RNA Isolation from Mammalian Cells Using Porous Polymer Monoliths: An Approach for High-Throughput Automation

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2010

Impact Factor: 5.64 · DOI: 10.1021/ac100063f · Source: PubMed

6 AUTHORS, INCLUDING:

Anirban Chatterjee
Boston University
17 PUBLICATIONS **395** CITATIONS

SEE PROFILE

Alexis F. Sauer-Budge
Fraunhofer-Gesellschaft zur Förderung der…
22 PUBLICATIONS **331** CITATIONS

SEE PROFILE

Anal. Chem. **2010,** *82,* 4344–4356

RNA Isolation from Mammalian Cells Using Porous Polymer Monoliths: An Approach for High-Throughput Automation

Anirban Chatterjee,[†,‡] Paul L. Mirer,[‡] Elvira Zaldivar Santamaria,[‡] Catherine Klapperich,[†,§] Andre Sharon,[†,‡] and Alexis F. Sauer-Budge*[,‡,§]

Departments of Mechanical Engineering and Biomedical Engineering, Boston University, Boston, Massachusetts 02215, and Center for Manufacturing Innovation, Fraunhofer USA, Brookline, Massachusetts 02446

The life science and healthcare communities have been redefining the importance of ribonucleic acid (RNA) through the study of small molecule RNA (in RNAi/siRNA technologies), micro RNA (in cancer research and stem cell research), and mRNA (gene expression analysis for biologic drug targets). Research in this field increasingly requires efficient and high-throughput isolation techniques for RNA. Currently, several commercial kits are available for isolating RNA from cells. Although the quality and quantity of RNA yielded from these kits is sufficiently good for many purposes, limitations exist in terms of extraction efficiency from small cell populations and the ability to automate the extraction process. Traditionally, automating a process decreases the cost and personnel time while simultaneously increasing the throughput and reproducibility. As the RNA field matures, new methods for automating its extraction, especially from low cell numbers and in high throughput, are needed to achieve these improvements. The technology presented in this article is a step toward this goal. The method is based on a solid-phase extraction technology using a porous polymer monolith (PPM). A novel cell lysis approach and a larger binding surface throughout the PPM extraction column ensure a high yield from small starting samples, increasing sensitivity and reducing indirect costs in cell culture and sample storage. The method ensures a fast and simple procedure for RNA isolation from eukaryotic cells, with a high yield both in terms of quality and quantity. The technique is amenable to automation and streamlined workflow integration, with possible miniaturization of the sample handling process making it suitable for high-throughput applications.

With the completion of the genome sequencing projects, the focus of the life science and healthcare industry has shifted to "functional genomics". Efforts are now concentrated on the finer details of genomic information content and its possible use in healthcare and for drug development purposes.[1−7] New paradigms in the biological sciences, like the discovery of micro (mi)RNA as potential biomarkers, drug targets developed from messenger (m)RNA based gene expression analysis,[8] ribosomal (r)RNA analysis for evolutionary studies,[9] and RNA silencing (siRNA)[10] have increased the importance of RNA in the life science industry. Removal of government sanctions on stem cell research in U.S. academic laboratories is likely to further increase the demands for RNA based research.[11] These factors have contributed to the need for improved isolation techniques for mRNA and total RNA (including mRNA, rRNA, and tRNA-(t)RNA), as well as for novel technologies and methods for isolation of small molecule RNA and micro (mi)RNA.[12] As the downstream research moves toward high-throughput methods, large research centers and pharmaceutical and biotechnology companies will move to automated solutions to meet the goals of rapid and inexpensive isolation methods for nucleic acid sample preparation.

Quantitative RNA isolation remains a sensitive and critical process due to the ubiquitous presence of RNA-degrading enzymes (RNases) in aqueous buffers, on labware, and introduced via human handling (bare hands, dandruff, etc.). The remarkable stability of some forms of RNase even after regular sterilization

* Corresponding author. E-mail: asauerbudge@fraunhofer.org.
† Department of Mechanical Engineering, Boston University.
‡ Fraunhofer USA.
§ Department of Biomedical Engineering, Boston University.

(1) Toft, J. H.; Lian, I. A.; Tarca, A. L.; Erez, O.; Espinoza, J.; Eide, I. P.; Bjorge, L.; Draghici, S.; Romero, R.; Austgulen, R. *J. Matern. Fetal Neonatal Med.* **2008**, *21*, 267–273.
(2) An, C.; Saha, S.; Jenkins, J. N.; Scheffler, B. E.; Wilkins, T. A.; Stelly, D. M. *Mol. Genet. Genomics* **2007**, *278*, 539–553.
(3) Alvarez, H.; Corvalan, A.; Roa, J. C.; Argani, P.; Murillo, F.; Edwards, J.; Beaty, R.; Feldmann, G.; Hong, S. M.; Mullendore, M.; Roa, I.; Ibanez, L.; Pimentel, F.; Diaz, A.; Riggins, G. J.; Maitra, A. *Clin. Cancer Res.* **2008**, *14*, 2631–2638.
(4) Hu, Y. F.; Kaplow, J.; He, Y. *Curr. Mol. Med.* **2005**, *5*, 29–38.
(5) Bilello, J. A. *Curr. Mol. Med.* **2005**, *5*, 39–52.
(6) Bremnes, R. M.; Sirera, R.; Camps, C. *Lung Cancer* **2005**, *49*, 1–12.
(7) Myler, P. J. *Adv. Exp. Med. Biol.* **2008**, *625*, 133–140.
(8) Mandruzzato, S. *Adv. Exp. Med. Biol.* **2007**, *593*, 12–18.
(9) Cole, J. R.; Wang, Q.; Cardenas, E.; Fish, J.; Chai, B.; Farris, R. J.; Kulam-Syed-Mohideen, A. S.; McGarrell, D. M.; Marsh, T.; Garrity, G. M.; Tiedje, J. M. *Nucleic Acids Res.* **2009**, *37*, D141–145.
(10) Kleinman, M. E.; Yamada, K.; Takeda, A.; Chandrasekaran, V.; Nozaki, M.; Baffi, J. Z.; Albuquerque, R. J.; Yamasaki, S.; Itaya, M.; Pan, Y.; Appukuttan, B.; Gibbs, D.; Yang, Z.; Kariko, K.; Ambati, B. K.; Wilgus, T. A.; DiPietro, L. A.; Sakurai, E.; Zhang, K.; Smith, J. R.; Taylor, E. W.; Ambati, J. *Nature* **2008**, *452*, 591–597.
(11) Kim, K. S.; Kim, J. S.; Lee, M. R.; Jeong, H. S.; Kim, J. *FEBS J.* **2009**, *276*, 2140–2149.
(12) Dineva, M. A.; MahiLum-Tapay, L.; Lee, H. *Analyst* **2007**, *132*, 1193–1199.

10.1021/ac100063f
Published on Web 05/05/2010

methods such as autoclaving and 70% ethanol washes[13,14] also contributes to the difficulty in successful handling of RNA in the laboratory. To overcome the problems associated with RNA isolation, several kits are commercially available for safe and easy RNA isolation from a diverse variety of samples. The largest suppliers of such kits are Qiagen (Dusseldorf, Germany), Ambion (Austin, TX), Invitrogen (Carlsbad, CA), Roche Applied Science (Indianapolis, IN), and Promega (Madison, WI). The different kits can be used to isolate different types of RNA molecules (e.g., total RNA, miRNA, or mRNA) from different sources (e.g., cultured mammalian and nonmammalian cells, animal tissues, blood and other biological fluids, fungi, plant cells, bacteria) and provide very similar RNA yield in terms of quality and quantity.[15]

Most of the existing kits either use silica-based solid phase extraction (SPE) technology or a magnetic-bead based extraction technology.[16] Such processes are nondiscriminatory for DNA or RNA, and in most cases the isolation of pure RNA is achieved by a secondary enrichment, either through enzymatic removal of DNA (DNase treatment) or by a second set of columns specific to mRNA or miRNA.[17] In the case of mRNA isolation, the most widely used method is to use the poly-A tails of eukaryotic mRNA by complementary base pairing through a poly-T affinity column.[18] Also available are phenol-based RNA isolation reagents[19] such as Trizol (Invitrogen), which have higher yields than the SPE kits. However these phenol-based products are extremely hazardous and difficult to automate for high-throughput purposes. New microfluidic approaches, such as arrays of columns similar to 96-well microtiter plates,[20] are also being developed.

The advantage of the nucleic acid isolation kits based on SPE columns is the ability to automate[21] them by developing equipment to replace the human user. Several such kits or kit-based techniques have been adopted for medium-throughput automation (up to a few hundred samples a day).[22] Qiagen has introduced an automated nucleic acid isolation device "Qiacube", which can handle up to 12 samples at a time using already-existing Qiagen kits for nucleic acid isolation. Qiagen also has a series of magnetic bead based nucleic acid isolation platforms like the Biorobot MDX (8 channels at a time, 96 samples) and the Biorobot Ez1 advanced (14 samples).[23,24] Similar automated instruments are available from other companies including the iPrep (Invitrogen), Maxwell 16 (Promega), and ABI 6700 (Applied Biosystems, Foster City, CA,). All of these instruments use existing nucleic acid isolation kits from their parent companies, with inherent limitations in the yield efficiencies, the inability to handle small samples, and the moderately long cycle times. For some of the instruments, significant parts of the process still require manual steps (e.g., centrifugation, cell lysis, and tissue homogenization), thus limiting the scope of the automation. Such problems have not only limited throughput levels for DNA or RNA isolation samples but have also limited researchers' abilities to analyze rare samples (small stem cell colonies, small cancerous growths, etc.).

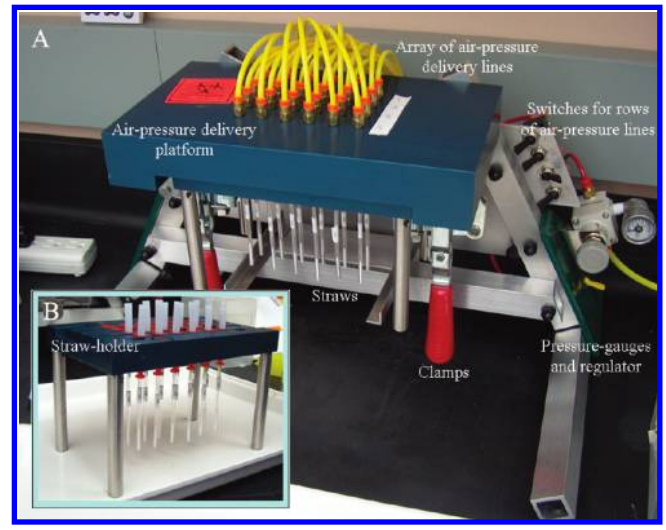

Figure 1. HT-SNAP pressure array (A) with straw holder engaged for pressurization. (B) Straw holder with attached straws and reservoirs disengaged for sample and buffer introduction.

Recent research has focused on modifying solid-phase DNA[25] or RNA isolation processes[26] to suit four major needs of the life science industry: higher throughput (parallel sample processing), higher yield, complete automation, and smaller sample handling capability. Some of the novel research in this area has been in microchip-based silica-embedded channels,[27,28] various chemical methods for nucleic acid precipitation,[29] silica nanoparticles,[30] and magnetic particles on a robotic platform.[31] None of these efforts have, however, tried to meet all of these needs in one system.

In this article, we present a new method for RNA purification from mammalian cells, which combines a low-cost solid phase extraction technology, a new pressure array design, and a different approach in sample handling to address all four of these needs (parallel sample processing, high yield, automatable, and small sample sizes). The system comprises a proof of concept pressure array called the "high-throughput system for nucleic acid purification" (HT-SNAP, Figure 1) driven by compressed air and a set of disposable low-cost plastic cylinders or "straws" for sample handling. The following work is broadly divided into four parts, (1) engineering of the HT-SNAP assembly and straws, (2)

(13) Skrypina, N. A.; Timofeeva, A. V.; Khaspekov, G. L.; Savochkina, L. P.; Beabealashvilli, R. *J. Biotechnol.* **2003**, *105*, 1–9.
(14) Mutiu, A. I.; Brandl, C. J. *J. Biomol. Tech.* **2005**, *16*, 316–317.
(15) Guarino, H.; Goyal, S. M.; Murtaugh, M. P. *New Microbiol.* **1997**, *20*, 319–324.
(16) Boom, R.; Sol, C. J.; Salimans, M. M.; Jansen, C. L.; Wertheim-van Dillen, P. M.; van der Noordaa, J. *J. Clin. Microbiol.* **1990**, *28*, 495–503.
(17) Chen, J.; Lozach, J.; Garcia, E. W.; Barnes, B.; Luo, S.; Mikoulitch, I.; Zhou, L.; Schroth, G.; Fan, J. B. *Nucleic Acids Res.* **2008**, *36*, e87.
(18) Rosenthal, L. J. *Nucleic Acids Res.* **1976**, *3*, 661–676.
(19) Robins, H. I.; McNutt, W. S. *Anal. Biochem.* **1974**, *57*, 636–639.
(20) Witek, M. A.; Hupert, M. L.; Park, D. S.; Fears, K.; Murphy, M. C.; Soper, S. A. *Anal. Chem.* **2008**, *80*, 3483–3491.
(21) Ullmann, S.; Hage, T.; Draheim, R.; Egerland, U.; Oelmuller, U.; Brune, K.; Pahl, A. *J. Biomol. Screen.* **2004**, *9*, 95–102.
(22) Hilbert, H.; Lauber, J.; Lubenow, H.; Dusterhoft, A. *DNA Seq.* **2000**, *11*, 193–197.
(23) Anslinger, K.; Bayer, B.; Rolf, B.; Keil, W.; Eisenmenger, W. *Leg. Med.* **2005**, *7*, 164–168.
(24) Mitsunaga, S.; Fujimura, K.; Matsumoto, C.; Shiozawa, R.; Hirakawa, S.; Nakajima, K.; Tadokoro, K.; Juji, T. *Transfusion* **2002**, *42*, 100–106.
(25) Tian, H.; Huhmer, A. F.; Landers, J. P. *Anal. Biochem.* **2000**, *283*, 175–191.
(26) Peirson, S. N.; Butler, J. N. *Methods Mol. Biol.* **2007**, *362*, 315–327.
(27) Hagan, K. A.; Bienvenue, J. M.; Moskaluk, C. A.; Landers, J. P. *Anal. Chem.* **2008**, *80*, 8453–8460.
(28) Satterfield, B. C.; Stern, S.; Caplan, M. R.; Hukari, K. W.; West, J. A. *Anal. Chem.* **2007**, *79*, 6230–6235.
(29) Lee, M.; Huh, N.; Kim, J. H. *Anal. Biochem.* **2008**, *381*, 160–162.
(30) Tan, W.; Wang, K.; He, X.; Zhao, X. J.; Drake, T.; Wang, L.; Bagwe, R. P. *Med. Res. Rev.* **2004**, *24*, 621–638.
(31) Jacobsen, N.; Nielsen, P. S.; Jeffares, D. C.; Eriksen, J.; Ohlsson, H.; Arctander, P.; Kauppinen, S. *Nucleic Acids Res.* **2004**, *32*, e64.

optimization of mammalian cell lysis using the HT-SNAP, (3) optimization of RNA isolation using HT-SNAP, and (4) mammalian RNA isolation using the combined cell-lysis/RNA isolation process compared to a commercially available kit.

The solid-phase extraction straws contain columns of porous polymer monolith (PPM) embedded with silica microspheres for nucleic acid binding.[32,33] PPM technology for the extraction of nucleic acids has been developed for the microscale format,[27,28,32−39] highlighting the utility of PPM for microfluidic applications, including reduction of associated assay costs and time. This work has been focused on individual microfluidic channels embedded in chips for lab-on-a-chip applications. In contrast, here we present a form factor that can be used in arrays for high-throughput applications. We have focused on small samples (<1 μg RNA) for high-throughput applications that typically require small sample usage to be economical. A set of buffers, reagents, and working protocols for cell lysis and nucleic acid binding are presented to complete the RNA isolation process from mammalian cells. We demonstrate superior RNA extraction (both quality and quantity) for small samples of mammalian cells as compared to a commonly used commercial kit in a form factor amenable to high-throughput automation.

EXPERIMENTAL SECTION

Reagents. All buffers used for the experiment were prepared with sterilized water treated overnight with diethylpyrocarbonate (DEPC). The compositions of common buffers are mentioned here; all specific buffers are mentioned in the relevant methods section. These include Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), RNA elution buffer (TE buffer with RNAsecure, Ambion), cell lysis buffer (0.1% Triton X-100 in RNA elution buffer). Other reagents used in the process include RNase-free DNase and RNase reaction buffer (Ambion), 70% ethanol, 2-propanol, and 3 M ammonium acetate solution pH 5.2 (Sigma-Aldrich, St. Louis, MO). All surfaces were cleaned with RNaseZAP solution (Ambion). All tips, microfuge, and centrifuge tubes used were guaranteed DNase-, RNase-, and pyrogen-free (Thermo Fisher Scientific, Waltham, MA).

Pretreatment of Straws. The PPM is formed as a porous cylinder inside an extruded cylindrical "straw" (Figure 2A−D). The straw forms a channel to deliver sample and buffers to the PPM. Each straw is 120 mm long and 3 mm in outer diameter; with either a 1 mm or 2 mm inner diameter. The straw is made from Zeonex 690R (Zeon Chemicals, Japan), a cyclic olefin polymer which is transparent to both visible and UV light and which is compatible with bioassays.[40,41] The material was extruded into straws by a commercial extruder (Argos Corp, Wareham, MA).

To prepare the straws for PPM polymerization, the extruded straws are first annealed to reduce internal stresses that form from rapid cooling during the extrusion process. The straws craze during UV curing without annealing. To anneal, the straws are heated in an oven to 165 °C and held at that temperature for 1 h. The oven is switched off and allowed to cool for 2−3 h before the straws are removed. Grooves are introduced to the inside surface of the annealed straws by scoring with a drill to retain the PPM when pressure is applied.

PPM Embedded Straw Manufacturing. The PPM was manufactured as previously published[33,34] and is briefly described here. The PPM is made in a two-step process that first prepares the channel side walls with a thin layer of grafted polymer, followed by filling the channel with a UV-cured monolith. Butyl methacrylate (99%, BuMA), ethylene dimethacrylate (98%, EDMA), ethylene diacrylate (90%, EDA), methyl methacrylate (99%, MMA), 1-dodecanol (98%), cyclohexanol (99%), benzophenone (99%), and 2,2-dimethoxy-2-phenylacetophenone (99%, DMPAP) were purchased from Sigma-Aldrich. The grafting solution was prepared with 3.88 mL of MMA and 120 mL of melted benzophenone. The grooved surface of the straws are coated with the grafting solution,[33] which is then polymerized (cured) by ultraviolet (UV) irradiation via a photoinitiated reaction for 10 min at 3mW/cm^2 in a CL-1000 cross-linker (UVP, Inc., Upland, CA). Next, the straws are attached to the HT-SNAP pressure array (described in the next section) and pressurized at 30 psi to remove excess grafting solution. After washing, the straws are dried with airflow at 30 psi.

Three different types of PPM were used for RNA isolation: (1) solid phase extraction (SPE) with embedded silica particles, (2) SPE-like PPM but without any silica used as a support for oligo-dT cellulose,[42] and (3) lysis PPM without any silica particles. The SPE monomer mixture was prepared by mixing BuMA (960 μL), EDMA (640 μL), 1-dodecanol (1680 μL), cyclohexanol (720 μL), and melted DMPAP (16 μL). An aqueous solution (4.0 mL) of silica (0.7 or 3 μm) microspheres (Polysciences, Inc., Warrington, PA) were centrifuged at 2500*g* for 10 min, the supernatant decanted, and then dried at 120 °C for 1 h to a hard pill. The pill was broken into a powder, mixed with the SPE monomer mixture, and sonicated in a Branson sonicator until silica dispersed (2−3 min). The SPE straws for mRNA isolation are similar to the SPE straws for total RNA isolation, but they lack the silica particles in the PPM emulsion. The lysis monomer mixture was prepared with BuMA (600 μL), EDMA (400 μL), 1-dodecanol (2100 μL), cyclohexanol (450 μL), and melted DMPAP (45.2 μL). A volume of 450 μL of carbon nanotubes (0.22% weight by volume) was then suspended by sonication to create an emulsion. The carbon nanotubes were included to allow a direct comparison to previous work, but their utility was not studied here.

Lysis straws and SPE straws are prepared by pipetting the lysis emulsion[33] (16 μL) or solid-phase extraction emulsion[33] (32 μL)

(32) Kulinski, M. D.; Mahalanabis, M.; Gillers, S.; Zhang, J. Y.; Singh, S.; Klapperich, C. M. *Biomed. Microdevices* **2009**, *11*, 671–678.
(33) Bhattacharyya, A.; Klapperich, C. M. *Anal. Chem.* **2006**, *78*, 788–792.
(34) Sauer-Budge, A.; Mirer, P.; Chatterjee, A.; Klapperich, C.; Chargin, D.; Sharon, A. *Lab Chip* **2009**, *9*, 2803–2810.
(35) Mahalanabis, M.; Al-Muayad, H.; Kulinski, M. D.; Altman, D.; Klapperich, C. M. *Lab Chip* **2009**, *9*, 2811–2817.
(36) Bhattacharyya, A.; Klapperich, C. M. *Sens. Actuators, B: Chem.* **2008**, *129*, 693–698.
(37) Wen, J.; Guillo, C.; Ferrance, J. P.; Landers, J. P. *Anal. Chem.* **2006**, *78*, 1673–1681.
(38) Wen, J.; Guillo, C.; Ferrance, J. P.; Landers, J. P. *Anal. Chem.* **2007**, *79*, 6135–6142.
(39) Wu, Q.; Bienvenue, J. M.; Hassan, B. J.; Kwok, Y. C.; Giordano, B. C.; Norris, P. M.; Landers, J. P.; Ferrance, J. P. *Anal. Chem.* **2006**, *78*, 5704–5710.
(40) Diaz-Quijada, G. A.; Peytavi, R.; Nantel, A.; Roy, E.; Bergeron, M. G.; Dumoulin, M. M.; Veres, T. *Lab Chip* **2007**, *7*, 856–862.
(41) Nikolov, I. D.; Ivanov, C. D. *Appl. Opt.* **2000**, *39*, 2067–2070.
(42) Bhattacharyya, A.; Klapperich, C. M. In *31st Annual EMBS Conference*, Proceedings of the IEEE, Minneapolis, MN, September 2−6, 2009.

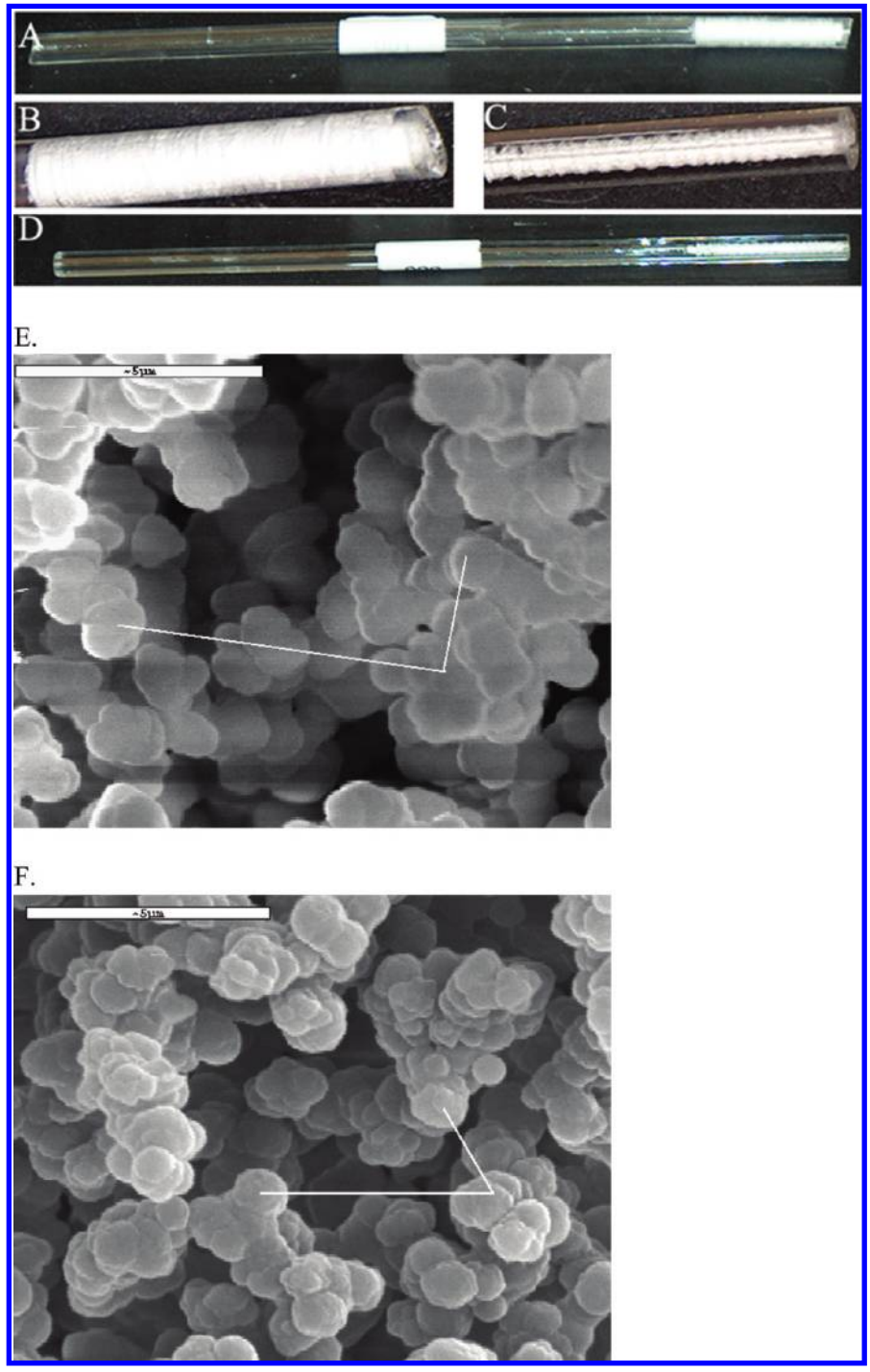

Figure 2. SPE straw (A) and close-up of SPE PPM (B) with a 2 mm inner diameter. Close-up of lysis PPM (C) and lysis straw (D) with a 1 mm inner diameter. Scanning electron micrograph of lysis PPM (E). Lines indicate pores in the monolith structure with pore sizes (in comparison to the bar) between 5−10 μm. Scanning electron micrograph of SPE PPM (F). Lines indicate pores in the monolith structure with pore sizes (in comparison to the bar) between 1 and 3 μm.

into the grafted region of the straws and curing by UV-irradiation for 15 min per side at 3mW/cm^2. Lysis emulsion is used in straws with 1 mm inner diameter, and SPE emulsion (with or without silica) is used in straws with 2 mm inner diameter. The straws are next attached to the HT-SNAP pressure array and each straw is washed with methanol (200 μL) at 30 psi pressure to remove excess unreacted material. The straws are finally air-dried by blowing air through the pressure array for 2 min at 30 psi of pressure. All straws were treated with 0.1% DEPC overnight to remove any RNase contamination and sterilized by autoclaving before use. After creation of the silica-free monolith, the mRNA isolation straws were packed with an oligo-dT cellulose bead (100 μL) solution (Ambion).

HT-SNAP Pressure Array. The HT-SNAP pressure array is designed to drive liquids through the PPM straws using compressed air. To operate the pressure array, the user loads a straw by pushing it through a one-touch fitting (SMC Corporation of America, Noblesville, IN), a standard component used to connect

pneumatic tubes. The fitting is specially modified by drilling through the stop, so that the straw may pass entirely through the fitting. The fitting locks the straw in place and withstands pressures up to 200 psi. A short section of Teflon tubing is then placed over the top of the straw to form a cuplike reservoir. The reservoir has a capacity of 200 μL and is filled with sample or buffer using a standard micropipet. The reservoir fits in a pressure chamber, sealed with o-rings, and is connected to a source of pressurized air, which is controlled by a regulator. When air pressure is turned on, the liquid is pressurized and forced down into the straw and through the PPM into a standard microtiter plate (96-well or 24-well) for further processing. The HT-SNAP can hold up to 24 straws, which allows the user to conduct multiple experiments in parallel.

Cell Culture. To standardize the process of total RNA and mRNA isolation from mammalian cells, the Madin-Darby Canine Kidney (MDCK) epithelial cell line was selected, as its adhesive properties make it a good model for cultured mammalian cells. The MDCK cells were grown to about 80% confluency at 37 °C in Modified Eagle's Media (MEM) supplemented with 10% fetal bovine serum (FBS), amino acids (1×), penicillin G (100 U/mL) and streptomycin (100 μg/mL), sodium bicarbonate (0.15%), and sodium pyruvate (2 mM) (Lonza, Allendale, NJ). The pH was controlled through 5% CO_2 atmosphere.[43] The media was aspirated off, and the cells were washed with phosphate buffered saline (PBS) and treated with trypsin solution at 37 °C for 5 min. The cells released by trypsin treatment were resuspended in MEM for RNA isolation. To quantify cell numbers, cells were counted on a hemocytometer with trypan blue staining.

Fluorescence Microscopy. Harvested MDCK cells were washed with phosphate buffered saline (PBS) and surface stained with Alexafluor-488 (Invitrogen) in 100 mM carbonate/bicarbonate buffer at pH 9.3 (30 min at room temperature). The stained cells were fixed with 2% paraformaldehyde solution in PBS (2 min on ice) and permeabilized with 0.1% Triton X-100 solution in PBS (2 min on ice). The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific).

Fluorescence microscopy was performed on cells (or cell lysates) using an Olympus IX81 microscope with a color imaging system. Results were analyzed using the ImageJ software available free of cost from the U.S. National Institutes of Health.

Total RNA Isolation. Harvested MDCK cells (typically 100 μL) were lysed by mixing with lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and RNAsecure RNase inhibitor reagent (Ambion) and then passing the mixture through lysis straws at 50 psi. The cell lysate was heated to 60 °C for 10 min to activate the RNase inhibitor in a 96-well microtiter plate, followed by treatment with DNase 1 (New England Biolabs, Ipswich, MA) for 20 min at 37 °C to degrade contaminating genomic DNA. To bind the total RNA with the silica particles in the solid-phase PPM, the lysate is mixed with an equal volume of total-RNA-loading buffer (95% 2-propanol, 5% 3 M ammonium acetate, pH 5.2) and 200 μL of mixed lysate were passed through SPE straws at a pressure of 5−10 psi. Straws are washed with RNA-washing buffer of 70% ethanol (600 μL) at 10 psi pressure. The total RNA is incubated with elution buffer (10 mM Tri-HCl pH 8.0, 1 mM EDTA, 1× RNAsecure reagent) for 2−3 min and eluted with two aliquots of 50 μL of elution buffer at 10 psi. After elution, the sample is heated to 60 °C for 10 min to activate the RNase inhibitor.

RNA samples were separated on a 6% urea-polyacrylamide denaturing gel (Invitrogen) in the presence of Tris-borate-EDTA buffer as per manufacturer's protocol. A 100 bp ladder was used as a molecular weight standard, and the gels were stained with SYBR-green (II) RNA-staining dye (Invitrogen).

RNA concentrations were measured by the UV-absorbance at 260 nm (with control measurement for protein contaminations at 280 nm) using a Nanodrop spectrophotometer (NanoDrop ND-1000 UV−vis spectrophotometer, Thermo Fisher Scientific).

mRNA Isolation. Harvested MDCK cells were lysed as described earlier in the total RNA isolation section. Treated cell lysate was mixed with an equal volume of mRNA-loading buffer (1 M NaCl, 20 mM Tri-HCl pH 8.0, 2 mM EDTA, and 2× RNAsecure). The mixed lysate was passed through SPE straws at a pressure of 5−10 psi to bind with oligo-dT cellulose beads packed inside straws. The straws were washed with 600 μL of mRNA-washing buffer (500 mM NaCl, 10 mM Tri-HCl pH 8.0, 1 mM EDTA, 1× RNAsecure reagent) at 10 psi. The mRNA is incubated with elution buffer (10 mM Tri-HCl pH 8.0, 1 mM EDTA, 1× RNAsecure reagent) for 2−3 min and eluted with two aliquots of elution buffer (50 μL) at 10 psi. The sample was heated to 60 °C for 10 min to activate the RNase inhibitor.

cDNA Synthesis and Real-Time PCR. cDNA synthesis was performed on the purified RNA samples (both from total RNA and mRNA) using either the M-MuLV reverse transcriptase cDNA synthesis kit (New England Biolabs) or the Superscript II cDNA synthesis kit (Invitrogen), using 18−23mer oligo-dT primers, as per the manufacturer's instructions. The final volume was 20 μL with 5 μL of RNA sample in each reaction mix. Samples mixed with real-time PCR reagents without reverse transcriptase were used to control for genomic DNA contamination.

We chose the canine β-actin gene as our target for MDCK RNA analysis. The primer sequences were 5′-GACTCAGATCAT-GTTCGAGACGTT-3′ and 5′-GGACAGCACAGCCTGGAT-3′ and that of the probe is 5′-FAM-CAACACCCCAGCCATGTAT-NFQ-3′. PCR was designed using the custom primer design tool for Taqman assays (Applied Biosystems) for a 75bp product. The Taqman assay primers and the Taqman assay master mix were purchased from Applied Biosystems, and the PCR reactions were run on an ABI7300 (Applied Biosystems) real-time PCR instrument as per the manufacturer's recommended protocol as follows: a 50 °C hold for 3 min, a 95 °C hold for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s, and a final hold at 4 °C. The PCR results were analyzed using the automated threshold cycle (C_T) value calculation algorithm in the SDS v1.4 software (Applied Biosystems). PCR products were electrophoresed on a 6% polyacrylamide gel (Invitrogen) in the presence of Tris-borate-EDTA buffer as per the manufacturer's protocol, with a 25 bp DNA ladder (Invitrogen) as a molecular weight standard, and stained with SYBR green(I) DNA binding dye.

(43) Cho, M. J.; Thompson, D. P.; Cramer, C. T.; Vidmar, T. J.; Scieszka, J. F. *Pharm. Res.* **1989**, *6*, 71–77.

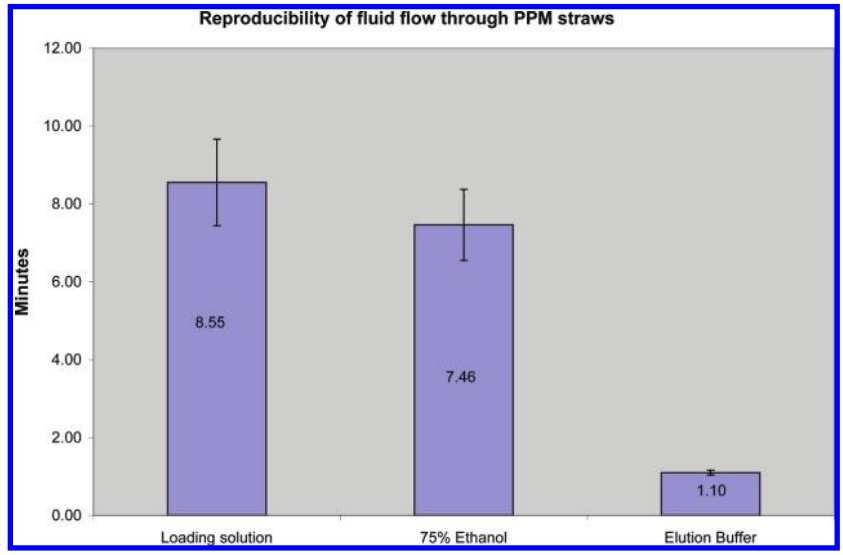

Figure 3. Various fluids involved in the RNA extraction protocol were passed through 24 different SPE channels made in 2 different batches, and the flow time was recorded to calculate flow rate variations. The loading solution (3 M GuSCN, 50% isopropanol) showed the largest variation with a standard deviation of 1.1 min, followed by the washing buffer (75% ethanol, standard deviation = 0.9 min) and the elution buffer (1× TE) having a standard deviation of 0.06 min.

RESULTS AND DISCUSSION

Engineering of HT-SNAP Assembly and Straws. *HT-SNAP Pressure Array.* The HT-SNAP pressure array (Figure 1) was designed as a proof-of-concept for an automated instrument to operate arrays of disposable "straws" containing PPM columns to isolate nucleic acids in a high-throughput manner. To date, the work with mini-solid phase extraction columns based on PPM[27,28,32−39,44−46] has been focused on integrated microfluidics and not on a form factor that allows parallel sample processing. The simple design of the pressure array allowed us to test and prove the concept of using pressurized air for driving liquids (sample and buffers) through the PPM in the straw form factor. The use of 10−100 psi of pressurized air allows for easy cell lysis, sample loading, washing, and elution of the isolated nucleic acids. To eliminate the risk of contamination, the system is designed so that the sample never comes into contact with the pressure array itself. Rather, the samples are contained within single use, disposable straws and reservoirs. This approach separates the sample from the rest of the reagents and will reduce or eliminate contamination.

To use the HT-SNAP, the straw holder (Figure 1B) is loaded with disposable straws and reservoirs. The liquid (sample, wash buffer, elution buffer, etc.) to be passed over the straws is pipetted into the reservoirs. The straw holder is then mounted to the HT-SNAP frame by two clamps. Alignment and placement is assisted by magnets on the underside of the frame. Once the straw holder is clamped in place, pressure is applied with pressurized air. The pressure is controlled by a pressure gauge and four switches that allow the rows of straws to be individually addressed. The liquid that passes through the straws can be discarded as waste or captured in a 96-well plate placed beneath the straw array. The array was designed so that the spacing of the straws would match the pitch of a 96-well plate. When the user wishes to pass the next solution over the array, the pressure is turned off by flipping the switches and the straw holder is removed after disengaging the clamps. The next solution can then be pipetted into the reservoirs.

Path to High-Throughput Sample Isolation. The straws and the reservoir are envisioned to be combined into a single molded disposable component. The array is capable of parallel handling of multiple samples, 24 in the proof-of-concept HT-SNAP, and easily expandable to 96 or 384. The form factor of a straw, driven by a pressure array, allows us to integrate the fluidics needed for reagent delivery with pipetting action required for sample handling from polymerase chain reaction (PCR) or assay plates. The same pressure array can thus be utilized for pipetting, dispensing, and pressurizing for cell lysis/RNA isolation. One can easily envision an instrument that would automate the loading of straw arrays, collection of waste and eluted purified nucleic acids, and all relevant fluid handling. The operation of the pressure array requires no off-line steps like centrifugation or organic phase separation, making it suitable for easy automation and scale-up.

Reproducibility of Fluid Flow through PPM. Since all downstream actions and performances of the array are dependent upon uniform fluid flow through the straw system, reproducibility of flow-rates was assessed for various fluids on a set of 24 different SPE channels made in 2 different manufacturing batches (Figure 3). The variation in time between the different porous polymer monoliths was largest for the loading solution (10 ng of *Bacillus subtilis* genomic DNA, 3 M GuSCN, 50% isopropanol) with a standard deviation of 1.1 min, followed by that of the 75% ethanol (standard deviation of 0.9 min), and the smallest variation with the elution buffer (1× TE) having a standard deviation of 0.06 min. The variation in flow rate is a function of the length of the column, the reagent, the structure of the PPM, the variation caused by the scratching of the sidewall, etc. Continuing optimization in reaction components and manufacturing methods suggest that the manufacturing reproducibility could be further improved,

(44) Hagan, K. A.; Meier, W. L.; Ferrance, J. P.; Landers, J. P. *Anal. Chem.* **2009**, *81*, 5249–5256.
(45) Easley, C. J.; Karlinsey, J. M.; Bienvenue, J. M.; Legendre, L. A.; Roper, M. G.; Feldman, S. H.; Hughes, M. A.; Hewlett, E. L.; Merkel, T. J.; Ferrance, J. P.; Landers, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 19272–19277.
(46) Gillers, S.; Atkinson, C. D.; Klapperich, C.; Singh, S. K. *Gastroenterology* **2008**, *134*, A-578.

and in high volume manufacturing designs, we would replace cylindrical straws with conical (pipet tip like) straws which do not need the scratching of the sidewalls.

Implications of Different Porous Polymer Monolith Morphologies. By varying the proportion of monomers and the organic solvents in the preparation of the monolith, one can adjust the average pore size of the resulting PPM. Previous work describes the pore size distribution of the SPE PPM[32] as 2.56 μm ± 1.00, which was designed for bacterial cell lysis. Initial experiments using the published PPM composition resulted in a high failure rate due to clogging, primarily because of the larger size of mammalian cells (>10 μm) compared to the PPM pore size (1−3 μm). Thus, we modified composition of the lysis straws to have a larger average pore size by varying the ratio of the BuMA and EDMA monomers. The scanning electron micrograph (SEM) of the lysis PPM (Figure 2E) shows an estimated pore size distribution of 5−10 μm in the lysis PPM. We postulate that the MDCK cells are squeezed through the 5−10 μm pore sizes of the lysis PPM, and the detergent stressed membranes are disrupted by the abrasions caused by moving through the lysis PPM.

After lysis, the lysate is passed over a SPE PPM and the released nucleic acids bind to the silica particles. The composition of the SPE PPM was the same as previously described.[32−34] Two major parameters influencing binding are the available silica particle surface area and the interaction time between the nucleic acids and the silica particles. The SEMs of the SPE PPM used here confirmed the pore size of 1−3 μm (Figure 2F) as expected.[32] The smaller pore sizes of the SPE PPM as compared to the lysis PPM result in slower flow rates at a given pressure thereby increasing the interaction time between the nucleic acids and the silica. We have optimized the SPE straw pressurization to maximize RNA yield. Future optimizations will include varying the silica particle size (and thus surface area to volume ratio), silica particle concentration, and other parameters effecting binding (i.e., hydrophobicity and ionic strength of the binding buffer, temperature, etc.).

Optimization of Mammalian Cell Lysis. *Buffer Selection.* Mammalian cell lysis is the important first step in RNA isolation process. Ideally, the cell membrane and the nuclear membrane would be lysed without disrupting the chromosome structure, thereby extracting most of the RNA material while limiting genomic-DNA contamination. Since most of the processed mRNA is present in the cytoplasm, even a cytoplasmic lysis (without nuclear lysis) would yield most of the important mRNA population. Previously, we have used a strong chemical stress (chaotropic buffers like guanidine thiocyanate) to augment the bacterial cell lysis.[34] Replacement of chaotropic reagents (e.g., guanidium, urea) or harsh detergents (e.g., SDS) with a mild detergent (e.g., 0.1% Triton X-100) should ensure the integrity of the chromosome structures while disrupting the cell and nuclear membranes. In the present study, several approaches were tried for cell lysis, including strong chemical lysis (3 M guanidine thiocyanate/GuSCN), weak chemical lysis (0.1% Triton X-100), mechanical lysis (pressurized through a PPM), and a combination of mild chemical and mechanical lysis (0.1% Triton X-100 and pressurization through a lysis PPM). The resulting lysates were analyzed by fluorescence microscopy and unlysed cells/nuclei were counted to ascertain lysis efficiency (Figure 4A). Efficiency was calculated as the fraction of intact/unlysed cells in a treatment condition compared to the control (TE buffer). The results show that the combination of a mild chemical and a mechanical lysis step (i.e., 0.1% Triton X-100 solution in TE buffer and pressurizing through a lysis PPM) gives the best results in terms of cell lysis and, as discussed below, minimizes contamination with genomic DNA.

Choice of PPM and Lysis Steps. Earlier published literature has reported the use of a single column for nucleic acid purification, including cell lysis (e.g., refs 27, 34, and 39). Initially, a single straw method was attempted for RNA isolation, using straws containing SPE PPM and a 3 M GuSCN lysis buffer similar to the DNA isolation process.[33,34] However, the results showed several limitations in the process, including clogged straws (cell debris), genomic DNA contamination, and low RNA yield. To address these issues, two different approaches were tried: (1) a single straw process with a lysis PPM containing silica particles (same lysis buffer, 3 M GuSCN) and (2) a two-step process with one straw for cell-lysis and another straw for nucleic acid binding. The two-straw method of RNA isolation involves the use of one straw with a specific type of PPM material for cell-lysis and a second straw with a Si-embedded PPM-material for nucleic acid binding. In the two-straw method, we compared several different approaches for cell lysis (TE, TE and Triton X-100, and GuSCN). All RNA samples were treated with reverse transcriptase enzyme (New England Biolabs), and the resulting cDNA was used for a RT-PCR assay for the MDCK actin gene. A separate set of the same samples were treated by the reverse transcriptase reaction mix, without the reverse transcriptase enzyme, as controls for genomic DNA contamination in the RNA sample. The results for four different methods are presented in Figure 4B, each for 50 000 MDCK cell samples: (i) lysing the MDCK cells with a lysis straw in presence of TE buffer and 0.1% Triton X-100 detergent (lanes 4−6, DNA contamination controls in lanes 1−3); (ii) lysing the MDCK cells with a lysis straw in the presence of TE buffer only (lanes 10−12, DNA contamination controls in lanes 7−9); (iii) lysing the MDCK cells with a lysis straw in the presence of 3 M GuSCN buffer (lanes 17−19, DNA contamination controls in lanes 14−16); and (iv) a single straw method (lanes 23−25, DNA contamination controls in lanes 20−22) using a PPM with features of both the lysis straw and the SPE straw where a lysis PPM (larger pore size for cell lysis) was spiked with 3 μm Si-microspheres (for RNA binding). A solution of 3 M GuSCN in TE buffer was used for the single straw process. Sample 13 is a positive control.

Using this data set, we can qualitatively assess the presence of contaminating genomic DNA and the RNA yield. Treatment (i) shows negligible amounts of contaminating genomic DNA, with high yields of RNA. Treatment (ii) shows larger amounts of genomic DNA contaminations and low RNA yield. Treatment (iii) shows almost no genomic DNA contamination but also very low yield of RNA. The results of the single straw method, treatment (iv), show low RNA extraction efficiency and the presence of genomic DNA contaminations. This experiment clearly elucidated the superiority of technique (i) where MDCK cells were lysed with a lysis straw in the presence of TE buffer containing 0.1% Triton X-100 detergent over the other three processes.

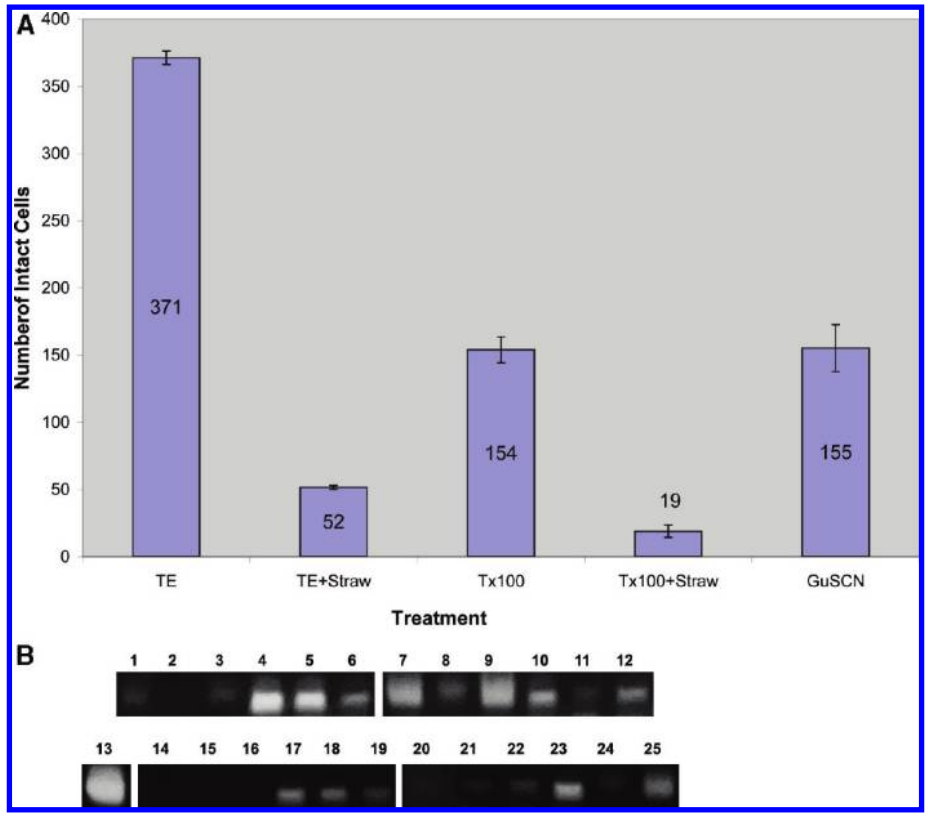

Figure 4. (A) Mammalian cell lysis efficiency of different methods was compared by counting the unlysed cells via fluorescence microscopy. Cells were surface stained with fluorescent dyes and nuclear stain DAPI and subjected to (i) TE buffer only as the control, (ii) pressurization through a lysis PPM in TE buffer, (iii) 0.1% Triton X-100 in TE buffer, (iv) a combination of 0.1% Triton X-100 in TE buffer and pressurization through a lysis PPM, and (v) 3 M guanidium thiocyanate (GuSCN) in TE buffer. Error bars are a single standard deviation from the cell counting by three different researchers. (B) Performance comparison of various two-straw methods (one for lysis and one for SPE) for RNA isolation (samples 1−12 and 14−19) with a single straw method (samples 20−25). Samples 1−6, lysis straw with 0.1% Triton X-100 in TE buffer treatment; samples 1−3 without reverse transcriptase and samples 4−6 with reverse transcriptase; samples 7−12, lysis straw with TE buffer only; samples 7−9 without reverse transcriptase; and samples 10−12 with reverse transcriptase. Sample 13, PCR positive control. Samples 14−19, lysis straw with GuSCN buffer; samples 14−16 without reverse transcriptase and samples 17−19 with reverse transcriptase. Samples 20−25 show results for the single straw method with a lysis PPM composition, containing 3 μm Si-microspheres, and sample mixed with GuSCN buffer; samples 20−22 without reverse transcriptase and samples 23−25 with reverse transcriptase.

Optimization of RNA Extraction Process. *Total RNA Extraction Using SPE-Straws.* To optimize the RNA extraction protocol, different reagent compositions were tested based upon existing RNA extraction protocols for other solid-phase extraction processes and previous DNA extraction work with the SPE-PPM.[27,33−39,45,46] Variations in RNA extraction efficiency were measured by RNA extraction through SPE-PPM, cDNA synthesis, and end-product PCR assay (based on a fragment of the canine actin gene). PCR was performed on cDNA synthesized from the purified total RNA and on the control set (without reverse transcriptase) to look for genomic DNA contaminations in the purified RNA.

Two different sizes (0.7 and 3 μm) of silica particles were used to prepare the SPE PPM to understand the effect of different silica surface area on nucleic acid capture. However, we did not see any significant changes in the capture in terms of total RNA extraction yield for either the 0.7 or 3 μm silica. Various organic solvents (100% ethanol, 66% isopropanol, 60% butanol, 45% sulfolane) and different concentrations of salts (50, 100, and 200 mM of sodium acetate or ammonium acetate pH 5.2) were tested for efficiency of solid phase extraction of RNA onto the SPE-PPM columns. All the organic solvents worked reasonably well, with extraction efficiencies ranging from 60 to 75%; the best results were observed with a composition of 66% isopropanol and 100 mM sodium-acetate (pH 5.2) in TE buffer (Supplemental Figure 1 in the Supporting Information). For the elution buffer composition, DEPC treated water, DEPC treated TE buffer, and TE buffer with three different RNase inhibitor reagents (RNAsecure from Ambion, RNase-inhibitor from New England Biolabs, and RNaseout from Invitrogen) were tested. The best results were observed with a solution of RNAsecure in TE buffer preheated to 60 °C, although the differences between different elution efficiencies were marginal (data not shown).

To optimize the effect of buffer volumes in the RNA extraction procedure, different volumes of the reagents were used, loading buffer (100, 200, and 400 μL), washing buffer (200, 400, and 800 μL), and elution buffer (50, 100, and 200 μL) for equal numbers of cells (10^5). We reasoned that varying the relative concentration of chaotropic agent to the sample during loading could improve the RNA capture efficiency. Increasing the wash buffer volume could improve the purity of the eluted RNA by removing any residual contaminations. The optimal volume of the elution buffer is a balance between complete elution and the final RNA concentration. In the case of the low wash volume (200 μL), the quality of eluted RNA was compromised, hamper-

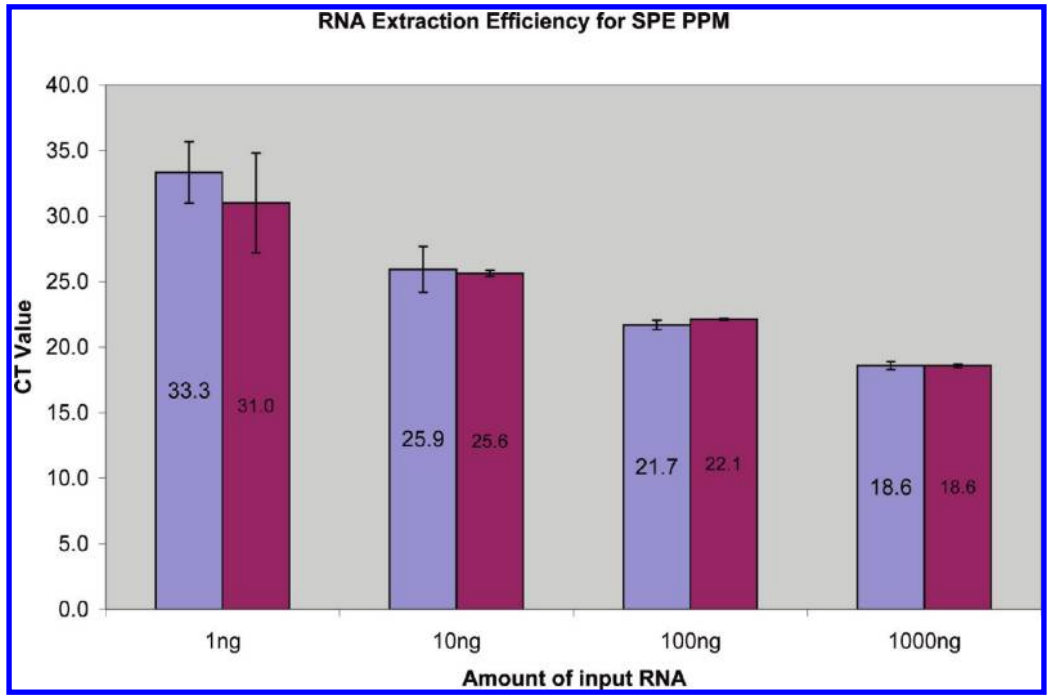

Figure 5. RNA extraction efficiency of the SPE PPM was analyzed by repurifying previously quantified purified RNA. Different amounts (1000, 100, 10, and 1 ng) of repurified RNA (blue bar) and control concentrations (starting material, red bar) were subjected to a quantitative PCR assay and the resulting C_T values recorded.

ing downstream cDNA synthesis process and resulting PCR results (data not shown). For the rest of the volume changes, RNA extraction was largely unaffected in terms of RNA quality or quantity, indicating that the user has considerable flexibility in choosing those parameters for the downstream assays.

DNase treatment was necessary to remove the presence of residual contaminating DNA from the purified RNA samples. However, using DNase after RNA elution was found to be detrimental for the downstream cDNA synthesis process, so DNase treatment was optimized for the cell lysates before loading the sample onto the SPE straws. A 20 min treatment with DNase was used as a conservative precautionary measure, although shorter incubations may also have similar results.

RNA Extraction Efficiency and Capacity. To calculate the RNA extraction efficiency of the SPE PPM, commercially available purified RNA (TaqMan GAPDH Control Reagents, Applied Biosystems) was repurified with the SPE PPM containing straws. The RNA was loaded onto the straws containing SPE-PPM and the RNA extraction steps of loading, washing, and elution were performed. The SPE PPM purified RNA was subjected to a quantitative one-step RT-Taqman assay as per manufacturer's protocol (Applied Biosystems). The same amounts of the source RNA were used as the positive controls (not passed through the SPE PPM) in the PCR assay (Figure 5). To calculate the extraction efficiency from the quantitative PCR results, we derived an equation comparing the difference in threshold cycle (C_T) values to the ratio of starting material.

The equation governing the relationship between the final concentration of amplicon (C_F) and the starting concentration of the target gene (C_0) is

$$C_F = C_0(1 + E)^x \tag{1}$$

where E is the efficiency of the RT-PCR reaction and x is the number of cycles. We designate the RNA passed over the column with a prime and the control reaction (RNA added directly to the RT-PCR mixture) with a double prime. Thus the final concentration of amplicon for the RNA passed over the column is described by the equation

$$C_F' = C_0'(1 + E)^{x'} \tag{2}$$

and that of the purified RNA added directly to the RT-PCR mixture as

$$C_F'' = C_0''(1 + E)^{x''} \tag{3}$$

When the final concentration of amplicon is equal to the threshold concentration

$$C_F' = C_F'', \quad \text{then} \quad x' = C_T' \quad \text{and} \quad x'' = C_T'' \tag{4}$$

Thus,

$$C_0'(1 + E)^{C_T'} = C_0''(1 + E)^{C_T''} \tag{5}$$

Rearranging the equation solving for the ratio of starting target concentrations, we derive that

$$C_0'/C_0'' = (1 + E)^{(C_T'' - C_T')} \tag{6}$$

To determine the RT-PCR efficiency, we plotted the logarithm of the starting concentration for 1, 10, 100, and 1000 ng of prequantified commercially available purified RNA versus their corresponding C_T values. We performed a linear regression to the

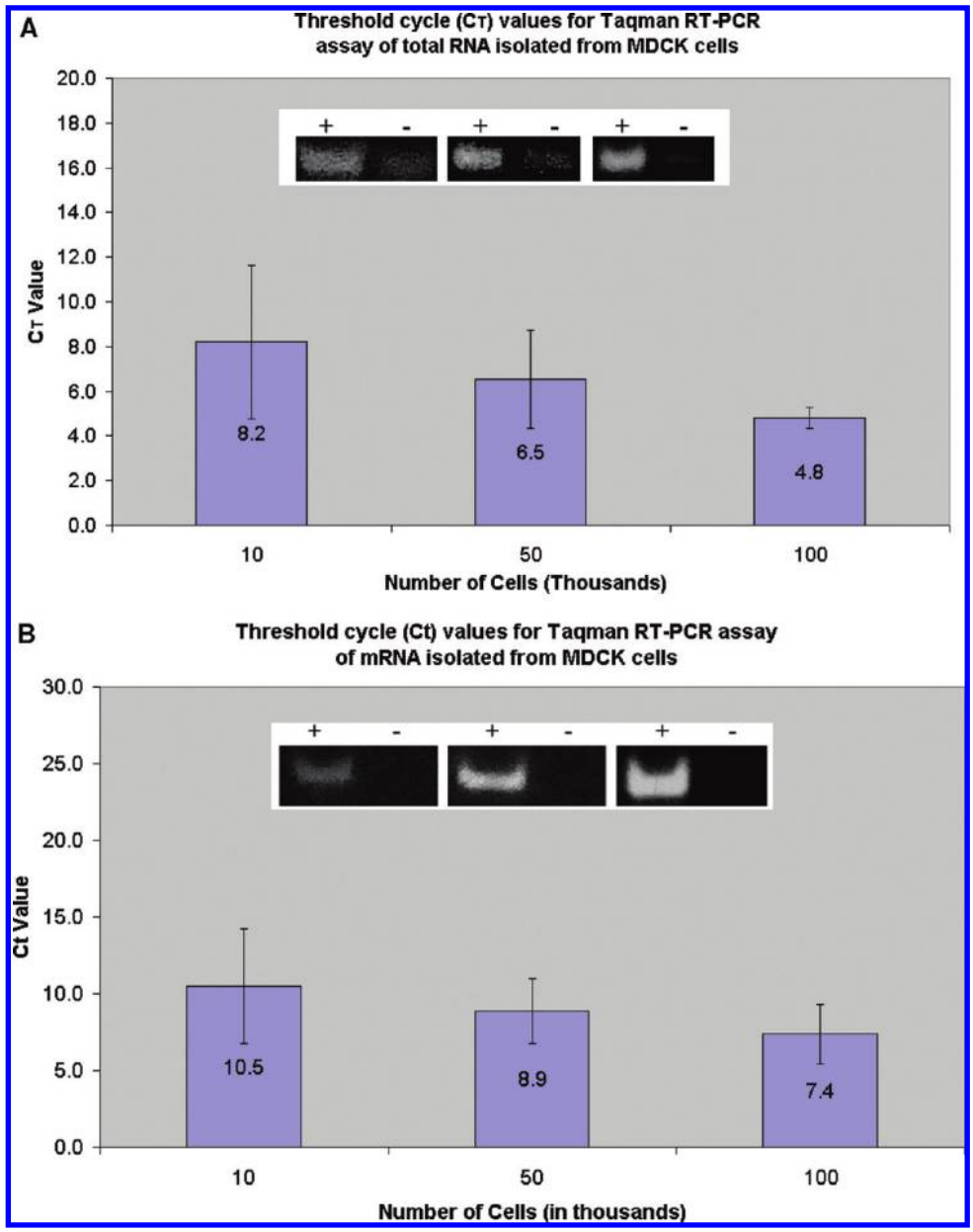

Figure 6. (A) Taqman RT-PCR assay for canine β-actin gene fragment was conducted using cDNA synthesized (Invitrogen Superscript II cDNA synthesis kit) from purified MDCK total RNA (10^4, 5×10^4, and 10^5). Each data point represents averages of 5−6 replicates; error bars represent standard deviation from the average. The inset shows the SYBR-green stained band of the PCR product. (B) Taqman RT-PCR assay for canine β-actin gene fragment using cDNA synthesized from purified MDCK mRNA. Each data point represents averages of 9−12 replicates; error bars represent standard deviation from the average. The inset shows the SYBR-green stained band of the PCR product.

data and determined the slope of line. The slope of this line, m, is used to calculate the RT-PCR efficiency (E) with the following equation

$$E = 10^{(-1/m)} - 1 \tag{7}$$

For this reaction, m is −4.0746 (R^2 value for the linear fit is 0.988) and the efficiency of the reaction was calculated to be 0.76. Using the calculated RT-PCR efficiency, we can determine the extraction efficiency from the data in Figure 5 and eq 6. The percentage of recovered material through the straws ranges from 100% at 1000 ng of input RNA to 27% at 1 ng of input RNA. The error on these measurements is dominated by the error in the C_T values which is ~1−2 C_T units and is higher for the smaller initial RNA inputs. Because of the geometric amplification of PCR, this corresponds to an average error of 34% in the extraction efficiencies. Overall, the method works very well for high nanogram concentrations and still performs admirably for single digit nanogram concentrations. As demonstrated in the comparison with an existing kit (Comparison of HT-SNAP Process with a Commercial Kit), the extraction efficiency clearly surpasses the commercially available kit at low concentrations. Note that the capacity of the straws is greater than 1000 ng of RNA, but due to our focus on performance for small sample sizes we have not yet ascertained the maximum capacity.

Quantitative Results of Total RNA Isolation from Mammalian Cells. Total RNA isolation was attempted from mammalian cells (MDCK cell line) using the HT-SNAP pressure array and the straws. For this experiment, MDCK cells (10^4, 5×10^4, and 10^5) were subjected to the total RNA protocol as described in the Experimental Section. Real-time PCR was performed on cDNA synthesized from the total RNA (Figure 6A). Quantitative PCR of total RNA is sensitive to the presence of contaminating genomic DNA. As such, the presence of genomic DNA contamination was always a consideration and a control set of samples without reverse transcriptase treatment were included for all RT-PCR assays. The C_T values show a quantitative increase in RNA amount with increasing cell numbers indicating a similar yield for all the cell concentrations used in the experiments. For a perfectly efficient extraction and RT-PCR process, one would expect a difference of 3.3 in C_T values for a 10-fold difference in starting sample concentration. The observed C_T value difference between 10^4 starting cells and 10^5 starting cells is 3.4, demonstrating the linearity of the RNA extraction efficiency in this cell concentration range.

The real-time PCR products were then electrophoresed, and the resulting gel bands at 75 bp length validated the amplification of the expected size of DNA fragment from the synthesized and are displayed as insets to the respective bar plots (Figure 6A). The data show consistent and quantitative real-time PCR results indicating successful total RNA isolation from mammalian cells by HT-SNAP.

Quantitative Results of Poly-A RNA Isolation from Mammalian Cells. Encouraged by the high yield and purity of the total RNA isolated by our method, we were interested in developing a similar method for isolation of poly-A RNA as direct poly-A RNA (mostly mRNA or mRNA) isolation is useful for targeted gene expression studies. To adapt our method for mRNA isolation, we replaced the silica-based SPE columns with oligo-dT cellulose packed on top of PPM of the same composition as the SPE straws but without embedded silica particles. Using these straws, mRNA isolation was performed from 10^4, 5×10^4, and 10^5 MDCK cells, followed by cDNA synthesis and real-time PCR. The C_T values for real-time PCR (Figure 6B) show that mRNA had been successfully isolated from MDCK cells. The changes in C_T values for different cell concentrations also indicate similar extraction efficiencies in this cell concentration range. The PCR products were validated by electrophoresis as before (insets Figure 6B).

Comparison of HT-SNAP Process with a Commercial Kit. *RNA Quality.* To ascertain the RNA quality, total RNA isolation was performed from 4×10^5 MDCK cells using the HT-SNAP pressure array. Total RNA was also isolated from a equal number of MDCK cells using a commonly used commercial kit, Ambion RNAqueous Micro Kit, to benchmark our process. For the commercial kit, half of the samples were subjected to the optional DNase treatment and both untreated and treated samples were used for further analysis. Purified RNA samples were electrophoresed under denaturing conditions (Figure 7). The electropherograms show the isolation of total RNA free of contaminating genomic DNA in our method (lanes A and C) and the presence of contaminating genomic DNA in the Ambion kit method without (lanes D and E) or with (lanes F and G) the optional DNase treatment. Sharper rRNA bands (28S, 18S, and precursors) in the electropherograms indicate that the RNA isolated by the HT-SNAP method was of better quality compared to the commercial kit. Also, the presence of precursor forms of rRNA in our method (absent in the Ambion kit samples) in addition to the processed 28S and 18S rRNA bands indicate the presence of nuclear RNA in the PPM purified RNA samples. We postulate that the Triton X-100 based lysis method allows the purification of RNA without disrupting the chromosome structure thereby reducing genomic DNA contamination. The method used for lysing cell membranes was borrowed from standard cell biology practices where mild detergent treatment is used to keep the nuclear structure intact, while increasing the porosity of the cell membrane.

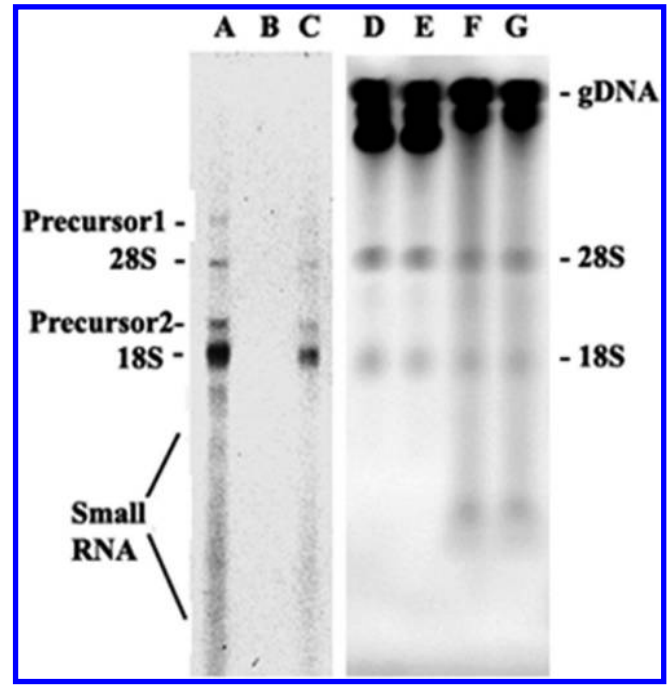

Figure 7. MDCK RNA purified from 400 000 cells by PPM straws (A,C), PPM straw negative control (B), and MDCK RNA from 400 000 cells from Ambion RNAqueous Micro Kit without DNase (D,E) and with DNase treatment (F,G).

Quantitative Comparison. To quantitatively compare our method to a commercial kit, total RNA was isolated from different numbers of MDCK cells (10^3, 10^4, and 10^5) using HT-SNAP and RNAqueous Micro Kit (Ambion). The purified RNA samples were eluted in identical volumes of respective elution buffer for comparable concentration/yield measurements. RNA concentrations were quantified by absorbance measurements. The results show the RNA yield for the HT-SNAP method to be about 1000 times better than those isolated with the RNAqueous-Micro kit for lower (10^3) cell numbers and about 20 times better at higher (10^5) cell numbers (Figure 8A). The standard deviations for the HT-SNAP are also better than those for the RNAqueous-Micro kit for lower sample numbers (10^3-10^4 cells). At higher sample numbers (10^5 cells), the HT-SNAP method has higher standard deviations indicating the possibility of matrix saturation, which may be overcome by increasing the size of the PPM column in the straws.

Comparing the methods by real-time PCR, the C_T values for total RNA isolated by HT-SNAP and by the benchmark kit,

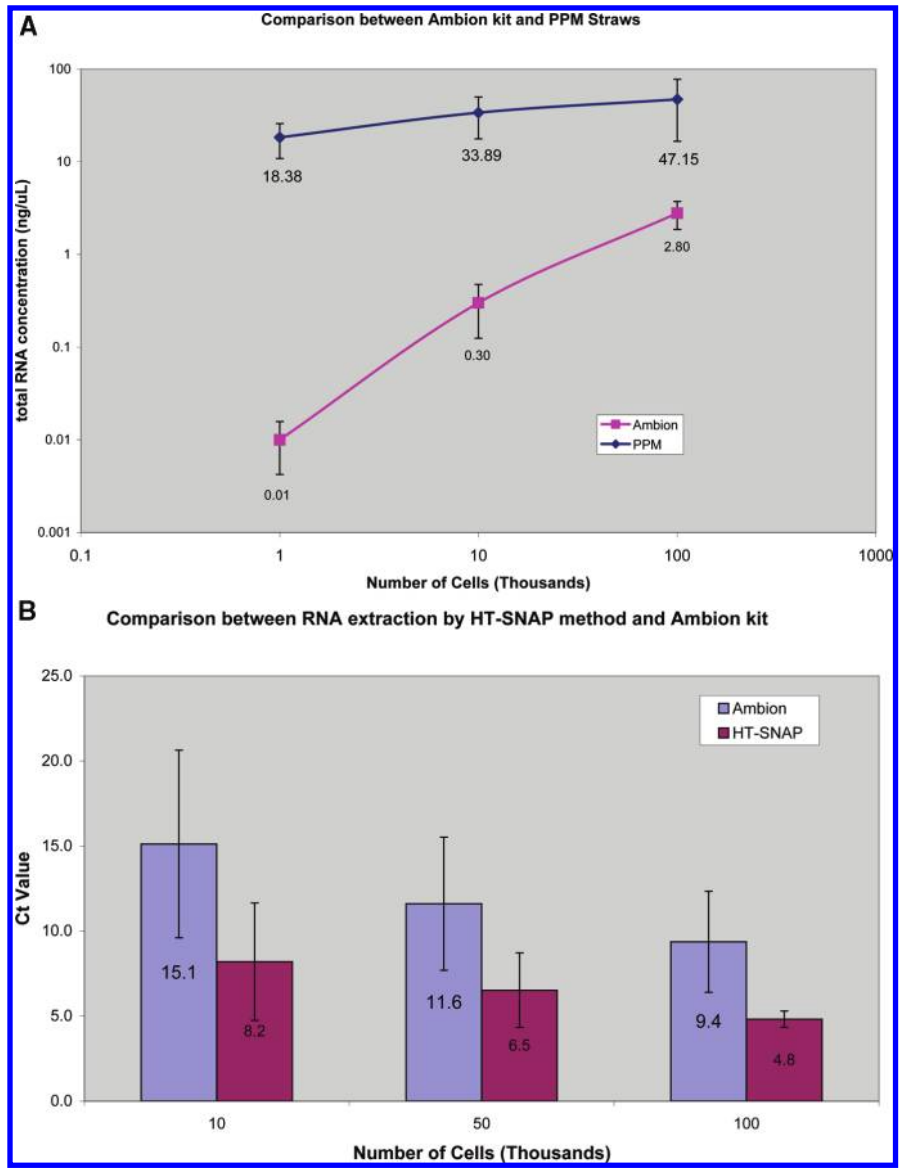

Figure 8. (A) Concentration of total RNA isolated from 10^3, 10^4, and 10^5 MDCK cells using our method compared to the total RNA isolated from same number of cells using the Ambion kit, measured on the nanodrop UV-spectrophotometer. Each data point represents averages of 5−6 replicates; error bars represent standard deviation from the average. (B) C_T values of real-time PCR performed on cDNA synthesized (New England Biolabs kit) from MDCK total RNA (from 10^4, 5×10^4, and 10^5 cells) isolated using HT-SNAP and compared with the Ambion kit. Each bar represents averages of 3−10 replicates; error bars represent standard deviation from the average.

RNAqueous-Micro (Ambion), consistently showed our method to have lower C_T values for the same number of input cells. The difference in C_T values (Figure 8B) is consistent with those measured by absorbance (Figure 8A). For example, at 10^4 cells, the difference in C_T's between our method and the Ambion kit is 6.9. Assuming a perfectly efficient PCR reaction, that would translate to a $2^{6.9} = 119$-fold difference in the starting concentrations of RNA. Figure 8A shows a 113-fold difference between the starting concentrations. Likewise, at 10^5 cells, the real-time PCR results indicate a 23-fold difference in performance, while the absorbance results show a 17-fold difference. Note that the experimental data presented in parts A and B of Figure 8 are from independent data sets indicating significant reproducibility of the method. For these experiments, only 10% the RNA samples isolated by the Ambion kit were uncontaminated by genomic DNA, whereas 60% of total RNA samples isolated by our method were free of contaminating genomic DNA. The results used in the figures all reflect experiments with uncontaminated RNA samples. The reader may note that the C_T values presented in Figure 8B are somewhat different for the C_T values for Figure 6A, although the average values are still within the ranges of 1 standard deviation from each other. We believe such differences are due to the use of two different cDNA synthesis kits (the Invitrogen Superscript II kit and the New England Biolabs First Strand cDNA synthesis kit) and reflect slight differences in the RT-PCR efficiencies of the two reactions.

CONCLUSIONS

As we have demonstrated in this article, the HT-SNAP pressure array and the PPM-based straws can purify both total RNA and mRNA from mammalian cells. Mammalian cells and nuclei are lysed by using a combination of chemical lysis (mild detergent)

and physical lysis (pressurization through the PPM column), possibly without disrupting the chromatin structure. The cell extract is suitably treated for the RNA molecules to bind to the SPE column and subsequently washed and extracted from there. The whole process is driven by a unique proof-of-concept pressure array (HT-SNAP) with varying amounts of pressurized air and does not require any centrifugation or vacuum manifold application, hence can be easily automated to a high-throughput design.

At smaller sample amounts, the PPM method described here clearly outperforms the commercial kit in terms of both quantity and quality. Since the existing commercial instruments use similar kits, we can postulate that the HT-SNAP system would definitely match and may probably outperform the existing commercial instruments for RNA isolation. The same pressure array and similar straws packed with oligo-dT cellulose are also effective for mRNA isolation. The cost per sample is lower than comparable automated processes due to the use of smaller reagent volumes, very little hazardous chemicals, and cheap plastic disposable components. Most importantly, the method and the system are ideally suited for high-throughput automation to purify and analyze large numbers of samples. We are currently developing an automated version of the HT-SNAP system with simultaneous reagent dispensing and pipetting features to further reduce human intervention and variability observed in the current system.

ACKNOWLEDGMENT

We thank Boston University and Fraunhofer Gesellschaft for funding this work through the BU-Fraunhofer Alliance for Medical Devices, Instrumentation, and Diagnostics Program. We also thank Nina Hoenig and Melanie Zimmermann for their technical assistance and Dr. Maxim Kalashnikov for helpful comments on the manuscript.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 14, 2009. Accepted April 10, 2010.

AC100063F