Applications

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Automated high-throughput isolation of total RNA from tissue culture cells using the Promega Wizard® SV 96 Total RNA Isolation System on the Eppendorf epMotion® 5075

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Abstract

A new, automated method for high-throughput RNA isolation from tissue culture cells has been developed on the Eppendorf ep*Motion*® 5075 VAC using the Promega Wizard® SV 96 Total RNA Isolation System. Total RNA is purified from cultured Jurkat cells with no detectable cross-contamination during the automated procedure. Yield and purity of RNA obtained using this method are determined to be of excellent quality as assessed quantitatively using the Quant-iT™ RiboGreen® RNA Assay and spectrophotometrically by relative absorbance measurements (i.e., A_{260}/A_{280}). Furthermore, successful RT-PCR amplification confirms compatibility of RNA isolated using this methodology for downstream applications.

Introduction

The need for a high-throughput system capable of isolating high-quality RNA, free of genomic DNA and protein contaminants, is increasing for many researchers in molecular biology. This system ideally should be simple to use, able to process many samples simultaneously, and capable of RNA isolation in a relatively short amount of time without contamination between samples. The system presented in this article fits this need nicely. It combines the chemistry of the Promega Wizard SV 96 Total RNA Isolation System with the instrumentation of the Eppendorf ep*Motio*n 5075 VAC.

The Promega Wizard SV 96 Total RNA Isolation System provides a high-throughput technique to prepare intact RNA from tissue and cultured cells. The system 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.

The Eppendorf ep*Motion* 5075 VAC is a flexible, modular automated pipetting system that can be adapted to various liquid handling tasks. The concept of exchangeable tips and the “Free Jet Dispensing” technology enables the system to dispense liquids contact-free and makes it ideal for implementing complex work steps where precision and reliability are required.

Materials and Methods

- **Eppendorf ep*Motion* 5075 VAC equipped as follows:**
  - Gripper
  - Dispensing tools TM1000-8 and TM300-8
  - Vacuum with manifold
  - Reservoir Rack
  - Height spacers 85 mm and 40 mm
  - Vac Frame 2
  - Waste Tub
- **Eppendorf consumables**
  - Reagent Reservoirs: 30 ml & 100 ml
  - epT.I.P.S. Motion Filtertips: 300 µl & 1000 µl
- **Promega Wizard SV 96 Total RNA Isolation System**
  - RNA Lysis Buffer
  - Beta-mercaptoethanol
  - RNA Wash Solution
  - DNase I (lyophilized)
  - $MnCl_2$, 0.09 M
  - Yellow Core Buffer
  - DNase Stop Solution
  - Nuclease-Free Water
  - SV 96 Binding Plate
  - 96-well Elution Plate
  - Plate Sealers
- **Fresh tissue culture cells, Jurkat cell line**
- **96-well Flat-bottom Tissue Culture Sample Plates.**

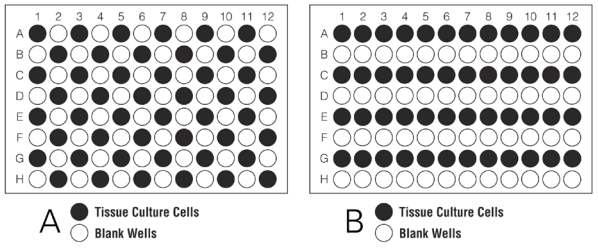

Figure 1: Sample plate layouts, checkerboard (A) and striped (B) patterns.

Cell, Reagent, and Worktable Preparation

Jurkat cells were dispensed at $1x10^5$ cells per well in two flat-bottom 96-well tissue culture Sample Plates (Corning, USA) in a checkerboard and a striped pattern (Figure 1 A and B, respectively). Select wells were intentionally left blank to serve as a control for method cross-contamination. Reagents were prepared and dispensed into 30 ml and 100 ml Reagent Reservoirs as described in Table 1. Labware was placed onto the ep*Motion* 5075 Worktable, as shown in Figure 2 and Table 2.

Table 1: Contents of the Reagent Reservoirs in the Reagent Reservoir Rack.

Reservoir Rack Position	Reservoir Size	Reservoir Contents	Reagent Preparation Instructions*
1	100 ml	12 ml RNA Lysis Buffer	Add 1 ml BME to 50 ml RNA Lysis Buffer
2	30 ml	3.125 ml DNase Solution	Add 312.5 µl Nuclease-Free Water to DNase I and swirl gently to mix
3	100 ml	22 ml DNase Stop Solution	Add 20 ml 95 % EtOH to DNase Stop Solution
4	100 ml	100 ml RNA Wash Solution	Add 95 % EtOH to the RNA Wash Solution bottle as directed on the label
5	100 ml	12 ml Nuclease-Free Water	

*See Promega electronic protocol EP003 for further details on reagent preparation.

Figure 2: Screenshot from the ep*Motion* Editor showing the ep*Motion* 5075 VAC worktable setup for the automated method.

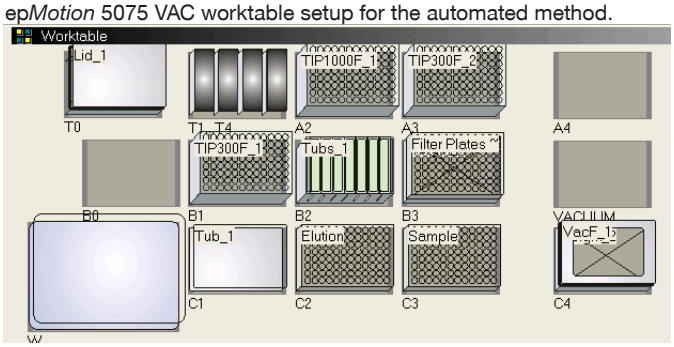

Table 2: ep*Motion* 5075 Worktable setup, details by position.

Worktable Position	Labware
A2	1000 µl epT.I.P.S. Motion Filtertips
A3	300 µl epT.I.P.S. Motion Filtertips
A4	Empty
B0	Empty
B1	300 µl epT.I.P.S. Motion Filtertips
B2	Reservoir Rack with 5 Reagent Reservoirs
B3	Binding/Filter Plate atop 85 mm Height Spacer
Vacuum	Empty
C1	Waste Tub with quarter wall separators
C2	96-well Elution Plate
C3	96-well Flat-bottom Tissue Culture Sample Plate
C4	Vac Frame 2 atop Vac Frame Holder

Automated Method Overview

The automated method uses the Promega Wizard SV 96 Binding Plate to purify total RNA from samples in a 96-well format. The method begins by dispensing 100 µl RNA Lysis Buffer to each well of the Sample Plate. The cells are disrupted in the lysis buffer which contains guanidine thiocyanate and beta-mercaptoethanol to rapidly denature RNases and maintain the integrity of the RNA. The cell lysates are mixed and transferred to the binding plate atop the vacuum manifold apparatus. In the presence of guanidine thiocyanate, nucleic acids in the cell lysates bind to the silica filter membrane at the bottom of each well. A vacuum is applied and cell lysates are pulled through the binding plate in each well by vacuum filtration. This vacuum process eliminates the need for centrifugation steps.

After vacuum filtration, 500 µl RNA Wash Solution, a low salt ethanol-based wash solution, is dispensed to each well of the binding plate. The vacuum is again applied and the wash solution is pulled through the binding plate. Next, 25 µl RNase-free DNase Solution is added to each well of the binding plate and incubated at room temperature for 10 minutes to remove genomic DNA. After the 10 minute incubation, the DNase is inactivated by adding 200 µl DNase Stop Solution to each well of the binding plate. A vacuum is applied and the stop solution is drawn through the binding plate.

Next, a second 500 µl RNA Wash Solution is dispensed to each well of the binding plate and the vacuum is reapplied. The DNase, along with contaminants such as proteins, salts and other cellular impurities, are removed by this wash. The wash step occurs while the binding plate remains on the vacuum. No disassembly of the manifold is required during binding and washing steps to remove filtrate waste. The vacuum remains on for an additional 5 minutes to remove any residual ethanol from the binding plate.

After the vacuum step, the Gripper tool disassembles the vacuum manifold stack by removing the binding plate and manifold collar from the vacuum manifold to holding position. The Gripper tool then moves the Elution Plate into the vacuum manifold and reassembles the vacuum manifold stack by moving the binding plate and Vac Frame 2 back onto the vacuum.

Lastly, 100 µl Nuclease-Free Water is added to each well of the Binding Plate and allowed to incubate at room temperature for 1 minute. A vacuum is then applied, and the Nuclease-Free Water is pulled through the Binding Plate, eluting the total RNA into the Elution Plate. The total processing time for the automated method is less than 1 hour. The average volume eluted is approximately 75 µl per well of a 96-well Elution Plate. The automated method was run separately for the checkerboard plate and the striped plate.

Analysis of Purified Total RNA

Analysis of the isolated total RNA samples from both Elution Plates was performed. RNA yields were quantitated using the Quant-iT RiboGreen fluorescent RNA assay (Invitrogen, USA). One microliter volumes from each of the RNA samples were placed directly onto a ND-1000 Spectrophotometer (Nanodrop, USA) and assessed spectrophotometrically by relative absorbance measurements for yield and purity (i.e., A_{260}/A_{280}). In addition, reverse transcription and polymerase chain reaction (RT-PCR) amplification was performed using the Access RT-PCR System (Promega, USA) with ß-actin primers (Integrated DNA Technologies, USA) on 20 randomly selected purified RNA samples. Ten samples were taken from the checkerboard elution plate and ten from the striped elution plate, half from blank wells and half from tissue culture cells wells. For RT-PCR, a negative control was performed. The thermal cycling conditions were as follows: 1 cycle 45 °C for 45 minutes, 1 cycle 94 °C for 2 minutes, 40 cycles of 94 °C for 30 seconds, 60 °C for 1 minute, 68 °C for 2 minutes, and 1 cycle 68 °C for 7 minutes. Following amplification, 10 µl of each reaction product was separated on a 1.2 % agarose gel and visualized by ethidium bromide staining.

Results

The average yield of total RNA determined spectrophotometrically at 260 nm, where 1 absorbance unit (A_{260}) equals 40 µg of single-stranded RNA per milliliter, and quantitatively with the RiboGreen RNA Assay was found to be 0.38 ± 0.07 µg for $1x10^5$ Jurkat cells, and 0.01 ± 0.07 µg for blank wells (Table 3). The expected total RNA yield from $1x10^5$ Jurkat cells should be approximately 0.3 µg. These results suggest that the automated system effectively isolated RNA with practically zero cross-contamination between wells of the same plate.

The average purity of total RNA determined spectrophotometrically from the relative absorbances at 260 and 280 nm (i.e., A_{260}/A_{280}) was also measured. For $1x10^5$ Jurkat cells, the average A_{260}/A_{280} ratio was 2.31 ± 0.68 (Table 3). Pure RNA should exhibit an A_{260}/A_{280} ratio of 2.0. This A_{260}/A_{280} ratio suggests that RNA isolated with this automated system is substantially free of DNA and contaminating protein.

Table 3: Average yield and purity of total RNA isolated from $1x10^5$ Jurkat cells and blank wells, as assessed by Nanodrop ND-1000 absorbance measurements and RiboGreen RNA quantitation.

Samples	Average RNA Yield (µg)	Average A_{260}/A_{280}
$1x10^5$ Jurkat Cells	0.38 ± 0.07	2.31 ± 0.68
Blank Wells	0.01 ± 0.07	4.73 ± 26.4

RT-PCR amplification products obtained using the purified RNA isolated from Jurkat cells with the upstream and downstream ß-actin primers were 285 bp in size and were readily visible by UV transillumination of the ethidium bromide-stained gel (Figure 3, odd-numbered lanes). Amplification products were not observed for the negative control (-C) or for the total RNA isolated from blank wells (Figure 3, even-numbered lanes). RT-PCR amplification of the specific target RNA (ß-actin) highlighted the integrity of the RNA isolated using this automated method as successful reverse transcription is dependent on the integrity and purity of the mRNA used as the template. These results suggest that the starting RNA template was of good quality.

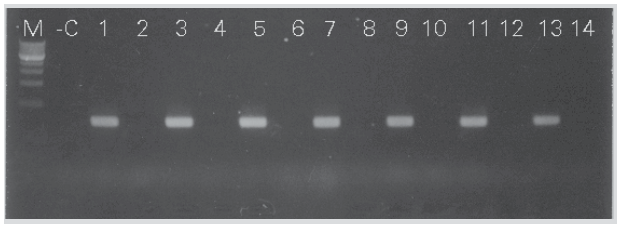

Figure 3: RT-PCR amplification products (ß-actin) separated on a 1.2 % agarose gel and visualized by ethidium bromide staining.
Expected size for ß-actin is 285 bp. A 1 kb sizing ladder (M) and RT-PCR negative control (-C) were included. Lanes 1, 3, 5, 7, 9, 11, and 13 correspond to samples taken from Jurkat cell wells of checkerboard (CB) and striped (S) Elution Plate locations CB-A11, CB-B2, CB-C5, CB-F4, S-C4, S-E9, S-G1, respectively. Lanes 2, 4, 6, 8, 10, 12, and 14 correspond to samples taken from blank wells of plate locations CB-B5, CB-C6, CB-F1, CB-G12, S-D2, S-F4, S-H12, respectively. Amplification products were clearly observed from RNA isolated from Jurkat cells, as shown in odd-numbered lanes. No PCR product was observed from RNA isolated from blank well samples, as shown in even-numbered lanes.

Conclusion

The automated system described in this article incorporating the Promega Wizard SV 96 Total RNA Isolation System to purify total RNA from cultured Jurkat cells ($1x10^5$ cells per well of a 96-well plate) using the epMotion 5075 VAC results in the successful isolation of high-quality RNA. Advantages of this combined system include isolation of total RNA from 96 samples in less than one hour, automated precision and consistency in the treatment of each sample processed, and comparable yields and purity to RNA isolated using other automated or manual RNA isolation procedures. In addition, RNA isolated using this system is also substantially free of DNA and contaminating protein, may be used directly for downstream applications including RT-PCR, and has no cross-contamination between wells of the same 96-well plate. This integrated system fits the needs of researchers requiring considerable sample sets of total RNA for downstream applications in molecular biology.

Eppendorf Ordering Information

Product	Order no. International	Order no. North America
ep*Motion* 5075 VAC (vacuum chamber included)	5075 000.164	960020014
30 ml Reagent Reservoirs	0030 003.993	960050100
100 ml Reagent Reservoirs	0030 126.513	960051017
Reservoir Rack	5075 754.002	960002148
1000 µl epTIPS Motion Filtered Tips	0030 126.513	960050100
300 µl epTIPS Motion Filtered Tips	0030 003.993	960050061

Promega Ordering Information

Product	Order no.
Wizard® SV 96 Total RNA Isolation System	Z3505
Nuclease-Free Water	P119A
Access Quick PCR Master Mix	A1260
BenchTop 1kb DNA Ladder	G754A

Promega

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