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Application Note

Evaluation of RNA Isolation Efficiency of Pall AcroPrep™ Advance 96-Well Long Tip Filter Plate for Nucleic Acid Binding

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1. Introduction

High throughput gene expression analysis is used in current genetic research to provide insights into normal, abnormal, or pathological cellular processes. Transcription of genes into RNA is an important step in the synthesis of functional gene products, which can be functional RNA species themselves or protein products formed after translation of messenger RNAs. In many high throughput gene expression analysis studies, cultured mammalian cells are exposed to different reagents or growth conditions, and reverse-transcription quantitative PCR (RT-qPCR) approaches are used to measure the influence of the various conditions on the expression of genes of interest. These types of studies are greatly aided by the availability of multi-well filter plates with silica based media that allow high throughput total RNA isolation from cultured mammalian cells in a robust fashion.

Here we evaluate the performance of the Pall AcroPrep Advance 96-Well Filter Plates for Nucleic Acid Binding (Pall NAB plate), which contain a silica-based quartz glass fiber media for the isolation of total RNA from cultured mouse bEnd.3 endothelioma cells (BEND3). The performance of the Pall NAB plate is compared to that of a commercially available plate from a total RNA isolation kit using the manufacturer's recommended protocol. Robustness of the Pall NAB plate as an RNA isolation media is further demonstrated through use of a protocol that relies on standard reagents that can readily be obtained and prepared in house by the researcher in an economically favorable manner. The latter protocol was also implemented in an industrial process to isolate RNA from mouse embryonic fibroblasts at a range of cell concentrations, which was subjected to one-step RT-qPCR assays for three genes. We show that RNA isolated with both methods is of high quality and suitable for a common downstream application, RT-qPCR, allowing detection of both rare and abundant messages.

2. Materials and Methods

2.1 Cell Culture

Mouse bEnd.3 endothelioma cells (ATCC CRL-2299◆) were maintained at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in humid atmosphere with 5% CO_2. On the day of the experiment, cells were harvested by a brief rinse with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution before adding 5 mL of Trypsin-EDTA solution. Once the cells began to detach, 25 mL of complete growth medium was added and cells were further dislodged and aspirated by gentle pipetting. Cell concentration was determined by hemocytometer and after appropriate dilution in DMEM w/o FBS, the cells were transferred to a 96-well V-bottom polypropylene microplate (Corning, PN 3357) at densities ranging from 3.1 to 400 x 10^3 cells/well. Just before commencing the RNA isolation protocol, cells were pelleted by centrifugation at 300 x g after which the medium was removed by careful aspiration.

2.2 Total RNA Isolation from Cultured Cells

2.2.1 Protocol 1 (Commercial Protocol)

Total RNA was isolated using the protocol from a commercially available kit for total RNA isolation. The protocol included a DNase I step to reduce possible contaminating genomic DNA. RNA was collected in a total elution volume of 140 µL. RNA samples were stored at -80 °C until further use.

2.2.2 Protocol 2 (Standard Reagent Protocol)

Cells were pelleted by centrifugation at 300 x g after which the medium was removed by careful aspiration. Lysis was achieved by addition of 150 µL of GTC Lysis Buffer (Thermo Fisher Scientific; 4M guanidine isothiocyanate, 50 mM Tris-HCl [pH 7.5], 25 mM EDTA) to each well of the microplate, followed by vigorously shaking the plate back and forth while keeping the microplate flat on the bench. One volume (150 µL) of 70% ethanol was added to the lysate and mixed thoroughly after which the entire volume was transferred to wells of the Pall NAB plate. RNA was allowed to bind to the media by vacuum filtration (15 in Hg). Two washes were carried out by adding 170 µL/well RNA wash buffer (60 mM Potassium Acetate, 10 mM Tris-HCl [pH7.5], 60% Ethanol) followed by vacuum filtration to clear buffer. DNase digestion was performed while RNA was bound to the membrane by pipetting 80 µL/well of 0.5 U/µL DNase I (Thermo Fisher Scientific) in 40mM Tris pH 7.5, 10 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$ onto the membrane and subsequently incubating it at room temperature for 20 min. The DNase was removed by two washes with 170 µL/well GTC Wash Buffer (0.25X GTC Lysis Buffer) followed by vacuum filtration to clear the buffer. Four additional washes were performed with 170 µL/well RNA Wash Buffer followed by vacuum filtration to clear buffer. The plate was then centrifuged until dryness for 2 min at (1,500 x g) in a Beckman Coulter Allegra◆ 6KR Centrifuge. RNAse free water (100 µL/well) was added and incubated for at least 1 minute after which RNA was eluted by centrifugation.

2.3 Evaluation of RNA samples

2.3.1 Concentration, Yield and Quality

RNA yield was determined fluorimetrically by means of the Quant-iT◆ RiboGreen◆ RNA Assay Kit (Thermo Fisher Scientific). RNA quality was assessed by separating samples on a PerkinElmer HT DNA 5K/RNA/Charge Variant Assay LabChip◆ with a PerkinElmer LabChip GX II Touch HT Instrument. The instrument's GX Touch software evaluated the RNA integrity by performing smear analyses on the electropherogram traces, and expressed the result as RNA Integrity Numbers (RIN).

2.3.2 RT-qPCR Analysis

Mouse bEnd.3 endothelioma cells are characterized by expression of Vascular cell adhesion molecule 1 (*Vcam1*). One-step RT-qPCR analysis was performed with AgPath-ID◆ One-Step RT-PCR reagents (Thermo Fisher Scientific) and PrimePCR◆ probe assays (Bio-Rad; Table 1) to determine message levels of *Vcam1* and of the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

Table 1

PrimePCR probe assay reagents

Type	Unique Assay ID	Description
Reference Gene Assay	qMmuCEP0039581	Gapdh, Mouse Glyceraldehyde-3-Phosphate Dehydrogenase
Experimental Gene Assay	qMmuCEP0055934	Vcam1, Mouse Vascular Cell Adhesion Molecule 1

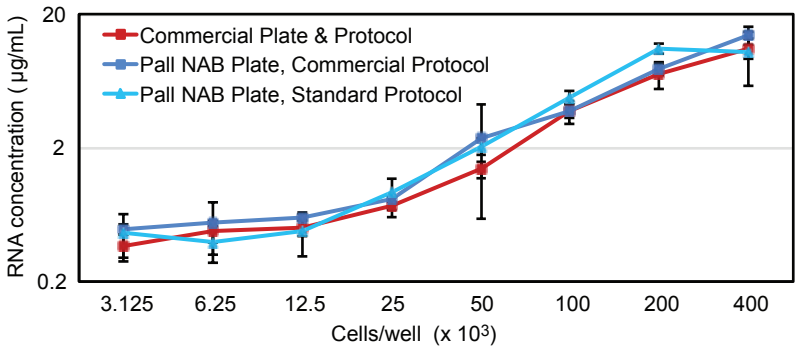
3. Results and Discussion

3.1 RNA Isolation

RNA isolation performance from cultured mouse bEnd.3 endothelioma cells was evaluated over a range of cell amounts employing two isolation protocols: the first with commercial reagents and the second with standard reagents prepared in house. Results of the RNA concentration measurements using the Quant-iT RiboGreen RNA assay are shown in Figure 1. The results indicate that regardless whether commercial reagents or standard reagents were used with the Pall NAB plate, RNA yields were similar to those obtained with the commercial kit.

Figure 1

RNA concentrations of samples isolated from mouse BEND3 cells



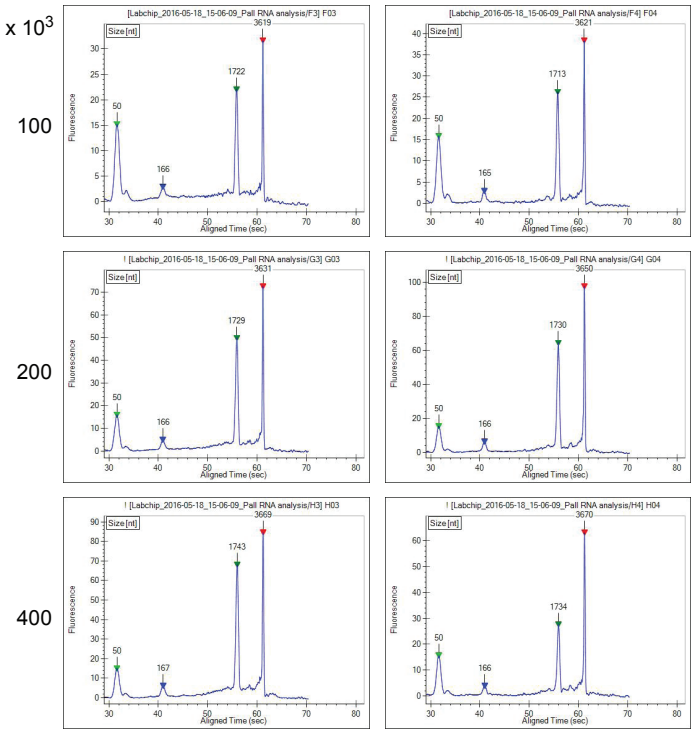
RNA concentrations of samples isolated from 3.1 - 400 x 10^3 cells were determined via Quant-iT RiboGreen RNA assay. Samples were isolated with Pall NAB plate using either a commercial reagent protocol (blue squares) or standard reagent protocol (blue triangles) or with a commercially available bundled plate/reagent kit (red squares).

RNA samples isolated with Pall's NAB plate were separated on a DNA 5K/RNA/CZE LabChip in the LabChip GX II Touch HT instrument to determine sample integrity. The instrument's GX Touch software further evaluated the RNA integrity by performing a smear analysis of the electropherogram traces and used an internal algorithm to calculate the RNA Integrity Number (RIN).The electropherograms show two distinct RNA peaks corresponding to 18S and 28S rRNA with little to no evidence of a smear to indicate RNA degradation (Figure 2A). For samples derived from 100 - 400 x 10^3 cells, RIN numbers varied from 8.0 to 9.6 (Figure 2B), indicating that the RNA is of high quality. For lower cell numbers, distinct peaks were observed, but the reduced signal to noise ratio did not allow accurate quality determination (data not shown).

Figure 2

LabChip Analysis demonstrating quality of BEND3 RNA isolated with Pall NAB plate.

A



B

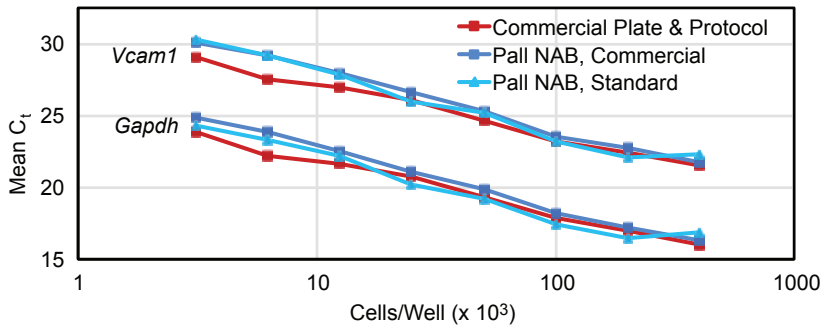
Cells ($\times 10^3$)	RIN number	
	Commercial Protocol	Standard Reagent Protocol
100	8.0	8.9
200	8.7	9.4
400	8.6	9.6

Panel A. LabChip electropherograms of RNA from 100 - 400 x 103 BEND3 cells isolated with either the Commercial Reagent Protocol or the Standard Reagent Protocol. Panel B. RNA integrity number

RNA integrity alone does not guarantee successful amplification in RT-qPCR application as co-purified inhibitors can impede reverse transcription and/or PCR efficiency. Mouse BEND3 cells are characterized by the expression of cellular adhesion protein *Vcam1*. The average C_t values showed a linear inverse relationship with increasing BEND3 cell numbers. In samples isolated with either protocol or plate, both the abundant housekeeping gene *Gapdh* message and the less abundant *Vcam1* message were detected and the slopes of the curves through points of RNA samples isolated from increasing numbers of cells were equal indicating that no inhibitory components copurified.

Figure 3

Expression of Gapdh and Vcam1 in mouse BEND3 cells as determined via RT-qPCR.

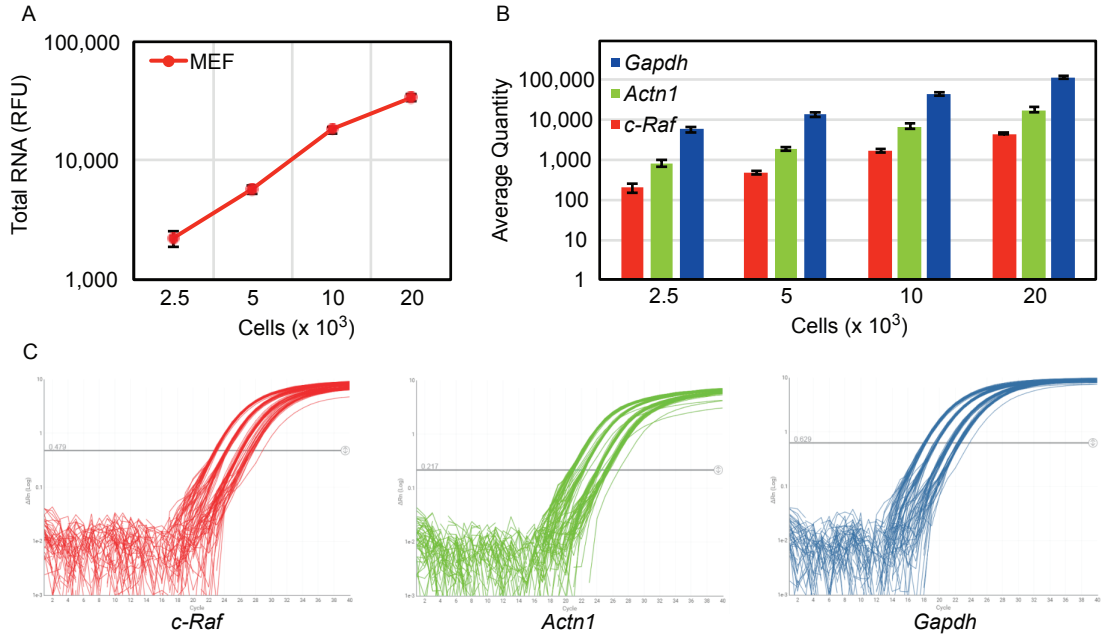


One-step RT-qPCR was carried out to detect messages for genes Gapdh and Vcam1 on mouse BEND3 cell RNA samples derived from 3.1 - 400 x 103 cells, isolated with Pall AcroPrep Advance Plates for Nucleic Acid Binding using either a commercial reagent protocol (blue squares) or standard reagent protocol (blue triangles) or with a commercially available bundled plate/reagent kit (red squares).

The standard reagent protocol was also implemented in an industrial process to isolate total RNA from mouse embryonic fibroblasts at a range of cell concentrations, which was subjected to one step RT qPCR assays to detect expression of genes *Gapdh, c-Raf* and *Actn1*. A linear relationship was observed between the cell amount and amount of total RNA (Fig. 4A). The amplification plots in the one-step RT-qPCR multiplex assays for *Gapdh, c-Raf* and *Actn1* show a tight grouping in four groups (Fig. 4C) corresponding to the number of cells used. The cell number dependent relative message increase is similar for the three genes (Fig 4B).

Figure 4

Expression of Gapdh, C-raf and Actn1 in mouse embryonic fibroblasts (MEF) as determined in automated application.



Panel A. RNA Concentrations expressed in relative fluorescent units (RFU) of samples isolated from mouse embryonic fibroblasts (MEF). Panel B. Expression of Gapdh, Actn1*, and* c-Raf *in MEF cells as determined via one step RT qPCR assays. Panel C. Amplification plots for* Gapdh, Actn1, *and* c-Raf *show tight grouping for different cell numbers. Data courtesy A. Watt, Ionis Pharmaceuticals*

4. Summary

- Pall AcroPrep Advance 96-well Long Tip Filter Plates for Nucleic Acid Binding can be used to purify total RNA of high quality from cultured mammalian cells.
- Pall AcroPrep Advance 96-well Long Tip Filter Plates for Nucleic Acid Binding demonstrate robust performance using total RNA isolation protocols relying on either commercial reagents or standard reagents that can be commonly obtained and prepared economically in house.
- RNA isolated with Pall AcroPrep Advance 96-well Long Tip Filter Plates for Nucleic Acid Binding is excellent for use in downstream applications as RT-qPCR allowing detection of both rare and abundant messages.

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