An Injection-Molded Device for Purification of Nucleic Acids From Whole Blood Using Isotachophoresis

Lewis A. Marshall,1 Anita Rogacs,2 Carl Meinhart,3 and Juan G. Santiago2
(1) Chemical Engineering, Stanford University, Stanford, CA, (2) Mechanical Engineering, Stanford University, Stanford, CA, (3) Mechanical Engineering, UCSB, Santa Barbara

Abstract

We present a novel microchip device for purification of nucleic acids from 25 µL biological samples using isotachophoresis (ITP). The device design incorporates a custom capillary barrier structure to facilitate robust sample loading. The chip uses relatively large channel dimensions to reduce processing time, minimize Joule heating, and achieve high extraction efficiency. To reduce pH changes in the device due to electrolysis, we incorporated a buffering reservoir physically separated from the sample output reservoir. To reduce dispersion of the ITP-focused zone, we used optimized turn geometries. The chip was fabricated by injection molding PMMA and COC plastics through a commercial microfluidic foundry; this fabrication approach is compatible with mass-production. The extraction efficiency of nucleic acids from the device was measured using fluorescent quantification, and an average recovery efficiency of 81% was achieved for nucleic acid masses between 250 ng and 250 pg. The devices were also used to purify DNA from whole blood, and the extracted DNA was amplified using qPCR to show the PCR compatibility of the purified sample.

Introduction

Isotachophoresis (ITP) for nucleic acid purification is a sample preparation technique with a growing portfolio of applications. ITP has been used to isolate DNA from human blood,1–3 serum, and plasma,4,5 as well as RNA purification from bacteria in urine7 and blood,8 and purification of small RNA from total RNA from kidney cells.6 ITP purification is a valuable alternative to conventional solid-phase extraction and liquid-liquid extraction techniques for nucleic acid purification as it can extract from very low abundance samples and does not require centrifugation or toxic chemistries.

Important early work on ITP purification of nucleic acids was performed by V.N. Kondratova and co-workers, who concentrated and isolated extracellular DNA from blood plasma and urine in agarose gels. Their devices were capable of delivering efficient extraction in limited time.4,5 However, their isolation procedure used centrifugation and overnight dialysis as crucial steps in removing all cells (and potentially PCR inhibitors), each prior to ITP. In addition, their ITP isolation procedure yielded DNA within an agarose gel slab, which required further purification prior for analysis. Their use of slab- and tube-based gels also makes their approach difficult to automate and miniaturize.

Microfluidic chips are an alternative platform for nucleic acid isolation using ITP. Glass3 and plastic2 microfluidic chips have been used for ITP purification, but to date these devices have been unable to provide high nucleic acid yield compared to the total nucleic acid load delivered to the chip. Typically, less than 1% of nucleic acids applied to a chip have been recovered.

Here, we demonstrate an injection-molded plastic microfluidic chip for ITP purification of nucleic acids that achieves high extraction efficiencies and is capable of processing 25 µL of blood lysate in a single experiment. We describe the design features of this chip, and analyze its performance using fluorescence quantification and quantitative PCR (qPCR).

The higher recovery efficiency demonstrated here is primarily due to two factors: A separation channel volume which is on the order of the sample volume, and the use reservoirs containing buffering
solutions free of sample for electrolysis. We achieve high volume separation channel with a 2 x 0.15 x 100 mm separation channel which maintains sufficient heat rejection to avoid unwanted effects of Joule heating. We placed the electrodes in dedicated buffering reservoirs, which are free of sample and contain high local buffer concentration. These reservoirs are effective in buffering the pH insults caused by electrolysis, even when large volumes are processed. The design draws on scaling analyses for ITP purification as presented by Marshall et al.9

Experimental

Device Design and Features
We generated the geometry of our custom microchip using commercial computer aided drawing (CAD) software (AutoCAD, AutoDesk, San Rafael, CA). The design has a nominal channel depth of 150 µm, a nominal width of 2 mm, and a total channel length (sum of sample and separation sections) of 20 cm. The channel is divided into a sample channel, with a total volume of 25 µL, and a separation channel, with a total volume of 30 µL. The design includes four reservoirs with access to the fluidic channel, and each reservoir can hold a total volume of 70 µL. Each reservoir is designed to be compatible with Leur lock connectors.

Our microfluidic device design was fabricated by a commercial microfluidic foundry (Microfluidic Chipshop Gmbh, Jena, Germany). The devices were fabricated by injection molding the fluidic layer and reservoirs. These devices were manufactured both in poly-methyl methacrylate (PMMA) and Topas, a cyclic olefin copolymer (COC). The devices were sealed with plastic films with thickness of 140 µm (COC) or 175 µm (PMMA).

The polymers here were chosen for their surface properties, optical quality, and compatibility with injection molding. COC has a reported equilibrium water contact angle of around 97°, while the PMMA is more hydrophilic, with a reported water contact angle of 60°.10 The finished device is shown in Figure 1.

Figure 1. Image of the finished device, with channels loaded with water dyed with blue and red food coloring. The sample channel (on the left half of the 25.5 by 75.5 mm chip) is orange, while the separation channel is blue. A more detailed view of the junction between these two channels and the structure of the connected air outlet channel is shown Figure 2. The device was manufactured using injection molding of COC and PMMA. Only the reservoirs in the top left and the two on the bottom right are used and connected to the channels (as highlighted in Figure 4).

Each channel corner in the device is an optimized 90° turn designed by Molho et al. to minimize electrokinetic dispersion due non-uniform electric fields in the turn.11 These turns have a constriction ratio of 0.5 and a recovery ratio of 1.0, as defined by Molho.

The reservoirs in the device are arranged so that the two electrode-containing reservoirs can be configured for extremely high buffering capacity without affecting the chemistry of either the sample or the extracted nucleic acids.12 To achieve this, the sample is loaded directly into the sample channel section, and not into the trailing electrolyte (TE) buffering reservoir. Further, the leading electrolyte (LE) buffering reservoir is separate from the sample extraction reservoir, and connected to the latter by a short channel. See loading process below for more details.
We designed and introduced specialized loading structures at the junction between the sample and separation channels to allow creation of a sharp interface between the sample and the leading electrolyte solutions. These structures also enable loading of the sample into the device without wasting liquid into the vacuum port. These structures operate in a manner similar to the phase-guide described by Vulto et al.\textsuperscript{13} or more commonly applied microfluidic capillary valve structures.\textsuperscript{14} We use ramps and a sudden expansion in channel height to achieve what we term capillary barriers. The ramps reduce the height of the channel from 150 µm to 75 µm over a distance of about 2 mm along the channel. This ramp rises from the bottom wall of the channel and terminates in a sharp step in channel height back to 150 µm. Liquid wicking up to this structure faces an energetic barrier associated with expanding past the ramp (as additional liquid surface area is required for the liquid to advance). These valve structures are oriented so that the liquid stopped by the capillary barrier can be wetted by liquid from an adjacent channel, creating a bubble-free liquid-to-liquid interface. We used two capillary barriers: One at the sample-to-separation buffer interface, and a second inside the connected vacuum outlet channel (labeled “Final fluid stop” in Figure 2). The filling and liquid-to-liquid mating operation enabled by these structures is summarized in Figure 2.

**Figure 2.** Stages in capillary-barrier-aided loading visualized using food coloring in water. a. The blue liquid, simulating separation buffer, is initially loaded into the separation channel. -0.1 psig vacuum is applied at the vacuum port, and the separation buffer flows to the capillary barrier, where it stops at the precise edge of the expansion downstream of the ram. b. The red liquid, simulating the sample solution, enters through the sample channel. c. The sample solution forms a liquid-to-liquid interface (repeatedly free of bubbles) with the separation buffer. The two liquids volumes then flow in parallel toward the air outlet. d. The volume near the liquid-to-liquid interface is run up against and stopped by the second capillary barrier inside the vacuum port. This avoids wasting either liquid into the vacuum. A sharp interface is preserved between the two liquid zones and the ITP process is ready to begin.

**Visualization**

We monitored ITP by fluorescence imaging using either an epifluorescence microscope or a stereoscope and adsorption filters. The upright epifluorescent microscope (BX40, Olympus) was equipped with a 2x objective (PlanApo NA=0.08, Olympus), and a collimated blue LED (M470L2 Thor Labs). We filtered the fluorescent signal using a filter cube designed for FITC (excitation/emission of 485/535 nm, Omega, VT). The images were collected through a 0.63x demagnification lens (model RD060-CMT, Diagnostic Instruments, MI).
The stereoscope (Olympus SZ40) with adjustable magnification was used for imaging, and was optionally fitted with 0.3x or a 1.5x Barlow lenses to adjust the magnification. This resulted in a total magnification as low as 0.19x, which allowed the stereoscope to include the entire custom chip in the field of view. For fluorescence visualization in the stereoscope, the chip was illuminated by the collimated blue LED, and the light was filtered using a single emission filter (Semrock, 550/32 BrightLine® Bandpass Filter, 25 mm) placed in the camera light path. Fluorescent images were captured using a monochrome CCD camera (MicroMax, Roper Scientific). For color imaging, ambient light was used for illumination, and no filter was used, and for Figure 2 the Barlow lens was removed. These images were captured on a color CCD camera (CoolSnap cf, Photometrics, Tuscon, AZ).

**Experimental Chemistry**

To perform fluorescence quantification of extraction efficiency on these devices, we used an idealized chemistry with samples composed of salmon sperm DNA diluted in buffer. To create the calibration curves, we diluted the sodium salt of salmon sperm DNA (Sigma Aldrich, St. Louis, MO) in an aqueous buffer composed of 20 mM Tris, 10 mM HEPES, with 1x SYBR Green I (Invitrogen, CA), pH = 8.2. We used DNA concentrations ranging from 100 pg/µL to 10 ng/µL. To perform ITP with these same samples, we prepared a LE composed of 100 mM Tris, 50 mM hydrochloric acid (HCl), and 1x SYBR Green I, pH = 8.2. We used a buffering leading electrolyte (BLE) with 500 mM Tris, 250 mM HCl, and 25% w/v Pluronic F-127. The buffering trailing electrolyte (BTE) was composed of 500 mM Tris, 250 mM HEPES, and 25% w/v Pluronic F-127. The Pluronic F-127 in both the BLE and BTE acted as a temperature-sensitive gel that acts as a liquid at 0 °C and a solid at 25 °C. This helped stabilize the device to pressure driven flow. As such, we placed both the BLE and BTE on ice until they were pipetted onto the chip.

To perform ITP purification of blood with these devices, we used an LE composed of 90 mM Tris and 60 mM HCl (pH=7.9). The BLE and BTE solutions are the same as used for fluorescence quantification. We prepared blood lysate by mixing 10 µL of whole blood anticoagulated with acid-citrate-dextrose (ACD) with 190 µL lysis buffer, composed of 25 mM Tris, 17 mM HCl, and 1% Triton x-100, and proteinase K, 1x SYBR Green, 0.1% PVP, and 40 mM dithiothreitol (DTT). The whole blood was purchased from the Stanford Blood Center and stored frozen at -20°C prior to use.) This sample was then held at 65°C for 10 min. The blood lysate was then placed on ice until it was pipetted onto the chip as the sample.

We also performed experiments in which we separated fluorescent dyes in the device. We separated fluorescein (FL) and Alexa Fluor 488 (AF488), which emit fluorescence in the same wavelength region (excitation at 495 nm, emission at 519 nm), but have different mobilities at pH below 7. For these experiments, we used an LE composed of 70 mM ε-aminocaproic acid and 35 mM HCl with 0.1% w/v PVP (pH=4.6). The sample was composed of 30 mM ε-aminocaproic acid and 15 mm ascorbic acid, 0.1% w/v PVP, 5 µM AF488, and 100 µM fluorescein (pH = 4.6). The BLE was composed of 200 mM ε-aminocaproic acid, 100 mM HCl, and 20% Pluronic F-127. The BTE was composed of 200 mM ε-aminocaproic acid, 100 mM Ascorbic acid, and 20% Pluronic F-127.

Lastly, we used commercial food coloring for preliminary visualizations of the channels in the device, as shown in Figures 1 and 2. In these cases, we mixed Super Red and Super Blue food coloring (Ateco, Sea Cliff, NY) with deionized water until the desired color saturation was achieved.

**Loading Process**

Microchips were received dry from manufacturer and kept dry until use. In each experiment, the liquids were loaded in the same order. First, we pipetted 50 µL LE into the extraction reservoir. Vacuum
(-0.1 psig) was applied at the buffering LE reservoir until the channel adjacent to it was filled, then 25 µL BLE solution was added to the buffering LE reservoir. We then applied vacuum (-0.1 psig) at the air outlet until the LE filled the entire separation channel (the liquid stopping at the capillary barrier). Then, we added the 25 µL sample to the buffering TE reservoir. Vacuum was again applied at the air outlet until the sample wetted the LE interface. Finally, BTE solution was immediately pipetted into the buffering TE reservoir to arrest pressure driven flow. This loaded chip was then used for ITP.

**ITP Purification**

We performed purification by placing 2 cm platinum wire electrodes in the LE buffering reservoir and the TE buffering reservoir. We applied a constant current of 100-250 µA between these two reservoirs using a Kiethley 2410 sourcemeter (Keithley Instruments, Cleveland, OH). The current was deactivated when the nucleic acid zone reached the extraction reservoir.

**Off-Chip PCR**

We monitored the DNA zone in the channel during ITP by fluorescently imaging the SYBR-labeled DNA using a fluorescence microscope. When the ITP interface fully eluted into the extraction reservoir, we gently mixed the liquid in the reservoir (~25 µL) by pipetting, then collected it into an Eppendorf tube using the same pipettor.

For each collected sample, we ran triplicate PCR reactions. To set up each PCR reaction, we pipetted 2 µL ITP-purified DNA into a PCR tube containing 10 µL of 2x Fast SYBR Green I master mix (Applied Biosystems, CA), and 200 nM primers (Invitrogen, CA). The primers were designed to amplify a 201 bp segment of the human BRAC2 gene. We used DNAse free water to adjust the volume of each reaction to 20 µL. We then performed off-chip quantitative PCR (qPCR) using a miniOpticon qPCR thermocycler (Bio-Rad, Hercules, CA). We performed the reaction with the following thermal profile: 20 s initial hold at 95 °C and 40 cycles composed of 3 s denaturation at 95 °C and 30 s annealing and extension at 60 °C. We then obtained post-amplification melting curves between 55 and 85°C using the same instrument. We also ran negative control reactions using the LE and the unpurified whole blood lysate as template.

**Results and Discussion**

**Separation Demonstration**

We demonstrated separation in the device using a model system composed of two dyes, AF488 and FL. We separated them at pH 4.4, where fluorescein has a mobility of $12 \times 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$, while AF488 has a mobility of $36 \times 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$. We recorded the separation of this system using a stereo scope. Figure 4 shows this system near the end of the separation process. The AF488 is focused to the ITP interface. The FL remains unfocused, in a wide zone behind the ITP zone. There is a dye-free zone, approximately 3.5 cm long, between the AF488 peak and the leading edge of the FL. This is the separation distance that allows collection of the AF488 peak without contamination by FL. This model system is illustrative of the separation that takes place to purify nucleic acids from lower-mobility contaminating species like proteins.
Figure 4. Demonstration experiment showing separation between FL and AF488 on the chip. The FL (red) is electromigrating in a long zone well behind the ITP interface. The AF488 (dark green) has collected to the ITP interface. The dye images are shown here with false color, AF488 in green and fluorescein in red. The focused AF488 zone is in the image about to elute into the extraction reservoir, allowing it to be selectively collected, while the FL remains behind, unfocused and distributed throughout a large portion of the separation channel.

In this system, the LE has a separation capacity of approximately 173 mC (charge processed), and the sample has a separation parameter (charge required to completely process sample) of 24 mC. The separation capacity is greater than the separation parameter, and so AF488 can be collected at the ITP interface with high efficiency (prior to its arrival at the extraction well). The separation was performed at 100 µA, and took approximately 20 min to complete.

Quantification Demonstration
We measured the recovery of salmon sperm DNA with the chip using fluorescence quantification. First, we filled the channel with known concentrations of salmon sperm DNA suspended in LE, and established a calibration curve relating the fluorescent signal collected by the CCD camera to DNA concentration (Figure 5). We then performed ITP from samples of salmon sperm DNA of known concentrations suspended in LE. We used a device configuration in which the sample had a separation parameter of 55 mC, and the device had a separation capacity of 159 mC, so that the processing efficiency is not limited by the flux of nucleic acid to the ITP interface. A set of 10 images of the ITP zone were taken near the extraction reservoir, and the average integrated fluorescence signal of the zone in these images was used to quantify total DNA amount. The DNA recovery was computed using the DNA calibration curve. As shown, recovery efficiency is consistently 76-86%, for the entire 250 pg to 250 ng of DNA range explored.
**Figure 5.** Fluorescence quantification of DNA. The fluorescence calibration curve of DNA dyed with SYBR Green I, measured by fluorescent signal from the CCD camera. The data points were fit with a linear curve over 2 orders of magnitude with a regression value of $R^2=0.9997$. **Inset a.** The estimated recovery efficiency of known concentrations of salmon sperm DNA spiked onto the chip. The efficiency estimate is based on fluorescence quantification, computed from the calibration curve. **Inset b.** A representative image of DNA focused to the ITP interface and electromigrating through the device. This DNA band is approximately 8 mm from the extraction reservoir.

**DNA purification from Whole Blood**

Finally, we purified DNA from whole human blood samples using this device. While nucleic acid purification from blood samples has been demonstrated,1-3,5,8 the results have so far been complicated by the dilution factor of the extracted sample into the qPCR reaction. For example, typical previous ITP extraction studies have diluted blood samples $10^3$ to $10^4$-fold as the liquid is dispensed into the PCR reaction. The latter dilution also dilutes inhibitors, and so it becomes interesting to test less aggressive dilutions of the extracted DNA.

In this demonstration, blood was diluted 10x when preparing the blood lysate. DNA from the 25 µL blood lysate sample was then extracted via ITP and eluted into the 25 µL volume of our chip’s extraction reservoir. Therefore, both the chip and extraction processes contribute to no change of lysate sample volume (no dilution). After extraction, the DNA sample was diluted 10x into the PCR master mix. The total dilution factor of the entire process (the lysing, extraction, and PCR solution preparation) was 100x, more directly showing the power of ITP as a purification method.

**Figure 6.** qPCR analysis of DNA purified from whole human blood using ITP on the new microfluidic device. DNA extracted using ITP, and control samples were amplified in the presence of a primer for the human gene BRAC2. The log of fluorescence signal versus cycle number is plotted. The negative control samples, (template-free LE buffer and unprocessed blood lysate) have fluorescent signals that remain below the threshold, indicating negligible PCR amplification. DNA extracted from blood using ITP amplifies leading to fluorescence well above threshold. The melting temperature of the amplicon (not shown) from all amplified samples was 74°C. This temperature matches approximate theoretical predictions from the Promega amplicon melting tool.17

**Conclusions**

We have demonstrated for the first time a microfluidic chip capable of achieving highly efficient ITP purification of nucleic acids from 25 µL biological samples. We designed this chip by taking into
account principles of ITP separation capacity, throughput, pH buffering capacity, and dispersion minimization. The chip incorporates high aspect-ratio channels to improve heat dissipation, and optimized turn geometries to reduce dispersion around corners. The chip uses separate buffering reservoirs to decouple the buffering capacity of the device from the sample and extraction reservoir chemistry. The chip incorporates a capillary barrier structure to allow sequential loading of the fluids without loss of fluid into the vacuum port. These structures provide robust, repeatable loading using easily achievable vacuum levels. Lastly, the chip was designed for and fabricated in common COC and PMMA using injection molding, which shows the possibility for simple and scalable fabrication of these devices. This chip design can act as a platform for future studies of ITP purification by allowing practical sample volumes to be processed in about 20 min, without wasting precious sample volume.

**Acknowledgements**

We gratefully acknowledge funding from Defense Advanced Research Projects Agency (DARPA) under contract no. HR0011-12-C-0080, program manager Daniel J. Wattendorf.

**References**