Anal. Chem. **2008,** *80,* 8453–8460

Microchip-Based Solid-Phase Purification of RNA from Biological Samples

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Having previously detailed a method for chip-based extraction of DNA (*Anal. Chem.* 2003, *75*, 1880−1886.), we describe here a microchip-based solid-phase extraction method for purification of RNA from biological samples is demonstrated. The method involves the use of silica beads as a solid phase, and the capacity of the device containing silica beads for RNA, RNA in the presence of protein, and DNA was determined. The capacity of the device for RNA binding in the presence of protein is 360 ng, which demonstrates sufficient capacity of the device for complete genetic analysis. An extraction of RNA can be performed on the device in as few as ∼9 min (analytical time), a time comparable to that of a commercial extraction method, but with less reagent consumption. The microchip-based extraction is also performed in a closed system, unlike the commercial extraction method, which provides the advantage of decreased opportunity for the introduction of RNases and contaminants—essential for the sensitive RNA-based analyses presented in this work. RNA purified using the device was shown to be amplifiable using reverse transcription PCR (RT-PCR), allowing for translation of the method to the purification and subsequent amplification of biological samples. RNA was purified using the microchip-based method from neat semen, a mock semen stain, and cultured cells from a common pediatric cancer, alveolar rhabdomyosarcoma.

With the goal of continuing the advancement of enhanced systems for the isolation, purification, and analysis of nucleic acids from biological samples, our previous work characterized the use of the microfluidic platform for DNA extraction.[1] In contrast to DNA, RNA presents unique challenges due to its susceptibility to degradation by ubiquitous exonucleases (RNases). RNases present in the bacteria and molds found in abundance in human skin and dust particles, are extremely active enzymes, making the isolation of RNA from common clinical and forensic samples challenging.[2] Conventional, open-system methods for RNA isolation and purification do not effectively guard against these enzymes, requiring multiple transfer and reagent addition steps that inherently expose the sample RNA to the environment and predispose it to contamination and degradation by RNases.[3] Although several commercially available, automated platforms exist for RNA extraction, there is not currently an automated microscale method for the purification of RNA that would permit the integration of all sample processing steps. Consequently, the need for a closed, RNase-free, rapid, automated, and effective purification system that can produce high quality, pristine, nondegraded RNA remains unfulfilled. The development and thorough characterization of a microchip-based extraction method for the purification of RNA would provide a closed, rapid, and automatable system that could enable more facile and efficient processing for samples of both clinical and forensic interest.

Although a well-characterized, silica-based DNA purification method has been established and exploited for use in microfluidic purification systems, where reduced sample and reagent consumption, as well as a reduction in analysis time, has been achieved,[1,4−8] the use of miniaturized, silica-based purification technology has not been extended to the isolation and recovery of RNA. A closed microfluidic, silica-based purification system would represent a significant improvement to current methodologies for RNA extraction, which often involve time- and reagent-consuming organic extractions, by decreasing opportunity for the introduction of contaminants and RNases, as well as reducing the amount of sample, reagents, and analytical time required to perform this delicate isolation. Furthermore, a microchip-based method could be easily incorporated into a μ-TAS (similar to that described by Easley et al.) for mRNA expression analysis,[4] which would ultimately allow for a substantial decrease in analysis time and reagent volumes compared to conventional methods for RT-PCR, separation, and detection and, in turn, act as a faster platform for mRNA expression analysis for either forensic or clinical use.

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10.1021/ac8011945 CCC: $40.75
Published on Web 10/15/2008

Recent work from the Ballantyne group[9,10] has detailed a method for using mRNA expression to identify body fluids for forensic analysis, an important and often challenging feat when using traditional testing methodologies. Additionally, detection of RNA and mRNA expression can be used for clinical diagnostics, as the presence of specific chromosomal translocations from tumor tissue[11] or increased levels of expression of specific mRNAs in the plasma and sera of patients can be indicative of cancer.[12] These methods are based on the inherently variable mRNA expression from different cell types, producing gene-specific patterns which can be verified by the presence of a unique mRNA expression pattern.[13] In order to obtain mRNA for the transcription and amplification that is necessary for both clinical and forensic gene expression analysis, however, the RNA must first be isolated and purified from the biological source of interest. Consequently, the development of a robust system for the purification of RNA has become essential as mRNA expression analysis methods evolve for application to forensic body fluid identification[9,14,15] and clinical diagnostics.[12,16−18] The work presented here attempts to thoroughly characterize and develop a closed microfluidic system for the isolation of large molecular weight RNA for potential application to forensic and clinical mRNA expression analysis, with a view toward the inclusion of this optimized method in a μ-TAS.

This report describes the essential development and optimization of a microchip-based silica extraction method for the purification of RNA as a preliminary step toward development of a total integrated device for the extraction, amplification, separation, and detection involved in mRNA expression analysis. Device capacity experiments for RNA and DNA binding in the presence and absence of protein are detailed, as well as method development for isolation and amplification of RNA. This method was evaluated successfully for the purification of RNA from crude biological samples including neat semen and semen stains, as well as an alveolar rhabdomyosarcoma (ARS) cell line. Similar to the other integrated genetic analysis devices,[4] the RNA extracted using this method was proven to be amplifiable via RT-PCR, demonstrating the effectiveness of this protocol as a front-end purification method for gene expression analysis, with possible application to forensic body fluid identification and clinical diagnostics. The microchip method allows for the purification of RNA more rapidly than conventional organic extractions, and in a time comparable to commercially-available spin-column extractions. The microchip-based system also consumes less reagents than commercial extraction kits and provides an improved concentration enhancement of the extracted RNA, which elutes in a smaller volume (4 μL). Perhaps most advantageous, the microchip methodology, in contrast to the commercial spin-column extraction method, is an inherently closed system providing less opportunity for the introduction of exogenous contaminants and RNases. A microchip-based methodology also enables more facile integration with other analytical processes (e.g., PCR and microchip electrophoresis), for integrated and automated genetic analysis. The thorough development of a single-process RNA purification device represents the first step toward an integrated μTAS capable of total mRNA profiling for forensic and clinical applications.

MATERIALS AND METHODS

Reagents. Hyperprep silica beads (15−30 μm) were purchased from Supelco (Bellefonte, PA). Guanidine hydrochloride (GuHCl), 2-amino-2-(hydroxymethyl)propane-1,3-diol-HCl (Tris-HCl), 2-(4-morpholino)ethanesulfonic acid (MES), and diethylpyrocarbonate (DEPC)-treated water were purchased from Fisher (Fairlawn, NJ). EDTA was from Amresco (Solon, OH), and ethanol was purchased from AAERB Alcohol and Chemical Co. (Shelbyville, KY). Quant-iT RiboGreen RNA Assay Kit containing Quant-iT RiboGreen RNA reagent fluorescent nucleic acid stain, 20× Tris-EDTA (TE), and rRNA standard (16S and 23S rRNA from *Escherichia coli*) was purchased from Molecular Probes (Eugene, OR). TaqMan Control Total RNA (human) was purchased from Applied Biosystems (Foster City, CA). λ-phage DNA from *E. coli* was purchased from Sigma-Aldrich (St. Louis, MO). SuperScript III One-Step RT-PCR System with platinum *Taq* and platinum *Taq* DNA polymerase were purchased from Invitrogen (Eugene, OR). RETROscript First Strand Synthesis Kit for RT-PCR was purchased from Ambion (Austin, TX). Primers for the 353-bp human β-actin mRNA gene, 153-bp protamine 1 (PRM1) mRNA gene, 200-bp c-ABL gene, and 373-bp ARS consensus gene were purchased from MWG-Biotech, Inc. (High Point, NC). All solutions were prepared in DEPC-treated water, with the exception of the TE buffer (pH 7.9) and 100 mM MES (pH 4.0), which were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA) and used to titrate the 6 M GuHCl to a final pH of 6.1.

Microchip Preparation. Microdevices were fabricated with Borofloat glass (Telic Co., Valencia, CA) using standard photolithographic techniques.[19] The resulting channel dimensions were 1.5 cm effective length, 225 μm deep, with a top width of 650 μm, and a bottom width of 200 μm. A 1.1-mm-diameter diamond-tip drill bit (Crystallite Corp., Lewis Center, OH) was used to drill access holes at both ends of the channel. A borofloat glass cover plate was cut to fit the device and thermally bonded to the etched bottom plate. The distance from the top of the weir to the cover plate was ∼5−20 μm. Following fabrication, silica beads, 15−30 μm, were suspended in DEPC-treated water and packed against the weir in the channel using vacuum. The channel was filled with new silica beads prior to each extraction. The microchip is shown in Figure 1.

Apparatus. The microchip-solid phase extraction (SPE) apparatus consisted of a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Holliston, MA) with a 250 μL Hamilton

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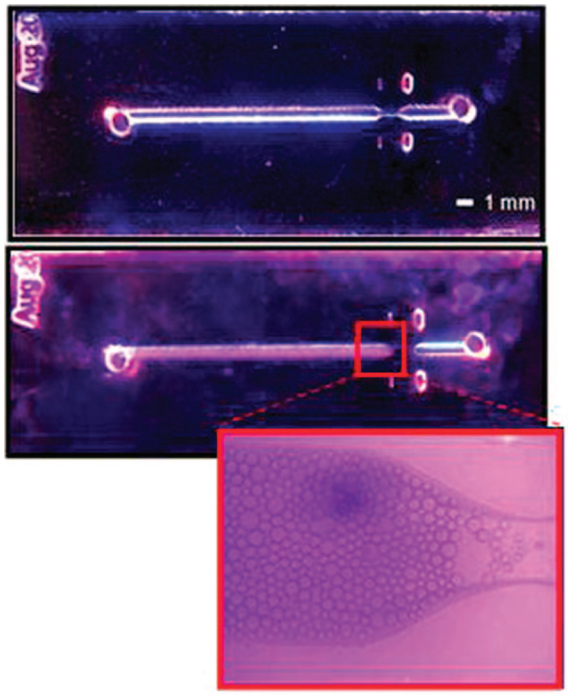

Figure 1. Glass microdevices for RNA SPE. (a) Unfilled; (b) filled with 30 μm silica phase. Inset shows close-up of 30 μm beads held in place by the weir.

gastight syringe (Hamilton, Las Vegas, NV). The syringe was connected to the microchip using PEEK tubing and mini-tight fittings (Upchurch Scientific, Oak Harbor, WA).

Microchip Solid-Phase Extraction Procedure. Microdevices filled with silica beads were conditioned with 6 M GuHCl (pH 6.1) for 10−30 min at a flow rate of 7.0 μL/min prior to each extraction. The extraction procedure consisted of pressure-driven load, wash, and elution steps, each performed at a flow rate of 7.0 μL/min. First, a 25 μL solution containing prepurified RNA or biological sample (neat semen, or 35−105 μL solution when using a semen stain, 50−165 μL solution containing ARS cell line; range represents a smaller volume load solution, which accordingly loads in less time, and a 3-fold larger volume load solution, 3-fold more dilute than the small-volume solution, which loads an equivalent amount of semen or ARS cell line in a longer time period, but avoids clogging the device) in a lysis/binding buffer solution of 6 M GuHCl was loaded onto the silica bed. When semen samples were used, a final concentration of 40 mM dithiothreitol (DTT) was included as a reducing agent in the lysis/loading buffer to achieve complete cell lysis.[5,20] When the ARS cell line was used, 10 μL (30 μL when loading the 3-fold larger volume) of proteinase K (20 mg/mL, Qiagen, Valencia, CA) was included in the load solution. A 25 μL wash of 80% ethanol (80/20 (v/v) ethanol/water) was flowed over the silica bed to simulate removal of any unbound material, proteins, or other potential PCR inhibitors when prepurified RNA was used in the extraction. When biological samples were used, 70 μL of 80% ethanol was used for the wash step to ensure complete removal of inhibitors. Finally, the purified RNA was eluted from the silica with DEPC-treated water, and 2−5 μL fractions were collected in PCR reaction tubes for subsequent fluorescent or RT-PCR analysis. DNA digestion on all biological samples postextraction was accomplished using the TURBO DNA-free" digestion kit (Ambion) in accordance with manufacturer's instructions.

Fluorescence Detection and Sample Amplification. Fluorescence detection was performed on a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE) using a RiboGreen fluorescence assay. RT-PCR studies using prepurified RNA were performed using a master mix consisting of Superscript III One-Step RT-PCR System components for amplification and executed on an Applied Biosystems thermocycler using the following protocol: 50 °C for 30 min, 94 °C for 2 min (cDNA synthesis and predenaturation), 40 cycles of denaturing at 94 °C for 15 s/annealing at 55 °C for 30 s/extension at 72 °C for 30 s, followed by 72 °C for 7 min (final extension). RT-PCR of purified biological samples was performed using a master mix consisting of RETROscript First Strand Synthesis Kit for RT-PCR components for cDNA synthesis and adapted from Juusola et al.[14] Thermal cycling was performed on a Bio-Rad MyCycler (Hercules, CA) using the following protocol: 95 °C for 1 min (initial denaturation), 35 cycles of denaturing at 94 °C for 20 s/annealing at 55 °C for 30 s/extension at 72 °C for 40 s, followed by 72 °C for 5 min (final extension). All RT-PCR products were analyzed via microchip gel electrophoresis on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) using the commercially available DNA Series II kit according to the manufacturer's instructions.

RESULTS AND DISCUSSION

RNA Purification. Methods for microchip silica-based solid-phase extraction and purification of DNA have been described in the literature[1,5,7,8] and are becoming well established. In order to test the effectiveness of a method similar to that described in the literature for DNA extraction (detailed in the Microchip Solid-Phase Extraction Procedure above), with an eye toward its inclusion in a μ-TAS, it was critical to ascertain whether the RNA: (1) adsorbs comprehensively to the silica phase driven by 6 M GuHCl in a manner previously optimized for DNA purification, (2) is effectively retained on the silica phase during the subsequent ethanol wash/protein removal step, and (3) can be effectively eluted using DEPC-treated water, without loss of RNA to RNase-mediated degradation. In order to evaluate these three conditions, an RNA elution profile was generated by determining the presence of RNA in the eluate during each phase of the extraction (depicted in Figure 2). Twenty-five microliters (containing a total mass of 200 ng, to promote effective visualization) of prepurified rRNA was loaded onto the bed, as described in the Microchip Solid-Phase Extraction Procedure. During the load and wash steps, 5 μL aliquots were collected from the outlet reservoir into PCR reaction tubes and 2 μL aliquots were collected during the elution phase of the extraction. Following completion of the extraction in a total analytical time of ~9 min, the amount of RNA present in each fraction collected from the load, wash, and elution was then quantified using a fluorescence assay. As shown in Figure 2, RNA was not detected in the load and wash steps, indicating complete adsorption and retention on the silica bed during both steps. In addition, the majority of the RNA desorbs in the first 6−8 μL of the elution step, with the most-concentrated portion eluting within a 4 μL fraction of this range. This represents both successful binding and retention of RNA, as well as effective, concentrated elution of the nucleic acids from the device: essential

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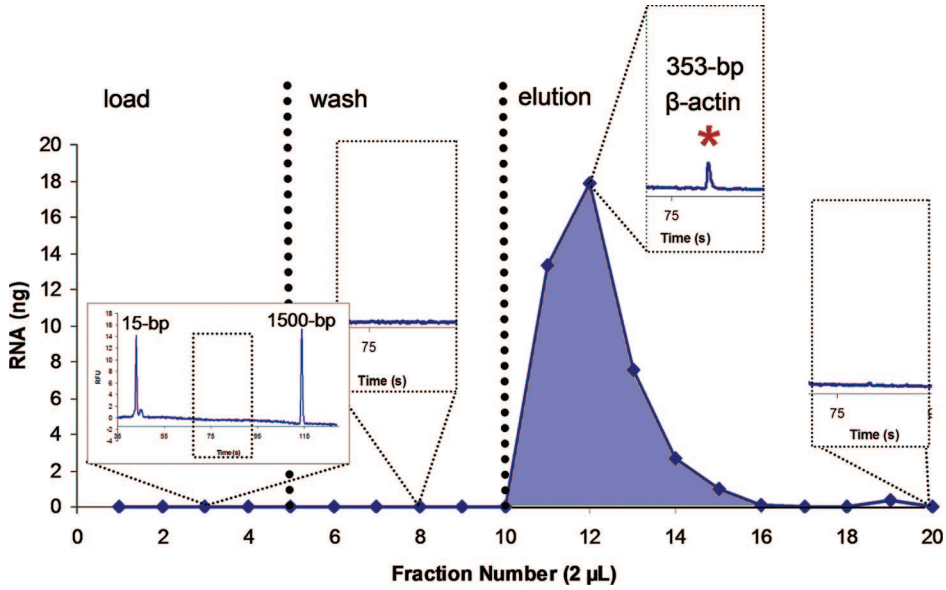

Figure 2. Extraction profile for prepurified RNA on 30-μm silica in a microdevice. Amplifiable RNA elutes in a concentrated plug in the first 6−8 μL of the elution (shaded area). (No RNA is fluorescently detected or amplified in the load and wash steps).

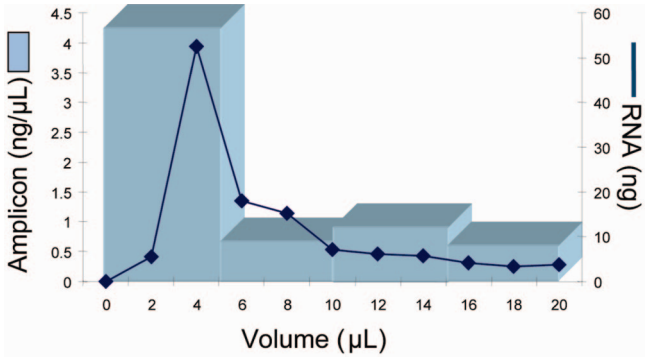

Figure 3. Correlation of mass of RNA extracted from the microdevice as determined by fluorescence (diamonds) and RT-PCR (histogram) assays.

for successful application of this method and device to the purification of RNA from biological samples, and for integration with other microfluidic sample processing steps.

Additionally, the insets in Figure 2 show the amplification of sample fractions from the load, wash, and elution steps of a representative extraction, as it is important to demonstrate the absence of PCR inhibitors in the elution fractions containing RNA for downstream analysis using RT-PCR. For this experiment, a solution containing 50 pg of total mRNA was loaded onto the extraction bed, followed by a 35 μL (5-min) wash of 80% ethanol. During the subsequent elution, ten 2 μL fractions were collected in 48 μL of RT-PCR master mix and amplified using primers for a 353-bp fragment of the highly expressed β-actin housekeeping gene. The results demonstrate that, in addition to successful retention and elution of RNA during the appropriate phase of the purification, the resultant purified RNA is amplifiable and suitable for further genetic analysis, critical if this technique is to be utilized for both clinical and forensic applications.

Figure 3 further illustrates the nuances of the elution of RNA from the device and the amplifiability of the resultant RNA. An elution profile obtained as described above (from 300 ng of total RNA loaded, again, to promote visualization) was overlaid with final amplicon concentration of four fractions collected after purification on the microdevice and amplified using primers for the 353-bp β-actin gene. As can be seen in this figure, the mass of amplicon generated tracks well with the amount of RNA eluted when visualized fluorescently. These results further indicate that, in addition to the largest mass of RNA being effectively eluted with DEPC-treated water within the first 8 μL, the eluted RNA clearly generates amplifiable product in each fraction. These results further highlight the appropriateness of the method for inclusion in an integrated system.

Capacity Studies. Having determined that the standard three-step, silica-based purification strategy was successful, it was important to ascertain that the binding capacity of the device as designed for RNA extraction was high enough to permit complex genetic analysis. In order to determine the binding capacity of this microchip device for RNA, a solution of 30 ng/μL purified rRNA was continuously loaded onto the bed at a flow rate of 7.0 μL/min, while a total of fourteen 2 μL fractions were collected from the outlet reservoir. The concentration of RNA in each fraction was subsequently measured using a commercial fluorescence assay by comparison to a calibration curve made from the same stock rRNA. The breakthrough point (where binding sites on the solid phase are saturated and RNA passes through unperturbed), indicative of the capacity of the device, occurred at the 13th fraction (26 μL). From these results, the capacity of the device for purified rRNA was calculated to be 760 ng ($n = 3$) using the derivative of a third-order polynomial approximation of the data—this mass of RNA exceeds what is required for a typical forensic or clinical analysis. If however, a larger mass of RNA is required for a specific application, the capacity of the device can be increased by simply increasing the size of the bed containing the solid phase—a trivial design change for this type of system.

Furthermore, in order to interrogate possible differences in device capacity with different classes of nucleic acids, a second capacity study was undertaken using a load solution of 30 ng/μL purified λ-phage DNA, utilized as an inexpensive polynucleic acid alternative to human genomic DNA. The experiment was carried

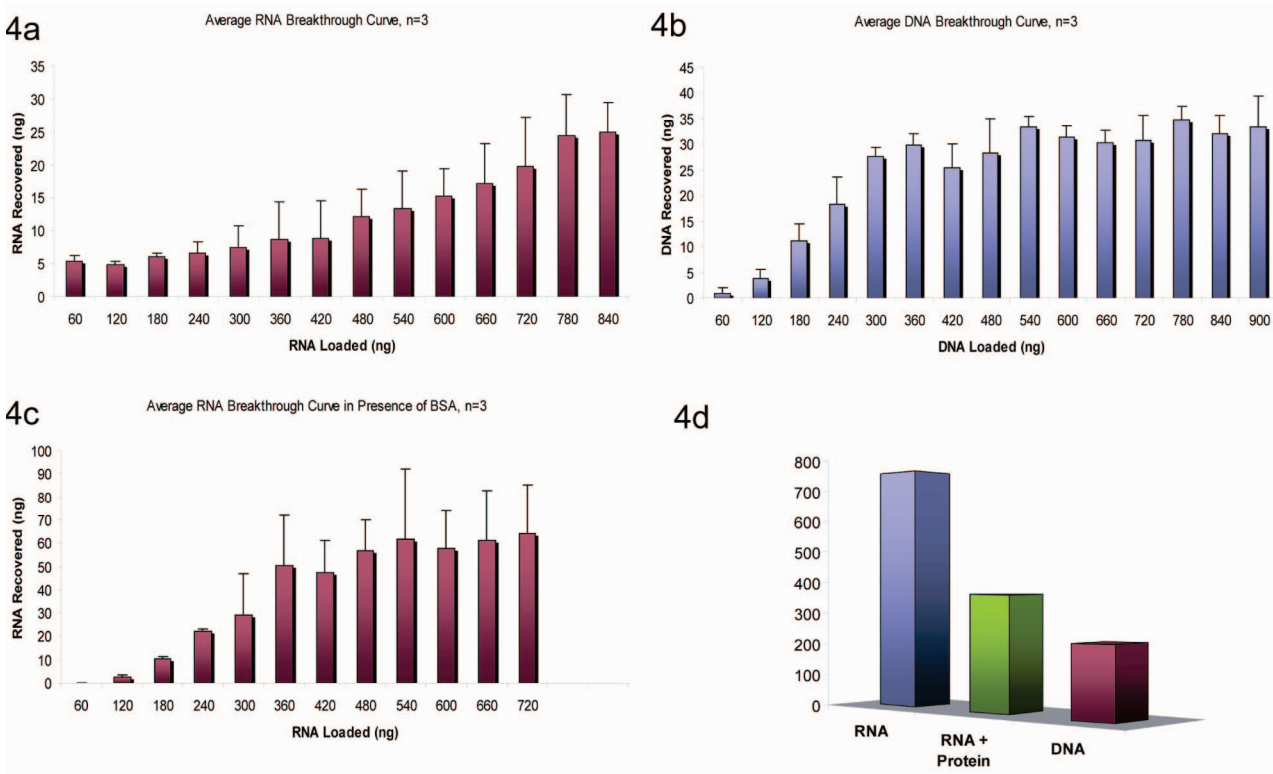

Figure 4. Capacity studies for RNA (±protein) and DNA binding to silica phase in a microdevice. Breakthrough curves from which capacity was determined to be for (a) RNA, 760 ng; (b) DNA, 223 ng; and (c) RNA in the presence of protein, 360 ng. (d) Comparison of capacities for RNA, RNA in the presence of protein, and DNA.

out in a fashion identical to that described above ($n = 3$), substituting DNA for RNA in the sample load step. Figure 4b shows that the breakthrough point for λ-phage DNA occurred at ~7.5 μL (fraction 3.72) indicating the capacity of the device and phase for DNA is ~223 ng. Interestingly, this capacity is ~3-fold lower than that for RNA, potentially signifying that the binding mechanisms of DNA and RNA to silica beads may differ—a suggestion also proffered by Melzak et al.[21]

Finally, a third capacity study using a model biological sample was undertaken to investigate the effect of competitive protein binding on the RNA capacity of the microdevice. Protein in biological samples can compete effectively for nucleic acid binding sites on the silica solid phase, particularly when the protein concentration dwarfs that of the nucleic acids, reducing the overall capacity of the system.[22] For these experiments, a solution of 30 ng/μL rRNA and 150 ng/μL BSA was used as the load solution, modeled after the RNA/protein ($R_{RNA:Pr}$) ratio found in prokaryotic organisms (typically 0.2 on average[23]). The experiment was carried out as previously described above, in a fashion identical to that for RNA and DNA, ($n = 3$) and as depicted in Figure 4c, the breakthrough point occurs at fraction 6 (~12 μL). The resultant calculated capacity was found to be 360 ng, roughly half the 760 ng capacity for RNA alone, demonstrating that the expected shift to a lower capacity does occur as proteins are introduced to the silica bed. A capacity of 360 ng in the presence of proteins, however, is more than sufficient for most genetic analyses, and consequently, the chip design and capacity were deemed appropriate for further investigation with more complex biological samples. Figure 4d highlights the compiled results of these three extractions, illustrating the capacity measurements for the device with these various different loading conditions. The results from these initial characterization experiments are essential for the continued successful development of an mRNA μ-TAS.

Purification of RNA from a Biological Sample. To illustrate that amplifiable RNA could be extracted from a crude biological sample and that the resultant RNA was suitable for amplification using RT-PCR, RNA purification from neat semen (obtained from anonymous donors following a procedure approved by the University's Institutional Review Board), for possible extension to forensic body fluid identification, was carried out. Microchip solid-phase purification was performed on neat semen samples, followed by RT-PCR analysis. A load solution (100 μL total volume: 96 μL of 6 M GuHCl and 4 μL of semen, with a final concentration of 40 mM DTT) was prepared and introduced onto the silica bed at a flow rate of 7.0 μL/min such that 1 μL of semen was effectively loaded. Following the sample load step, a 10 min wash with 80% ethanol was performed for removal of all interfering proteins. Finally, during elution with DEPC-treated water, four 5 μL fractions were collected and DNA in each sample was digested postextraction to eliminate possible amplification of contaminating DNA during downstream RT-PCR analysis. It is worth mentioning that for some applications, a larger total eluate volume or mass of RNA might be advantageous, in which case the eluate fractions can be collected and pooled into one or more larger volume aliquots for downstream processing. Total analytical time for the microchip-

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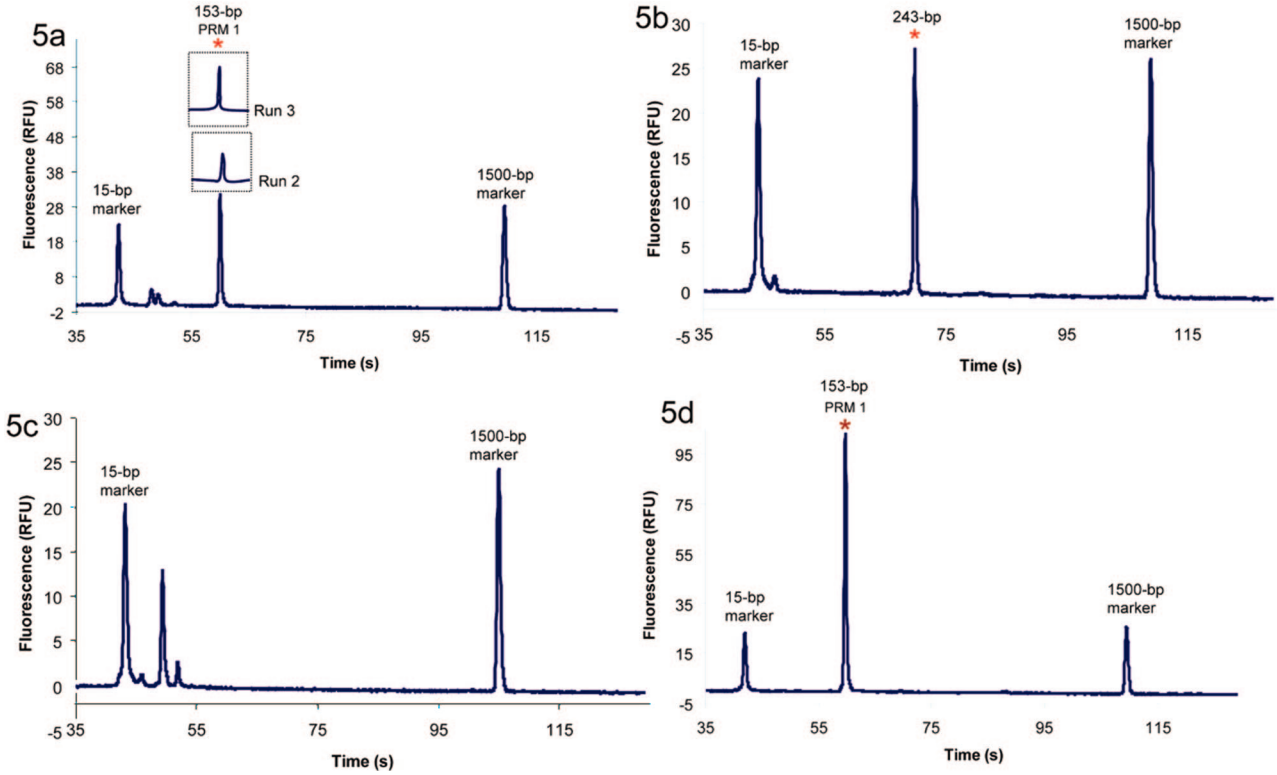

Figure 5. Microdevice-based purification of RNA from human semen. (a) After purification, amplification of eluate results in the presence of a 153-bp amplicon (asterisk) indicative of the semen-specific protamine 1 (PRM 1) gene. (b) When human genomic DNA is amplified using the same primers for PRM 1, the resulting amplicon is 243 bp in length. (c) No amplicon is observed after amplification of samples lacking reverse transcriptase. (d) A 153-bp amplicon is present after amplification of RNA purified from neat semen using a commercial extraction method.

based extraction from neat semen was ~16 min. Following digestion, 5 μL aliquots were distributed into two PCR reaction tubes for each of the four digested fractions (eight samples total). As a control to ensure that DNA was successfully digested in the purified samples, four of the samples were amplified without reverse transcriptase present. The primers used for this reaction, which can be used for body fluid identification as described by Juusola et al.,[14] were gene-specific to mRNA present only in semen. As expected, a 153-bp amplicon indicative of the semen-specific protamine 1 (PRM1) gene was seen in each RNA sample amplified with reverse transcriptase present (Figure 5a) in three replicate experiments.

Additionally, with these primers, when a human genomic DNA positive control (1 ng of hg-DNA) is amplified, the resulting target amplicon peak is 243-bp in length (depicted in Figure 5b). Consequently, if contaminating DNA was present in a sample and not digested, the resultant amplicon would be larger than the 153-bp peak expected for RNA and would be present in those samples amplified without reverse transcriptase. There was no amplicon present in samples lacking reverse transcriptase (Figure 5c), demonstrating successful removal of contaminating DNA by digestion, making the identification of semen as the body fluid in question, based on the presence of the gene protamine 1, unambiguous.

To further demonstrate the effectiveness of the microchip-based purification protocol, the μSPE purification method was compared with a commercial silica-based kit (Qiagen RNeasy MinElute kit, Qiagen Inc.). Three replicate purifications were performed on neat semen samples (1 μL of semen diluted to a total volume of 450 μL with DEPC-treated water and Qiagen lysis buffer) using the commercial extraction method, with a final concentration of 40 mM DTT added to the provided lysis buffer to promote effective sperm cell lysis. Three 2 μL aliquots of each of the three replicate RNA extracts were amplified by RT-PCR. The expected 153-bp amplicon can be seen in the electropherogram representative of a Qiagen-extracted RNA sample in Figure 5d. As indicated by the amplicon detected, the microchip-based SPE method was utilized to successfully extract and purify RNA from a crude biological sample with results consistent with those achieved by utilizing this comparable conventional technique. An advantage of the microchip-based method is that it yields a more concentrated eluate with the majority of the concentrated RNA eluted in a volume of 4 μL that could easily be isolated from the first 6−8 μL of eluate for future integrated processes, as opposed to a larger 14 μL total volume of eluate yielded by the commercial method. Furthermore, as this work represents the initial characterization of this method for inclusion in a μ-TAS, these results indicate that this method, with its smaller elution volumes, can be easily incorporated into an integrated analysis system.

Purification of RNA from a Semen Stain. To demonstrate the effectiveness of the proposed μSPE method for forensic applications, where body fluid identification would often be performed on sexual assault evidence, purification of RNA from a semen stain, a sample more typical of forensic evidentiary materials, was attempted. Mock semen stains were created by pipetting 50 μL of neat semen onto 10.5-cm^2 squares of white 100% cotton cloth, which were then exposed to air, and allowed to dry overnight at room temperature. A 6-mm^2 patch was cut from the

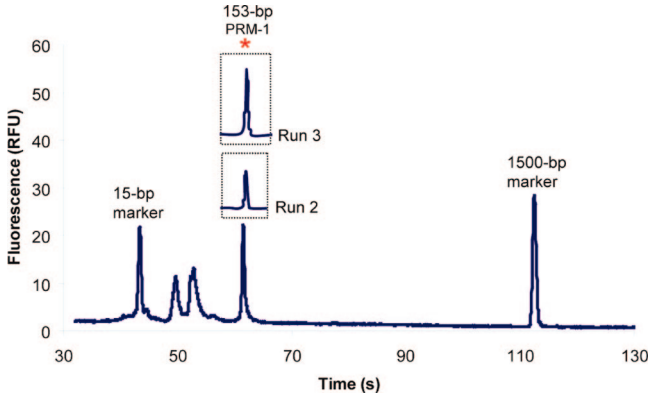

Figure 6. Microdevice-based purification of RNA from a semen stain. A 153-bp amplicon indicative of the semen-specific protamine 1 gene is present after amplification of purified RNA extracted from a semen stain using the microchip-based SPE method.

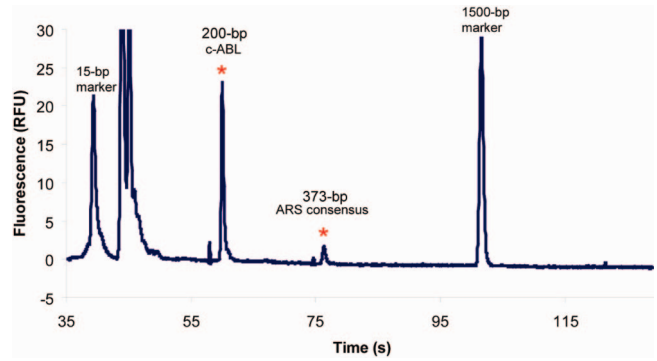

Figure 7. Purification of RNA from a cancer cell line using microdevice RNA SPE. The microchip-based SPE method was used to purify RNA from an alveolar rhabdomyosarcoma cell line. The extracted RNA was amplified and amplicon at 200 and 373 bp resulted, indicating successful purification and amplification of RNA from a cancer cell line using μ-SPE.

center of the dried stain and placed in a solution of 6 M GuHCl containing 40 mM DTT for cell lysis. Prior to extraction, the microchip filled with 15−30 μm silica beads was conditioned with the lysis/binding buffer. The lysed sample was subsequently loaded onto the bed of the microchip as described. Following a wash with 80% ethanol for 10 min to remove proteins and other cellular debris, the RNA was eluted with DEPC-treated water. 20 μL of eluate was collected (four 5 μL fractions), and 5 μL of DEPC-treated water added to each sample prior to digestion of the DNA present in the sample. The extraction of RNA from this mock semen stain was completed in as few as 18 min (analytical time). The 6 μL aliquots of the resulting DNA-free solution were placed into four PCR reaction tubes, amplified by RT-PCR for the semen-specific protamine 1 gene, and the resultant PCR products separated using microchip electrophoresis. In each of three replicate extractions performed, a 153-bp peak indicative of the PRM1 gene was present; a representative electropherogram from the first fraction of each replicate can be seen in Figure 6. These results highlight the effectiveness of the rapid μSPE method for the purification of amplifiable amounts of RNA from a semen stain, representing a typical forensic sample and the potential application of this technology to forensic body fluid identification. The extraction procedure utilizes smaller elution volumes than a traditional commercial extraction method, concentrating the recovered DNA in the eluate. Additionally, microchip-based SPE is performed in a closed environment with fewer sample transfer steps and fewer opportunities for the introduction of contaminants to the system, imperative for forensic analyses and for sensitive RNA assays. These advantages show that the microchip-based SPE method for the isolation and purification of RNA lends itself well to the field of forensics, where reduced analysis costs and less opportunity for sample contamination are important issues to consider. The integration of this SPE method with downstream sample processing steps, such as RT-PCR, separation, and detection, will allow for more timely identification of body fluids present in forensic evidence, leading to expedited processing of evidence and potentially, decrease the time required for an investigation.

Purification of RNA from a Cancer Cell Line. Application of the microchip-based SPE method to the purification of RNA for clinical diagnostics was also demonstrated by extracting RNA from a cancer cell line to show pertinence of this method in clinical diagnosis. In ARS, the most common pediatric sarcoma and the sixth most common cancer in children, the oncogene PAX3:Fkhr, a fusion gene present in 90% of children with ARS, results in inappropriate muscle development. A protocol used for the diagnosis of ARS, as well as other cancers present in children, involves a RT-PCR fusion transcript analysis, where primer sequences specific for a fusion transcript of interest are used. When certain amplicons are detected, a diagnosis of the cancer can be made or confirmed,[11] and these protocols rely on the recovery of purified RNA from the malignant cells. In order to explore the effectiveness of the μSPE RNA purification method, samples of an ARS cell line were obtained and evaluated.

For these proof-of-concept experiments ($n = 3$), a load solution was made from an aliquot of ARS cultured cells mixed with proteinase K, and heated at 56 °C for 10 min. The appropriate volume of a stock solution of 6−8 M GuHCl, pH 6.1, was then added to this solution (to ensure a final concentration of at least 4.8 M GuHCl, for sufficient binding to the silica phase[1]) to make a 400 (±100) cells/μL load solution, and this mixture was heated again at 56 °C for 10 min. The sample pretreatment heated incubation steps allowed for more facile flow onto the microdevice. Following loading, a wash of 80% ethanol was again used to remove cellular debris, and the RNA subsequently eluted with DEPC-treated water. Four 5 μL fractions were collected during elution and diluted with 5 μL of DEPC-treated water for DNase treatment. The complete extraction process of RNA from the ARS cultured cells was successfully performed in a total analytical time of 20 min. From each of the four fractions, a 6 μL aliquot was transferred to a PCR tube for cDNA synthesis and RT-PCR using the RETROscript First Strand Synthesis Kit for RT-PCR. A duplex RT-PCR reaction was carried out using c-ABL primers, which allow identification of the presence of amplifiable RNA in a sample and ARS consensus primers, which indicate the presence of ARS in the sample of interest. According to the protocol from the Molecular Genetics Laboratory at Children's Hospital (Columbus, OH), results for a patient sample are only reported if successful amplification with c-ABL primers, along with the ARS consensus primers, is achieved. As shown in a representative electropherogram depicted in Figure 7, amplicon is present at 200- and 373-bp, indicating RNA from the c-ABL and ARS consensus genes was amplified successfully. These results illustrate the potential that the microchip-based SPE method for the purification of RNA

could be integrated with downstream analytical processes and used in a point-of-care device for clinical diagnostics, as shown by the positive results obtained from a representative cell line. The results from the microchip-based SPE method highlight the advantages over a conventional commercial extraction method, permitting purification of RNA with smaller reagent volumes in a closed environment free of RNases and contaminants. Furthermore, the microchip-based SPE method can be incorporated in the future into a point-of-care device, integrated with other sample processing steps such as PCR and ME, allowing for more rapid analysis time as well as more timely diagnosis.

CONCLUSIONS AND FUTURE WORK

A microchip SPE method has been established for the purification of RNA from biological samples. An elution profile was obtained for the solid-phase extraction of RNA using silica beads (30 μm), which provided ample RNA binding capacity (both in the presence or absence of proteins). The RNA purified using the μSPE method was proven to be suitable for downstream RT-PCR analysis, as an amplifiable mass of RNA was extracted from neat semen, mock evidentiary semen stains, and a cancer cell line. As proof of concept, this work demonstrates the broad utility of a μSPE silica-based method for further application to RNA purification from biological samples. There are ultimately two great advantages of the microchip-based method over commercial spin-column methods for RNA extraction: (1) The purification is performed in a closed environment during μSPE, allowing less opportunity for the introduction of exogenous contaminants and RNases to the sample, and (2) the μSPE process can be easily integrated with downstream analytical methods for complete sample processing as a portable, contamination-free system. The work presented here has been tailored toward forensic applications for potential use in mRNA expression analysis for body fluid identification, as well as to clinical applications for potential use in cancer diagnostics. Future endeavors will involve pursuing other clinical applications, as well as more diverse forensic protocols, to demonstrate the breadth of the μSPE method for RNA purification. Subsequent work will detail the purification of RNA from other common biological samples and, ultimately, demonstrate the utility of a silica-based μSPE method for mRNA expression analysis in integrated micrototal analysis systems.

ACKNOWLEDGMENT

K.A.H. and J.M.B. contributed equally to this work. The authors acknowledge Agilent Technologies, Inc. for the DNA 1000 Series II kits used in this work.

Received for review June 12, 2008. Accepted July 30, 2008.

AC8011945