RNA Purification: A Rapid and Versatile Protocol for the Isolation of Total RNA

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Promega's new SV Total RNA Isolation System[a] offers a complete system for the isolation of quality RNA from small amounts of starting material. This system provides a fast and simple technique for the preparation of purified and intact total RNA from tissues, cultured cells and whole blood samples. This purification is achieved without the use of phenol/chloroform extractions and employs a simple DNase step to eliminate genomic DNA efficiently. No genomic DNA contamination is seen when the RNA is analyzed by reverse transcription-PCR[b] (RT-PCR). In this article, we demonstrate that RNA isolated using this system is highly pure and is ideally suitable for applications such as RT-PCR.

[a]*Patent Pending.*

[b]*The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.*

INTRODUCTION

RNA is central to the flow of genetic information within a cell and is required for the translation of proteins into structural and regulatory components of the cell. The analysis of RNA is important, for example, to understanding how cells respond to environmental stimuli, developmental programs and diseased states. Specific RNA expression can be an indication of a viral infection or a diseased state and can be specific to a certain type of cell or stage in development. Many RNA analysis methods are available to determine the size, expression level and structure of the RNA molecule of interest. Methods such as Northern analysis, for determining the size and expression level of a specific RNA molecule, cDNA cloning and RT-PCR require intact RNA molecules in order to provide meaningful results.

With the exception of *in situ* techniques, all methods of RNA analysis require the isolation of RNA away from cellular components and contaminants. The most commonly used methods of total RNA isolation involve organic extraction of RNA from homogenized tissues or cells. These methods work well but require the use of toxic compounds and are cumbersome, especially when a large number of samples are processed. Often, organic solvent contaminants remain with the purified RNA, requiring further labor-intensive methods to yield pure RNA. These contaminants can affect the performance of the sample in downstream applications.

In this article, a convenient, new, silica membrane-based isolation system for the rapid isolation of total RNA is described. The new SV Total RNA Isolation System offers a complete system for the isolation of intact total RNA from tissues, cultured cells and blood samples (using the SV RNA Red Blood Cell Lysis Solution, Cat.# Z3141), without the need for organic extractions (1). Like the Wizard® *Plus* SV DNA Purification System[a] (Cat.# A1330), either a spin or vacuum ("SV") purification protocol can be used (2). The SV Total RNA Isolation System provides proven and optimal performance.

PURIFICATION FORMAT

The SV Total RNA Isolation System is designed for both spin (microcentrifuge) and vacuum formats for RNA purification. The design of the Spin Column Assemblies allows them to be spun at 14,000 x *g* (i.e., top speed for most microcentrifuges). For the vacuum format, Miniprep Vacuum Adapters[a] (Cat.# A1331) are used to attach the columns to a laboratory vacuum manifold (e.g., the Vac-Man® Laboratory Vacuum Manifold, Cat.# A7231 or the Vac-Man® Jr. Laboratory Vacuum Manifold, Cat.# A7660). The vacuum format is quicker and easier because wash steps pass through the Spin Basket without accumulating in the Collection Tube. Both formats yield high quality total RNA.

A number of convenient features are built into the SV Total RNA Isolation System. Two of the key solutions are distinguished by color, making it easy to keep track of the protocol steps. Removal of genomic DNA contamination is an integral part of the system and is accomplished on the silica membrane. This eliminates the need for further post-RNA isolation steps, commonly needed when isolating total RNA using other systems. The Spin Column Assemblies, consisting of a Spin Basket and a Collection Tube, and sterile Elution Tubes come in convenient packs to assure quality performance of the system.

The purification of total RNA begins with the preparation of a tissue or cell homogenate (Figure 1). The most difficult obstacles involved with RNA isolation are the stability of ribonucleases (RNases) and the lack of cofactor requirements for some RNases. In order to isolate intact RNA, endogenous RNases must be inactivated immediately during lysis of tissues and cells. The SV Total RNA

Isolation System utilizes the combination of the disruptive and protective properties of guanidine thiocyanate (GTC) and beta-mercaptoethanol (BME) to inactivate the ribonucleases present in cell extracts (3). Samples are homogenized in SV RNA Lysis Buffer, which contains both GTC and BME. GTC, which is one of the most effective protein denaturants known (3), acts in association with SDS to disrupt nucleoprotein complexes. This allows the RNA to be isolated essentially free of protein.

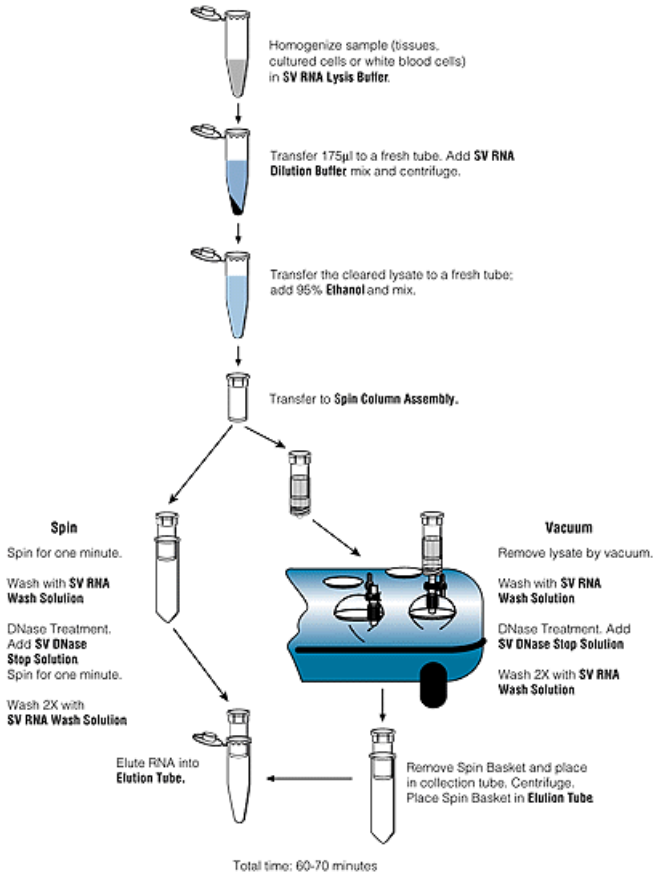

Figure 1. Schematic representation of the SV Total RNA Isolation System.

A common problem seen with a comparable, commercially available system is clogging of the membrane during centrifugation. This problem has been solved in the SV Total RNA Isolation System by preparing a cleared lysate prior to passing the RNA mixture through the membrane[a]. SV RNA Dilution Buffer, colored blue for easy identification, is added to the cell homogenates. Dilution of the homogenized samples in the presence of GTC causes selective precipitation of cellular proteins to occur while the RNA remains in solution. Samples are centrifuged to clear the diluted homogenates of precipitated proteins and cellular debris. Binding of RNA to the silica membrane is made possible by adding ethanol to the cleared lysate. The cleared lysate/ethanol mixtures are added to the Spin Basket, and the RNA binds to the membrane as the mixture passes through the Spin Basket. Because the lysates have been cleared effectively of precipitated proteins and cellular debris, they may be passed through the membrane by either centrifugation or vacuum filtration. Salts and impurities are removed by a simple washing step.

In order to remove contaminating genomic DNA, RNase-Free DNase I is applied directly to the silica membrane. Genomic DNA can interfere with amplification-based methodologies and is a common contaminant in many RNA isolation procedures. During the DNase treatment step, DNA is digested directly on the membrane. The SV DNase Incubation Buffer is colored yellow so that complete coverage of the membrane can be visualized. To ensure that the isolated RNA is not contaminated with DNase, SV DNase Stop Solution is added to inactivate the DNase. The bound total RNA is purified further by simple washing steps to remove contaminating salts, proteins and cellular impurities. The total RNA is eluted from the membrane by the addition of Nuclease-Free Water. The purified RNA is ready to use and does not require ethanol precipitation, eliminating the difficulties associated with solubilizing precipitated RNA. This system makes it easy for both the novice and the experienced user of RNA isolation procedures to prepare high quality total RNA rapidly and efficiently.

PROCESSING CAPACITY

The SV Total RNA Isolation System has been developed and optimized for total RNA isolations from tissues 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. Larger tissue samples can be processed by performing multiple isolations as necessary to supply the desired amount of total RNA. The system

works well for tissues containing both relatively high and low levels of endogenous RNase, such as spleen and liver, respectively. RNA has been isolated easily from many tissues (Table 1), yielding RNA that is free of detectable genomic DNA in RT-PCR analysis (Figure 2).

Table 1. Yields, Average A_{260}/A_{230} Ratios and Average A_{260}/A_{280} Ratios of Total RNA Isolated from Tissues and Cells[d].

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}
Liver	30mg	133	4.4	2.4	1.9
Kidney	20mg	46	2.3	2.1	1.9
Heart	60mg	16	0.3	1.8	2.1
Spleen	15mg	79	5.3	2.3	1.9
Brain	60mg	39	0.7	2.1	2.1
Lung	60mg	36	0.6	2.0	2.1
Muscle	30mg	22	0.7	1.8	2.1
RAW264.7 Cell Line	5×10^6 cells	51	NA	2.0	2.0

NA: not applicable.

[d]*The values in this table represent means of results achieved at Promega. Yields will depend on the type of tissue, cultures and metabolic state of the samples. The means for the cell line and spleen samples are the average of two and four determinations, respectively. The means for all other samples are the average of at least six determinations. Means were calculated using both spin and vacuum formats for all samples listed, except kidney, liver and the cell line. The vacuum format works well for all sample types listed.*

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

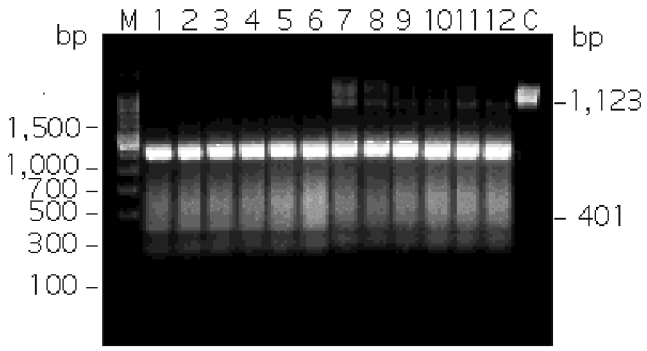

Figure 2. Analysis of RNA purity and genomic DNA contamination by RT-PCR analysis. RNA was isolated from 30mg of mouse liver using both the SV kit and a competitor's kit. Twenty microliters (20% of the preparation) of each sample was reverse transcribed using AMV Reverse Transcriptase (Cat.# M5101), Recombinant RNasin® Ribonuclease Inhibitor[c] (Cat.# N2511) and an Oligo(dT)$_{15}$ Primer (Cat.# C1101). One-sixth of this cDNA reaction was amplified using primers for IL-1beta, a rare cytokine message, using *Taq* DNA polymerase (Boehringer Mannheim) (primer sequences and PCR conditions provided by Dr. A.L. Oaklander, Johns Hopkins University). The expected result from a pure RNA sample using these primers is a 401bp product, which spans an intron in the mouse IL-1beta gene. Any genomic contamination will be detected as a 1,123bp PCR product generated from the genomic DNA rather than from the cDNA. Lanes 1-6 are the PCR products from SV RNA samples, while lanes 7-12 are the RT-PCR products from the competitor's samples. Lane C is a control of the IL-1beta PCR product generated from Mouse Genomic DNA (Cat.# G3091). The size of Promega's 100bp DNA Ladder (lane M, Cat.# G2101) is indicated.

[c]*U.S. Pat. No. 5,552,302 has been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI).* Inhibitors of Angiogenin, *which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.*

TOTAL RNA ISOLATION FROM SMALL AMOUNTS OF STARTING MATERIAL

The SV Total RNA Isolation System was used to isolate RNA from titrated amounts of tissues and cells. The yield of total RNA was

found to be nearly linear for mouse liver, kidney and tissue culture cells (Figure 3). Mouse liver, a tissue rich in RNA expression, and kidney, which is a dense tissue, show proportional yields of RNA based on the amount of starting material. However, we find that starting amounts of greater than 20mg of kidney tissue may result in genomic DNA contamination in RT-PCR. Therefore, we recommend not exceeding the tissue amounts suggested in Table 1. When the suggested tissue amounts are used in the SV Total RNA Isolation System, most purified RNA samples should not show genomic DNA contamination in RT-PCR. However, since a finite amount of DNase is used for 15 minutes in the protocol, dense tissues or cultures may contain too much DNA to eliminate completely. If DNA contamination is a problem in a sample or if a tissue is not listed in Table 1, we recommend using <=15mg tissue or <=1 x 10^6 cultured cells. The sensitivity of RT-PCR detection for a rare cytokine message, IL-1beta, from RNA isolated from mouse liver is similar to that seen with the RNAgents® Total RNA Isolation System (data not shown).

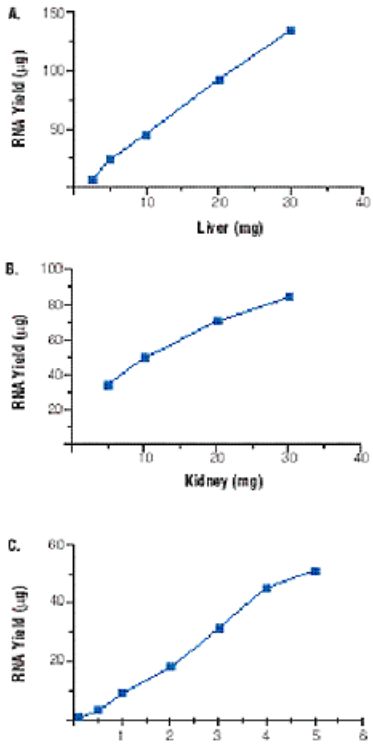

Figure 3. Liver, kidney and tissue culture cell titrations. Mouse liver **(Panel A)** and kidney **(Panel B)** lysates were prepared at an initial concentration of 30mg (wet weight) per 175µl of Lysis Buffer and diluted to the lower concentrations shown. Triplicate or quadruplicate preparations for each concentration were performed, and the average yield was calculated in total micrograms per preparation as predicted by A_{260}. **Note:** Kidney tissue lysates at the concentrations of 30mg and 25mg are presented for RNA yield demonstration only. We recommend using 20mg or less per preparation of kidney tissue as at the higher concentrations genomic DNA contamination might be observed. **Panel C:** RAW264.7 cells were lysed at an initial concentration of 5 x 10^6 and serially diluted to the lower cell numbers shown. Duplicate preparations for each concentration were performed, and the average yield as predicted by A_{260} was calculated.

ELUTION VOLUMES

For certain downstream applications such as Northern analysis, concentrated RNA samples are required. Therefore, we investigated the volume of Nuclease-Free Water into which the purified RNA could be eluted. RNA was isolated from 30mg of mouse liver, and volumes of 25-200µl of Nuclease-Free Water were used for elution of the RNA from the Spin Basket membranes. For volumes in the range of 50-200µl, the results show that an increase in the volume of water used did not result in an increase in RNA yield (Table 2). This decreased yield could be due to a limitation in the solubility of the RNA or to incomplete wetting of the membrane. Therefore, we recommend using elution volumes of 50-100µl. The RNA concentration resulting from elution with 50-100µl of water generally is sufficient for RT-PCR analysis. If more concentrated RNA samples are needed, the RNA can be vacuum-dried at room temperature without precipitation and resuspended in the desired volume.

Table 2. Elution Volumes.

Elution Volume	Total RNA Yield	Concentration of RNA (µg/µl)	A_{260}/A_{230}	A_{260}/A_{280}
25µl	101.4µg	4.1	2.25	2.01
50µl	136.9µg	2.7	2.33	1.95
100µl	137.9µg	1.4	2.39	1.85

2 x 100µl	141.0µg	0.71	2.43	1.75
200µl	131.0µg	0.66	2.44	1.73
The values in this table represent the average of duplicate samples.				

YIELD, PURITY, QUALITY AND SIZE RANGE OF RNA

The SV Total RNA Isolation System gives higher RNA yields than a comparable, commercially available system (Figure 4). Using 30mg of liver as the starting tissue, the SV System yielded 46% more RNA than was isolated using the competitor's kit. The purity of the RNA is very high, as measured by UV absorbance ratios. Pure preparations of RNA and DNA have an A_{260}/A_{280} ratio between 1.7 and 2.0. If there is protein contamination in the sample, this ratio will be significantly lower (4). The A_{260}/A_{280} ratios of samples isolated with the SV Total RNA Isolation System are greater than 1.7, indicating that the RNA is pure. A_{260}/A_{230} ratios lower than 1.5 generally indicate carryover contamination by guanidine thiocyanate. The A_{260}/A_{230} ratios for samples isolated with the SV Total RNA Isolation System are greater than 2.25 indicating high purity of the RNA (Table 1). Denaturing formaldehyde agarose gel electrophoresis demonstrates the integrity of the RNA (Figure 4). The ratio of 28S to 18S eukaryotic ribosomal RNA (rRNA) should be approximately 2:1 by ethidium bromide staining. In RNA samples that have been degraded, this ratio will be reversed since the 28S rRNA characteristically is degraded to an 18S-like species. In the SV Total RNA preparations, the ribosomal RNA bands are clean and sharp, and the 28S upper rRNA band shows greater intensity of staining than the lower 18S rRNA band. An analysis of the size range of RNA capture and elution by the system demonstrates that all RNA bands between 0.28-6.58kb (RNA Markers, Cat.# G3191) are purified equally (100µl elution, data not shown).

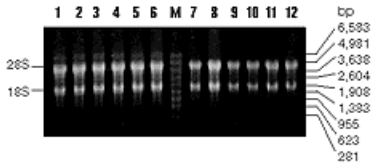

Figure 4. Formaldehyde gel electrophoresis of RNA isolated from liver tissue. RNA was isolated from 30mg of liver tissue following the SV isolation protocol (lanes 1-6) and a competitor protocol (lanes 7-12). Ten percent of the final preparation volume of 100µl (10µl) for each sample was analyzed by electrophoresis in a 1% MOPS-EDTA-formaldehyde agarose gel (9) and visualized by including 0.1µg/µl ethidium bromide in the sample loading buffer. SV samples yielded an average of 108µg and were very consistent with a coefficient of variation (% CV) of 4.36 (data not shown). The competitor samples yielded an average of 74µg with wide variability as shown by a % CV of 21.56 (data not shown). The sizes of Promega's RNA Markers, 0.28-6.58kb (lane M, Cat.# G3191) are indicated.

LACK OF GENOMIC DNA CONTAMINATION IN RT-PCR

The total RNA purified using the SV Total RNA Isolation System was shown to be free of detectable genomic DNA contamination in coupled reverse transcription and PCR amplification (RT-PCR) (Figure 2). IL-1beta, a rare cytokine message, was amplified from 20% (20µl) of the final RNA preparations isolated using either the SV Total RNA Isolation System or a competitor's kit (Figure 2). The expected RT-PCR product from IL-1beta cDNA is 401bp (5). The IL-1beta primers span an intron and result in a 1,123bp PCR product when genomic DNA is present in the sample (lane C, genomic DNA control). The SV Total RNA samples yielded a strong IL-1beta cDNA signal, as indicated by the amplified band of 401bp. No genomic DNA contamination was detected in these samples. In contrast, RT-PCR reactions using RNA samples that were isolated with the competitor's kit produced both the 401bp and 1,123bp bands, indicating the presence of contaminating genomic DNA when using the competitor's kit (lanes 7-12).

FREE OF DNASE CARRYOVER

A DNase exonuclease assay was performed to determine if any of the DNase used during purification of the RNA was carried over to the final product. The assay consists of incubating [^{3}H]-labeled DNA substrate with the sample at 37°C overnight. Upon incubation, the samples were tested for the percentage of tritium released into the supernatant after precipitation. If a sample contains contaminating DNase, the substrate will be degraded, releasing tritium into the supernatant.

Table 3 lists the results of incubation of each of the following samples with the [^{3}H]-labeled DNA substrate. The Positive Control (A) is a mock sample in which all of the DNase used in the purification is added to 100µl of Nuclease-Free Water, and 5µl are tested. The average percentage release of these samples is 36%. The Minus DNase Stop Solution Control (B) is an RNA sample isolated using the SV Total RNA Isolation System in which the SV DNase Stop Solution step is omitted. With this step omitted, the percentage release is 0.11%, indicating that the membrane does not favor the binding and release of DNase in the elution. In the Negative Control (C), RNA samples are prepared according to the procedure of the SV Total RNA Isolation System with the DNase omitted from the incubation mix. The samples in (C) represent the expected background before using DNase in the protocol. The Standard Preparation (D) samples are processed using the SV Total RNA Isolation System procedure, and the level of DNase carryover in these samples is not significantly different from the Negative Control (C).

Table 3. DNase Carryover.	
Type of Sample	**[^{3}H]% Release**
(A) Positive Control	36
(B) Minus DNase Stop Solution Control	0.11
(C) Negative Control	-0.07
(D) Standard Preparation	-0.03
The values in this table represent the average of triplicate samples. Values are expressed as percentages of total counts subtracting a background (blank) determination.	

ISOLATION OF TOTAL RNA FROM BLOOD

Isolation of RNA from peripheral blood leukocytes and analysis by RT-PCR is a powerful technique that is useful for a variety of research and clinical/diagnostic applications. However, the use of RT-PCR in a high-throughput format requires the rapid isolation of RNA from multiple (blood) samples. Whole blood contains extremely high concentrations of serum proteins and derivatives of hemoglobin that can interfere with efficient RNA extraction and subsequent amplification (6,7). In addition, techniques routinely used for isolation of RNA from blood require relatively large quantities of starting material to isolate peripheral blood mononuclear cells prior to RNA extraction. This is time-consuming and not appropriate for high-throughput screening of relatively small amounts of blood.

We have used Promega's SV Total RNA Isolation System to develop rapid RNA isolation protocols that utilize small amounts (less than 1ml) of human whole blood or blood leukocytes as starting material (1). For isolation of total RNA from blood, the white blood cells were enriched by first lysing the red blood cells using the SV RNA Red Blood Cell Lysis Solution (Cat.# Z3141). Blood cells were collected by centrifugation at 400 x *g* and treated with SV RNA Red Blood Cell Lysis Solution to remove erythrocytes. The remaining peripheral blood leukocytes were used as starting material in the standard protocol. The SV Total RNA Isolation System produced typical yields of approximately 0.75-1.5µg RNA/ml of blood. The RNA samples obtained were analyzed by coupled reverse transcription and PCR amplification (RT-PCR) using Promega's single-tube, coupled Access RT-PCR System[(e)] (Cat.# A1250, reference 8). RT-PCR using a primer pair designed to amplify human beta-actin mRNA or a less abundant mRNA encoded by the *Adenomatous Polyposis Coli (APC)* gene yielded the expected products of 511 and 937 base pairs, respectively (Figure 5). These techniques enable the rapid and efficient isolation of RNA from whole blood and could readily be scaled to the high throughput formats required for screening and diagnostic applications.

[(e)]*The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this system is recommended for persons that either have a license to perform PCR or are not required to obtain a license.*

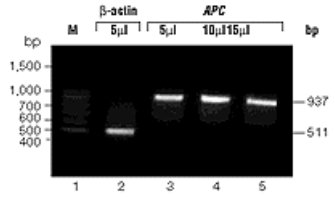

Figure 5. RT-PCR amplification of RNA isolated from human blood. Total RNA was isolated from 1ml of human blood using Promega's SV Total RNA Isolation System (Cat.# Z3100) and the protocol for blood supplied with the system (1). RT-PCR reactions using the indicated volumes of eluted RNA and primers complementary to human ß-actin RNA (lane 2) or human *Adenomatous Polyposis Coli (APC)* RNA (lanes 3-5) were performed using the Promega Access RT-PCR System as described in the System protocol (8). RT-PCR yielded the expected products of 511 and 937 base pairs from the beta-Actin mRNA and *APC* mRNA, respectively. Ten microliters of the beta-actin reaction and 20µl of the APC reactions were resolved in a 1.5% agarose gel. The gel was scanned with an AMBIS™ Image Acquisition and Analysis System. The size of Promega's 100bp DNA Ladder is indicated (M).

SUMMARY

The SV Total RNA Isolation System procedure yields high quality total RNA after a single round of purification without organic extractions or precipitations. The procedure is easy to perform with small quantities of tissue, cultured cells or whole blood and can be used to process multiple samples simultaneously. Unlike other comparable, commercially available systems, Promega's columns do not clog during centrifugation, total RNA yields are higher, genomic DNA contamination is not present in RT-PCR analysis and the results are more reliable as seen by a lower % CV. The protocol is easy to perform and can be completed in as little as one hour. The ability to purify the RNA by vacuum as well as by spin format further simplifies the procedure. In addition, two key buffers are distinguished by color to help track protocol steps. The system provides a valuable alternative to the use of organic extractions for the preparation of high quality total RNA.

RNA purified with the SV Total RNA Isolation System is suitable for many molecular biology applications, including RT-PCR analysis, Northern blot hybridization and RNase protection assays. For more information on downstream applications, see the Promega *Protocols and Applications Guide*, Third Edition (9), and the Promega *RNA Applications Guide* (10).

REFERENCES

1. *SV Total RNA Isolation System Technical Manual* #TM048, Promega Corporation.
2. Brisco, P. *et al.* (1996) *Promega Notes* **59**, 10.
3. Chirgwin, J.M. *et al.* (1979) *Biochemistry* **18**, 5294.
4. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual,* 2nd edition, Cold Spring Laboratory, Cold Spring Harbor, NY.
5. Telford, J.L. *et al.* (1986). *Nucl. Acids Res.* **14**, 9955.
6. Higuchi, R. (1992) In: *PCR Technology: Principles and Applications for DNA Amplification,* Erlich, H.A., ed., Stockton Press, 31.
7. Akane, A. (1996) *BioTechniques* **21**, 392.
8. *Access RT-PCR System and Access RT-PCR Introductory System Technical Manual* #TB220, Promega Corporation.
9. *Protocols and Applications Guide,* Third Edition (1996) Promega Corporation.
10. *RNA Applications Guide* (1997) Promega Corporation.

Ordering Information

Product	Size	Cat.#
SV Total RNA Isolation System	50 preps	Z3100
SV Total RNA Isolation System, Trial Size	10 preps	Z3101
Miniprep Vacuum Adapters	20 each	A1331
SV RNA Red Blood Cell Lysis Solution	200ml	Z3141
Vac-Man® Laboratory Vacuum Manifold	20-sample capacity	A7231
