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 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 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 12,000–14,000 × g for 1 minute is recommended. Depending on the number of input cells and RNA expression levels, a second elution may yield as much as 10–20% of additional RNA.

5. Determination of RNA Yield and Quality

Yield and Purity

The ReliaPrep™ RNA Cell Miniprep System can be used to isolate intact RNA from a variety of cell 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 230, 260 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 , 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 can be determined using the QuantiFluor™ RNA System (Cat.# E3310).

5. Determination of RNA Yield and Quality (continued)

RNA isolated with the ReliaPrep™ RNA Cell 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 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.

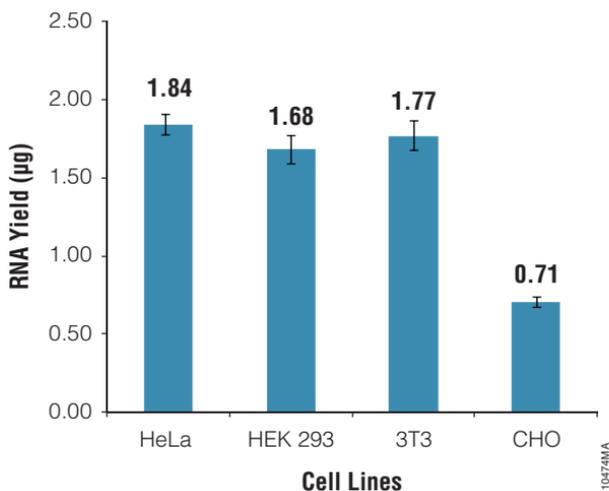


Figure 2. Average total RNA yield obtained from four cultured cell lines using the ReliaPrep™ RNA Cell Miniprep System. One hundred-thousand cells were used for each purification, with 4 replicates processed per cell line. RNA yield was calculated from the concentration obtained at 260nm using a NanoDrop®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

6. Troubleshooting

Symptoms	Possible Causes and Comments
Low A_{260}/A_{280} ratios	<p data-bbox="486 244 899 454">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.</p> <hr/> <p data-bbox="486 470 899 685">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.</p>
Low A_{260}/A_{230} ratios	<p data-bbox="486 702 899 911">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.</p> <hr/> <p data-bbox="486 928 899 1217">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 $10,000 \times g$ 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.</p>

6. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Low A ₂₆₀ (low RNA yield)	<p>Cell lysates 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.</p> <hr/> <p>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.</p> <hr/> <p>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 BL + TG Buffer should be stored at -20°C to -70°C.</p> <hr/> <p>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.</p> <hr/> <p>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.</p> <hr/> <p>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.</p>

6. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Low A ₂₆₀ (low RNA yield) (continued)	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 for lysis to improve yield and stability if overheating is a problem.
Genomic DNA contamination seen when performing PCR	<p>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.</p> <p>The sample may contain too much genomic DNA. For cultured cells, do not exceed 5 x 10⁶ cells per column.</p> <p>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.</p>
Genomic DNA contamination	<p>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.</p> <p>MnCl₂ 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 MnCl₂, 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.</p>

6. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Genomic DNA contamination (continued)	<p>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.</p> <p>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.</p>
Clogged Binding Columns	<p>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 before adding RNA Wash Buffer. Use no more than 500µl of lysate per column.</p>
Lysate too viscous; does not pipet easily	<p>The initial lysate is too viscous. Dilute the lysate with BL + TG Buffer.</p> <p>Lysate becomes too viscous while sitting on ice. Briefly mix by drawing up through a 20-gauge needle to shear genomic DNA.</p>
RNA degradation	<p>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.</p> <p>RNA was degraded during sample prep. Work quickly during sample preparation (see comments under "Low A₂₆₀").</p>

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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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. 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 Section 8.B.)

- BL + TG Buffer
 - 1X PBS
1. Lysates may be prepared rapidly in the culture plate or flask by the addition of BL + TG Buffer. Recommended volumes of wash (PBS) and BL + TG Buffer (lysis) to add per well are shown in Table 3, below.

Table 3. 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 media and wash the cells with ice-cold, sterile 1X PBS (Section 8.B). Add BL + TG Buffer, gently rock the plate or flask to cover adherent cells. Rinse the cells by repeated pipetting of the lysate over the well surface, 7–10 times. Collect the lysate and transfer it to a new microcentrifuge tube.

8.A. Harvesting Procedure for Adherent Cells (continued)

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 500 μ l.

3. Proceed to Step 5 in Section 4.B. Preparation of Cell Lysates.

Note: If you prefer to harvest cells by conventional trypsinization methods, harvest cells and proceed to Step 3 in Section 4.B. Preparation of Cell Lysates.

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.

BL + TG Buffer

4M Guanidine thiocyanate
0.01M Tris (pH 7.5)
2% 1-Thioglycerol

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)

8.C. Related Products

Total RNA Isolation from FFPE Samples

Product	Size	Cat.#
ReliaPrep™ FFPE Total RNA Miniprep System	10 preps	Z1001
	100 preps	Z1002

Total RNA Isolation Systems

Product	Size	Cat.#
SV 96 Total RNA Isolation System	1 × 96 each	Z3500
	5 × 96 each	Z3505
MagneSil® Total RNA mini-Isolation System	4 plates	Z3351
PureYield™ RNA Midiprep System	10 preps	Z3740
	50 preps	Z3741
SV 96 Total RNA Isolation System	1 × 96 each	Z3500
	5 × 96 each	Z3505

mRNA Isolation from Total RNA

Product	Size	Cat.#
PolyAtract® mRNA Isolation System I	3 isolations	Z5210
PolyAtract® mRNA Isolation System II	3 isolations	Z5200
PolyAtract® mRNA Isolation System III	15 isolations	Z5300
PolyAtract® mRNA Isolation System IV	15 isolations	Z5310

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All prices and specifications are subject to change without prior notice.

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