Anal. Chem. **2009,** *81,* 9507–9511

Technical Notes

Purification of Nucleic Acids from Whole Blood Using Isotachophoresis

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We present and demonstrate a novel technique for the purification of nucleic acids from biological samples using isotachophoresis (ITP). We demonstrate a simple and rapid method to achieve ITP-based extraction, preconcentration, and purification of DNA from nanoliter volumes of whole blood. We show that ITP purification yields genomic DNA samples which can be quantitated with fluorescence measurements and are immediately compatible with polymerase chain reaction (PCR) (e.g., a PCR-friendly solution free of significant inhibitors). We hypothesize ITP purification is applicable to processing of a wide range of complex biological samples.

Microfluidics has become an alternative to traditional techniques for biological and medical analysis and offers the use of small reagent volumes, fast analyses, and the potential for parallelization.[1] Polymerase chain reaction (PCR),[2] capillary electrophoresis,[3] immunoassays,[4] and many other analytical techniques used in biology and medicine have been successfully miniaturized. However, sample preparation is often still a challenge and a limiting factor in the capability of many devices,[5] so that most miniaturized systems have used prepurified, ideal samples as analyte. One important application is the purification of nucleic acids (NA) from complex biological samples. We here demonstrate a simple, fast, efficient, and sensitive technique for the purification of NA from whole blood which leverages the physicochemistry of isotachophoresis (ITP).

The standard method for NA purification is based on solid phase extraction (SPE). For example, commonly used QIAGEN (Valencia, CA) purification columns rely on the adsorption of NA on silica membranes.[6] Extensive work by Landers and co-workers has shown successful microchip integration of SPE with application to purification of DNA[7] and RNA[8] and successful integration with on-chip PCR.[9] While micro-SPE shows excellent efficiency and throughput,[6] the process requires specialized materials and fabrication (e.g., micropillars or packing of silica beads). Further, the typical SPE protocol involves three successive steps (loading, washing, elution), requires bulk flow control, and uses a PCR inhibiting chemistry (e.g., chaotropic agents, organic solvents).[6]

ITP is a well established separation and preconcentration technique.[10,11] It leverages a heterogeneous buffer system to generate strong electric field gradients, allowing simultaneous focusing and separation of ionic species based on their effective electrophoretic mobilities (see schematic in Figure S-1 in the Supporting Information).[12] ITP has been marginally used as a sample purification method.[13,14] For instance, Caslavska et al.[15] used ITP to simultaneously purify and isolate proteins. Kondratova et al.[16] concentrated and isolated extracellular DNA from blood plasma and urine by agarose gel ITP with applications to cancer diagnosis. This ITP isolation procedure yields DNA in an agarose gel slab which requires further purification steps prior to analysis. More recently, Schoch et al.[17] extracted micro-RNA from cultured cells by ITP in a pluronic sieving matrix. To our knowledge, ITP has never been applied to sample preparation from biological fluids for genomic analysis or to sample preparation of blood.

We here present and demonstrate an ITP-based purification method for extracting genomic DNA from a biological sample. We

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10.1021/ac901965v CCC: $40.75
Published on Web 10/15/2009

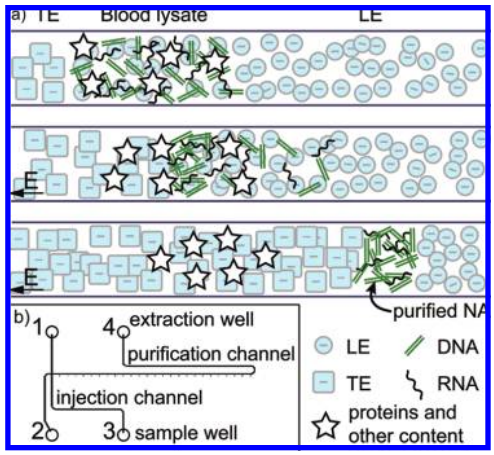

Figure 1. (a) Schematic of the ITP-based NA purification from a complex biological sample like blood lysate. We select LE and TE with mobilities, respectively, larger and smaller than NA. The TE needs to have larger mobility than proteins (and other contents) present in blood lysate. We inject a finite plug of lysate between TE and LE. Upon application of an electric field, NA focus between LE and TE, while proteins cannot focus as they travel slower than the ITP interface. After sufficient time, the ITP zone contains only pure NA extracted from the lysate. In (b), we show the design of the chip used to perform the ITP-based purification. Sample is injected from reservoir 3 by applying a vacuum at 2.

perform ITP focusing in free solution and use a small injected volume (order of 10 nL) of whole blood lysate as sample. We leverage the selectivity of ITP focusing to concentrate NA in a sharp zone while rejecting proteins and other unwanted compounds. We first determine extraction efficiency to evaluate the performance of the technique. We then demonstrate ITP purification of genomic DNA from whole blood and assess its performance with this difficult sample. Finally, we recover the genetic material and perform off-chip PCR to assess the quality of the ITP purification process. Our method uses ITP to purify DNA from whole blood in free aqueous solution and yields genomic DNA ready for analysis.

EXPERIMENTAL SECTION

Microchip Preparation. We performed on-chip experiments in a microchip with 90 μm wide by 20 μm deep borosilicate microchannels in a simple cross geometry (model NS12A, Caliper Life Sciences, CA, cf. Figure 1b). We treated the channels with the silanizing agent Sigmacote (Sigma, MO) as follows. We first rinsed the channel 10 min with a 1:1 methanol/hydrochloric acid solution, followed by 10 min of concentrated sulfuric acid. We then rinsed the channels with deionized water for 2 min or more and dried them thoroughly with a vacuum. Next, we applied the silanizing solution for about 10 min. We then rinsed the channels with hexane and deionized water. To avoid cross contamination, we rinsed the chip between each experiment as follows: 2 min with a 1:10 (v/v) household bleach solution (Clorox, CA), 2 min with deionized water, and 2 min with leading electrolyte buffer (see below).

Sample Lysis. Blood samples from a healthy donor were collected in heparin tubes and stored in 2 mL aliquots at −80 °C. Before each set of experiments, we thawed one blood aliquot and prepared a stock of lysis buffer containing 1% Triton X-100 (Sigma, MO) in 50 mM Tris hydrochloride at pH = 8.2. We diluted 10 μL of whole blood and 4 μL of proteinase K (RNA grade, Invitrogen, CA) in 86 μL of lysis buffer. We then incubated the lysate 10 min at 56 °C in a hot bath. In the case

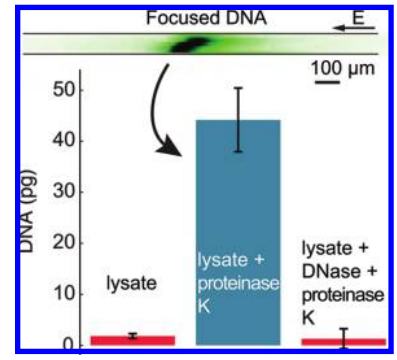

Figure 2. Experimental demonstrations of ITP-based NA purification from human blood. The bars show mean values of NA mass purified from blood lysate, as calculated from the fluorescence intensity profile. We show results from three sets of experiments. ITP purification of pure lysate yields little or no signal and, therefore, little or no extraction of NA. The lysate pretreated with proteinase K shows significant signal enhancement over the previous case. We show an image of ITP-focused DNA zone above the bar graph. The amount of focused DNA is 44.2 ± 6.2 pg. As a control, we performed the extraction with a lysate treated with first DNase and then proteinase K, which significantly lowers the focused DNA mass. Together, these show that the ITP purification method efficiently extracts DNA from human blood lysate. We injected 2.5 nL of blood, which contains between 65 and 162 pg of DNA, so that our purification efficiency for a blood sample ranges between 30% and 70%. Uncertainty bars represent uncertainty in measured mass of DNA with a 95% confidence interval. The three sets are results from $N = 2$, 9, and 2 repetitions, respectively.

of the second control in Figure 2 (third bar in the plot), we added 4 U of deoxyribonuclease I (DNase I, amplification grade, Invitrogen, CA) to the lysate and incubated for 15 min at room temperature prior to proteinase K treatment. To quantify the ITP extraction efficiency, we diluted a commercial standard solution of λ-DNA (0.333 mg mL^{-1}, Invitrogen, CA) in lysis buffer and used this as a standard sample. All solutions were prepared with DNase/RNase free deionized water (Gibco, CA).

Isotachophoresis-Based Purification. Leading (LE) and trailing electrolytes (TE) were, respectively, 50 mM Tris titrated with hydrochloric acid to pH = 8.2 and 50 mM Tris titrated with HEPES to pH = 7.8. LE and TE each contained 1× SYBR Green I (Invitrogen, CA) for fluorescence visualization and on-chip DNA quantitation. We obtained the best results adding also 0.1% Triton X-100 to reduce electroosmotic flow and protein adsorption (in conjunction with silanization treatment).

For each experiment, we first filled all four channels and reservoirs (cf. Figure 1b) with LE. We emptied reservoir 3 with a vacuum and pipetted 1 μL of lysate into that reservoir. We then applied a vacuum to reservoir 2 to fill the injection channel with lysate (~25 nL). We carefully removed the lysate remaining in reservoir 3 and then rinsed and replaced it with TE. We then immediately applied an electric field between 3 and 4 (500 V) with a sourcemeter (model 2410, Keithley, OH) to carry out the purification. We used the current signal to locate the ITP interface in the channel (see Figure S-2 in the Supporting Information). We used this same injection protocol for the extraction efficiency quantitations performed with λ-DNA.

Visualization. We performed on-chip visualization on an inverted epifluorescent microscope equipped with a 4× (Plan APO, N.A. = 0.2, Nikon, Japan) or a 10× objective (Plan APO, N.A. =

0.45); a mercury light source (Ushio, Japan); a filter cube (exciter/emitter 485/535 nm, Omega, VT); and a 0.6× demagnification lens (model RD060-CMT, Diagnostic Instruments, MI). We acquired images with a CCD camera (Cascade 512F, Roper Scientific).

On-Chip Quantitation. We quantified the amount of DNA extracted from whole blood by first calibrating our fluorescence measurement. For the calibration, we used a control solution of genomic DNA purified from blood with the DNeasy blood and tissue purification kit (QIAGEN, CA). We measured its DNA concentration with a Nanodrop 1000 spectrophotometer (Thermo Scientific, MA) and prepared a 1.42 μg mL^{-1} standard solution stained with 1× SYBR Green I in LE. We acquired images of the fluorescent profile of this standard filling the purification channel (but without performing ITP). Using these images, we were able to relate peak areas to DNA mass in the ITP experiments. We show details of the procedure in Figure S-3 in the Supporting Information. We also performed this calibration with a solution of λ-DNA.

Off-Chip PCR. We tracked the position of the focused zone in the channel by directly visualizing the focused species or by monitoring current transients (see Figure S-2 in the Supporting Information). After the ITP interface exited the purification channel, we collected the liquid from reservoir 4 (~2 μL) with a standard pipettor into a PCR tube containing 5 μL of 2× Fast SYBR Green I master mix (Applied Biosystems, CA) and 0.1 μM primers (Invitrogen, CA) targeting a 201 bp fragment of the BRCA2 gene (primer sequences provided in Supporting Information) and filled the rest of the reaction tube with deionized water up to 10 μL. We summarize this procedure in Figure S-4 in the Supporting Information. All PCR reactions were prepared in a UV-sterilized fume hood to avoid contamination and allow sensitive amplification without false positives. We performed off-chip real-time PCR on an ABI 7500 Fast thermocycler with the following thermal profile: 20 s initial hold at 95 °C and 40 cycles composed of 3 s denaturation at 95 °C followed by 30 s annealing and extension at 60 °C. We performed post-PCR dissociation curve analyses on the same instrument.

RESULTS AND DISCUSSION

Principle of ITP Purification. Our purification method relies on the ability of ITP to separate and focus species based on their effective electrophoretic mobilities.[12,18] We depict the ITP-based purification in Figure 1. We select LE and TE with effective mobilities,[19] respectively, higher and lower than that of the target nucleic acids (NA). Upon application of an electric field, NA molecules focus between TE and LE in a sharp concentrated zone. Species with smaller effective mobilities than the TE migrate into the channel but lag behind and do not focus. Faster species overspeed the sample zone and also do not focus. At moderate pH and in free solution, DNA has relatively large magnitude (negative) mobility compared to a vast number of polypeptides,[20,21] so the mobility of the TE effectively determines purification selectivity.

There are an abundance of PCR inhibitors in blood (e.g., hemoglobin, immunoglobulin G, lactoferrin), making it a challenging sample for a NA purification technique.[22] In our protocol, we use proteinase K to degrade proteins which potentially inhibit PCR into short polypeptides.[23] However, proteinase K is itself a PCR inhibitor.[24] We compensate for this by operating with an ITP chemistry (LE pH = 8.2) where proteinase K (pI = 8.9)[23] is positively charged and is, therefore, kept away from the sample zone as it electromigrates in the opposite direction (enters and remains in the TE reservoir). The combination of proteinase K and ITP-based purification effectively removes PCR inhibiting species and other polypeptides from the lysate.

Lysis. We carefully chose the chemical lysis agent to avoid possible contamination. The surfactant Triton X-100 is an efficient lysis agent for eukaryotic cells and has the advantage of being PCR friendly.[25] We have found Triton-X does not appreciably alter proteinase K function or modify protein mobilities. This is in contrast to, for example, the popular bacterial lysis agent sodium dodecyl sulfate (SDS). SDS disrupts cell membrane but also denatures proteins and significantly increases protein effective charge.[26] Our lysis buffer also avoids chaotropic agents which at significant ionic strengths can interfere with ITP dynamics (e.g., high concentration guanidine hydrochloride[27,28]).

Extraction Efficiency. In our purification procedure, there are at least two extraction efficiencies of interest: the fraction of DNA purified and focused via ITP from the amount injected into the channel and the amount of DNA extracted from the chip and delivered to PCR versus the amount of DNA in the lysate dispensed into the chip. To characterize the former, we applied our technique to a solution of λ-DNA of known concentration. We injected 10 pg of λ-DNA on-chip and performed the ITP purification as described in the Experimental Section. We determined the amount of focused DNA by quantifying SYBR Green I fluorescence and comparing it to the standard. We measured the mean fraction of extracted DNA (vs amount injected into the channel) as 1.03 ± 0.06 (N = 3). This approximately complete extraction is at least as good as traditional extraction methods (e.g., QIAGEN kits) and state-of-the-art microsolid phase extraction devices.[6] We estimate that we extract out of the chip (and deliver to the PCR) approximately all of the DNA which we focus on the chip.

Our injection protocol currently processes a small amount of the 1 μL lysate sample volume dispensed into the chip. We process only 25 nL of sample, which is 1/40th of the volume dispensed into the chip. We estimate a much higher sample fraction can be processed by modifying the chip design. For example, a custom chip with a relatively large 1 μL injection channel can be designed. Alternatively, the sample volume dispensed into the chip could

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be reduced by using a smaller reservoir and a nanoliter fluid dispenser.

Extraction Time. In ITP, migration velocity is directly proportional to current.[18] Consequently, the duration of purification depends on applied voltage and channel geometry. In our conditions, a typical purification lasts about 250 s for an applied voltage of 500 V (cf. Figure S-2 in the Supporting Information), which is fast compared to a typical micro-SPE process.[7] Increasing applied voltage can be used effectively to reduce this time. As a demonstration of the potential speed of the purification process, we also performed experiments with an applied voltage of 3 kV using lambda λ-DNA as sample. This higher applied potential yielded extraction in an average of 56 s ($N = 3$), without undue joule heating.

ITP-Based Purification from Whole Blood Results. We show examples of DNA purifications from a whole blood lysate in Figure 2. Here, we present purifications of DNA from about 2.5 nL of whole blood (25 nL of blood lysate). We show three sets of experiments: purified blood lysate, blood lysate treated with proteinase K, and blood lysate treated first with DNase and then proteinase K. We used SYBR Green I fluorescence measurements to estimate the amount of DNA recovered in the ITP zone (as in Figure S-3 in the Supporting Information). The amount of DNA recovered without proteinase K is negligible (on the same order as the negative control). The mass of focused DNA is significant when performing the assay on a lysate initially treated with proteinase K. The third set of results shows ITP purification of a lysate treated with DNase prior to proteinase K treatment. This control case shows low DNA recovery, as expected. We hypothesize that DNA binding proteins (in particular histones[29]) significantly reduce the electrophoretic mobility of DNA by increasing the hydrodynamic Stokes' drag of the complex. If the mobility of the DNA−protein complex is smaller than the mobility of the TE, DNA does not focus and cannot be purified. Proteinase K releases DNA from binding proteins allowing focusing and purification. Together, these experiments show that our fluorescence signal is due to focused DNA from the lysate samples and that the extraction process is suitably repeatable.

The fluorescence measurements from Figure 2 show that we focused about 44 ± 6 pg ($N = 9$) of DNA from the initial 2.5 nL of whole blood (25 nL of lysate). A nanoliter of blood from a healthy human contains 4−10 white blood cells, and each human diploid cell contains about 6.6 pg of DNA.[29] We, therefore, estimate that the efficiency of ITP purification of blood ranges between 30% and 70%, which competes with both batch and microchip-based SPE methods.[6]

Finally, we collected extracted genetic material (at reservoir 4 using a pipettor) and performed PCR analyses to verify that (i) we effectively purified DNA from the human blood sample and (ii) the purified NA are free of PCR inhibitors. We show real-time PCR amplification curves in Figure 3. Real-time fluorescence monitoring shows repeatable amplification signal with a threshold cycle of $C_t = 30.9 \pm 0.4$ ($N = 4$); while the negative control showed negligible amplification after 40 cycles. This is in contrast to results obtained with an equivalent, unpurified amount of blood lysate (25 nL, obtained by dilution), for which PCR was clearly inhibited (negligible amplification after 40 cycles).

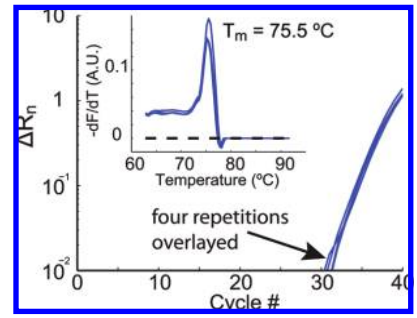

Figure 3. Real time PCR amplification of ITP purified DNA from human blood. We present a schematic of the extraction-PCR protocol in Figure S-3 in the Supporting Information. The amplification curve shows repeatable amplification of the extraction product (here four repetitions, threshold cycle $C_t = 30.9 \pm 0.4$). Negative controls and PCR from equivalent amount of lysate showed no amplification after 40 cycles. We, therefore, successfully purified DNA from whole blood to obtain PCR-ready NA in a PCR-friendly buffer. The inset shows the post-PCR dissociation curve (derivative of SYBR Green I fluorescence). The melting temperature of the PCR product equals that of the positive control. The dashed line corresponds to melting curves of negative controls.

We also performed dissociation curve analysis of the PCR products from all samples as additional identification of the target. The melting temperature of the PCR product of the ITP-purified samples and a positive control were equal ($T_m = 75.5$ °C, see dissociation curves on the inset of Figure 3). The experiments show that we successfully and repeatably purified DNA from whole blood and recovered genomic DNA free of PCR inhibitors.

CONCLUSIONS

For the first time, we have demonstrated ITP-based purification of nucleic acids from whole blood. We leveraged the focusing and separation power of ITP in free solution to purify nucleic acids from proteins and other sample contents and yielded a sample ready for PCR. After chemical lysis and proteinase K treatment, we extracted DNA from a few nanoliters of sample in just 3 min. The efficiency of the ITP-based purification (versus injected sample) ranges between 30% and 70% for whole blood and reaches 100% for λ-DNA, which rivals that of other microchip purification techniques. On-chip ITP-based purification is a fast and simple technique for the purification of NA from small volumes of biological samples (1 to 100 nL), results in negligible PCR inhibitors, and uses a PCR friendly chemistry. We here demonstrated the ability of this technique to extract DNA from 10 to 25 cells and, therefore, hypothesize that sample preparation from a single cell is possible. Finally, the ITP-based purification is performed in a single step, enabling automated and multiplexed analysis.

ACKNOWLEDGMENT

The authors thank Paul J. Utz and Pamela L. Clark for the blood samples and Marc Levenston for the use of the real time

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PCR thermocycler. We gratefully acknowledge support from the Micro/Nano Fluidics Fundamentals Focus (MF3) Center funded by Defense Advanced Research Projects Agency (DARPA) MTO Grant No. HR0011-06-1-0050 and contributions from MF3 corporate members. We also gratefully acknowledge the support of the National Science Foundation under the Contract No. CBET-0729771-002 with Marc S. Ingber as program manager.

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 31, 2009. Accepted October 3, 2009.

AC901965V