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Small-Sample Total RNA Purification: Laser Capture Microdissection and Cultured Cell Applications

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ABSTRACT

Gene expression studies require analysis of RNA, but isolation of total RNA from very small samples by traditional methods can be difficult and inefficient. The Absolutely RNA™ microprep kit provides a convenient method for isolating total RNA from small numbers of cells such as those harvested by laser capture microdissection (LCM). The protocol includes binding of RNA to a solid support, thus eliminating the need for organic extraction and alcohol precipitation. DNase digestion on the solid support reduces or eliminates DNA contamination and minimizes RNA handling. Efficient washing removes contaminants, and elution in a small volume of buffer results in high-purity RNA at a concentration appropriate for demanding applications such as RT-PCR. RNA isolated from as few as 200 laser capture microdissected brain tumor cells resulted in detection of low, medium, and highly expressed genes by conventional and real-time RT-PCR.

INTRODUCTION

There is significant interest in analyzing gene expression from distinct cell populations. Heterogeneous populations of cells within tissues of various types possess correspondingly different patterns of gene expression, and these cells must be separated from one another for accurate assessment of gene expression. Laser capture microdissection (LCM) is a particularly useful tool for recovering small cell samples and even enables researchers to collect individual cells from tissue sections (4). This method facilitates the separation of histologically different cells so that proteins, DNA, or RNA from these cells can be analyzed in isolation from the surrounding unwanted cells. In addition to LCM samples, some samples of cells in culture are available in limiting amounts, such as slow-growing clones or primary cells with a limited life span. Isolation of RNA from small cell samples can be challenging because of the inefficiency of protocols designed for larger samples. For example, yields can be low because of losses during phenol-chloroform extraction and/or ethanol precipitation. We have developed a method specifically for the purpose of isolating total RNA from small samples of cells. The protocol was designed for applications such as LCM, where samples of 100–1000 cells are common, but has been successfully applied from 1 cell up to 5×10^5 cells. The resulting RNA is of high quality and is ideal for demanding quantitative and conventional RT-PCR applications.

MATERIALS AND METHODS

All materials were supplied by Stratagene (La Jolla, CA, USA) unless indicated otherwise.

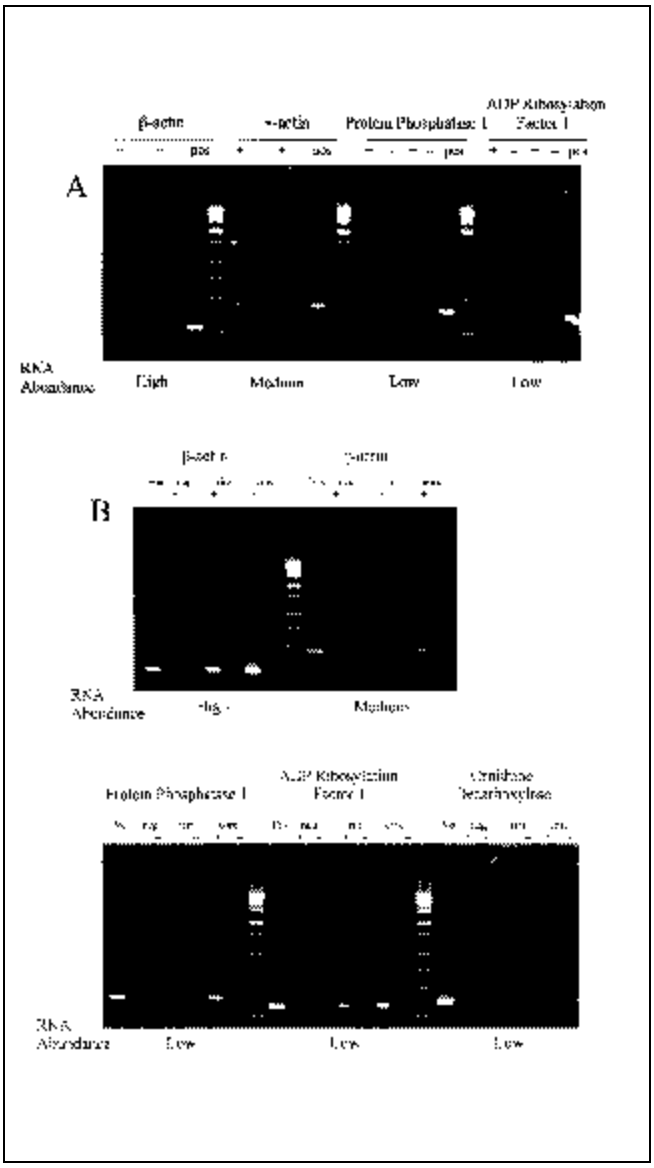

Figure 1. RT-PCR analysis of RNA isolated from human brain tumor LCM samples. LCM was used to harvest 200 cells from the core (A) and 1000 cells from the rim and core (B) of a brain tumor section. (A) Half of the RNA from 200 cells was used for cDNA synthesis. One-twentieth of the cDNA was used for PCR amplification of β-actin, one-fifth of the cDNA was used for γ-actin, and one-fourth of the cDNA was used for low-abundance RNAs (protein phosphatase 1 and ADP ribosylation factor 1). RT-PCR was performed using Stratagene's Primer Sets for RT-PCR allowing amplification of human genes of varying abundance levels. *Taq2000* DNA polymerase was used for the PCR in a RoboCycler 96 temperature cycler according to the instructions provided with the primers and as described in the Materials and Methods section. Half of each PCR product was electrophoresed in a 1.5% agarose gel, equivalent to the RNA from 2.5 cells (β-actin), 10 cells (γ-actin), and 13 cells (protein phosphatase 1 and ADP ribosylation factor 1). (B) Eight microliters of each 30-µL sample from 1000 cells were used for cDNA synthesis. One-tenth of the cDNA was used for detecting high- and medium-abundance RNA (β-actin and γ-actin), and one-fifth of the cDNA was used for detecting low-abundance RNAs (protein phosphatase 1, ADP ribosylation factor 1, and ornithine decarboxylase). Two-fifths of each PCR were electrophoresed on a 1.5% agarose gel. This is equivalent to 11 cells for the high- and medium-abundance RNAs and 21 cells each for the low-abundance RNAs. "Pos" in panels A and B represents a positive PCR control for each primer set using template provided with Stratagene's Primer Sets for RT-PCR. The designations (+) and (-) in panels A and B refer to the presence or absence of MMLV-RT in the RT-PCR for each primer set. PCR products present in the (-) lanes results from DNA contamination in the RNA sample. "Neg" in panel B refers to NTC reactions for each primer set, with (+) and without (-) MMLV-RT.

Laser Capture Microdissection

LCM was performed using CapSure™ transfer film with the PixCell II® LCM system (Arcturus Engineering, Mountain View, CA, USA). Cells were harvested from the invasive rim and the core of a frozen human brain tumor section (M. Berens, Barrow Neurological Institute, Phoenix, AZ, USA).

RNA Isolation

Total RNA was isolated using the Absolutely RNA™ microprep kit. Cells are disrupted in 100 µL of a lysis buffer containing guanidine thiocyanate, a strong protein denaturant, and 0.7 µL β-mercaptoethanol. Each sample is then mixed with an equal volume of 70% ethanol and applied to a spin cup where the RNA binds to a silica-based fiber matrix. After centrifugation and disposal of the flow-through, the fiber matrix is washed with 600 µL low-salt wash buffer and then dried with a 2-min centrifugation. DNase treatment is performed using 50 U DNase with 25 µL DNase digestion buffer in a 15-min incubation at 37°C to remove contaminating DNA. The fiber matrix is washed with 500 µL high-salt wash, followed by 600 and 300 µL low-salt wash to remove DNase and other contaminants. The spin cup is centrifuged at 13 000× *g* for 2 min to dry the fiber matrix. Highly pure RNA is eluted from the fiber matrix with 30 µL elution buffer (10 mM Tris, pH 7.5) (http://www.stratagene.com/manuals/400752.pdf).

Conventional RT-PCR

The ProSTAR™ first-strand RT-PCR kit was used for cDNA synthesis (http://www.stratagene.com/pcr/table/rt-pcr.htm). Each RNA sample was incubated with 60 ng random primers in a total volume of 16.4 µL at 65°C for 5 min, followed by slow cooling at room temperature (10 min) to allow the primers to anneal to the RNA. The remaining reaction components were then added to a final volume of 20 µL containing 1× first-strand buffer, 16 U RNase Block Ribonuclease Inhibitor, 1 mM each dNTP, and 20 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT), and the reaction was incubated at 37°C for 1 h. The reverse transcriptase was then inactivated by incubation at 90°C for 5 min. PCR was performed using Stratagene's Primer Sets for RT-PCR for genes of varying abundance levels (human) (http://www.stratagene.com/oligo/primer_probes.htm) using *Taq2000*™ DNA polymerase in a RoboCycler® 96 temperature cycler (http://www.stratagene.com/instruments/robocycler.htm) according to the instructions provided with the primers. Each PCR contained 1× *Taq* DNA polymerase buffer, 200 µM each dNTP, 25 pmol each primer, and 2.5 U *Taq2000* DNA polymerase. The reactions were run at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, followed by 10 min at 72°C. The samples were electrophoresed in 1.5% agarose gels.

Real-Time RT-PCR

RT-PCR analysis of total RNA was performed using the single-tube quantitative RT-PCR core reagent kit and the molecular beacon human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression analysis kit (http://www.stratagene.com/q_pcr/index.htm). Each 50-µL reaction contained 3

µL RNA, 5 µL 10× core RT-PCR buffer, 3.5 µL 50 mM $MgCl_2$, 2 µL human GAPDH primer mixture (30 pmol), 1 µL human GAPDH molecular beacon (25 pmol), 2 µL 20 mM dNTP mixture, 1.25 U StrataScript™ RT, 0.5 µL *Taq2000* DNA polymerase (2.5 U), and 300 mM final concentration of reference dye. The reactions were run in the ABI 7700 real-time thermal cycler (Applied Biosystems, Foster City, CA, USA) for 30 min at 45°C, 3 min at 95°C, and 40 cycles of 30 s at 95°C, 1 min at 55°C, and 30 s at 72°C.

Electrophoresis

Total RNA (200 ng) was electrophoresed in a 1.2% formaldehyde-agarose gel in a Joule Box™ electrophoresis apparatus (http://www.stratagene.com/instruments/joule_box.htm). The gel contained 1.2% agarose, 1× MOPS buffer (20 mM MOPS/8 mM sodium acetate/1 mM EDTA), 0.33 M formaldehyde. The RNA samples were mixed with 9 µL loading buffer [1× MOPS buffer, 50% formamide, 2.2 M formaldehyde, 10× loading dye (50% glycerol, 1 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), 20 µg/mL ethidium bromide], incubated at 60°C for 10–15 min and placed on ice before loading in the gel. Images of the gel were generated with the Eagle Eye® II still video system (http://www.stratagene.com/instruments/eagle_eye.htm).

RESULTS

Analysis of LCM Samples

Determination of gene expression patterns responsible for the invasiveness of a tumor is important for understanding tumor cell biology and development of cancer treatments. Comparison of gene expression in cells from the core relative to the invasive rim of a tumor can provide insight into genes important for tumor invasiveness. In a trial experiment to test the effectiveness of the Absolutely RNA method for small cell number samples, total RNA was isolated from LCM samples from the invasive rim and the noninvasive core of a human brain tumor section using the Absolutely RNA microprep kit. RNA of different abundance levels (1) was detected from samples of 200 and 1000 cells (Figure 1). For the 200-cell sample, high-abundance RNA (β-actin) was detected from a portion of the RT-PCR product equivalent to 2.5 cells, medium-abundance RNA (γ-actin) from a portion equivalent to 10 cells, and low-abundance RNA (protein phosphatase 1 and ADP ribosylation factor 1) from portions equivalent to 13 cells (Figure 1A). For the 1000-cell samples (Figure 1B), high- and medium-abundance RNA was detected from portions of the RT-PCR products equivalent to 11 cells, and low-abundance RNA (protein phosphatase 1, ADP ribosylation factor 1, and ornithine decarboxylase) was detected from portions equivalent to 21 cells. A comparison of cells from the invasive rim and the core of the brain tumor showed expression of these genes in both cell types (Figure 1B).

RNA from Tissue Culture Cells

RNA was isolated from 5×10^5 cells using the Absolutely RNA microprep kit and electrophoresed in a formaldehyde-agarose gel to check the integrity of the RNA. Cultured cell lines included human HeLa, THP-1 and HL-60 cells, mouse NIH/3T3 cells, and hamster CHO cells. All of the samples were intact, as observed by the distinct 28S and 18S ribosomal RNA bands (Figure 2).

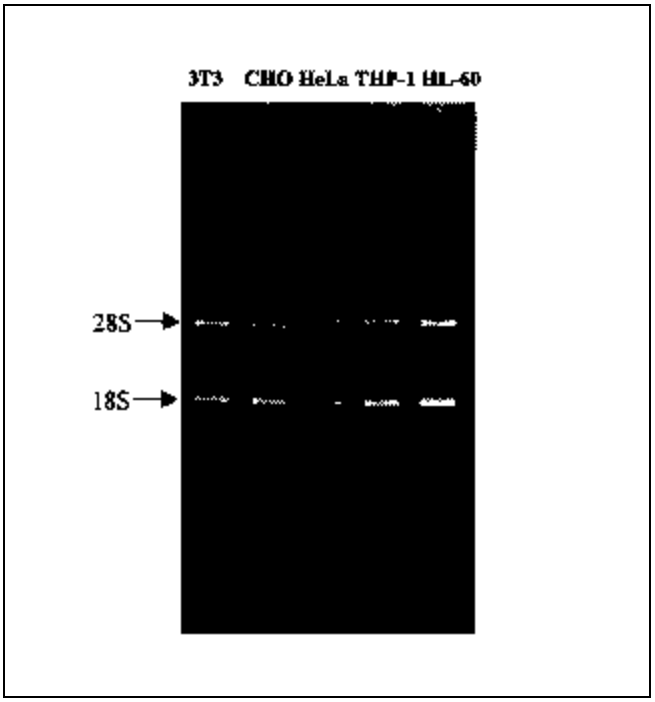

Figure 2. Formaldehyde-agarose gel electrophoresis of total RNA from different cell lines. RNA (200 ng) isolated from 5×10^5 cells was run in the gel in the presence of ethidium bromide and imaged using an Eagle Eye II still video system.

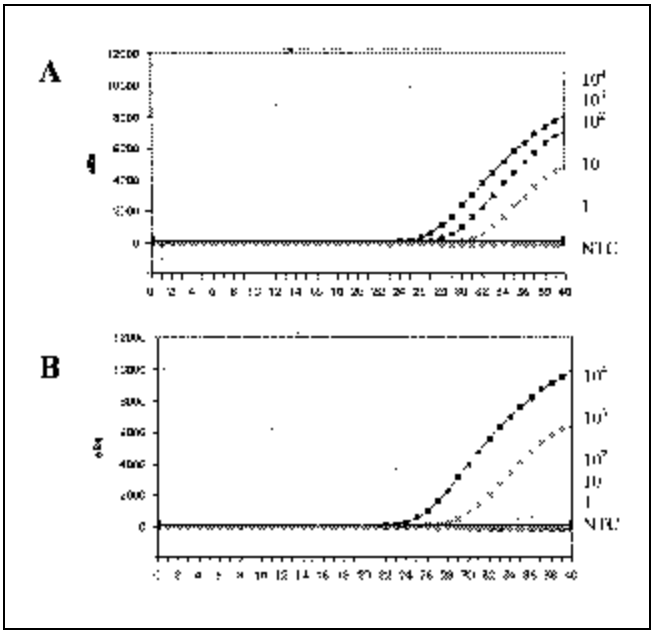

Figure 3. Real-time RT-PCR analysis of total RNA isolated from HeLa and THP-1 cells. One-tenth of the total RNA isolated from the equivalent of 10^4, 10^3, 10^2, 10, and 1 (A) HeLa and (B) THP-1 cells was used with the single-tube quantitative RT-PCR core reagent kit and the molecular beacon human GAPDH expression analysis kit. Aliquots equivalent to each cell number were taken from dilutions of cell lysates. ΔRn, total fluorescence; NTC, no template control.

Detection of RNA Using Real-Time RT-PCR

Quantitative, real-time RT-PCR is a powerful application for RNA analysis. In addition to high sensitivity, this method provides a means for comparing the quantity of a specific RNA sequence in different samples to each other and/or to a standard curve for absolute quantification. RNA from varying numbers of cultured HeLa and THP-1 cells was isolated using the Absolutely RNA microprep kit and subjected to real-time RT-PCR analysis by the molecular beacon method (8) using a fluorogenic probe (beacon) specific for human GAPDH. The beacon fluoresces where it hybridizes to the target sequence. The threshold cycle number (C_t) at which a fluorescence signal becomes detected is inversely proportional to the concentration of target sequence in the reaction (5). The signal for the first 3–10 cycles of the "no template" control (NTC) sample is designated as the baseline. The presence of the NTC signal as a line near zero indicates that no increase in background signal occurs over the course of the reaction, resulting in maximum sensitivity. The human GAPDH expression analysis kit specifically detects GAPDH mRNA sequences and not genomic DNA or pseudogene sequences (3). This kit can detect the human GAPDH mRNA target within 1 pg total RNA or 0.01 pg poly(A)$^+$ RNA (6). Using the Absolutely RNA microprep kit, GAPDH mRNA was readily detected from RNA samples isolated from the equivalent of one cell, with C_t values of 35 for HeLa and 36 for THP-1 cells. No signal was generated from the NTC sample (Figure 3). As expected, a consistent increase of C_t value was observed with decreasing cell numbers.

DISCUSSION

In this report, we demonstrate the suitability of the Absolutely RNA microprep kit for isolating RNA from small samples of cultured cells and LCM samples. The method generates pure intact RNA with ample yields for applications such as RT-PCR and gel electrophoresis.

Conventional RT-PCR is commonly used to detect the presence or absence of a particular RNA sequence, whereas real-time RT-PCR can measure the amount of a specific RNA with great sensitivity. Conventional RT-PCR was used for the detection of various RNAs in two regions of a brain tumor sample. Real-time RT-PCR was used to detect GAPDH RNA from the equivalent of one cell from two different cell lines. Considering that a typical cell contains approximately 10 pg RNA (7), these results show a high degree of sensitivity, reflecting the efficient recovery of pure RNA.

LCM is an effective method for harvesting individual cells from tissue sections. The method facilitates separation and isolation of adjacent cells within a tissue, thereby facilitating analysis of cellular components such as RNA. Because of the small size of these samples, efficient recovery of pure RNA is essential. Efficient RNA recovery was demonstrated by successful amplification using conventional RT-PCR of the RNA from as few as five cells and using molecular beacon quantitative RT-PCR from the equivalent of one cell. In addition, RNA isolated from malignant glioma cells using the Absolutely RNA microprep kit has been used in differential display experiments. These experiments identified several differentially expressed genes in the invasive rim of the tumor relative to the noninvasive tumor core (M. Berens, personal communication) (2).

Use of the Absolutely RNA microprep kit in conjunction with techniques such as LCM and RT-PCR provides the means for gene expression analysis of individual cells within a tissue. Furthermore, the use of such samples for microarray analysis will facilitate the generation of gene expression profiles for each distinguishable cell type within a tissue.

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