Microsystem for Isolation of Fetal DNA from Maternal Plasma by Preparative Size Separation

Thomas Hahn,† Klaus S. Drese,* and Ciara K. O’Sullivan*†

BACKGROUND: Routine prenatal diagnosis of chromosomal anomalies is based on invasive procedures, which carry a risk of approximately 1%–2% for loss of pregnancy. An alternative to these inherently invasive techniques is to isolate fetal DNA circulating in the pregnant mother’s plasma. Free fetal DNA circulates in maternal plasma primarily as fragments of lengths <500 bp, with a majority being <300 bp. Separating these fragments by size facilitates an increase in the ratio of fetal to maternal DNA.

METHODS: We describe our development of a microsystem for the enrichment and isolation of cell-free fetal DNA from maternal plasma. The first step involves a high-volume extraction from large samples of maternal plasma. The resulting 80-μL eluate is introduced into a polymeric microsystem within which DNA is trapped and preconcentrated. This step is followed by a transient isotachophoresis step in which the sample stacks within a neighboring channel for subsequent size separation and is recovered via an outlet at the end of the channel.

RESULTS: Recovered fractions of fetal DNA were concentrated 4–8 times over those in preconcentration samples. With plasma samples from pregnant women, we detected the fetal SRY gene (sex determining region Y) exclusively in the fragment fraction of <500 bp, whereas a LEP gene (leptin) fragment was detected in both the shorter and longer recovery fractions.

CONCLUSIONS: The microdevice we have described has the potential to open new perspectives in noninvasive prenatal diagnosis by facilitating the isolation of fetal DNA from maternal plasma in an integrated, inexpensive, and easy-to-use microsystem.

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Received March 25, 2009; accepted August 28, 2009.

Previously published online at DOI: 10.1373/clinchem.2009.127480

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lishing new tools for point-of-care diagnostics (20, 21) and to enable the development of complex biological-testing applications in forensic and general medical diagnostics (22–24).

Free fetal DNA is present at low concentrations in maternal plasma (2, 25, 26). Preconcentration steps after extraction of total DNA from a relatively large plasma sample (typically 1 mL) are essential to yield 50–100 μL of a DNA-containing eluate for processing on microfluidic platforms (27–29).

Several reports have described microscale preconcentration methods (30) and a number of preconcentration techniques, such as field-amplified sample stacking (31), temperature gradient focusing (32), and isotachophoresis (ITP) (33). A drawback of these techniques is that they allow only relatively small sample volumes (a few microliters) to be used, and the target molecules are not always nucleic acids. Transient ITP offers the advantage of size separation after preconcentration (34), which facilitates the concentration of a DNA sample before separation (35); however, transient ITP suffers from the drawback of requiring long channels that exceed the chip dimensions for samples > 10 μL. Other approaches for preconcentration have exploited filter structures or membranes (36, 37) and, recently, electrokinetic trapping (EKT) at polyethylene terephthalate (PET) membranes for stacking larger volumes of about 80 μL (38).

The microsystem we describe exploits the advantages of EKT at membranes combined with field-amplified sample stacking to concentrate the sample. This system may find application as a preparative tool for the analysis of cell-free fetal DNA circulating in maternal plasma.

Materials and Methods

MATERIALS AND REAGENTS

Acrylamide solution (400 g/L), polyethylene glycol (M, 50 000) and ammonium persulfate were purchased from Sigma–Aldrich. N,N-methylenebisacrylamide solution (20 g/L), glycine (>99% pure), and TEMED were provided by Carl Roth. Tris was purchased from Fluka. All chemicals were at least of analytical grade unless otherwise stated. PET membranes with a 100-nm pore size were purchased from Sterlitech Corporation and contained 4 × 10^6 pores/cm². A lyophilized 100-bp ladder was supplied by SERVA Electrophoresis. Fragments of 230 bp were generated by the PCR with the commercially available pGEM Vector as template DNA (Promega). SYBR Green I (Invitrogen) and GelStar (Lonza Group) were used for DNA staining in EKT experiments and in slab gels, respectively. Reagents for the PCR were purchased from Qiagen, and a real-time PCR kit with SYBR Green I was purchased from Bio-Rad Laboratories. HPLC-grade primers were obtained from Metabion. Frozen maternal plasma samples of third-trimester pregnancies with a male fetus were kindly provided by Ellen van der Schoot of the Sanquin Blood Supply Foundation (Amsterdam, the Netherlands).

CHIP FABRICATION AND ASSEMBLY

Poly(methylmethacrylate) chips were micromachined from templates of 64 × 43 × 2 mm and cleaned one time each with 2-propanol, 0.5 mol/L sodium hydroxide, and finally ultrasonically in deionized water for 2–3 min. A PET membrane with a 100-nm pore size was incorporated into the chamber via thermal bonding [Fig. 1A (4)]. The membrane area of 0.28 mm² contained approximately 1.1 × 10⁶ pores. The chip was sealed on both sides with adhesive polypropylene foil.

A solution of 90 g/L acrylamide and 1.5 g/L N,N-methylenebisacrylamide was prepared in LE (leading electrolyte: 50 mmol/L Tris/HCl, pH 8.5) and introduced into the separation channel [Fig. 1A, (10)] until the T-crossing valve [Fig. 1A, (9)] and polymerized with TEMED/ammonium persulfate. The anode channels [Fig. 1A, (8)] were flushed with TE (terminating electrolyte: 5 mmol/L Tris/glycine, pH 8.5) from anode reservoir I [Fig. 1A, (2)]. The buffer entered the sample reservoir and escaped through the cathode reservoir. Consequently, an air plug that formed between the separation matrix and TE served as a valve at the T-crossing [Fig. 1A, (9)]. In the sample reservoir [Fig. 1A, (3)], we introduced 80 μL deionized water containing 0.5 ng/μL of a 100-bp nucleic acid ladder, a 230-bp PCR product, or (for testing maternal plasma samples) an eluate derived from a DNA extraction. The sample was injected through an inlet [Fig. 1A, (5)]. The cathode reservoir [Fig. 1A, (1)] and anode reservoir I [Fig. 1A, (2)] were filled with 170 μL and 160 μL TE, respectively, and a platinum electrode was placed into each reservoir.

To avoid chemical disruption of the DNA due to pH changes during the assay, we separated the cathode from the sample by placing a comb structure at the cathode reservoir [Fig. 1A, (1)]. A recovery outlet [Fig. 1A, (7)] in the separation channel was used to recoup the DNA.

EKT AND TRANSIENT ITP

EKT was performed by applying 1000 V at anode reservoir I (Fig. 1B). The electrode in the cathode reservoir was set to ground while the electrode in anode reservoir II remained floating. A software-controlled ISEG 3-kV
unit served as the supply of direct-current power. The software was based on a script program for highly reproducible switching between the different modes.

During EKT, the air plug separating the matrix and the EKT buffer was removed, opening the valve at the T-crossing [Fig. 1A, (9)] by piercing the bonding foil. The volume flow due to electro-osmotic flow within the anode channels [Fig. 1A, (8)] increased the back pressure to produce a connection of TE and polyacrylamide at the T-crossing. Passage into ITP was allowed by setting the cathode reservoir and anode reservoir I electrodes to ground, and the anode reservoir II electrode was set to 250 V. The discontinuous buffer system consisting of TE and LE was chosen to lead the nucleic acids into the polyacrylamide matrix. Subsequently, the TE was removed via the inlet [Fig. 1A, (5)] and exchanged with LE to obtain a homogeneous buffer system for the nucleic acids entering the size-separation channel. The separation process was preceded with a potential of 180 V at anode reservoir II (Fig. 1C). All other electrodes were grounded. The processes of EKT and separation with their respective time intervals are summarized in Table 1.

**OPTICAL DETECTION**

During development of the microsystem, nucleic acids were stained with SYBR Green I (1:10000) and detected with an inverted fluorescence microscope (CKX41; Olympus Germany) equipped with a fluorescence mirror unit (U-MNB2), an excitation bandpass filter (470–490 nm), an emission high-pass filter (520 nm), and a camera (DX30; Kappa Optoelectronics) that focused on the membrane chamber or the separation channel. Fluorescence images of the DNA were taken at various time intervals at exposures of 192 ms; the signal was amplified 50-fold for EKT experiments.

**RECOVERY AND QUANTIFICATION**

Fractions of 2 µL were manually recovered every 30 s in the separation process from the LE-containing and matrix-free recovery outlet [Fig. 1A, (7)]. Fluid from anode reservoir II refilled the outlet because of the higher back pressure subsequent to any recovery. It was very important to hold the pipette at an angle of 90° and to carefully touch the ground of the recovery outlet to avoid shifts in the appearance of the fragments. For quantification, the fractions were loaded onto a 20-g/L agarose gel stained with GelStar. Slab gel images were saved as 16-bit files. The public-domain ImageJ software quantified nucleic acids on slab gels by integrating the peak area while comparing the peak to the unfractonated sample (39). For a reliable calibration curve, we loaded 3 different volumes (2, 4, and 6 µL) of the

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**Table 1. Summary of the step-by-step performance for EKT, ITP, and capillary electrophoresis (CE).**

<table>
<thead>
<tr>
<th>Processing time (s)</th>
<th>Applied method</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–300</td>
<td>EKT</td>
<td>Preconcentration in front of the membrane</td>
</tr>
<tr>
<td>301–350</td>
<td>ITP</td>
<td>DNA enters the matrix in the separation channel</td>
</tr>
<tr>
<td>351–360</td>
<td>Break</td>
<td>All electrodes are floating to exchange TE with LE</td>
</tr>
<tr>
<td>361–870</td>
<td>CE</td>
<td>Size separation and recovery every 30 s</td>
</tr>
</tbody>
</table>

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**Fig. 1. View from underside of the microchip for isolation of fetal DNA.**

(A) Shown are the cathode reservoir (1), anode reservoir I (2), sample reservoir (3), membrane chamber (4), sample inlet (5), anode reservoir II (6), recovery outlet (7), anode channels (8), T-crossing valve (9), and separation channel (10). The schematics show a potential application for EKT (B) and transient ITP for preparative separation of nucleic acids (C).
original sample on a slab gel along with the recovered fractions. The intensities of the bands in the recovered fractions and certain bands were related to the original sample to determine concentration factors. The calibration curve was linear at the DNA concentrations used, which allowed a reliable determination of concentration factors.

**MATERNAL PLASMA SAMPLES AND REAL-TIME PCR**

The manual Chemagic Viral RNA/DNA Kit (Chemagen) was used to extract total DNA from 1 mL of maternal plasma. The supplier’s protocol was followed and performed 5 times with 200-μL extractions. Total DNA was eluted in 5 × 60 μL of deionized water in 1.5-mL reaction tubes. After extracting the total DNA from a plasma sample obtained from a woman in a third-trimester pregnancy, we carried out the preconcentration and separation process in an automated fashion on the developed microsystem. Three eluates from DNA extractions were sufficient to perform 3 separation experiments [for LEP$^5$ (leptin) or SRY (sex determining region Y)] immediately after the extractions for each aliquot of the same plasma sample. Thoroughly rinsing the microchip with deionized water was required to completely remove any remaining salts that could affect the preconcentration at the membrane.

The following recovery fractions of a separation experiment were combined to obtain 6 μL/fraction: 9, 9.5, and 10 min (fraction 1); 10.5, 11, and 11.5 min (fraction 2); 12, 12.5, and 13 min (fraction 3); and 13.5, 14, and 14.5 min (fraction 4). Combining 3 fractions ensured sufficient numbers of molecules for the subsequent real-time PCR analysis. As a negative control at the beginning of an experiment, we used 6 μL from anode reservoir II (fraction NC).

A Bio-Rad Q5 column was used for the SYBR Green I–based real-time PCR analysis in combination with a melting-curve analysis. Primer sequences for a 105-bp LEP fragment and a 107-bp SRY fragment were used as previously described (18). LEP was amplified with forward primer 5′-CAGTCTCCTCACACAGAAATCA-3′ and reverse primer 5′-GTCATCTTGGATAAGGTCAGGA-3′, and SRY was amplified with forward primer 5′-AAAGGCAACGGGTCCAGGAGTCC-3′ and reverse primer 5′-TGA GTTTGCGATTCTGGGATT-3′. Real-time PCR protocols were as described elsewhere (21), with slight modifications [50 cycles (SRY) or 40 cycles (LEP) of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s]. For each PCR experiment, we used 6 μL template (fractions 1–4 and fraction NC) in a 15-μL total reaction volume.

**Results**

**SAMPLE PRECONCENTRATION**

Applications involving low copy numbers of nucleic acids routinely require a preconcentration step. After extracting total DNA from maternal plasma, we used a disposable and completely automatable polymer microsystem in a 2-step process to concentrate 80 μL of eluate prior to preparative separation of DNA fragments by size. First, the eluate containing nucleic acids was preconcentrated at a PET membrane by charge exclusion (Fig. 2). The entire sample was trapped in the

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$^5$ Human genes: LEP, leptin; SRY, sex determining region Y.
2.6-μL membrane chamber within 300 s, with electro-
omotive flow supporting the trapping process. A more
detailed description of this preconcentration process is
described elsewhere (38). Moreover, this electro-
omotive flow increases the reservoir height in the ca-
thodic compartment, leading to a higher back pressure
and producing a connection between the matrix in the
separation channel and the EKT fluid. At this stage, the
script-controlled program of the power supply initi-
atid the second preconcentration step by applying a
potential in anode reservoir II and grounding both an-
ode reservoir I and the cathode reservoir. The nucleic
acids were directed to leave the membrane chamber to
the second anodic compartment and to enter the poly-
merized acrylamide in the separation channel (Fig. 2).

The 80-μL samples were thus reduced to 200 nL to
fit within the dimensions of the separation channel
(Fig. 2). The use of matrices with a lower sieving ability
(e.g., 1%-2% polyethylene glycol in the separation
channel) permits the sample to occupy a minimal vol-
ume of <10 nL, which represents a theoretical precon-
centration factor of at least 8000. For the use of poly-
merized acrylamide, the ITP preconcentration step is
accompanied by separation and concomitant band broadening (Fig. 2). For optimal preconcentration, we
carried out ITP for 50 s. Combining the ability to trap
nucleic acids at membranes with ITP permits a suffi-
cient reduction in volume with a notably higher effi-
ciency than previously reported setups (33, 34) and
adequately prepares the sample for preparative separa-
tion by size.

SEPARATION OF THE 100-bp LADDER

Transient ITP has been used to stack a sample in the
upper part of a microchannel to prepare it for subse-
cquent size separation with a homogeneous buffer sys-
tem. For the system we describe, separation is achieved
after ITP preconcentration when the buffer in the ca-
thodic compartment is exchanged with LE. After the
application of nucleic acids, fractions of different sizes
separate and appear at specific times in the recovery
outlet. The cross-sectional area of the separation chan-
nel (300 × 600 μm) increases 10-fold at the end of the
recovery outlet to produce a proportional decrease in
the velocity of nucleic acids at this position to allow the
recovery of nucleic acids in 2-μL fractions.

Two single fractions at 9.5 min and 10 min con-
tained the majority of the fragments between 100 bp
and 300 bp (Fig. 3). Comparing the preconcentration
samples with the untreated sample revealed that the
recovered fractions had become concentrated by 4- to
8-fold (Fig. 3). For 8 experiments in 4 separate micro-
systems, we have confirmed that the pattern of appear-
ance of nucleic acids of certain sizes is time dependent,
with a maximum shift of ±30 s (Fig. 4), implying a
threshold of 1 for the concentration factor (Fig. 4).

APPLICATION OF THE MICROSYSYEM TO ANALYSIS OF
MATERNAL PLASMA SAMPLES

The results obtained for experiments with maternal
plasma samples are consistent with those previously
obtained with the 100-bp ladder. A LEP fragment of
105 bp was amplified in both shorter and longer DNA
fragments (Fig. 5A), an expected result because both
the fetal and maternal alleles are amplified, whereas we
detected SRY only in fractions 1–2 at a fragment size
<500 bp (Fig. 5B). These findings show the male fetus–
derived SRY fragment occurs in a range detectable in
the short-fragment recovery fraction. These data con-
cur with those of Chan et al., who evaluated the same
set of primers for size dependency of fetal DNA in ma-
ternal plasma and observed the 107-bp SRY fragment
only in fractions of <500 bp (18). LEP occurred in
fractions of smaller and larger nucleic acids in all ex-
periments. We surmise that LEP was not amplified in a
median fraction in the described experiments because
of a lack of nucleic acids in this fraction. Interestingly, we observed this result in all 3 experiments. Southern blot analysis of plasma DNA in the circulation with highly repetitive Alu probes has shown that freely circulating plasma DNA appears in regularly sized fractions (19).

The melting-curve analysis confirms the amplification of SRY fragments, with a melting point at 83 °C, a result that agrees with that for the positive control (unfractionated sample) (Fig. 5B). The microsystem we have developed thus enriches and preconcentrates DNA from maternal plasma samples and then preparatively separates the concentrated DNA molecules by size, thereby facilitating the recovery of DNA for subsequent analyses. The microdevice is easy to use, disposable, and inexpensive, and the entire process can be completed in <15 min, with an estimated production cost of €2.

Discussion

The discovery of fetal DNA circulating freely in the plasma of pregnant women is revolutionizing the field of noninvasive prenatal diagnostics, with much of the focus being on demonstrating the use of cell-free circulating DNA for the diagnosis of specific diseases and anomalies. New technologies that advance the capability for efficiently isolating fetal DNA fragments have not yet been reported, however. We have presented data on a polymeric microsystem that has the potential to expand the state of the art for methods of noninvasive prenatal diagnosis. The microsystem has the advantage of being cost-effective and automatable, while eliminating inherent problems of contamination and sterility. The device can be envisaged as part of an integrated microsystem that also incorporates DNA extraction or, alternatively, can be combined with state-of-the-art DNA-extraction robots. The drawback of
the requirement of small volumes has been overcome with a new preconcentration technique that preconcentrates a large sample at a membrane. We have demonstrated field-amplified EKT to be an effective tool for handling large sample volumes. Samples from plasma extractions can be readily extracted into deionized water, with no additional treatment necessary before processing within the microdevice. Future work is focusing on integrating the developed microsystem with an automated sample-introduction module, which we expect to considerably improve the microsystem’s performance. Moreover, we plan to test the long-term stability and preconcentration factors for plasma samples to confirm the preconcentration factors obtained for the 100-bp ladder.

An important element of this work was the preparation of the sample under low-salt conditions to avoid any interference of salts with the real-time PCR. Our data with maternal plasma samples prepared with the microdevice demonstrate that nucleic acids in the LE were amplifiable in a ratio of template sample to PCR reagents of 1:1.25, thus enabling us to lower the total volume of a PCR reaction to 15 μL. This reduction in volume represents a 70% reduction in reagents compared with macroscale experiments.

The microsystem we have described does not require an optical-detection unit to separate shorter nucleic acids (e.g., fetal DNA), thus facilitating the implementation of this disposable microsystem in a general clinical diagnostic laboratory. This microsystem has been demonstrated to enrich and preconcentrate DNA from a mixture of nucleic acids of different sizes and to permit preparative separation of the concentrated DNA by size, thereby facilitating the recovery of DNA or subsequent analysis in an easy-to-use and inexpensive disposable microdevice.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honorary: None declared.

Research Funding: Funding from the European Commission for the Special Noninvasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence (LSHB-CT-2004–503243), for which this study was fully funded, is gratefully acknowledged.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Aicha at Sousan and Ellen van der Schoot (Sanquin, Amsterdam) for providing maternal plasma samples.

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