TECHNICAL BULLETIN

SV 96 Total RNA Isolation System

Instructions for Use of Products
Z3500 and Z3505

Revised 8/16
TB294

SV 96 Total RNA Isolation 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 Bulletin.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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), RNase protection assays, primer extension, oligo(dT) selection of poly(A)+ RNA, in vitro translation and cDNA library construction. RT-PCR has emerged as a powerful method to identify and quantitate specific mRNAs from small amounts of total RNA. Also, RT-PCR allows cloning of cDNA products without the need for constructing and screening cDNA libraries. As use of amplification as a research tool has grown, the need for high-throughput methods to rapidly isolate high-quality RNA, substantially free of genomic DNA contamination, from small amounts of starting material (e.g., cultured cells) has also increased. The SV 96 Total RNA Isolation System addresses these needs.

1. Description (continued)

The SV 96 Total RNA Isolation System provides a fast, simple technique for the preparation of intact, purified RNA from tissue culture cells. This technique also allows automation on liquid-handling workstations such as the Beckman Coulter Biomek® 2000. Total RNA can be purified from 96 samples at once in less than an hour. 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 phenol/chloroform extraction or ethanol precipitation, and there is no detectable DNase carryover in the final RNA preparation. Figure 1 describes RNA isolation using the SV 96 Total RNA Isolation System.

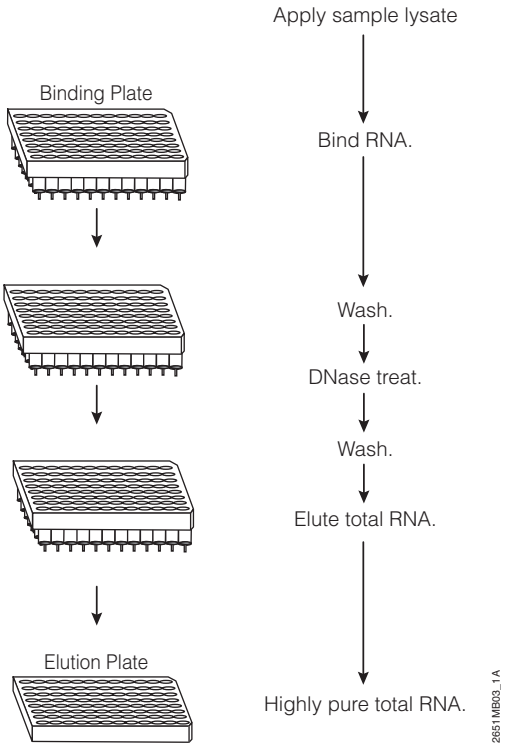

Figure 1. Total RNA isolation and purification using the SV 96 Total RNA Isolation System.

The SV 96 Total RNA Isolation System requires the use of the Vac-Man® 96 Vacuum Manifold (Figure 2) or similar 96-well manifold for manual total RNA purification. For total RNA purification using the Beckman Coulter Biomek® 2000, the Beckman Coulter vacuum manifold and collar are required (see Section 5). Total RNA is purified from cell lysates using 96-well vacuum filtration, eliminating the need for centrifugation. Washing the bound RNA requires no disassembly of the manifold, and filtrate waste products are delivered directly to a vacuum trap, eliminating the need to empty waste collection vessels during RNA purification. Total RNA is collected by elution into a 96-well plate.

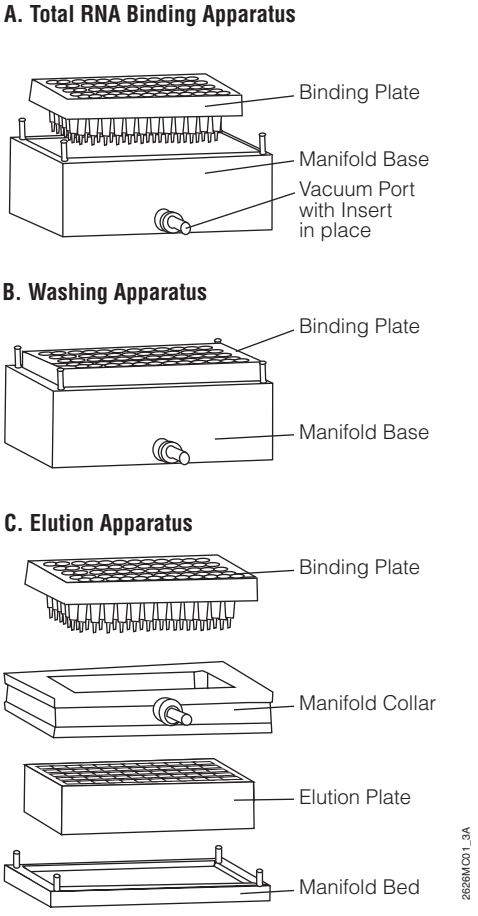

Figure 2. The Vac-Man® 96 Vacuum Manifold with the SV 96 Total RNA Isolation System components. Panels A, B and C show the manifold and plate combinations necessary to accomplish RNA binding, washing and elution, respectively, for manual total RNA purification. For automated total RNA purification, see the *SV 96 Total RNA Isolation System Automated Protocol* #EP003 (available at: **www.promega.com/protocols**) for additional information regarding required hardware and labware.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
SV 96 Total RNA Isolation System	**1 × 96 preps**	**Z3500**

Each system contains sufficient reagents for 96 isolations. Includes:

- 50ml RNA Lysis Buffer (RLA)
- 900µl β-Mercaptoethanol (97.4%)
- 1 vial DNase I (lyophilized)
- 750µl $MnCl_2$, 0.09M
- 2.5ml Yellow Core Buffer
- 13.25ml DNase Stop Solution (DSA)
- 58.8ml RNA Wash Solution (RWA)
- 13ml Nuclease-Free Water
- 1 Binding Plate
- 1 Elution Plate
- 3 Plate Sealers

PRODUCT	SIZE	CAT.#
SV 96 Total RNA Isolation System	**5 × 96 preps**	**Z3505**

Each system contains sufficient reagents for 5 × 96 isolations. Includes:

- 2 × 50ml RNA Lysis Buffer (RLA)
- 2 × 900µl β-Mercaptoethanol (97.4%)
- 5 vials DNase I (lyophilized)
- 5 × 750µl $MnCl_2$, 0.09M
- 5 × 2.5ml Yellow Core Buffer
- 5 × 13.25ml DNase Stop Solution (DSA)
- 5 × 58.8ml RNA Wash Solution (RWA)
- 5 × 13ml Nuclease-Free Water
- 5 Binding Plates
- 5 Elution Plates
- 6 Plate Sealers

Storage Conditions: Store the RNA Lysis Buffer with β-Mercaptoethanol (BME) added at 4°C. Store all other components at 15–30°C. For information on rehydration of DNase I, see Section 4.A.

Note: The Yellow Core Buffer is colored yellow to allow the user to visualize whether the membrane is completely covered by the DNase mixture in the DNase step. The dyes have no effect on quality or downstream performance.

Caution: Guanidine thiocyanate (a component of the RNA Lysis Buffer) 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.

Products Available Separately

PRODUCT	SIZE	CAT.#
Vac-Man® 96 Vacuum Manifold	each	A2291
RNA Lysis Buffer (RLA)	50ml	Z3051
RNA Wash Solution (RWA)	58.8ml	Z3091
Binding Plates	10 plates	A2271

3. General Considerations

3.A. Direct Purification of RNA

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

The SV 96 Total RNA Isolation System combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β-mercaptoethanol to disrupt nucleoprotein complexes and inactivate the ribonucleases present in cell extracts (1). The cell lysate is then applied to the Binding Plate. Binding of total RNA to the columns in the Binding Plate occurs rapidly due to the disruption of water molecules by chaotropic salts, 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, 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 in a 96-well, high-throughput format.

The Wizard® *Plus* SV DNA Purification System Buffers are not compatible with this system and should not be used.

The SV 96 Total RNA Isolation Protocol is a multistep procedure that requires that the correct reagents be used in the correct order.

3.B. Processing Capacity

The SV 96 Total RNA Isolation System is optimized for total RNA isolation from cultured cells with a broad spectrum of RNA expression levels. A maximum of 5×10^6 cells can be processed after lysis with 100µl of RNA Lysis Buffer. To process a larger number of cells, a larger volume of RNA Lysis Buffer will be required. Lysing greater than 5×10^6 cells with 100µl of RNA Lysis Buffer will result in a viscous lysate that will not pass easily through the Binding Plate. Low total RNA yields will result.

3.C. Downstream Applications

RNA purified with the SV 96 Total RNA Isolation System is suitable for many molecular biology applications, including RT-PCR. For more information on downstream applications, see the *Protocols and Applications Guide* (2).

3.D. Creating a Ribonuclease-Free Environment

Ribonucleases are extremely difficult to inactivate. Care should be taken 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 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 SV 96 Total RNA Isolation System. Gloves should be worn 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.
3. Treat nondisposable glass 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 water by adding diethyl pyrocarbonate (DEPC) to 0.1% and incubating overnight at room temperature. Autoclave for 30 minutes to remove any trace of DEPC. Use DEPC-treated water to prepare solutions used with RNA. Use only dedicated reagents when making RNA solutions.

! Do not treat solutions containing Tris with DEPC.

4. RNA Isolation and Purification Procedure

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.)

- ethanol, 95%, RNase-free (120ml per 96-well plate)
- 10X phosphate-buffered saline (PBS), sterile (for cultured cells)
- Vac-Man® 96 Vacuum Manifold (Cat.# A2291)
- vacuum trap for waste collection (e.g., Fisher Cat.# 10-182-50B, 1L size)
- vacuum pump capable of 15–20 inches of Hg (e.g., Fisher Cat.# 01-092-29)
- vacuum tubing
- single or multichannel pipettors capable of 10–1,000µl dispensings

! For best results, use fresh samples. If necessary, samples lysed in RNA Lysis Buffer may be stored at –20°C or –70°C. Due to the toxicity of the chemicals used in the RNA purification procedure and the prevalence of RNases, it is essential that gloves be worn throughout the lysis and purification procedure.

4.A. Preparation of Solutions

Prior to beginning the SV 96 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 96 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
RNA Lysis Buffer	Add 0.5ml of β-Mercaptoethanol (BME) to 50ml of RNA Lysis Buffer (RLA).	After adding BME, mark on the bottle that this step has been performed. Store the RNA Lysis Buffer at 4°C.
DNase I*	Add 275µl of Nuclease-Free Water (supplied) to the lyophilized DNase I.	Gently mix by swirling. Do not vortex. One vial is sufficient for one 96-well plate. If processing less than a whole plate, we recommend dividing the rehydrated DNase into working aliquots using sterile, RNase-free microcentrifuge tubes. 2.5µl of rehydrated DNase I is required per RNA purification. Store rehydrated DNase I at –20°C.
RNA Wash Solution	Add 100ml of 95% ethanol to the bottle containing 58.8ml of RNA Wash Solution (RWA).	After adding ethanol, mark on the bottle that this step has been performed. The RNA Wash Solution is stable at 15–30°C when tightly capped.
DNase Stop Solution	Add 20ml of 95% ethanol to the bottle containing 13.25ml of DNase Stop Solution (DSA).	After adding ethanol, mark on the bottle that this step has been performed. The DNase Stop Solution is stable at 15–30°C when tightly capped.

*If you are using the SV 96 Total RNA Isolation System in an automated format, add 312.5µl of Nuclease-Free Water to the lyophilized DNase I. See the *SV 96 Total RNA Isolation System Automated Protocol* #EP003 (available at: **www.promega.com/protocols**) for more details.

4.B. Lysis of Cultured Cells Grown in a 96-Well Tissue Culture Plate

Use the following protocol for lysis of cultured cells grown in a 96-well tissue culture plate. Use at least 1.5×10^3 cells to a maximum of 5×10^6 cells per purification. The number of cells may need to be adjusted depending on cell type, function and RNA expression levels at the time of harvest.

1. Wash the cells once with sterile 1X PBS.
2. Verify that BME has been added to the RNA Lysis Buffer. Add 100µl of RNA Lysis Buffer to the washed cells. Mix by pipetting.

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4.C. RNA Purification

1. Prepare the vacuum manifold as shown in Figure 2. Place the Binding Plate in the vacuum manifold base. To ensure that the samples and well numbers correspond on both plates, orient the Binding Plate in the vacuum manifold with the numerical column headers toward the vacuum port. Attach the vacuum line to the vacuum port on the Manifold Base.
2. Transfer the cell lysate to the wells of the Binding Plate. Apply vacuum until the cell lysate passes through the Binding Plate. Release vacuum.
3. Verify that ethanol has been added to the RNA Wash Solution. Add 500µl of RNA Wash Solution to each well of the Binding Plate. Apply vacuum until the RNA Wash Solution passes through the Binding Plate. Release vacuum.
4. For each isolation, prepare the DNase incubation mix by combining 20µl of Yellow Core Buffer, 2.5µl of 0.09M $MnCl_2$ and 2.5µl of DNase I per sample in a sterile tube (in this order). For an entire 96-well plate, combine 2ml of Yellow Core Buffer, 250µl of 0.09M $MnCl_2$ and 250µl of DNase I. Mix by gentle pipetting; do not vortex. Keep the DNase I on ice while it is thawed. Apply 25µl of this freshly prepared DNase incubation mix directly to the membrane of the Binding Plate.

   Note: If you are using this system in an automated format, see the *SV 96 Total RNA Isolation System Automated Protocol* #EP003 (**www.promega.com/protocols**) for more details.

   ! Use freshly prepared DNase incubation mix.
5. Incubate for 10 minutes at 20–25°C. After this incubation, add 200µl of DNase Stop Solution to each well of the Binding Plate.
6. Apply vacuum until the DNase Stop Solution passes through the Binding Plate. Release vacuum.
7. Add 500µl of RNA Wash Solution to each well of the Binding Plate. Apply vacuum until the RNA Wash Solution passes through the Binding Plate. After the wells have emptied, continue to apply vacuum for an additional 10 minutes to allow the binding matrix to dry.
8. Turn off the vacuum. Release the vacuum line from the Manifold Base and snap it into the vacuum port in the Manifold Collar. Remove the Binding Plate from the Manifold Base and blot by gently tapping onto a clean paper towel to remove residual ethanol.
9. Place a 96-well Elution Plate in the Manifold Bed and position the Manifold Collar on top. Orient the plate with the numerical column headers toward the vacuum port.
10. Position the Binding Plate on top of the Manifold Collar and Elution Plate as shown in Figure 2. The Binding Plate tips must be centered on the Elution Plate wells and both plates must be in the same orientation. Add 100µl of Nuclease-Free water to each well of the Binding Plate and incubate for 1 minute at room temperature. Apply a vacuum for 1 minute.
11. Release vacuum and remove the Binding Plate. Carefully remove the Manifold Collar, making sure that the Elution Plate remains positioned in the Manifold Bed. If droplets are present on the walls of the Elution Plate wells, briefly centrifuge the plate to collect the droplets on the bottom of the wells. Eluate volumes may vary but are generally 60–70µl. Samples can be stored at –20°C or –70°C by covering the plate tightly with a Plate Sealer.

    Note: Upon completion of this procedure, the Binding Plate can be stored at room temperature for later use of any unused wells.

4.D. Determination of RNA Yield and Purity

Yield and Purity

The yield of total RNA may be determined spectrophotometrically at 260nm, where 1 absorbance unit (A_{260}) = 40µg of single-stranded RNA/ml. The purity may also be estimated by spectrophotometry from the relative absorbances at 260 and 280nm (i.e., A_{260}/A_{280}).

RNA isolated with the SV 96 Total RNA Isolation System is substantially free of DNA and contaminating protein and may be used directly for any of the applications listed previously. Pure RNA will exhibit an A_{260}/A_{280} ratio of 2.0. However, because of 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 value is less than 1.7, refer to the troubleshooting section for possible causes and suggestions for improving the purity of the isolated RNA.

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 (3,4), or glyoxal (4,5) 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 significant degradation has occurred.

5. Automated RNA Isolation on Liquid-Handling Workstations

Fully verified and optimized automated SV 96 Total RNA Isolation methods are available for a variety of liquid-handling workstations. Information on obtaining these methods is available at: **www.promega.com/automethods/**

Automation of the SV 96 Total RNA Isolation System procedure is described in the *SV 96 Total RNA Isolation System Automated Protocol* #EP003, available online at: **www.promega.com/protocols**

The SV 96 Total RNA Isolation System automated methods have been optimized for purification of total RNA from tissue culture cells (HeLa, CHO, NIH/3T3 and 293 cells). For RNA isolation from sample sources other than tissue culture cells, the parameters of the automated methods may need to be adjusted.

6. Troubleshooting

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

Symptoms	Causes and Comments
Low A_{260}/A_{280} ratios	Typically due to protein contamination. Several methods may be used to remove contaminating protein from RNA solutions. The most expedient is phenol/chloroform extraction. This organic extraction should yield higher A_{260}/A_{280} ratios. However, loss of RNA (up to 40%) should be expected.
	An incomplete wash step with the RNA Wash Solution can result in protein contamination. Be sure to use the entire 500µl volume of RNA Wash Solution for each well of the 96-well plate.
	A_{260}/A_{280} ratios become less reliable as A_{260} and A_{280} values decrease below 0.1.
Low A_{260} (low RNA yield)	Using lysate stored at –20°C or –70°C. Cell lysate that has been stored frozen may have a decreased amount of total RNA. For optimal performance, purify the RNA as soon as the lysate is prepared.
	Cells low in total RNA. Cells vary in the total amount of RNA that can be purified based on wet weight. If total RNA yields are low, increase the amount of starting material processed.
	Poor sample integrity. Samples that were not homogenized or frozen immediately upon isolation may have decreased amounts of RNA with reduced integrity. Homogenized samples should be stored at –20°C or –70°C.
	Exceeded binding capacity of membrane in Binding Plate. If the lysate contains more RNA than the capacity of the Binding Plate (20µg/ml), the excess RNA will be washed away during the wash steps. When maximum recovery is essential, divide the lysate among multiple wells of the Binding Plate and perform multiple purifications. Pool the resulting RNA solutions.
	Steps not followed correctly or wrong reagents were used. The SV 96 Total RNA Isolation System is a multistep procedure that requires that the correct reagents are used in the correct order. This ensures that the RNA remains bound to the membrane during the purification process. The Wizard® *Plus* SV DNA Purification System Buffers are not compatible and should not be used with the SV 96 Total RNA Isolation System.

Symptoms	Causes and Comments
Low A_{260} (low RNA yield; continued)	Ethanol was not added to the DNase Stop Solution or the RNA Wash Solution. Prepare the solutions as instructed in Section 4.A before beginning the procedure.
	Lysate overheated during homogenization. Work as quickly as possible. Lysates can be placed on ice during sample preparation.
Genomic DNA contamination	Too much sample input into the PCR. Reduce the total RNA input to 50–100ng in the control PCRs. Generally, the RNA-specific product is seen from a rare message in RT-PCR using 50ng of total RNA.
	Too much genomic DNA in the sample. For cultured cells, do not exceed 5×10^6 cells per prep. When the suggested sample size is used, 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, we recommend performing a post-RNA isolation DNase treatment using RQ1 RNase-Free DNase (Cat.# M6101) followed by phenol/chloroform extraction.
	Resuspend and store the inactive lyophilized DNase I according to Section 4.A. Do not expose the DNase aliquots to more than 3 freeze-thaw cycles.
	$MnCl_2$ or DNase I not added to the Yellow Core Buffer. For each isolation, prepare the DNase incubation mix by combining 20µl of Yellow Core Buffer, 2.5µl of 0.09M $MnCl_2$ and 2.5µl of DNase I in a sterile tube just before use. Prepare the DNase incubation mix fresh for each set of RNA isolations. Do not vortex.
	DNase solution was not in full contact with the membrane during digestion. Visually inspect to ensure that the DNase I solution completely covers the membrane during DNA digestion.
	DNase I step omitted or not performed properly. The DNase step should be performed to eliminate the possibility of host DNA contaminating the system.
Clogged Binding Plate	Lysate was too concentrated. If the lysate is too viscous, dilute with RNA Lysis Buffer until it becomes easy to pipet. Then apply the entire lysate to a well of the Binding Plate.
Lysate too viscous to pipet easily	Initial lysate too viscous. Dilute lysate with RNA Lysis Buffer.
	Lysate becomes too viscous while sitting on ice. Briefly re-homogenize the sample to shear genomic DNA. Rehomogenization of lysates can result in lower RNA yields; therefore, only rehomogenize when necessary.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Vacuum steps are slow	Lysate too concentrated. If the lysate is difficult to pipet easily, the vacuum step to pass lysate through the Binding Plate may be slow. Continue to apply vacuum until lysate passes through the plate; however, extended vacuum steps may result in lower RNA yields.
	Vacuum pressure is insufficient. A vacuum pressure >15 inches of mercury is required to use the Binding Plate.
RNA degradation	RNase was introduced by 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 preparation. It is essential to work quickly during sample preparation.

7. Composition of Buffers and Solutions

PBS, 10X (per liter)

11.5g	Na_2HPO_4
2g	KH_2HPO_4
80g	NaCl

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

DNase Stop Solution (DSA)

5M	guanidine isothiocyanate
10mM	Tris-HCl (pH 7.5)

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

RNA Lysis Buffer (RLA)

4M	guanidine thiocyanate (GTC)
10mM	Tris (pH 7.5)
0.97%	β-mercaptoethanol (when added)

RNA Wash Solution (RWA)

162.8mM	potassium acetate
27.1mM	Tris-HCl (pH 7.5)

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

Yellow Core Buffer

22.5mM	Tris (pH 7.5)
1.125M	NaCl
0.0025%	yellow dye (w/v)

8. References

1. Chirgwin, J.M. *et al.* (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–9.
2. *Protocols and Applications Guide*, Online Edition (2009) Promega Corporation (**www.promega.com/paguide/**).
3. Lehrach, H. *et al.* (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical re-examination. *Biochemistry* **16**, 4743–51.
4. Ausubel, F.M. *et al.*, eds. (1993) In: *Current Protocols in Molecular Biology*. Greene Publishing Associates and John Wiley and Sons, New York, NY.
5. 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.

9. Related Products

Product	Size	Cat.#
PureYield™ RNA Midiprep System	10 preps	Z3740
	50 preps	Z3741
Wizard® SV Genomic DNA Purification System	50 preps	A2360
	250 preps	A2361
Wizard® SV 96 Genomic DNA Purification System	1 × 96 preps	A2370
	4 × 96 preps	A2371
Wizard® SV 96 PCR Clean-Up System	1 × 96 preps	A9340
	4 × 96 preps	A9341
	8 × 96 preps	A9342

10. Summary of Changes

The following changes were made to the 8/16 revision of this document:

1. The amount and concentration of the β-Mercaptoethanol component was changed, Sections 2 and 4.A.
2. The document design was updated.