Quantitative, Real-Time RT-PCR Expression Using the SV 96 Total RNA Isolation System

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Abstract

The SV 96 Total RNA Isolation System provides a fast, simple technique for the purification of intact, RNA from tissue culture cells. The system isolates high-quality RNA from small amounts of starting material using a 96-well format. Here we describe real-time RT-PCR analysis of RNA isolated with the SV 96 Total RNA Isolation System.

The SV 96 Total RNA Isolation System provides a high-throughput solution for users who want to isolate total RNA from cell lines for real-time RT-PCR analysis.

Introduction

As the focus of genomics research shifts from genome sequencing to analysis of gene function and expression, the need for reliable RNA isolation systems has increased. Researchers who would like to perform RNA analysis on cells cultured in multiwell plates need an easy, reliable way to purify the RNA. Most traditional RNA isolation protocols are designed to process small numbers of samples. These protocols can be cumbersome and make isolating RNA from tens or hundreds of samples an arduous task. Additionally, preparation of RNase-free reagents is difficult and time-consuming, and successful results cannot be guaranteed.

Promega's SV 96 Total RNA Isolation System[a] is designed to meet the various throughput needs of researchers. Up to 96 samples may be processed simultaneously with high yield and purity. The protocol is fast and easy to perform using Promega's Vac-Man® 96 Vacuum Manifold. A 96-well plate can be processed in approximately one hour. The system is designed to enable researchers to focus on RNA analysis rather than RNA isolation.

The SV 96 Total RNA Isolation System is based on the membrane technology of the SV Total RNA Isolation System[a,b]. Both systems use guanidine thiocyanate and β-mercaptoethanol to inactivate RNases (1). The SV Total RNA Isolation System provides researchers with a simple, versatile small-scale protocol for isolating total RNA from a variety of starting materials (2). In this "miniprep" format, researchers have a choice of spin or vacuum protocols. In contrast, the SV 96 Total RNA Isolation System is based upon a vacuum protocol and can be automated. The 96-well plate format is compatible with liquid-handling workstations such as the Beckman Coulter Biomek® 2000 and Biomek® FX (3). Both systems incorporate RNase-free DNase treatment while the RNA is bound to the SV membrane to reduce genomic DNA contamination. This is important, because genomic DNA can interfere with RNA quantitation and quantitative real-time RT-PCR[c] (4). The system requires no phenol/chloroform extractions or ethanol precipitations, and there is no DNase carryover in the final RNA preparation (2). The process reduces cross-well contamination below detectable limits, as demonstrated by end-point RT-PCR (3).

In this study we demonstrate that total RNA isolated with the SV 96 Total RNA Isolation System is suitable for immediate use in quantitative, real-time RT-PCR. Two specific mRNAs, β-actin and lamin A/C, were detected using the TaqMan® real-time RT-PCR assay. The results were dependent on the priming method chosen for first-strand cDNA synthesis. Random hexamer-primed cDNA synthesis consistently produced more sensitive results than oligo(dT)-primed synthesis under the conditions tested.

Real-Time RT-PCR

A key technique for analyzing purified RNA is expression analysis through quantitative, real-time RT-PCR (4–6)(for review, see reference 5). RT-PCR can be performed in either one or two steps, depending on the enzymes, primers and buffers used. The reverse transcription of RNA can be primed by a gene-specific primer, random hexamers or oligo(dT) to produce first-strand cDNA. In real-time RT-PCR, quantitation is performed during amplification of the cDNA. The signal produced in the early exponential phase of the reaction is proportional to the initial amount of template (7).

Real-time TaqMan® RT-PCR Assays have been developed to quantitate the expression of many human genes (ABI). The TaqMan® Assay uses a quenched fluorescent molecule that "reports" the amount of amplified material accumulating in each thermal cycle. Amplifications are performed in a specialized cycler, such as the ABI PRISM® 7700 Sequence Detection System, that is both a thermal cycler and a fluorometer. TaqMan® gene expression assays use a set of specific RT-PCR primers plus a specific reporter probe. The reporter probe binds to the desired amplimer and has both a fluorescent dye tag (i.e., 6FAM) and a fluorescence quencher molecule attached. As long as the tag and quencher molecule remain close together, the fluorescence is quenched. As the PCR primer is extended by *Taq* DNA polymerase, the reporter primer is degraded by the inherent 5′ to 3′

exonuclease activity of the enzyme. Degradation of the reporter primer releases the fluorescent tag from the influence of the quencher, and the tag can be detected. As more amplimer accumulates, more fluorescent tags are released. The amount of accumulated fluorescence is proportional to the log of the input quantity and the amount of specific amplimer at each time point, generating "real-time" data.

Reactions are not characterized by the total amount of product that has accumulated at the end of a specified number of cycles. Instead, the reactions are characterized by the point at which the fluorescence from the reporter significantly exceeds the background. This point is called the "threshold cycle" (C_T). The C_T is dependent upon the amount of starting material—the more copies of starting template there are in the reactions, the fewer cycles it will take to reach the C_T. Conversely, the lower the amount of starting template, the longer it will take to reach the C_T.

Results and Discussion

RNA isolated using the SV Total RNA Isolation System has been proven to perform well in quantitative, real-time PCR. Researchers have used the system on cultured cells (8–11), bacterial cells (12–14) as well as *Arabidopsis* (15) and *Drosophila* (16). Here we demonstrate that the SV 96 Total RNA Isolation System is also compatible with real-time RT-PCR. We chose to assay β-actin and lamin A/C from HeLa cells using TaqMan® Gene Expression Assays in accordance with the manufacturer's instructions. We tested both random hexamers and oligo(dT)$_{16}$ as reverse transcription primers.

We investigated the effect of cell number titration on the isolation and detection of specific mRNAs. Cultured HeLa cells were diluted into aliquots of 50,000, 25,000, 12,500, 6,250, 3,125, and 1,563 cells. Total RNA was isolated from each aliquot using the SV 96 Total RNA Isolation System. Two-step TaqMan® Gold RT-PCR assays with AmpErase® UNG (uracil-N-glycosylase) cross-contamination control were used for quantitation, in accordance with the manufacturer's instructions. With this approach, the expression of more than one gene can be quantitated from the same cDNA sample. The data should then represent similar reverse transcription efficiencies for each message, such as an experimental and an endogenous control (17).

The choice of RT primers is a consideration that can affect the design and outcome of an experiment. Although random hexamers will preferentially prime ribosomal RNA (unless the amount of reverse transcriptase is increased), they will prime any type of RNA or DNA, including partially degraded mRNA and genomic DNA. Oligo(dT), on the other hand, offers more specific priming from the poly(A)+ mRNA tail or from randomly dispersed poly(A)-rich regions, giving greater assurance that the mRNA is intact. However, oligo(dT) cannot be used to prime 18S rRNA, eliminating its use as a control. Some studies have suggested that 18S rRNA is a more reliable endogenous control for gene expression studies than commonly used housekeeping genes, such as GAPDH or β-actin (18–20). Other studies suggest normalizing data to multiple endogenous controls (21,22). ABI suggests using random hexamers to prime total RNA for TaqMan® gene expression assays.

The data shown in Figure 1 represents random hexamer priming. Oligo(dT) data showed similar results with respect to the HeLa cell titration (data not shown). Random hexamer-primed cDNA consistently produced a lower C_T value than oligo(dT)-primed cDNA. This is true for both the β-actin and lamin A/C reactions (Figure 2). As expected, the C_T values increase as RNA is isolated from fewer cells. Analysis of the C_T versus the log of the input number of cells gives a semi-log linear response. This indicates that the yield of total RNA is proportional to the number of input cells. It also indicates that the overall composition of the purified RNA samples (i.e., mRNA:rRNA ratio) remains the same as the amount of purified RNA decreases.

The reverse transcription conditions are slightly different for priming with random hexamers, as mentioned above. According to the manufacturer's recommendations, the amount of MultiScribe® RT should be increased from 1.25u/µl to 3.125u/µl when random hexamers are used. This is to compensate for the vast amount of ribosomal RNA that is primed along with the mRNA. Because we used TaqMan® Pre-Developed Assay Reagents (PDARs) and two-step RT-PCR, we were unable to test gene-specific primers. Lekanne Deprez *et al.* (22) reports that gene-specific cDNA synthesis yields the maximum sensitivity. Our findings suggest that it is not advisable to compare the data from an oligo(dT) reaction to that from a random hexamer reaction. Similar shifts in C_T values have also been seen when DNA is amplified with different TaqMan® PCR reagents (per manufacturer).

Summary

The SV 96 Total RNA Isolation System provides a robust, reliable method to isolate intact RNA from up to 96 samples at a time. The system provides a standardized high-throughput solution for users who want to isolate total RNA from cell lines for real-time RT-PCR analysis. Total RNA isolated from decreasing numbers of cells using this system performed well in the TaqMan® assay, yielding quantitative, semi-log linear results. We found that the RNA can be primed with either random hexamers or oligo(dT). Since the absolute results were dependent on the reverse transcription conditions, it is important to compare RT-PCR results only between reactions that were primed in the same manner.

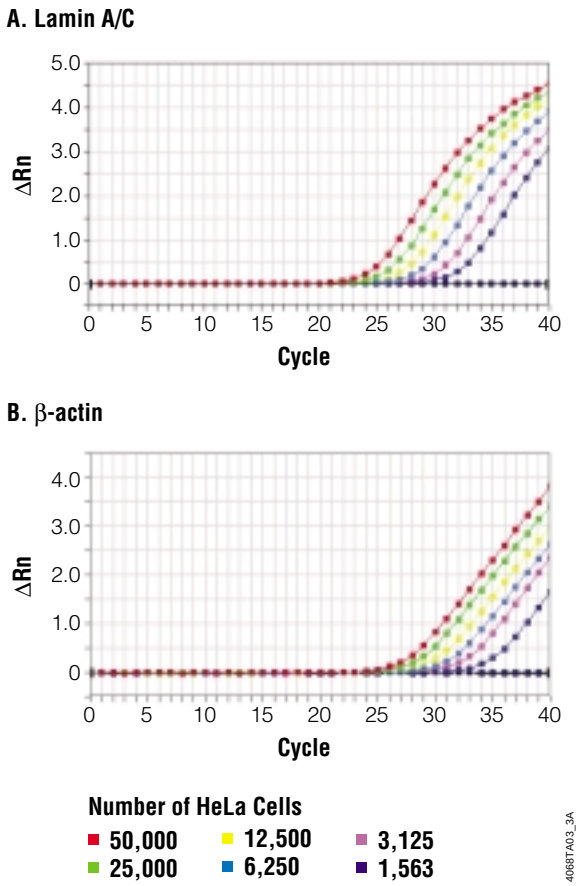

Figure 1. Real-time, quantitative RT-PCR analysis of human lamin A/C and β-actin mRNA levels. The SV 96 Total RNA Isolation System was used to isolate total RNA from serially diluted HeLa cells. The cells were diluted from 50,000 to 1,563 cells by a series of twofold dilutions as described in the Methods. The total RNA was eluted in 100µl Nuclease-Free Water. Twenty microliters of the eluted total RNA was converted to first-strand cDNA by random hexamer priming (100µl reaction). Quantitative, real-time PCR was performed using 5µl of the first-strand cDNA reaction. **Panel A.** Real-time, quantitative RT-PCR analysis of lamin A/C expression. **Panel B.** Real-time, quantitative RT-PCR analysis of β-actin expression. Controls (no template) were included with both sets. Reactions are corrected for background fluorescence.

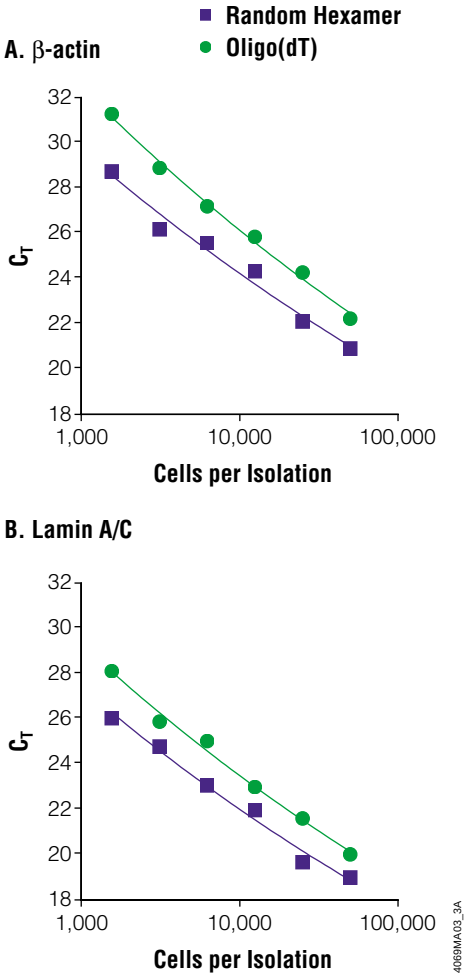

Figure 2. Threshold cycle values for β-actin and lamin A/C from decreasing amounts of HeLa cells. The SV 96 Total RNA Isolation System was used to isolate total RNA from serially diluted HeLa cells. RNA isolation and RT-PCR assays were performed as described in Figure 1 and the Methods. **Panel A.** Quantitative, real-time RT-PCR analysis of β-actin levels. **Panel B.** Quantitative, real-time RT-PCR analysis of lamin A/C levels. For both targets, the threshold cycle (C_T) values were plotted against the log of the number of cells in each reaction.

Methods

RNA Isolation: HeLa cells, grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, were diluted from 50,000 to 1,563 cells by a series of twofold dilutions with 1X PBS, then centrifuged. The cell pellets were washed once with 1X PBS and lysed in 100µl of SV RNA Lysis Solution plus β-mercaptoethanol. The RNA was purified with the SV 96 Total RNA Isolation System and the Vac-Man® 96 Vacuum Manifold as directed (1).

First-Strand cDNA Synthesis: Twenty microliters of the eluted RNA was reverse transcribed in a 100µl reaction using the ABI TaqMan® Reverse Transcription Reagents (ABI Cat.# N808-0234) and MultiScribe® Reverse Transcriptase (ABI Cat.# 4308228). Reactions using oligo(dT)$_{16}$ for first-strand synthesis used 1.25u/µl of MultiScribe® reverse transcriptase, and reactions using random hexamers used 3.125u/µl. The reverse transcription reactions were incubated at 25°C for 10 minutes followed by 60 minutes at 37°C. The reverse transcriptase was inactivated at 95°C for 5 minutes, then held at 4°C. Reactions were performed in a Perkin Elmer GeneAmp® PCR System 9600 Thermal Cycler. All materials were used in accordance with manufacturer's instructions.

Quantitative, Real-Time RT-PCR: Five microliters of the first strand cDNA synthesis reaction was amplified in a 50µl TaqMan® Gold PCR reaction using a TaqMan® PCR Core Reagent Kit (ABI Cat. #N808-0228). Each specific PCR amplification was performed independently using the reverse transcribed cDNA pools obtained from either random hexamer priming or oligo$(dT)_{16}$ priming. All reactions were quantified in the same run. Amplification and real-time detection took place in an ABI PRISM® 7700 Sequence Detection System. The CCD camera exposure time was set for 10msec. The amplification reactions were set up in a separate PCR plate, then carefully transferred to an Optical Plate (ABI Cat.# N801-0560). The plate was sealed with an Optical Adhesive Cover (ABI Cat.# 4313663).

TaqMan® assays were performed with 6FAM-MGB-labeled probes. β-Actin was detected using the Human ACTB (20X) Pre-Developed Assay Reagent (ABI Cat.# 4333762F), and lamin A/C was detected using the Human Lamin A/C Assays-on-Demand™ Gene Expression Reagent (ABI Cat.# 50244042-E). PCR included AmpErase® UNG. The temperature profile was: 50°C for 2 minutes (UNG[d] incubation), 95°C for 10 minutes (AmpliTaq Gold® DNA polymerase activation), denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute for 40 cycles. The reaction was held at 72°C to prevent amplicon degradation. Data were analyzed using Sequence Detection System v1.9 software (ABI).

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Protocols

- *SV 96 Total RNA Isolation System Technical Bulletin* #TB294, Promega Corporation. (www.promega.com/tbs/tb294/tb294.html)
- *SV Total RNA Isolation System Technical Manual* #TM048, Promega Corporation. (www.promega.com/tbs/tm048/tm048.html)
- *SV Total RNA Isolation System Automated Protocol* #EP003, Promega Corporation. (www.promega.com/tbs/ep003/ep003.html)

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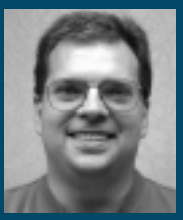

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Ordering Information

Product	Size	Cat.#
SV 96 Total RNA Isolation System[a]*	1 × 96	Z3500
	5 × 96	Z3505
Vac-Man® 96 Vacuum Manifold	1 each	A2291
ImProm-II™ Reverse Transcriptase*	100 reactions	A3802
	500 reactions	A3803
AMV Reverse Transcriptase (10u/µl)*	300u	M5101
	1,000u	M5108
AMV Reverse Transcriptase (20–25u/µl)*	600u	M9004
M-MLV Reverse Transcriptase, RNase H Minus	10,000u	M5301
SV Total RNA Isolation System[a,b]*	10 preps	Z3101
	50 preps	Z3100

* For Laboratory Use.

** Please contact your local Promega Branch Office or distributor for ordering information.

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[b] Australian Pat. No. 730718 and other patents and patents pending.

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