



Technical Manual

SV Total RNA Isolation System

INSTRUCTIONS FOR USE OF PRODUCTS Z3100, Z3101 AND Z3105.



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PRINTED IN USA.
Revised 10/09

Part# TM048

SV Total RNA Isolation System

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Technical Manual. Please contact Promega Technical Services if you have questions on use
of this system. E-mail: techserv@promega.com

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1. Description

The purity and integrity of RNA isolated from tissue or cultured cells are critical for its effective use in applications such as reverse transcription PCR (RT-PCR), quantitative reverse transcription PCR (qRT-PCR), RNase protection assays, Northern blot analysis, oligo(dT) selection of poly(A)⁺ RNA, in vitro translation and microarray analysis. In recent years, RT-PCR and qRT-PCR 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., tissue and cultured cells) has also increased. The SV Total RNA Isolation System has been designed to address these needs.

The SV Total RNA Isolation System^(a) provides a fast and simple technique for preparing purified and intact total RNA from tissues, cultured cells and white blood cells (see Section 8.A) in as little as one hour, depending on the number of samples to be processed. Like the Wizard[®] *Plus* SV Minipreps DNA Purification System (Cat.# A1330), either a spin or vacuum (“SV”) purification protocol can be used. Up to 60mg of tissue can be processed per purification, depending on the type, function and RNA expression levels of the tissue. 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, and there is no DNase carryover in the final RNA preparation.

The SV Total RNA Isolation System also can be used to isolate both genomic DNA and RNA from the same sample. For protocols and additional information on the use of this system for DNA isolation, please see reference 1 or visit the Promega web site at: www.promega.com/pnotes/69/

Selected Citations Using the SV Total RNA Isolation System

To view a list of peer-reviewed articles that cite the use of the SV Total RNA Isolation System, please visit: www.promega.com/citations/

2. Product Components and Storage Conditions

Product	Size	Cat.#
SV Total RNA Isolation System	250 preps	Z3105

For Laboratory Use. Each system contains sufficient reagents for 250 isolations of total RNA from tissue, cells or blood. Includes:

- 5 packs Collection Tubes (50/pack)
- 5 packs Elution Tubes (50/pack)
- 5 packs Spin Columns (50/pack)
- 250ml RNA Lysis Buffer (RLA)
- 96ml RNA Dilution Buffer (RDA) (blue buffer)
- 5.5ml β -mercaptoethanol (48.7%)
- 5 vials DNase I (lyophilized)
- 2 \times 750 μ l $MnCl_2$, 0.09M
- 11ml Yellow Core Buffer
- 26.5ml DNase Stop Solution (DSA) (concentrated)
- 206ml RNA Wash Solution (RWA) (concentrated)
- 2 \times 25ml Nuclease-Free Water

Product	Size	Cat.#
SV Total RNA Isolation System	50 preps	Z3100

For Laboratory Use. Each system contains sufficient reagents for 50 isolations of total RNA from tissue, cells or blood. Includes:

- 2 packs Collection Tubes (25/pack)
- 2 packs Elution Tubes (25/pack)
- 2 packs Spin columns (25/pack)
- 50ml RNA Lysis Buffer (RLA)
- 20ml RNA Dilution Buffer (RDA) (blue buffer)
- 2ml β -mercaptoethanol (48.7%)
- 1 vial DNase I (lyophilized)
- 250 μ l $MnCl_2$, 0.09M
- 2.5ml Yellow Core Buffer
- 5.3ml DNase Stop Solution (DSA) (concentrated)
- 58.8ml RNA Wash Solution (RWA) (concentrated)
- 13ml Nuclease-Free Water

Product	Size	Cat.#
SV Total RNA Isolation System, Trial Size	10 preps	Z3101

For Laboratory Use. Each system contains sufficient reagents for 10 isolations of total RNA from tissue, cells or blood. Includes:

- 2 packs Collection Tubes (5/pack)
- 2 packs Elution Tubes (5/pack)
- 2 packs Spin Columns (5/pack)
- 10ml RNA Lysis Buffer (RLA)
- 4ml RNA Dilution Buffer (RDA) (blue buffer)
- 2ml β -mercaptoethanol (48.7%)
- 1 vial DNase I (lyophilized)
- 250 μ l MnCl₂, 0.09M
- 2.5ml Yellow Core Buffer
- 5.3ml DNase Stop Solution (DSA) (concentrated)
- 11.8ml RNA Wash Solution (RWA) (concentrated)
- 1.25ml Nuclease-Free Water

Available Separately

Product	Size	Cat.#
Vacuum Adapters	20 each	A1331

Note: The Vacuum Adapters (Cat.# A1331) are required for the vacuum format of the SV Total RNA Isolation System and must be purchased separately.

Product	Size	Cat.#
RNA Lysis Buffer (RLA)	50ml	Z3051
Red Blood Cell Lysis Solution (CLB)	200ml	Z3141

For Laboratory Use.

Note: The RNA Dilution Buffer (RDA) and Yellow Core Buffer are colored blue and yellow, respectively, so they can be more easily distinguished. The yellow dye in the Yellow Core Buffer allows users to visualize whether the membrane is completely covered by the DNase mixture in the DNase step. The dyes have no effect on the quality or downstream performance of the RNA.

Storage Conditions: Store the RNA Lysis Buffer (RLA) with β -Mercaptoethanol (BME) added at 4°C. Cap tightly between uses. For information on rehydration of DNase I see Section 4.A, Preparation of Solutions. Store all other components at 22–25°C.

! **Do not** combine or replace components of the SV Total RNA Isolation System with components from any Wizard® Plus or Wizard® Plus SV DNA Purification System.

Caution: Guanidine thiocyanate and β -mercaptoethanol are toxic solutions. Wear gloves and follow standard safety procedures while working with these solutions. When processing human or infectious tissues or blood samples, follow standard procedures for handling and disposal of hazardous materials.

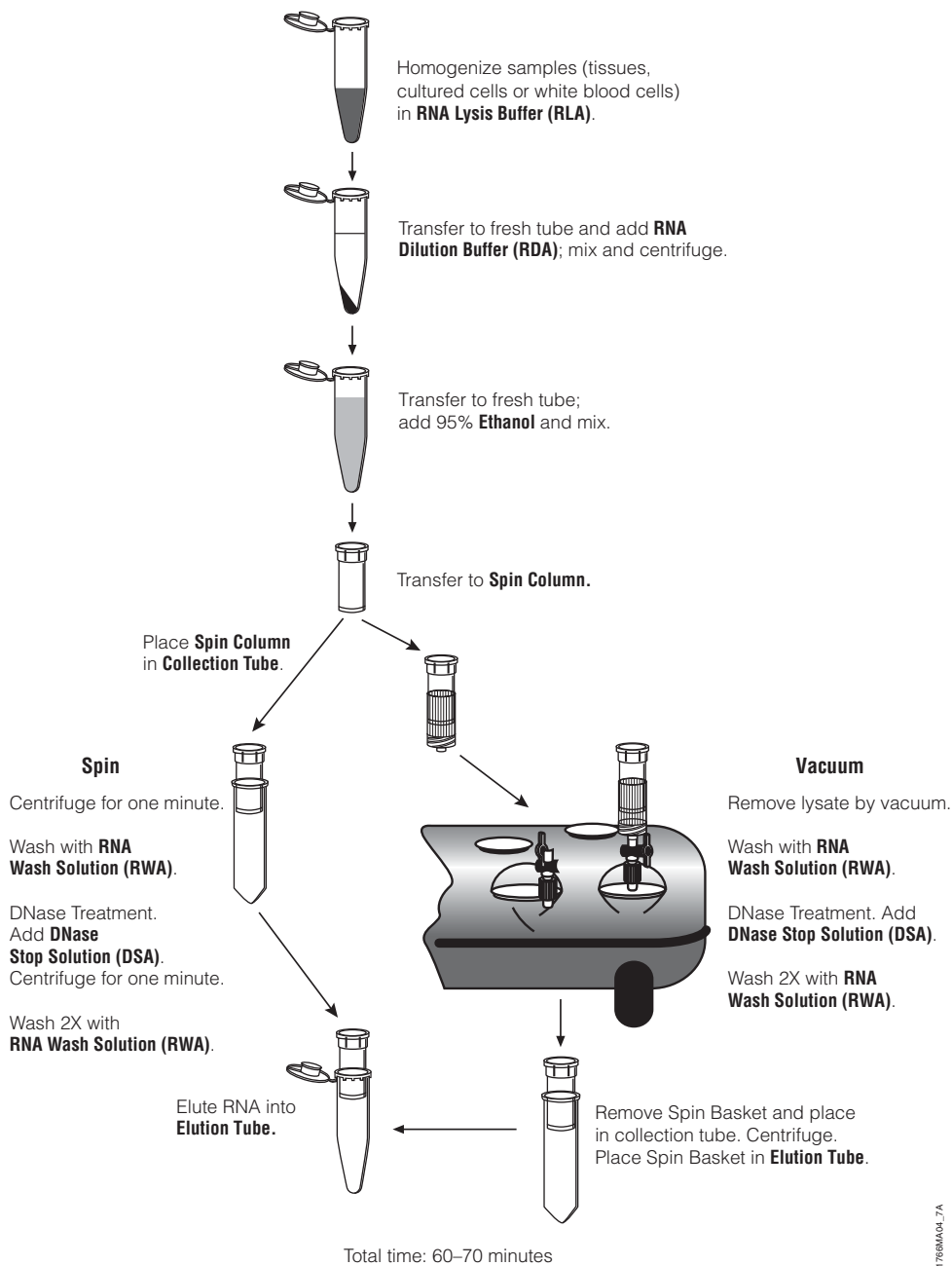


Figure 1. Schematic representation of the SV Total RNA Isolation System. For information on the protocol, please read Section 4 and Sections 8.A–D.

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.


The SV Total RNA Isolation System combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β -mercaptoethanol to inactivate the ribonucleases present in cell extracts (2). GTC, in association with SDS, acts to disrupt nucleoprotein complexes, allowing the RNA to be released into solution and isolated free of protein. Dilution of cell extracts in the presence of high concentrations of GTC causes selective precipitation of cellular proteins to occur, while the RNA remains in solution. After centrifugation to clear the lysate of precipitated proteins and cellular debris, the RNA is selectively precipitated with ethanol and bound to the silica surface of the glass fibers found in the Spin Basket. By effectively clearing the lysate of precipitated proteins and cellular debris, these cleared lysates may be bound to the Spin Baskets by either a centrifugation or a vacuum filtration method. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts, thus favoring adsorption of nucleic acids to the silica. RNase-Free DNase I is applied directly to the silica membrane to digest contaminating genomic DNA. The bound total RNA is further purified from contaminating salts, proteins and cellular impurities 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 tissue, blood or cultured cells, and it can be used to process multiple samples.

Processing Capacity

The SV Total RNA Isolation System has been developed and optimized for total RNA isolation from tissues, blood or cultured cells with a broad spectrum of RNA expression levels. For a tissue rich in RNA such as mouse liver, 30mg of fresh tissue can be processed per purification. When using a tissue such as lung, which has a lower RNA to tissue mass ratio, up to 60mg of tissue can be processed per purification (see Table 2, Section 5). **Excess tissue or RNA samples should not be processed because this leads to clogging of the membranes and poor purification.** The maximum volume of lysate that can be processed in each Spin Basket is 175 μ l. For larger tissue amounts, perform multiple isolations as necessary to supply the desired amount of total RNA. Recommended sample amounts of various tissues are listed in Table 1, Section 4.B.

3.B. Downstream Applications

RNA purified with the SV Total RNA Isolation System is suitable for many molecular biology applications, including RT-PCR, microarrays and Northern blot hybridizations. For more information on downstream applications, see the Promega *Protocols and Applications Guide* (3) and the Promega *RNA Applications Guide* (4).

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

3.C. Creating a Ribonuclease-Free Environment

Ribonucleases are extremely difficult to inactivate. 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. 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.
3. Treat nondisposable glassware and plasticware before use to ensure that it is RNase-free. Bake glassware at 200°C overnight, and thoroughly rinse plasticware with 0.1N NaOH, 1mM EDTA followed by RNase-free water.
4. Treat solutions supplied by the user by adding diethyl pyrocarbonate (DEPC) to 0.1% and then incubating overnight at room temperature. Autoclave for 30 minutes to remove any trace of DEPC.

Note: DEPC reacts rapidly with amines and cannot be used to treat Tris buffers.

 **Caution:** DEPC is a suspected carcinogen and should be used in a chemical fume hood.

4. RNA Isolation and Purification Procedure

For best results from this system, use fresh samples when processing tissue. Older samples may yield less total RNA. If necessary, freeze the samples immediately after collection in liquid nitrogen and store at -70°C for future use. Samples homogenized in RNA Lysis Buffer (RLA) may be stored at -20°C or -70°C. For valuable tissue samples, we suggest that a portion of each sample be reserved at

4. RNA Isolation and Purification Procedure (continued)

-70°C in the event that loss of a sample occurs during RNA purification. 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.G.)

- small tissue homogenizer (e.g., Brinkmann, Tissuemizer® or Omni Micro homogenizer)
- 95% ethanol, RNase-free
- microcentrifuge
- 10X phosphate-buffered saline (PBS), sterile (for cultured cells)
- sterile hypodermic syringe fitted with a sterile 20-gauge needle (for cultured cells)
- water bath or heating block, preheated to 70°C
- Laboratory Vacuum Manifold (e.g., Vac-Man®, Cat.# A7231, or Vac-Man® Jr. Laboratory Vacuum Manifold, Cat.# A7660) and Vacuum Adapters (purchased separately; Cat.# A1331) (required for RNA purification by vacuum)

4.A. Preparation of Solutions

Before beginning the SV Total RNA Isolation System protocol, four solutions must be prepared.

Note: Throughout this document, RNA Lysis Solution (RLA), RNA Wash Solution (RWA) and DNase Stop Solution (DSA) refer to the solutions supplied with the SV Total RNA Isolation System. Once prepared as described below, these solutions are referred to as RNA Lysis Buffer, RNA Wash Solution and DNase Stop Solution.

Solution	Preparation Steps	Notes
DNase I	For all prep sizes: 250 prep size (Cat.# Z3105), 50 prep size (Cat.# Z3100) and 10 prep size (Cat.# Z3101): Add Nuclease-Free Water (supplied) in the amount indicated on the DNase I vial to each vial of lyophilized DNase I.	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
RNA Lysis Buffer	<p>250 prep size (Cat.# Z3105): Add 5ml β-mercaptoethanol (BME) to 250ml RNA Lysis Buffer (RLA)</p> <p>or</p> <p>50 prep size (Cat.# Z3100): Add 1ml BME to 50ml RNA Lysis Buffer (RLA)</p> <p>or</p> <p>10 prep size (Cat.# Z3101): Add 200μl BME to 10ml RNA Lysis Buffer (RLA).</p>	<p>After adding the BME, mark on the bottle that you have performed this step. Store the RNA Lysis Buffer (RLA) at 4°C. Cap tightly between uses.</p>
RNA Wash Solution	<p>250 prep size (Cat.# Z3105): Add 350ml of 95% ethanol to the bottle containing 206ml concentrated RNA Wash Solution (RWA)</p> <p>or</p> <p>50 prep size (Cat.# Z3100): Add 100ml of 95% ethanol to the bottle containing 58.8ml concentrated RNA Wash Solution (RWA)</p> <p>or</p> <p>10 prep size (Cat.# Z3101): Add 20ml of 95% ethanol to the bottle containing 11.8ml concentrated RNA Wash Solution (RWA).</p>	<p>After adding the ethanol, mark on the bottle label that you have performed this step. The reagent is stable at 22–25°C when tightly capped.</p>
DNase Stop Solution	<p>250 prep size (Cat.# Z3105): Add 40ml 95% ethanol to the bottle containing 26.5ml concentrated DNase Stop Solution (DSA)</p> <p>or</p> <p>50 or 10 prep size (Cat.# Z3100 or Z3101): Add 8ml 95% ethanol to the bottle containing 5.3ml concentrated DNase Stop Solution (DSA).</p>	<p>After adding ethanol, mark on the bottle label that you have performed this step. The reagent is stable at 22–25°C when tightly capped.</p>

4.A. Preparation of Solutions (continued)

Sections 4.B, C and D provide step-by-step guides for lysis of variously sized tissue and cell samples. Section 4.B provides the protocol for lysis and homogenization of tissue samples ≤ 30 mg, and Section 4.C provides the protocol for lysis and homogenization of tissue samples >30 mg. Additional information on preparation of tissue lysates is presented in Section 8.E. For lysis of suspension or adherent cultures, use the protocol provided in Section 4.D. Sections 4.E and 4.F provide protocols for RNA purification from lysates using the spin or vacuum formats, respectively. For isolation of total RNA from white blood cells, plant tissue, yeast and bacterial cells, see Sections 8.A-D.

4.B. Preparation of Lysates from Small Tissue Samples (≤ 30 mg)

This protocol is for processing small tissue samples. In general, the mass of tissue to be lysed and homogenized in **175 μ l RNA Lysis Buffer** should be **≤ 30 mg** but may be adjusted for certain tissues (see Table 1 for tissue mass recommendations for different tissues).

1. Transfer **175 μ l of RNA Lysis Buffer** (with BME added) to a sterile microcentrifuge tube to which your tissue sample will be added. Use RNase-free pipettes, and wear gloves to reduce the chance of RNase contamination.
2. Weigh the tube containing the RNA Lysis Buffer and record the weight.
3. Quickly cut the tissue into small pieces with a sterile razor blade, freeze in liquid nitrogen, and grind in a mortar and pestle under liquid nitrogen. Transfer liquid nitrogen and ground tissue to an appropriately sized sterile tube, allow the liquid nitrogen to evaporate and then **immediately** transfer the tissue to the tube containing **175 μ l RNA Lysis Buffer**. Mix thoroughly by inversion.
4. Weigh the tube containing the tissue and RNA Lysis Buffer. Calculate the tissue mass by subtracting the weight obtained in Step 2 from this new weight. In general, the ratio of tissue mass to RNA Lysis Buffer should be approximately 30mg/175 μ l but may be adjusted for certain tissues (see Table 1 for recommendations for tissue mass to RNA Lysis Buffer ratios for different tissues). If necessary, add RNA Lysis Buffer to the tissue to achieve this ratio.

Note: Some lysed samples (e.g., spleen) contain a large amount of nucleic acid, cellular debris and protein, which cause a very thick lysate. If after adding RNA Lysis Buffer the sample is too viscous to pipet easily, dilute with additional RNA Lysis Buffer **before** adding the RNA Dilution Buffer in Step 5. Add the minimum amount of RNA Lysis Buffer required to make the lysate easy to pipet. The maximum volume of lysate that can be processed in each Spin Basket is 175 μ l. For larger volumes, use additional Spin Baskets. For tissues with lower RNA levels, more concentrated lysate may be used as long as the lysate is not difficult to pipet.

5. Add **350µl of RNA Dilution Buffer** (blue) to **175µl of lysate**. Mix by inverting 3–4 times. Place in a water bath or heating block at 70°C for 3 minutes. Incubating longer than 3 minutes may compromise the integrity of the RNA.
6. Centrifuge for 10 minutes at 12,000–14,000 × g. Proceed to Section 4.E (Spin Purification) or Section 4.F (Vacuum Purification).

Table 1. Recommended Amounts of Tissue and Cells for Preparation of Lysates.

Sample Material	Approximate Wet Weight of Organ from a 30g mouse	Maximum Tissue Mass or Number of Cells per 175µl of Lysis Buffer	Maximum Tissue Mass or Number of Cells per 1ml of Lysis Buffer ¹
Liver	940mg	30mg	171mg
Kidney	210mg	20mg	114mg
Muscle ²	—	30mg	171mg
Spleen	90mg	15mg	85mg
Heart	150mg	60mg	342mg
Brain	463mg	60mg	342mg
Lung	200mg	60mg	342mg
RAW264.7 cells	NA	1×10^5 – 5×10^6	5.7×10^5 – 2.8×10^7

The tissue amounts and cell numbers for given volumes of Lysis Buffer are recommended for optimal performance of the Spin Columns.

¹The maximum amount of lysate that can be efficiently processed is 175µl per Spin Basket. If a lysate contains more RNA than the capacity of the Spin Basket allows, some RNA will be lost during the wash steps. Note that for some organisms and tissues, less than 1ml of RNA Lysis Buffer will be required for processing the entire organ (e.g., mouse heart and lung). Use the recommended ratio (e.g., for a 154mg mouse heart, use approximately 450µl of RNA Lysis Buffer for homogenization) and adjust the viscosity of the lysate as required.

²An approximate weight for all types of muscle is not given.

NA: not applicable.

4.C. Preparation of Lysates from Tissue Samples >30mg

This protocol describes the lysis and homogenization of tissues using 1ml of RNA Lysis Buffer. Refer to Table 1 for recommended sample mass for different tissue types.

1. Transfer 1ml of RNA Lysis Buffer (with BME added) into the tube to which your tissue sample will be added. Use RNase-free pipettes, and wear gloves to reduce the chance of RNase contamination.
2. Weigh the tube containing the RNA Lysis Buffer; record the weight.
3. Excise the tissue of interest, and place it in the tube containing the RNA Lysis Buffer. Work as quickly as possible. Homogenize the tissue at high speed using a small homogenizer (such as a Tekmar Tissuemizer® homogenizer) until no visible tissue fragments remain. (See Section 8.E for additional information on preparing lysates.)
4. Weigh the tube containing the tissue and RNA Lysis Buffer. Calculate the tissue mass by subtracting the weight obtained in Step 2 from this new weight. In general, the ratio of tissue mass to RNA Lysis Buffer should be approximately 171mg/ml but may be adjusted for certain tissues (see Table 1 for recommendations for tissue mass to RNA Lysis Buffer ratios for different tissues). If necessary, add RNA Lysis Buffer to the tissue to achieve this ratio.

Note: Some lysed samples (e.g., spleen) contain a large amount of nucleic acid, cellular debris and protein, which cause a very thick lysate. If after adding RNA Lysis Buffer the sample is too viscous to pipet easily, dilute by adding additional RNA Lysis Buffer **before** adding the RNA Dilution Buffer in Step 5. Add the minimum amount of RNA Lysis Buffer required to make the lysate easy to pipet. The maximum volume of lysate that can be processed in each Spin Basket is 175µl. For larger volumes use additional Spin Baskets. For tissues with lower RNA levels, more concentrated lysate may be used as long as the lysate is not difficult to pipet.

5. Transfer 175µl of the tissue lysate to a 1.5ml microcentrifuge tube. Additional lysate should be frozen at -20°C or -70°C. Add 350µl of RNA Dilution Buffer (blue). Mix by inverting 3–4 times. Place in a water bath or heating block at 70°C for 3 minutes. Incubation for longer than 3 minutes may result in compromised integrity of the RNA.
6. Centrifuge for 10 minutes at 12,000–14,000 × g. Proceed to Section 4.E (Spin Purification) or Section 4.F (Vacuum Purification).

4.D. Lysis of Cultured Cells


Use the following protocol for lysis of cultured cells from suspension or adherent cultures. Use at least 1.5×10^3 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. For harvesting adherent cells, follow the protocol in the appendix (Section 8.F) prior to cell lysis. For suspension cells proceed to Step 2.
2. Collect 1.5×10^3 - 5×10^6 cells in a sterile 50ml conical centrifuge tube by centrifugation at $300 \times g$ for 5 minutes. Wash the cell pellet with 25ml of ice-cold, sterile 1X PBS (see Section 8.G for recipe). Centrifuge at $300 \times g$ for 5 minutes to collect the cells. Discard the supernatant.
3. Verify that BME has been added to the RNA Lysis Buffer. Add **175 μ l of RNA Lysis Buffer** to the washed cells, dispersing the pellet and mixing well by vortexing and/or pipetting.
4. Up to **1 x 10⁶ cells** will lyse easily in **175 μ l of RNA Lysis Buffer**.
1 x 10⁶-5 x 10⁶ cells will need to be passed through a 20-gauge needle to shear the genomic DNA. Repeat 4 to 5 times. Expel the lysate into a 1.5ml tube.
Note: If the lysate is too viscous to pipet easily, dilute with additional RNA Lysis Buffer before adding RNA Dilution Buffer in Step 5. Add the minimum amount of RNA Lysis Buffer required to make the lysate easy to pipet.
5. Add **350 μ l of RNA Dilution Buffer** (blue) to **175 μ l of lysate**. Mix by inverting the tube 3-4 times. Place in a water bath or heating block at 70°C for 3 minutes. Incubating longer than 3 minutes may compromise the integrity of the RNA.
6. Centrifuge at 12,000-14,000 $\times g$ for 10 minutes at 20-25°C. Proceed to Section 4.E (Spin Purification) or Section 4.F (Vacuum Purification).

4.E. RNA Purification by Centrifugation (Spin)

Wear gloves and open the pack carefully. Remove one Spin Column and Collection Tube for each sample to be processed. If you do not need the caps on the Spin Baskets, simply remove them using a twisting motion; the caps are designed to detach from the Spin Baskets. If the caps are left attached to the Spin Baskets, they must be **closed** during centrifugation steps. Label the Collection Tube and place the Spin Column Assembly in a microcentrifuge tube rack. It is important to label your tubes to maintain sample identity. Wear gloves when handling the tubes.

1. Transfer the cleared lysate solution to a fresh microcentrifuge tube by pipetting. Avoid disturbing the pelleted debris.

 Carryover of a small amount of pelleted debris is not detrimental to RNA purification. Sometimes debris will form a solid layer on top of the supernatant. Simply push this layer to the side of the tube with the pipette tip before pipetting the supernatant. The supernatant volume should be approximately 500 μ l but will depend on the amount of tissue mass in the lysate.

4.E. RNA Purification by Centrifugation (Spin; continued)

2. Add **200µl 95% ethanol** to the cleared lysate, and mix by pipetting 3–4 times. Transfer this mixture to the Spin Column Assembly. Centrifuge at 12,000–14,000 × *g* for one minute.
3. Take the Spin Basket from the Spin Column Assembly, and discard the liquid in the Collection Tube. Put the Spin Basket back into the Collection Tube. Verify that the RNA Wash Solution has been diluted with ethanol as described in Section 4.A. Add 600µl of RNA Wash Solution to the Spin Column Assembly. Centrifuge at 12,000–14,000 × *g* for 1 minute.
4. Empty the Collection Tube as before and place it in a rack. For each isolation to be performed, prepare the DNase incubation mix by combining **40µl Yellow Core Buffer, 5µl 0.09M MnCl₂ and 5µl of DNase I enzyme** per sample in a sterile tube (in this order). Prepare only the amount of DNase incubation mix required and pipet carefully. **Mix by gentle pipetting; do not vortex.** Keep the DNase I on ice while it is thawed. Apply **50µl** of this freshly prepared DNase incubation mix directly to the membrane inside the Spin Basket. Make sure that the solution is in contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to visualize.



Do not mix the Yellow Core Buffer and 0.09M MnCl₂ prior to Step 4. The Yellow Core Buffer and 0.09 MnCl₂ should be stored separately and mixed fresh for each set of RNA preparations.

5. Incubate for 15 minutes at 20–25°C. After this incubation, add **200µl of DNase Stop Solution** (as prepared in Section 4.A; verify that ethanol has been added) to the Spin Basket, and centrifuge at 12,000–14,000 × *g* for 1 minute. There is no need to empty the Collection Tube before the next step.
6. Add **600µl RNA Wash Solution** (with ethanol added) and centrifuge at 12,000–14,000 × *g* for 1 minute.
7. Empty the Collection Tube, and add 250µl RNA Wash Solution (with ethanol added); centrifuge at high speed for 2 minutes.
8. If you have not already done so, remove the cap from the Spin Basket by using a twisting motion.
9. For each sample, remove one capped 1.5ml Elution Tube. Transfer the Spin Basket from the Collection Tube to the Elution Tube, and add **100µl Nuclease-Free Water** to the membrane. Be sure to completely cover the surface of the membrane with the water. Place the Spin Basket Assemblies in the centrifuge with the lids of the Elution Tubes facing out. Centrifuge at 12,000–14,000 × *g* for 1 minute. Remove the Spin Basket and discard. Cap the Elution Tube containing the purified RNA and store at –70°C.

Note: Elution volumes of less than 100µl are not recommended. If the RNA needs to be concentrated, it can be vacuum-dried and resuspended in a smaller volume of water. If maximum recovery of RNA is essential, a


second elution into a second sterile tube with an additional 100µl of Nuclease-Free Water followed by centrifugation at 12,000-14,000 × g for 1 minute is recommended. Depending on the amount of input tissue and RNA expression levels, a second elution may yield as much as 10-20% additional RNA.

4.F. RNA Purification by Vacuum

Wear gloves and open the pack carefully. Remove one Spin Column and Collection Tube for each sample to be processed. **Detach the cap from the Spin Basket using a simple twisting action and discard.** The caps should be removed for the vacuum procedure, as they will interfere with use of the vacuum manifold. **Label the Collection Tube and save until Step 9.** It is important to properly label your tubes to maintain sample identity. Label the Spin Basket only on the side of the top band. Any markings placed on the side of the basket may be removed by ethanol during the wash steps. If the Spin Basket becomes clogged during the vacuum procedure, switch to the procedure for purification of RNA by centrifugation (Section 4.E).

Note: Vacuum Adapters (Cat.# A1331) are required for this protocol. When reusing Vacuum Adapters, thoroughly rinse them with 0.1N NaOH, 1mM EDTA and then with Nuclease-Free Water before use.

1. Attach one Vacuum Adapter with Luer-Lok® fitting to one port of the manifold. Gently press an RNA Spin Basket into the Vacuum Adapter until snugly in place.
2. Transfer the cleared lysate solution to a fresh microcentrifuge tube by pipetting. Avoid disturbing the pelleted debris.

 Carryover of a small amount of pelleted debris is not detrimental to RNA purification. Sometimes debris will form a solid layer on top of the supernatant. Simply push this layer to the side of the tube with the pipette tip before pipetting the supernatant. The supernatant volume should be approximately 500µl but will depend on the amount of tissue mass in the lysate.

3. Add **200µl 95% ethanol** to the cleared lysate, and mix by pipetting 3-4 times. Transfer this mixture to the Spin Column Assembly.
4. Apply a vacuum of at least 15 inches (37.5mm) of mercury and allow the lysate to pass through the Spin Basket. Verify that the RNA Wash Solution has been diluted with ethanol as described in Section 4.A. Add **900µl of RNA Wash Solution**, and allow it to pass through the Spin Basket.

4.F. RNA Purification by Vacuum (continued)

5. Turn off the vacuum source, and open an unused port to vent the manifold. **Make certain that all vacuum pressure is released before performing the next step!** It is important to turn off the vacuum at the source to prevent backflow into the column.
6. For each isolation to be performed, prepare the DNase incubation mix by combining **40µl Yellow Core Buffer, 5µl 0.09M MnCl₂ and 5µl of DNase I enzyme** per sample in a sterile tube (in this order). Prepare only the amount of DNase incubation mix required and pipet carefully. **Mix by gentle pipetting; do not vortex.** Keep the DNase I on ice while it is thawed. Apply **50µl** of this freshly prepared DNase incubation mix directly to the membrane inside the Spin Basket. It is important to make sure that the solution is in contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to visualize.



Do not mix the Yellow Core Buffer and 0.09M MnCl₂ prior to Step 4. The Yellow Core Buffer and 0.09 MnCl₂ should be stored separately and mixed fresh for each set of RNA preparations.

7. Incubate for 15 minutes at 20–25°C. After this incubation add **200µl of DNase Stop Solution** (as prepared in Section 4.A; verify that ethanol has been added) to the Spin Basket. Close the unused port, apply the vacuum and allow the solution to pass through the Spin Basket.
8. Add **900µl of RNA Wash Solution** (with ethanol added), and allow it to pass through the Spin Basket. Repeat this wash a second time.
9. Turn off the vacuum source and open an unused port to vent the manifold. Remove each Spin Basket from the manifold and place in the Collection Tube that was set aside earlier. Centrifuge the Spin Basket and Collection Tube at 12,000–14,000 × g for one minute to remove any remaining Wash Solution.
10. For each sample, remove one capped 1.5ml Elution Tube from the packaging. Transfer the Spin Basket from the Collection Tube to the Elution Tube.
11. To elute the RNA, add **100µl of Nuclease-Free Water** to the Spin Basket. Be sure to completely cover the surface of the membrane with the water.
12. Centrifuge at 12,000–14,000 × g for one minute. Remove the Spin Basket and discard. Cap the Elution Tube containing the purified RNA and store at -70°C.
Note: Using elution volumes of less than 100µl of Nuclease-Free Water may result in lower RNA yield. If the RNA needs to be concentrated, it can be vacuum-dried and resuspended in a smaller volume of water. If maximum recovery of RNA is essential, a second elution into a second sterile tube with an additional 100µl of Nuclease-Free Water is recommended. Depending on the amount of input tissue and RNA expression levels, a second elution may yield as much as 10–20% additional RNA.

5. Determination of RNA Yield and Quality

Yield and Purity

The SV Total RNA Isolation System can be used to generate intact RNA from a variety of tissues and cell sources. The yield of total RNA obtained may be determined spectrophotometrically at 260nm, where 1 absorbance unit (A_{260}) equals 40 μ g 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 SV Total RNA Isolation 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 exhibits a ratio less than 1.7, refer to Section 6 for possible causes and suggestions on improving the purity of the RNA. Table 2 lists representative yields and A_{260}/A_{280} ratios of total RNA isolated from a number of different cell and tissue sources using the SV Total RNA Isolation System. 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 (5,6) or glyoxal (6,7) 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.

Table 2. Average Yields of Total RNA Isolated from Tissues and Cells.

Samples	Maximum Amount to Process	Average Yield per Prep (µg)	Average Yield per mg Tissue (µg)	Average A ₂₆₀ /A ₂₃₀	Average A ₂₆₀ /A ₂₈₀
Mouse Tissues					
Liver	30mg	131	4.4	2.4	1.9
Kidney	20mg	44	2.2	2.1	1.9
Spleen	15mg	79	5.3	2.3	1.9
Brain	60mg	39	0.65	2.1	2.1
Muscle	30mg	22	0.73	1.8	2.1
Rat Tissues					
Pancreas	30mg	100	3.5	2.2	1.9
Heart	60mg	16	0.27	1.8	2.1
Lung	60mg	36	0.60	2.0	2.1
Bacteria					
<i>E. coli</i>	~1 × 10 ⁹ cells	36	NA	1.6	2.0
Yeast					
<i>S. cerevisiae</i>	~4 × 10 ⁷ cells	19	NA	1.7	2.1
Plant					
Tomato Leaves	30mg	4.6	0.15	1.4	2.0
Cell Line					
RAW264.7 cells	5 × 10 ⁶ cells	51	NA	2.0	2.1

NA: not applicable.

For information about additional sample types, visit:

www.promega.com/applications/dna_rna/sampletypes/svrnasampletypes.htm

The Table 2 values represent means of results achieved at Promega. Yields will depend on the type of tissue, cultures and metabolic state of the sample. The means for the cell line and spleen sample are the average of two and three determinations, respectively. The means for all other samples are the average of at least six determinations.

Means were calculated using both the spin and vacuum formats for all rat and mouse tissue samples listed, except kidney, liver, spleen and cell line. Bacteria, plant and yeast samples were evaluated using the spin format only.

RNA yields for all tissues but heart and lung were determined from mouse tissues; RNA yields for heart and lung were determined from rat tissues. The cell line used was RAW264.7, a mouse macrophage line grown to confluence in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1mM pyruvate.

6. Troubleshooting

Symptoms	Possible Causes and Comments
Low A_{260}/A_{280} ratios	<p>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>Low A_{260}/A_{230} ratios are typically due to guanidine thiocyanate contaminaton. 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.</p>
Low A_{260} (low RNA yield)	<p>Tissues or cell lysates that have been stored frozen (-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> <p>Tissue may not contain large quantities of total RNA. Tissues and cells vary in total amount of RNA that can be purified based on wet weight. If total RNA yields are low, increase the amount of starting material that is processed.</p> <p>Sample RNA may be of poor quality. Samples that were not homogenized or frozen immediately upon isolation may have decreased amounts of RNA with reduced integrity. Freeze tissues immediately in liquid nitrogen and store at -70°C if they cannot be processed immediately. Homogenized samples should be stored at -20°C or -70°C.</p> <p>The binding capacity of the membrane in the spin basket was exceeded. If the lysate contains more RNA than the capacity of the Spin Basket, 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>

6. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Low A_{260} (low RNA yield) (continued)	<p>The diluted homogenate may not have been heated at 70°C as described in Section 4.B, Step 5; Section 4.C, Step 5; or Section 4.D, Step 5. This mixture should be heated at 70°C for 3 minutes for optimal recovery of total RNA. Failure to heat this mixture will result in decreased yields.</p> <hr/> <p>The protocol may not have been followed correctly, or incorrect reagents may have been used. The SV Total RNA Isolation System is 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. Wizard® <i>Plus</i> SV DNA Purification System buffers are not compatible with this system and should not be used.</p> <hr/> <p>Ethanol may not have been added to the DNase Stop Solution or to the RNA Wash Solution. Prepare the solutions as instructed in Section 4.A before beginning the procedure.</p> <hr/> <p>Lysate was allowed to overheat during homogenization. Work as quickly as possible. Lysates can be placed on ice during sample preparation. Use ice-cold RNA Lysis Buffer for homogenization to improve yield and stability if overheating is a problem. Verify that the homogenizer head is properly covered with lysate during homogenization.</p>
Foaming of tissue lysate	<p>Some homogenizers generate foam when tissues are homogenized. Allow the foam to settle prior to pipetting. Only homogenize until visible tissue fragments are eliminated.</p>
Poor reproducibility between samples	<p>The cleared lysate was decanted. Decanting the cleared lysate may yield variable quantities of recovery and poor reproducibility. Optimal performance and reproducibility is achieved when <u>pipetting the full amount of the cleared lysate.</u></p> <hr/> <p>The diluted homogenate may not have been heated at 70°C as described in Section 4.B, Step 5; Section 4.C, Step 5; or Section 4.D, Step 5. This mixture should be heated at 70°C for 3 minutes for optimal recovery of total RNA. Failure to heat this mixture will result in decreased yields.</p>

6. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
<p>Genomic DNA contamination seen when performing PCR</p>	<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> <hr/> <p>The sample may contain too much genomic DNA. Reduce the mass of starting tissue in the preparation of the homogenate. Most tissues will not show a genomic DNA contamination problem at 30mg or less per prep. For kidney tissue, do not exceed 20mg tissue per prep, and for spleen tissue, do not exceed 15mg tissue per prep. For cultured cells, do not exceed 5×10^6 cells per prep.</p> <p>When the suggested tissue amounts are used in the system, most purified RNA samples do not show genomic DNA contamination in RT-PCR. However, dense tissues or 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>
<p>Genomic DNA contamination</p>	<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> <hr/> <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 40µl Yellow Core Buffer, 5µl 0.09M MnCl₂ and 5µl 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> <hr/> <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 Spin Baskets	<p>The lysate is too concentrated. If the homogenized lysate is difficult to pipet easily, the cell lysate is too concentrated, and the RNA Dilution Buffer will not effectively clear the lysate. Concentration of RNA will vary between tissues depending on their function. If the lysate is too viscous, simply dilute with RNA Lysis Buffer before adding RNA Dilution Buffer. Use only 175µl of lysate per prep.</p> <hr/> <p>Pellet was disturbed after clearing the lysate. Carefully pipet the cleared lysate after the centrifugation step. Avoid disturbing the pellet of precipitated proteins and cellular debris.</p>
Lysate too viscous to pipet easily	<p>The initial lysate is too viscous. Dilute the lysate with RNA Lysis Buffer.</p> <hr/> <p>Lysate becomes too viscous while sitting on ice. Briefly rehomogenize sample to shear genomic DNA. Rehomogenization of lysates can result in lower RNA yields; therefore, only rehomogenize when necessary.</p>
Spin Basket will not filter in the vacuum format	<p>The vacuum pressure is insufficient. A vacuum pressure of >15 inches of mercury is required to use the Spin Basket in the Vacuum Protocol. If the Spin Basket is not effectively cleared using the Vacuum Protocol, switch to the Spin Protocol and proceed.</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> <hr/> <p>RNA was degraded during sample prep. Work quickly during sample preparation. (See comments under "Low A₂₆₀".)</p>

7. References

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8. Appendix

8.A. Isolation of Total RNA from Leukocytes

Total RNA can be effectively isolated directly from 100µl of whole blood, plasma or serum using the standard SV Total RNA Isolation System protocol (Section 4.B). The first 5 steps of the following protocol are crucial to the effective isolation of RNA from white blood cells (leukocytes) when using >100µl of whole blood. Steps 6–10 are the same procedure as that used for isolation of RNA from tissue samples or cultured cells using the SV Total RNA Isolation System. Typical yields obtained at Promega are approximately 0.75–1.50µg total RNA per milliliter of blood. Note that the actual amount of RNA obtained will vary depending on the leukocyte count or pathogen titer of the sample.

The following example is for processing 1ml of blood in microcentrifuge tubes using the SV Total RNA Isolation System. This procedure may be scaled down to 100µl using microcentrifuge tubes.

Materials to Be Supplied by the User

(Prepare SV Total RNA Isolation System solutions as in Section 4.A. Solution compositions are provided in Section 8.G.)

- water bath or heating block preheated to 70°C
 - 95% ethanol, RNase-free
 - microcentrifuge
 - RNA Red Blood Cell Lysis Solution (CLB; Cat.# Z3141, or as prepared in Section 8.G)
1. Place 1ml of whole blood (collected in heparinized or EDTA-treated tubes) into a sterile tube.

Note: Up to 1ml of whole blood can be processed in a 1.5–2ml microcentrifuge tube. Larger volumes can be processed using 15ml conical tubes and a centrifuge.

8.A. Isolation of Total RNA from Leukocytes (continued)

2. Collect blood cells by centrifugation at 3,000rpm ($400 \times g$) for 5 minutes to generate a relatively clear supernatant (approximately 30% of the volume) and a **large** cell pellet (about 60–70% of the total volume). Remove the supernatant by carefully pipetting from the **top** of the sample. Be careful not to disturb the large cell pellet. Note that the plasma may be red due to the lysis of erythrocytes.
3. Add 1ml Red Blood Cell Lysis Solution and resuspend the pellet by carefully pipetting 4–5 times.
Note: Three volumes (relative to the original blood volume) of Red Blood Cell Lysis Solution will be sufficient to lyse the red blood cells. If larger tubes are being used that will accommodate the total lysis volume, one resuspension with three volumes of Red Blood Cell Lysis Solution will be sufficient.
4. Centrifuge at 3,000rpm for 5 minutes.
Note: A **distinct pellet may not be observed at this point**. Remove 1ml of supernatant by pipetting from the **top** and discard. Leave the remaining supernatant and cell pellet in the tube.
5. Repeat Steps 3 and 4 twice (for a total of 3 times).
Note: Because leukocytes constitute approximately 1% of total blood volume, the size of the cell pellet will decrease significantly during the red blood cell lysis procedure. Exercise caution to avoid loss of the white blood cell pellet in Steps 3–5.
6. Remove all but 100 μ l of supernatant from the tube, being careful to avoid the cell pellet. Verify that BME has been added to the RNA Lysis Buffer. Add 175 μ l of RNA Lysis Buffer to the cells. Pipet to resuspend and lyse the cells.
7. Add 350 μ l of RNA Dilution Buffer (blue). Mix by inverting 3–4 times.
8. Place in a water bath or heating block at 70°C for 3 minutes. Incubation for longer than 3 minutes might result in compromised integrity of RNA.
9. Centrifuge at 12,000–14,000 $\times g$ for 10 minutes at 20–25°C.
10. Transfer the cleared lysate to a sterile tube by pipetting. Proceed to Section 4.E, Step 2 (Spin Purification), or Section 4.F, Step 3 (Vacuum Purification).

Notes:

1. Abundant RNA species (e.g., β -actin) may be detected by RT-PCR using 1 μ l of the RNA preparation isolated from 1ml of blood in the reaction. The detection of less abundant RNAs may require the use of larger amounts (e.g., 10 μ l) of the eluted RNA preparation.
2. Determining the yield and purity of RNA from whole blood by spectrophotometry is not recommended due to low expected yields of RNA from blood samples.

8.B. Isolation of Total RNA from Plant Tissue

See Kobs, G. (1998) Isolation of RNA from plant, yeast and bacteria. *Promega Notes* 68, 28-9.

1. Freeze the plant tissue in liquid nitrogen and grind using a mortar and pestle.
2. Add 30mg of ground tissue to 175µl of RNA Lysis Buffer.
3. Add 350µl of RNA Dilution Buffer. Mix by inversion and centrifuge at maximum speed in a microcentrifuge for 10 minutes.
4. Proceed as directed in Section 4.E, Steps 1-9.

8.C. Isolation of RNA from Gram-Positive (*B. subtilis*) and Gram-Negative (*E. coli*) Bacteria

See Kobs, G. (1998) Isolation of RNA from plant, yeast and bacteria. *Promega Notes* 68, 28-9.

Notes:

The following procedure has been evaluated using only the centrifugation (spin) format.

For certain *Staphylococcus* species, a mixture of 60µl of 10mg/ml lysozyme and 60µl 10mg/ml lysostaphin is required for efficient lysis. However, many Gram-positive bacterial strains (e.g., *Bacillus subtilis*, *Micrococcus luteus*, *Arthrobacter luteus*, *Nocardia oititidscaviarum*, *Rhodococcus rhodochrous* and *Brevibacterium albidium*) lyse efficiently using lysozyme alone.


1. Grow an overnight bacterial culture in the appropriate media and at the appropriate temperature. The following day, dilute the culture 1:50 and grow until the OD₆₀₀ is 0.6-1.0. This should only take a few hours. If growth is too slow, reduce the dilution factor.

 Do not use the overnight culture for RNA isolation.

2. Transfer 1ml of culture to a 1.5ml microcentrifuge tube. Centrifuge for 2 minutes at 14,000 × g.
3. Carefully remove the supernatant, leaving the pellet as dry as possible.
4. Resuspend the pellet in 100µl of **freshly prepared** TE containing lysozyme. For Gram-positive bacteria use 3mg/ml lysozyme; for Gram-negative bacteria, the final concentration should be 0.4mg/ml lysozyme. Tap gently to mix.
5. Incubate the resuspended pellet at room temperature. For Gram-positive bacteria, incubate the pellet for 5-10 minutes; for Gram-negative bacteria, incubate the pellet for 3-5 minutes.
6. Add 75µl of RNA Lysis Buffer.

8.C. Isolation of RNA from Gram-Positive (*B. subtilis*) and Gram-Negative (*E. coli*) Bacteria (continued)

7. Add 350µl of RNA Dilution Buffer. Mix by inversion. **Do not centrifuge.**

 **Do not centrifuge** bacterial RNA preparations at this stage. The RNA will be lost if the lysate is centrifuged at Step 7.

8. Proceed as directed in Section 4.E, Steps 1-9.

8.D. Isolation of RNA from Yeast

See Kobs, G. (1998) Isolation of RNA from plant, yeast and bacteria. *Promega Notes* **68**, 28-9.

1. Grow an overnight culture in the appropriate media and at the appropriate temperature. The following day, dilute the culture 1:50 and grow until the OD₆₀₀ is 0.6-1.0. This should only take a few hours.

2. Centrifuge the culture for 2 minutes at 14,000 × g in a microcentrifuge.

3. Resuspend the pellet in 100µl of the following solution*:

1M sorbitol

0.1M EDTA (pH 7.4)

Just before use add 0.1% β-mercaptoethanol and 50 units of lyticase or zymolase. (*Solution reagents are available from Sigma Chemical Company.)

4. Incubate at 30°C for 15-30 minutes until the solution appears clear.

5. Add 75µl of RNA Lysis Buffer. Mix gently.

6. Add 350µl of RNA Dilution Buffer (blue). Mix by inversion and centrifuge at maximum speed for 10 minutes in a microcentrifuge.

7. Proceed as directed in Section 4.E, Steps 1-9.

8.E. Further Tips on Preparing Lysates

1. Some homogenizers generate foam when tissues are homogenized. To make pipetting easier, allow the foam to settle before pipetting. Homogenize only as long as necessary to eliminate visible tissue fragments.

2. Do not allow the lysate to overheat because lower total RNA yields will result. Work as quickly as possible. Lysates can be placed on ice during sample preparation. Use ice-cold RNA Lysis Buffer for homogenization to improve yield and stability. In general, an overhomogenized sample smells burned and turns brown. Verify that the homogenizer head is properly covered with lysate during homogenization.

3. If a lysate becomes too viscous to pipet while sitting on ice, dilute the lysate with RNA Lysis Buffer, and briefly rehomogenize the sample to shear the genomic DNA. Rehomogenization of lysates can result in lower RNA yields, so it should be performed only when warranted. Table 1 (Section 4.B) gives guidelines for preparing lysates from various tissues. When in doubt, prepare lysates at a concentration of ≤30mg/175µl and dilute if necessary.

8.F. Adherent Culture Cells

Use one of the following two protocols for harvesting adherent cells. The first protocol uses a trypsin-EDTA solution to detach cells. The second protocol is for harvesting cells by manual scraping.

Trypsinization Procedure for Removing Adherent Cells

Materials to Be Supplied by the User

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

- 1X trypsin-EDTA solution
 - 1X PBS
1. Wash the cells with ice-cold, sterile 1X PBS (see Section 8.G for recipe). Prepare a sterile trypsin-EDTA solution in 1X PBS. After removing the last wash (1X PBS), add just enough of the trypsin solution to cover the cell monolayer: 2ml for a 150mm flask, 1ml for a 100mm plate. Rock the culture vessel to distribute the trypsin solution evenly. Place the plates or flask in a 37°C incubator until the cells just begin to detach (usually 1–2 minutes).
 2. Once the cells begin to detach, quickly remove the trypsin solution (tilt the plate or flask to remove as much solution as possible with a pipette). Strike the bottom and sides of the culture vessel sharply with the palm of your hand to dislodge the remaining adherent cells. Rinse cells with 1X PBS.
 3. Collect the cells in a sterile centrifuge tube by centrifugation at $500 \times g$ for 5 minutes. Discard the supernatant.
 4. Proceed to Step 3 in Section 4.D, Lysis of Cultured Cells.

Scraping Procedure for Harvesting Adherent Cells

For adherent cells grown in multiwell plates, a yield of 3.5×10^4 to 1×10^6 cells per well is possible depending on the cell type and well size.

1. Lysates may be prepared rapidly by manually scraping the cells from the culture vessel in the presence of RNA Lysis Buffer. Recommended volumes of RNA Lysis Buffer to be added per well are 175 μ l for 6- to 96-well plates and a minimal volume (e.g., 1–2ml) for a flask. Remove the culture media and wash the cells with ice-cold, sterile 1X PBS (see Section 8.G for recipe). Add RNA Lysis Buffer, scrape and pipet to mix.
Note: For 6- to 12-well plates, pipet the RNA Lysis Buffer over the bottom of the well to contact all of the cells. Scraping is not required for individual wells.
2. Shear genomic DNA by passing through a 20-gauge needle if the lysate is too viscous. Transfer 175 μ l of the cell lysate to a 1.5ml screw-cap or microcentrifuge tube.
3. Proceed to Step 5 in Section 4.D, Lysis of Cultured Cells.

8.G. Composition of Buffers and Solutions

PBS buffer, 10X (per liter)

11.5g Na₂HPO₄
 2g KH₂PO₄
 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 Lysis Buffer (RLA)

4M GTC
 0.01M Tris (pH 7.5)
 0.97% β-Mercaptoethanol
 (when added)

Red Blood Cell Lysis Solution (CLB) (for isolation of RNA from blood)

5mM MgCl₂
 10mM NaCl
 10mM Tris-HCl (pH 7.0)

DNase Stop Solution (DSA), (concentrated)

5M GTC
 10mM Tris-HCl (pH 7.5)

After dilution with ethanol, the final concentration (approximate) is 2M guanidine isothiocyanate, 4mM Tris-HCl (pH 7.5) and 57% ethanol.

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.H. Related Products

Product	Size	Cat.#
One-Way Luer-Lok® Stopcocks	10 each	A7261
Vac-Man® Laboratory Vacuum Manifold	20-sample capacity	A7231
Vac-Man® Jr. Laboratory Vacuum Manifold	2-sample capacity	A7660

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 plate	Z3351
PureYield™ RNA Midiprep System	10 preps	Z3740
	50 preps	Z3741

For Laboratory Use.

mRNA Isolation from Total RNA

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

For Laboratory Use.

Magnetic Separation Products

Product	Size	Cat.#
MagneSphere® Technology Magnetic Separation Stand (two-position)	0.5ml	Z5331
	1.5ml	Z5332
	12 x 75mm	Z5333
MagneSphere® Technology Magnetic Separation Stand (twelve-position)	0.5ml	Z5341
	1.5ml	Z5342
	12 x 75mm	Z5343
PolyATtract® System 1000 Magnetic Separation Stand	1 each	Z5410

^(a)Australian Pat. No. 730718, Singapore Pat. No. 64532, Korean Pat. No. 486402 and other patents pending.

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