Analytical Biochemistry **283,** 175–191 (2000)
doi:10.1006/abio.2000.4577, available online at http://www.idealibrary.com on IDEAL®

Evaluation of Silica Resins for Direct and Efficient Extraction of DNA from Complex Biological Matrices in a Miniaturized Format

Huijun Tian,* Andreas F. R. Hühmer,*[,1] and James P. Landers†[,‡,2]

**Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260; †Department of Chemistry and ‡Department of Pathology, McCormick Road, University of Virginia, Charlottesville, Virginia 22901*

Received December 8, 1999

For DNA purification to be functionally integrated into the microchip for high-throughput DNA analysis, a miniaturized purification process must be developed that can be easily adapted to the microchip format. In this study, we evaluate the effectiveness of a variety of silica resins for miniaturized DNA purification and gauge the potential usefulness for on-chip solid-phase extraction. A micro-solid-phase extraction (μSPE) device containing only nanograms of silica resin is shown to be effective for the adsorption and desorption of DNA in the picogram–nanogram mass range. Fluorescence spectroscopy as well as capillary electrophoresis with laser-induced fluorescence detection is employed for the analysis of DNA recovered from solid-phase resins, while the polymerase chain reaction (PCR) is used to evaluate the amplifiable nature of the eluted DNA. We demonstrate that DNA can be directly recovered from white blood cells with an efficiency of roughly 70%, while greater than 80% of the protein is removed with a 500-nl bed volume μSPE process that takes less than 10 min. With a capacity in the range of 10–30 ng/mg of silica resin, we show that the DNA extracted from white blood cells, cultured cancer cells, and even whole blood on the low microliter scale is suitable for direct PCR amplification. The miniaturized format as well as rapid time frame for DNA extraction is compatible with the fast electrophoresis on microfabricated chips.

Key Words: **DNA purification; PCR suitable DNA template; solid-phase extraction; protein and DNA quantitation; micro-solid-phase extraction; silica.**

Miniaturization of analytical methods and instrumentation for biomedical and clinical research is an area of burgeoning interest. In many cases, the reduction in size of an analytical procedure or technique often translates to a reduction in analysis time and costs. Miniaturization of analytical methods often paves the way for the use of established technologies in high-throughput applications. Great efforts have been made to develop fast, cost-effective, high-throughput separation methods for DNA analysis. Capillary electrophoresis (CE) with its speed, automation, and small volume requirements provides an attractive DNA separation alternative over traditional, labor-intensive slab gel electrophoresis. To improve throughput, capillary array electrophoresis (96 capillaries) has been developed for DNA sequencing, sizing DNA fragments, and genotyping (1–4). In recent years, microfabricated chips with even faster separation times and smaller volume requirements than CE have been developed to further miniaturize the DNA separation process. Sizing PCR products, DNA sequencing, and mutation detection on microfabricated chips have recently been reported (5–12). In DNA analysis or molecular diagnostics, DNA purification, DNA amplification by the polymerase chain reaction (PCR), and DNA separation are generally needed (13). While the sizing of DNA fragments can be completed in less than 100 s (5–8) on an electrophoretic microchip, macroscale DNA purification and amplification still requires several hours, minimizing the significance of fast DNA separation.

[1] Current address: Texas Instruments, Inc., Analytical Sensor Group, 13536 N. Central Expressway, MS945, Dallas, TX 75243. E-mail: a-huhmer1@ti.com.

[2] To whom correspondence should be addressed at Department of Chemistry, McCormick Road, University of Virginia, Charlottesville, VA 22901. Fax: 412-243-8852. E-mail: jpl5e@virginia.edu.

0003-2697/00 $35.00

The microchip platform has the potential for integrating sample pretreatment, target amplification, and detection in a single device. Combination of these processes into a single device (i.e., create the elusive "lab-on-a-chip") can minimize sample loss and contamination problems as well as reduce analysis times substantially—all of which would improve DNA analysis in molecular diagnostics. While our laboratory (14, 15) and others (16–21) have been working toward miniaturizing PCR for expedited amplification of DNA in the microchip format, less effort has been exerted toward miniaturizing DNA purification methods. Currently, preelectrophoresis sample preparation is the rate-limiting step in the diagnostic procedure, due to the very laborious and time-consuming processing of complex biological specimens (e.g., serum, lymphocytes). There is little doubt that miniaturization of the entire process (DNA extraction, PCR, electrophoresis) would be beneficial—high-throughput capabilities would result as well as low analysis cost due to the small volume of sample and reagents required for the analysis. However, for this to occur, effective on-chip DNA extraction will be required, because efficient and reliable amplification of target DNA sequences is dependent on the presence of a sufficient amount of relatively pure template DNA. This is of particular importance in clinical diagnostics. The DNA isolated from patient samples constitutes the primary source of information, consequently, efficient DNA sample preparation is critical.

A fully integrated process for the preparation of samples and the amplification of DNA on microfabricated structures has been attempted, but it is clearly not sufficiently reliable for processing a variety of different samples (18–21). While the functional integration of sample preparation, DNA amplification, and sample analysis in a single electrophoretic microchip has obvious advantages, the potential of a miniaturized DNA purification method extends beyond reducing analysis time in molecular diagnostics. A miniaturized DNA purification method would have further utility in a number of other areas including separating PCR product from reaction by-products, purifying DNA fragments prior to sequencing, and desalting primers of DNA hybridization targets. An effective miniaturized DNA sample preparation methodology could also be interfaced with conventional capillary electrophoresis, integrated electrophoretic microchips or pipettes capable of micro-solid-phase extraction (μSPE).[3]

It is obvious that classic DNA purification methods (precipitation in organic solvents, centrifugation) designed to purify large quantities of DNA are incompatible with miniaturized DNA purification methods. A miniaturized DNA purification protocol must accomplish extraction and purification of DNA in as few steps as possible and should minimize solvent volume, lower dilution effects, and reduce the possibility of contamination. Additionally, if the method is to be used for molecular diagnostic purposes, it should also be applicable to a number of clinically relevant DNA sources. For example, blood is the universal source of DNA, but consists of a complex mixture of cells, proteins, and peptides of diverse molecular weight, lipids, carbohydrates, and other low-molecular-weight compounds, all of which interfere with most analyses. A variety of organic and inorganic compounds, such as heme and its degradation products, urea, bile acids, humic acids, and polysaccharides, have been reported to inhibit the amplification of nucleic acids by PCR (22, 23). While human blood is a convenient DNA source in molecular diagnostics, it also contains a panorama of potent inhibitors, which must be removed before effective PCR can be carried out. These include hemoglobin (23) as well as a factor found in eosinophils that has been shown to specifically inhibit reverse transcription–PCR (RT–PCR) (24). Several conventional methods have been defined for removing these components from a variety of biological samples (including blood), ultimately yielding PCR-suitable DNA (22, 23, 25). However, most of these require multistep methodology (including centrifugation, organic solvent extraction or solid phase extraction) to obtain high-quality, PCR-suitable DNA.

Methods that exploit the proclivity of certain materials for adsorbing DNA (e.g., silica, glass fibers, anion exchange resins and modified magnetic beads) have been developed for purifying DNA (26–35). The major advantages of such DNA binding matrices are that they circumvent problems of physical and biochemical degradation of DNA during purification. Immobilized DNA is also less susceptible to digestion by DNA-degrading enzymes. In combination with protein denaturants and ribonuclease inhibitors, degradation of DNA can be minimized, while degradation of RNA and proteins is maximized in the DNA purification process.

A large body of literature describes the use of silica resins, celite particles, and glass beads for the purification of DNA from blood, agarose gel, or PCR products (26–28), but little information is available that assists in the design and operation of a miniaturized DNA purification method based on silica resins. Specific problems associated with a microSPE (μSPE) device include: (i) the total capacity of the μSPE device, (ii) the ability of the device to retain large (kb) fragments, (iii) the loss of DNA due to shearing and irreversible binding, (iv) the compatibility of the retained DNA fraction with PCR applications, and (v) the reproduc-

[3] Abbreviations used: μSPE, micro-solid-phase extraction; GuHCl, guanidine hydrochloride; WBCs, white blood cells; TE, Tris/EDTA; LIF, laser-induced fluorescence; GuSCN, guanidine thiocyanate; SiO_x, silica.

ibility of the DNA extraction method with complex clinical samples.

In this report, we discuss the results from the in-depth evaluation of silica particle-based μSPE for the effective adsorption of human genomic DNA from various biological matrices. We describe the development of a methodology to reproducibly prepare μSPE devices via the quantitative evaluation of the effectiveness of various silica particles for adsorbing and recovering human genomic DNA. We extend our evaluation of the efficiency of μSPE methodology for extracting DNA from complex biological matrices by exploiting fluorescence-based methods to quantify the adsorption/desorption of both DNA and proteins during the process. We demonstrate the effectiveness of this optimized method by successfully extracting DNA directly from purified white blood cells, cultured cancer cells, and whole blood with a relatively simple process. The PCR-suitable nature of the DNA extracted from these sources by μSPE is confirmed by PCR-amplifying DNA fragments from β-globin, BRCA1 and BRCA2 genes. In doing so, these results establish μSPE as a viable microminiaturized DNA extraction method that may be extended to a variety of applications in genetic analysis or clinical diagnostics.

MATERIALS AND METHODS

Materials

Supelco irregular-shaped silica particles were removed from a silica solid-phase extraction cartridge in Supelclean SPE Tubes Methods Kit A (Bellefonte, PA). Hyperprep silica (size, 30–40 μm; pore size, 120 Å; surface area, 200 m^2/g) was purchased from Supelco (Bellefonte, PA). Merck G-60 silica (size, 40–63 μm; pore size, 60 Å; surface area, 550 m^2/g) and hydroxylethylcellulose (HEC, M_r 250,000) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Glass fibers were removed from the bed support of a disposable spin column (36–40). GeneAmp thin-walled PCR tubes, AmpliTaq DNA polymerase with buffer, and GeneAmp lambda Control Reagent kit were from Perkin–Elmer (Norwalk, CT). The 100 mM dNTP (deoxyribonucleotide triphosphate) stock solutions were supplied by Gibco BRL (Gaithersburg, MD). TaqStart antibody (7 mM, 1.1 μg/μl) was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). EDTA, sodium borate, tris(hydroxymethyl)aminomethane (Tris), guanidine hydrochloride (GuHCl), isopropanol, methanol, pBR322 *Hae*III digest, sodium dodecyl sulfate (SDS), Triton X-100, and Tween 20 were from Sigma Chemical (St. Louis, MO). Polyimide was from Alltech Associates, Inc. (Deerfield, IL). Polyethylene tubing (i.d. 0.38 mm, o.d. 1.09 mm, No. 427405) was from Becton–Dickinson and Co. (Sparks, MD). [3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde] (CBQCA), PicoGreen, and YO-PRO-1 were from Molecular Probes (Eugene, OR). FC (fluorocarbon)-coated capillaries were from J&W Scientific, Inc. (Folsom, CA). Two-milliliter disposable polystyrene cuvettes were from Fisher Scientific, Inc. (Pittsburgh, PA).

Prepurified human genomic DNA samples and human white blood cells were provided by Molecular Diagnostics Laboratory, Department of Pathology, University of Pittsburgh Medical Center (Pittsburgh, PA).

Prostate cancer cells (DU-145) and breast cancer cells (MCF-7) used for DNA extraction were obtained from the American Type Culture Collection (Manassas, VA), grown in Dulbecco's modified Eagle medium (DMEM), collected, washed, suspended in PBS, submitted for cell counting, and then stored at −70°C until use (41).

Methods

Preparation of white blood cells and prepurified human genomic DNA. Blood for the preparation of white blood cells and prepurified genomic DNA was drawn from a healthy volunteer by venapuncture in a glass tube containing EDTA and was divided into two fractions. The smaller fraction was used to obtain white blood cells (WBCs), while the other fraction of the blood was used to extract DNA (as prepurified genomic DNA) by phenol/chloroform extraction method (25). WBCs were separated by centrifugation in a Ficoll gradient, washed by PBS, and submitted for cell counting in a standard cell counter (Beckman–Coulter). The white blood cell count was 3.3×10^6 cells/ml with a purity of 99%. The concentration of prepurified human genomic DNA was measured using the PicoGreen dsDNA quantitation assay (42).

Preparation of a micro-solid-phase extraction device. The μSPE devices were essentially made as described previously (36–40). μSPE devices of similar size and quality were made containing 0.2–0.3 mg of the solid-phase materials. The beads were retained in a polyethylene sleeve between two glass fiber frits. One side of the polyethylene sleeve was connected to a 1.5-cm-long fused silica capillary (50 μm i.d.). Another side of the sleeve was connected with a 1.5-cm-long fused silica capillary (50 μm i.d.) or with a 50-μm i.d., 365 μm o.d. × 17-cm fused silica capillary (Polymicron Technologies, Phoenix, AZ). The fractions from the μSPE devices were collected into the microcentrifuge tubes via the capillary connected to the μSPE device for the microliter scale or via the outlet of the cartridge when the μSPE device was directly coupled to the capillary in the CE cartridge for the submicroliter scale (Figs. 1A–1C). Polyimide glue was used to hold the polyethylene sleeve and the capillaries in place. The total volume of the packed μSPE device was typically around 460–570 nl. The μSPE devices were washed

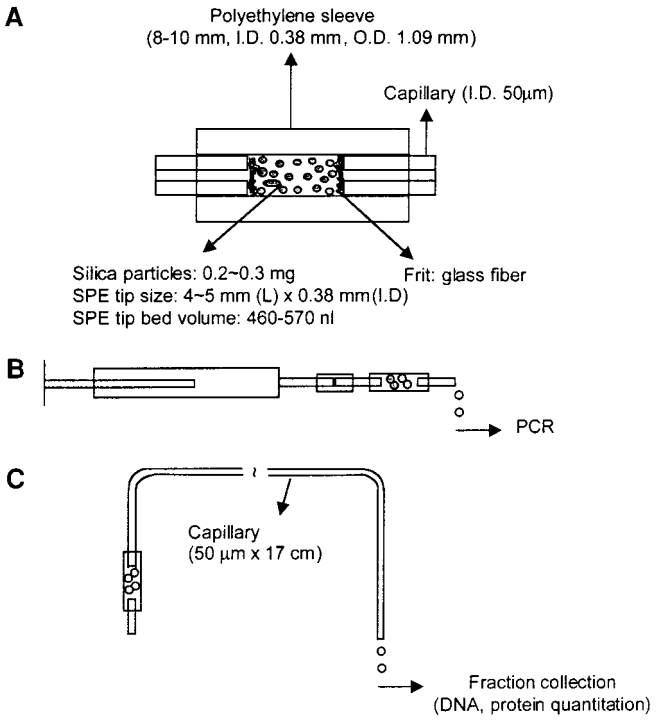

FIG. 1. Schematic diagrams. (A) Micro-solid-phase extraction device (μSPE device); (B) off-line μSPE; (C) on-line μSPE.

with 50 μl 10 mM TE or water and 50 μl methanol before use.

Purification of DNA by micro-solid-phase extraction. For the μSPE processes, λ DNA (Perkins–Elmer, Norwalk, CT) and prepurified human genomic DNA were diluted in 8 M GuHCl, which was dissolved in 10 mM TE [10 mM tris(hydroxymethyl)aminomethane, 1 mM EDTA, pH 6.7], with 6 M GuHCl as the final concentration; the DNA loading concentrations were 0.1 and 0.3 ng/μl, respectively. Human lysed WBC loading solutions were prepared by mixing 65 μl human WBCs (3.3×10^6 cells/ml), 350 μl 10% Triton X-100 and diluting to 3500 μl using 6 M GuHCl (in 10 mM TE, pH 6.7). For quantitation of μSPE process, the DNA and protein concentrations of lysed WBC loading solution were analyzed by PicoGreen assay (0.4 ng/μl) and CBQCA (84 ng/μl). The human whole blood or cancer cell loading solution was made by mixing 65 μl thawed human whole blood or cancer cells, 350 μl 10% Triton X-100 and diluting to 3500 μl using 6 M GuHCl (in 10 mM TE, pH 6.7).

Purification of DNA by the μSPE method involved three steps: DNA loading, washing, and elution. During the loading step, 10 μl solution containing DNA diluted with GuHCl-based loading buffer was pushed through the μSPE device. 80% isopropanol (20 bed volume) was used to wash the device. DNA was eluted by 10 mM TE at pH 8.4 (3–18 bed volume) from the μSPE device. A syringe pump (Cole Parmer, Vernon Hills, ID) and a 100-μl gas-tight syringe (Hamilton, Reno, NV) were used to control the flow on the microliter scale. Based on the recoveries of DNA fragments (123, 267, 504 bp) at different pumping flow rates (data not shown), the pump flow rate at 200 μl/h was selected for all μSPE experiments carried out "off-line," shown in Fig. 1B. For DNA purification by μSPE "on-line" shown in Fig. 1C, the μSPE device was coupled to the capillary in a CE instrument. Based on the flow rates, the volume of different solutions used in μSPE was controlled by the pumping of solutions via a positive pressure (20 psi) supplied by the Beckman 5510 P/ACE instrument. The flow rates of all the solutions used were measured based on the ratio of the elution volume over the time when 20 psi pressure was applied. For on-line experiments, the flow rates for 6 M GuHCl, 80% isopropanol, 10 mM TE, and methanol were 5.2–5.6, 2.6–3.0, 8.1–9.7, and 12.8–14.2 μl/min, respectively. The flow rate for each solution was assessed from the time needed to push a constant volume through the device at constant pressure. Based on the measured flow rates of different solutions, pressure was applied for the appropriate amount of time to deliver the required volume.

For the on-line μSPE experiments, the elution of DNA and protein was monitored using PicoGreen dsDNA and CBQCA protein quantitation assays, respectively (42–44); 10 μl prepurified human genomic DNA or lysed human WBC solution in 6 M GuHCl–10 mM TE was loaded on the μSPE device (μSPE fractions 1–4); 20 bed volume 80% isopropanol was used to wash the device (μSPE fractions 5 and 6). One bed volume plus the capillary volume (500 nl) of 10 mM TE was pushed through the capillary (μSPE fraction 7). A total volume of 7.5 μl of 10 mM TE (pH 8.4) (equivalent to 5 times the fraction collection volume of 1.5 μl which was roughly 3 bed volumes) was used to elute the DNA from the device (μSPE fractions 8–12 or 13 if using 18 bed volume). Each of 12–13 fractions during the μSPE process was collected in 48 μl 10 mM TE (pH 7.5) solution for DNA quantitation or in 48 μl 0.1 M borate buffer for protein quantitation (used immediately or stored at −20°C until use).

Amplification of bacteriophage and human DNA by the polymerase chain reaction. Primers used in this study were listed in Table 1 and all were ordered from Life Technologies (Gaithersburg, MD) except the primers for amplification of 500-bp fragment of λ-DNA, which were provided by the Perkins–Elmer GeneAmp PCR reagent kit. PCR-ready mixtures for amplification were made by combining the following components: 10 mM Tris–HCl (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.25 μM each of forward and reverse primer listed in Table 1, 2.5 units *Taq* + Ab (2.5

TABLE 1

PCR Primers and Conditions Used for Amplifying DNA Eluted from μSPE Devices

Name	Primers	Size (bp)	PCR conditions
λ-DNA	Forward: 5′-GATGAGTTCGTGTCCGTACAACTGG-3′ Reverse: 5′-GGTTATCGAAATCAGCCACAGCGCC-3′	500	95°C for 5 min; 35 cycles of 94°C for 0.15 min, 68°C for 1.0 min; 72°C for 10 min; 4°C until analyzed. (14)
β-globin	Forward: 5′-AACTGTTGCTTTATAGGATTTT-3′ Reverse: 5′-AGGAGCTTATTGATAACTCAGAC-3′	650	95′C, 5 min; 30 cycles of 94°C for 1.0 min, 55°C for 1.0 min, 72°C for 1.5 min; 72°C for 3 min; 4°C until analyzed. (13)
Exon 2, BRCA1	Forward: 5′-GAAGTTGTCATTTTATAAACCTTT-3′ Reverse: 5′-TGTCTTTTCTTCCCTAGTATGT-3′	258	94°C for 5 min; 5 cycles of 93°C for 0.5 min, 63°C for 3 min; 5 cycles of 93°C for 0.5 min, 60°C for 3 min;
Exon 20, BRCA1	Forward: 5′-ATATGACGTGTCTGCTCCAC-3′ Reverse: 5′-GGGAATCCAAATTACACAGC-3′	399	5 cycles of 93°C for 0.5 min, 57°C for 3 min; 5 cycles of 93°C for 0.5 min, 54°C for 3 min; 25 cycles of 93°C for 0.5 min, 54°C for 1 min,
Exon 11, BRCA2	Forward: 5′-CACCTTGTGATGTTAGTTTGGA-3′ Reverse: 5′-TGGAAAAGACTTGCTTGGTACT-3′	201	65°C for 1 min; 65°C for 5 min; 4°C until analyzed. (56)

units/μl of *Taq* and 0.55 μg/μl TaqStart Ab), 1.0–10.0 μl of DNA as template (λ DNA solution or prepurified human genomic DNA or fractions from eluted steps of the μSPE experiment), adding autoclaved H_2O to bring the total volume to 25 μl. A Progene thermocycler (Model Techne, Duxford Cambridge, UK) was used in this study. The temperature conditions are also listed in Table 1.

Since PCR is capable of amplifying even trace quantities of template DNA, control experiments (loading buffer without DNA subjected to the μSPE procedure) were conducted routinely to ensure that the PCR products obtained in our experiments were not the results of contaminant DNA from any of the materials used in the experiments.

Detection of PCR products by capillary electrophoresis. The PCR product solutions were directly analyzed by capillary electrophoresis without dilution or desalting. Laser-induced fluorescence (LIF) detection was used with the excitation at 488 nm and emission at 520 nm. Capillary electrophoresis conditions were as follows: an FC-coated capillary was 50 μm (i.d.) by 27 cm (effective length of 20 cm); the running buffer was 1% HEC-TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.6, 1% w/v hydroxylethylcellulose) containing 1 μM YO-PRO-1 as the dsDNA fluorescent intercalator. Samples were injected electrokinetically for 20 s at 6.5 kV flanked by a 3-s injection of water at 3.5 kV on both sides. The separation was carried out at 5 kV (185 V/cm; 13 μA) with the poles reversed (inlet as cathode and outlet as anode) and the capillary was maintained at 20°C. Between runs, the capillary was flushed with water for 2 min and rinsed with the buffer for 4 min. For the first run of the day, this latter step was replaced with a fresh buffer rinse for 20 min.

Quantitation of double-stranded DNA using a fluorescence assay. Fractions collected from the μSPE were mixed with 1 ml diluted PicoGreen dye (1:400 in TE buffer) and incubated for 5–30 min at room temperature protected from light. A benchtop fluorometer TD 700 from Turner Design (Sunnyvale, CA) was used for fluorescence measurements (Ex/Em, 486 nm/520 nm). The calibration curves were primarily obtained through a previously described protocol (42), except that 2.5 μl of loading buffer (1% Triton X-100 in 6 M GuHCl) or 5.7 μl wash solution (80% isopropanol) was added into the standard series (50–2000 pg/ml) to account for any interferences to the colorimetric assay associated with components of the loading and washing buffer. Under our conditions, detection of DNA as low as 50 pg in one fraction was possible, corresponding to a concentration limit of detection limit of 50 pg/ml in each fraction.

Protein quantitation in μSPE using a CBQCA quantitation assay. For protein assay, fractions from the μSPE process were collected in 48 μl 0.1 M borate buffer (pH 9.3). Into each fraction from the μSPE process, 85 μl 0.1 M borate buffer, 5 μl 20 mM KCN and 10 μl 4 mM CBQCA were added. The solutions were mixed and rotated for a total of 1.5 h incubation time. Before fluorescence measurements, the solutions were then diluted to 1 ml by 0.1 M borate buffer.

Since GuHCl, Triton X-100, isopropanol, and Tris/EDTA affected the CBQCA protein assay (43, 44), independent calibration curves were created using an already defined protocol (43, 44) except that 2.5 μl of loading buffer (1% Triton X-100 in 6 M GuHCl), 5.7 μl of wash solution (80% isopropanol), or 1.7 μl elution buffer (10 mM TE) was added into the standard series (100–400 ng/ml) to account for possible interferences to the colorimetric assay associated with components of

the loading, washing, and elution buffer. Under our conditions, detection of protein as low as 100 ng in one fraction was possible, corresponding to a concentration limit of detection limit of 100 pg/μl in each fraction.

Equilibrium capacity experiments. The maximum loading capacity of silica particles under equilibrium conditions was determined by adding 180 μl of 0.57 ng/μl prepurified DNA (dissolved in the loading buffer, 6 M GuHCl) to ~2.2 mg silica particles. The particles were incubated in the buffer containing DNA for 1 h (mixing several times during this period) and subsequently centrifuged at 2000*g* for 5 min and the supernatant was used to quantify the unbound DNA. The silica particles were rinsed twice with 80 μl 80% isopropanol. The wash solution was separated from the silica particles by centrifugation, combined, and used for DNA quantitation. The loading capacity was calculated by dividing the mass of adsorbed DNA (subtracting the DNA amount in loading and wash solutions from the total DNA loading amount) with the weight of silica particles used.

RESULTS AND DISCUSSION

A popular method of DNA purification is to bind DNA to silica particles using commercially available resins, diatomaceous earth or glass fibers. The binding of DNA and RNA to silica, celite or glass powder in the presence of chaotropic agents such as sodium iodide, sodium perchloride, guanidine hydrochloride (GuHCl), and guanidine thiocyanate (GuSCN) is well known (26–28). DNA or RNA is selectively absorbed by the surface of silica, celite, or glass fiber in the presence of high concentrations of chaotropic agents. The purification of DNA by silica-based solid-phase extraction is accomplished by the elution of nucleic acids with solutions of low ionic strength or even water, with the eluted DNA directly amenable to a variety of applications without additional concentration or precipitation steps. The relative ease of eluting DNA with low ionic strength buffers offers a considerable advantage in situations in which the DNA purification process is followed by a high-resolution electrophoresis or by a salt-sensitive reaction such as PCR. A quick, one-step treatment of plasma or serum with a silica-based solid-phase material that yields highly purified DNA is, therefore, particularly attractive for protocols that combine DNA extraction with PCR applications.

Optimized Conditions for DNA Adsorption on Silica Surfaces

A miniaturized DNA extraction method based on the interaction of DNA with the silica (SiO_x) surface requires carefully controlled experimental conditions to obtain efficient DNA adsorption in a simple two-step process. Several purification goals must be accomplished simultaneously to recover biologically active DNA from cells and adsorb it on SiO_x in a single step. Cell lysis, DNA and protein denaturation, and effective DNA binding to SiO_x are accomplished in a loading buffer, thereby making the miniaturized purification protocol as simple and effective as possible.

While the phenomenon of DNA adsorption to silica surfaces is well-known (26) and has been utilized to purify DNA from biological sources (26, 45, 46), little or no information is available to aid in determining optimal binding conditions for DNA on silica surfaces. Based on reports in the literature (26, 45) and information obtained from reagents used in commercial DNA purification kits (47), initial conditions to selectively adsorb DNA on silica were chosen as follows: DNA was dissolved in 10 mM TE at pH 7.5 containing 6 M GuHCl. In a series of experiments with the pH of the solution varied between 6.7 and 7.5, it was determined that the adsorption of DNA on Supelco silica was more efficient with a lower pH loading buffer, i.e., pH 6.7. While the process of DNA adsorption on a silica surface is not comprehensively understood, our results, and a proposed mechanism for DNA–silica surface interaction (48), suggest that a high-ionic-strength buffer system with a pH at or below the pK_a of the surface silanol groups provides the highest adsorption efficiency.

Solutions of high ionic strength promote the adsorption of DNA on silica as a result of a number of interactions. At higher solution ionic strength, the negative potential at the silica surface is reduced, thereby decreasing the electrostatic repulsion between the negatively charged DNA molecule and the silica surface. However, high concentrations of salts, e.g., 4 M NaI, 4 M $NaClO_4$, or 6 M GuHCl, reduce the activity of water by forming hydrated ions, thereby dehydrating DNA and the silica surface and driving the DNA polymer to the silica. The driving force for the DNA adsorption phenomenon is presumably the increase in entropy of the system during binding of the DNA polymer to the silica surface which results from the loss of water structure near the DNA molecule (48). In addition to the effect on the activity of water, chaotropic agents also affect the DNA structure by disrupting the hydrogen bonding in the double-stranded (ds) DNA to produce single-stranded (ss) DNA. The liberated bases in the ssDNA are then able to hydrogen bond with silica, a driving force that overcompensates for the weak electrostatic repulsion between the DNA (sorbate) and the sorbent.

This electrostatic repulsion is further reduced if the DNA is dissolved in a loading buffer below the p*K*a of the silica where increasing protonation of the surface silanol groups reduces the surface charge. However, extremely acidic loading buffer conditions will affect

the solubility of proteins and lipids and may induce their precipitation—this could ultimately lead to the failure of the μSPE device. Consequently, a 10 mM TE buffer titrated to pH 6.7, containing 6 M GuHCl and 1% Triton X-100 (the function of which is discussed below) was chosen to test the effectiveness of DNA adsorption on the silica resins.

In addition to providing high-ionic-strength conditions for the adsorption of DNA onto the silica surface, the presence of GuHCl conveys additional functionality useful in the μSPE of DNA. GuHCl is known to lyse cells, denature cellular components (including proteins), inhibit nuclease activity, and prevent the enzymatic degradation of DNA during the purification procedure (26). Following lysis and denaturation, a wash solution consisting of 80% isopropanol provides sufficient solvent strength to remove non-DNA impurities from the μSPE device without affecting bound DNA. It is noteworthy that binding of heme impurities to the silica resins was observed (as evidenced by coloration of the μSPE device during the loading step). This was not problematic since the heme impurities were effectively desorbed (discoloration) during the washing step with isopropanol. In contrast to the loading and elution buffers, the washing solution exhibited a slightly higher (but not problematic) back-pressure due to the high viscosity of isopropanol. Finally, the recovery of DNA was accomplished in a low-ionic-strength elution buffer consisting of 10 mM TE at pH 8.4. Combined, these conditions should allow for μSPE devices to be loaded with sample, and DNA recovered in a reproducible manner using microliter volumes—this is conducive to DNA extraction in a miniaturized format.

Micro-Solid-Phase Extraction of λ DNA and Its Amplification by PCR

A method that will find widespread applicability for extraction of DNA from sources as complex as WBCs or whole blood must be robust in the presence of reagents that are necessary to process such samples. Detergents such as sodium dodecyl sulfate, Triton X-100, and Tween 20 are commonly used to accelerate cell lysis and increase the solubility of lipophilic cellular components that could potentially bind to the silica and interfere with DNA extraction. An additional caveat is that some of these detergents are also known to interfere with the PCR, even at low concentration (22, 23). Therefore, any residual detergent not removed from the silica resin during the washing step could coelute with the DNA and inhibit the PCR process. This was evaluated by carrying out PCR with DNA recovered from the μSPE devices using elution buffers containing low concentrations of the common cell lysis detergents: Triton X-100, Tween 20, and SDS. λ DNA was chosen as the template DNA for these experiments and the μSPE devices constructed as illustrated in Fig. 1. Micro-solid-phase extraction experiments were conducted with 100 pg of λ dsDNA dissolved in 18 device volumes (total of 10 μl) of loading buffer (6 M GuHCl). In each case, DNA was loaded onto the device at 3.3 μl/min, washed with 40 device volumes (20 μl) of 80% isopropanol and the λ DNA eluted with 10 μl of elution buffer, containing either 1% Triton X-100, 1% Tween 20, or 0.01% SDS. The eluent was collected and added to the PCR mixture, which was thermocycled in a conventional instrument to amplify a 500 bp fragment of λ DNA. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was used to detect the λ DNA PCR products.

As shown in Fig. 2, the intensity of the fluorescence signal from amplification products eluted with a buffer containing nonionic detergents, i.e., 1% Triton X-100 (Fig. 2C) or 1% Tween 20 (Fig. 2D), was indistinguishable from the PCR controls which included 100 pg λ DNA not subjected to μSPE (Fig. 2A), or DNA eluted with 10 mM TE with the μSPE process (Fig. 2B). In contrast, the fluorescent signal from the product amplified following elution of DNA with 10 mM TE containing 0.01% SDS was significantly lower, indicating either the incomplete elution of DNA from the μSPE device or the inhibition of PCR by SDS (Fig. 2E). Since it has been well established that ionic detergents inhibit the PCR reaction (22, 23), no further investigation was carried out to discern the cause of the decreased product signal. The observation that 1% Triton X-100 could be added to the loading buffer without interfering with the adsorption of DNA to silica and without downstream effects on the highly sensitive PCR was important. Since Triton X-100 is known to be an effective cell lysis agent, its presence will mediate the rupture of cells added directly to the loading buffer. In addition, this detergent can induce the release of DNA from the cell nucleus as well as solubilize lipid and membrane-bound components. This is important since the solubilization of lipophilic components may prevent occlusion of microchannels in the miniaturized system.

Retention of Human Genomic DNA Fragments on Silica Particles

For an on-line purification method that aims to extract DNA directly from a biological matrix, the solid-phase material must be capable of extracting human genomic DNA fragments (>50 kb) with reasonable efficiency. This was investigated using a human genomic DNA preparation that was prepurified by a conventional organic solvent extraction method (49) and consists of DNA fragments >50 kb (Huschenbett-Hühmer, J., personal communication). Three nanograms of the prepurified human genomic DNA was dissolved in

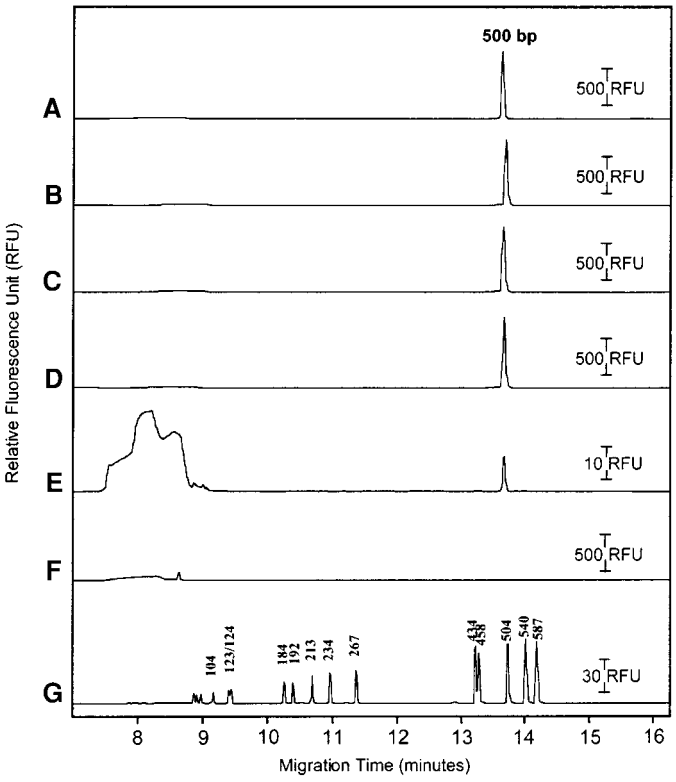

FIG. 2. Retention of λ DNA by μSPE and the effect of elution buffers on its amplification—The electropherogrmas from CE-LIF analysis of the PCR products (500 bp). In all the μSPE cases, 10 μl of 0.01 ng/μl λ DNA in 6 M GuHCl was loaded on the irregular-shaped silica μSPE device, 20 μl of 80% isopropanol was used to wash the device before using 10 μl of 10 mM TE containing different detergents to elute the DNA from the device. A syringe pump was used to control the flow. CE-LIF conditions were described in the text. (A) The positive control, 0.1 ng λ DNA was used, no treatment by μSPE. The elution conditions were as follows: (B) 10 mM TE, (C) 10 mM TE containing 1% Triton X-100, (D) 10 mM TE containing 1% Tween 20, (E) 10 mM TE containing 0.01% SDS, (F) blank control, μSPE, no DNA loaded. The absence of a 500-bp peak in the electropherogram F confirms that the PCR product in A–E represent specific amplification and was not the result of λ DNA contamination. (G) DNA size markers (pBR 322 *Hae*III digest).

loading buffer, applied to the silica μSPE device (containing Supelco irregular-shaped silica particles), and eluted with 10 column volumes of elution buffer (10 mM TE, pH 8.4). The μSPE device eluent was used to amplify a 650-bp fragment of the β-globin gene by the PCR (24). Using CE-LIF, the product was identified by comparing the migration time of the 650-bp β-globin gene amplicon with a DNA size standard. Figure 3A shows the PCR product generated in a positive control experiment in which 3 ng of DNA purified conventionally (no μSPE treatment) was directly thermocycled to amplify the 650-bp fragment of the β-globin gene. PCR amplification in the absence of prepurified DNA failed to generate a product (blank control; Fig. 3B). Figure 3C shows that a single 650-bp PCR product peak was observed by CE-LIF, confirming that sufficient prepurified genomic DNA template (fragments ~50 kb or greater) was effectively retained by the silica. This contrasts a previous report that suggested that retention of large DNA fragments on silica surfaces was inefficient (47). In fact, we observed that the extraction of DNA from cells lysed *in vitro,* which presumably contained large, unsheared DNA fragments, did not exhibit any difference in binding in comparison with smaller DNA fragments (e.g., λ DNA). Comparison of the fluorescence signal strength for the PCR product in Fig. 3C with that of the positive control (Fig. 3A) indicates that a slightly smaller amount of template DNA was available for thermocycling following μSPE. Since we cannot ascertain that both PCR reactions were performed with the same efficiency, a definitive conclusion regarding the quantity of the template available for PCR from these data is not possible. However, at the

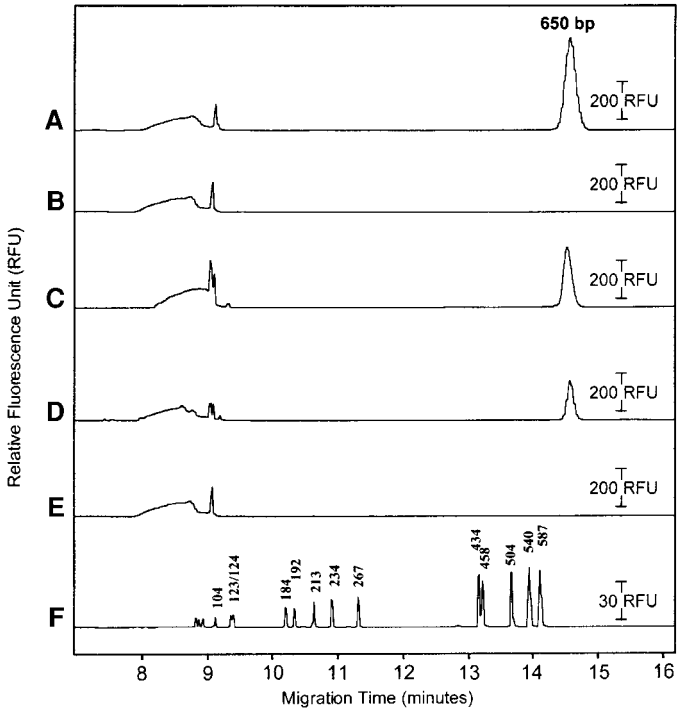

FIG. 3. Amplification of β-globin gene from μSPE-purified DNA—The electropherograms from CE-LIF analysis of the PCR products (650 bp). In all μSPE experiments, irregular-shaped silica particles were used to make the μSPE devices, 20 μl 80% isopropanol and 10 μl of 10 mM TE was used in the wash and elution steps, respectively. The eluted DNA was added into the PCR mixture for amplifying 650 bp fragment of β-globin gene. (A) Positive control, no μSPE, 3 ng purified human genomic DNA was used for PCR amplification. (B) Negative control, no DNA loaded during the μSPE. (C) 10 μl of 0.3 ng/μl purified human genomic DNA was loaded. (D) 10 μl lysed white blood cell solution in 6 M GuHCl containing 1% Triton X-100 (~630 cells) was loaded. (E) 10 μl lysed white blood cells (~630 cells in PBS containing 1% Triton X-100) was directly added into the PCR mixture for PCR amplification. (F) DNA size markers (pBR 322 *Hae*III digest).

very least, it is obvious that sufficient DNA template was retained by the silica and that an appreciable PCR product signal could be observed. Since a more quantitative evaluation of this process would be useful in method optimization, more intricate experiments were conducted to investigate the quantitative aspects of DNA extraction by μSPE.

Quantitative Evaluation of Different Silica Particles: μSPE of Prepurified Human Genomic DNA

The results discussed above establish that silica materials can be utilized to retain PCR-suitable human genomic DNA. However, to investigate the exact DNA binding capacity of silica and the physicochemical parameters that define the binding capacity in a miniaturized extraction device, we quantified the DNA retained on each of the three silica materials using a fluorescence-based assay. The exact character and quality of the silica materials used for nucleic acid purification in a number of commercial kits is mostly unknown and, at best, poorly defined (47, 50). While details on the physicochemical properties of silica material used for microscale extraction of DNA are scarce in the literature, it can be assumed that the surface properties are critical for effective DNA extraction from hundreds or even fewer cells. For effective DNA binding, the particle size and shape, the pore diameter and volume, and the packing and resin porosity are of major significance to the overall effectiveness. Consequently, several silica resins with a variety of properties were evaluated to determine which parameters were critical for effecting the purification of DNA in a miniaturized format.

In addition to the irregular-shaped particles (Supelco) described above, two well-defined porous silica materials (Hyperprep and Merck G-60) were investigated. These spherical-shaped silica resins were selected based on particle size, pore size, surface chemistry, and surface area, as well as the degree to which the resin had been characterized. Their average particle size was 30–64 μm, a size range that should facilitate effective packing in the devices and avoid the high back-pressure associated with tightly packed devices. Both silica resins were porous (one with 60-Å pores, the other with 120-Å pores) to increase the available surface binding area but with pores small enough to avoid the trapping of DNA fragments.

Carefully prepared μSPE devices packed with the Supelco, Hyperprep, or Merck G-60 silica (~200 μg of each; ~500 nl bed volume) were connected to a capillary mounted in a CE instrument and the three-step μSPE procedure was carried out using a hydrostatic pressure of 20 psi. For each experiment, 3 ng of prepurified human genomic DNA was loaded onto the devices and at least 12 fractions were collected into 10

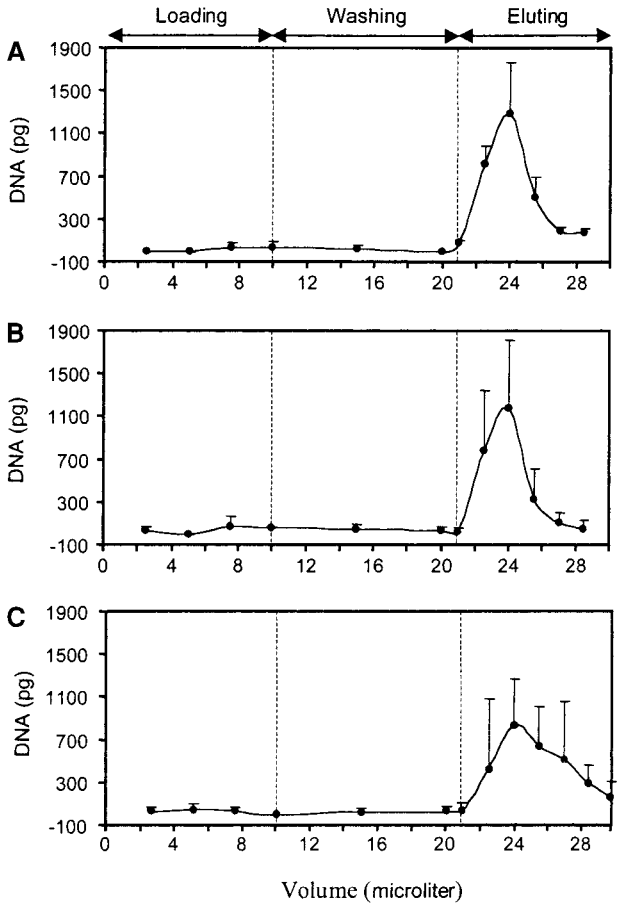

FIG. 4. Quantitative evaluation of retaining purified human genomic DNA by silica particles—Comparison of irregular-shaped silica, Hyperprep silica, and G-60 silica materials. In all the μSPE experiments, 10 μl of 0.3 ng/μl purified human genomic DNA was loaded, and 12 or 13 fractions were collected in 48 μl of 10 mM TE for DNA quantification by PicoGreen assay. Fractions 1–4, loading step; fractions 5–7, washing step; fractions 8–12 or 8–13, elution step. (A) Irregular-shaped silica SPE devices; (B) Hyperprep silica SPE devices; (C) G-60 silica SPE devices. Each point was the average (X) of three experiments with the sample standard deviation (s) as the error bar.

mM TE (pH 8.4). Fractions 1–4 constituted the loading buffer, fractions 5–7 contained molecules eluted with the wash solution, and fractions 8–13 were the elution fractions. Figure 4 shows the DNA elution profiles for the three different silica-based solid-phase materials. All elution profiles displayed a peak with maxima at approximately fraction 9, with >50% of the DNA being eluted in the 3-μl volume associated with fractions 9 and 10. In terms of the most reproducible behavior, the irregular-shaped silica was clearly superior (Fig. 4A), followed by the spherical Hyperprep resin which showed elution profiles for DNA that were similar to the irregular-shaped silica (Fig. 4B). The Merck G-60 was the least impressive, showing poor device-to-device reproducibility, slightly shifted elution profiles,

and DNA eluting over a larger number of fractions (Fig. 4C). This may correlate with the fact that the Merck G-60 silica was more difficult to pack during μSPE device assembly, and may be due to the broad distribution of particle sizes with this resin.

The optimal device for DNA purification by surface adsorption-mediated binding will have a large surface-area to volume ratio so that the adsorption of DNA is greatly enhanced. The total surface area of the silica material in the device ultimately determines the total binding capacity of the μSPE device. In this study and in others by our group (36–40), we have demonstrated the capability to assemble microdevices with a relatively large surface-to-volume ratio for the extraction of analytes. For a 500-nl device, a surface area of 420 cm^2 was achieved with the Hyperprep silica and 1100 cm^2 with the G-60 silica. The surface area can be distinguished as area accessible to all analytes, including ssDNA or surface area in the pores that is only accessible to smaller analytes. The pore size with both materials (60 and 120 Å) was small in comparison to the size of the ssDNA purified. The size exclusion limits expected with such pore sizes would be in the 40- to 60-bp range for ssDNA. Thus, only fragments smaller than this could be trapped and probably is not of concern for most applications. Device parameters such as porosities and total surface area can be estimated using the physical properties of silica as well as the specifications of the silica resins as provided by the manufacturer (see Materials and Methods for details)—details on these relationships can be found in the literature (51, 52). The DNA-accessible surface area for our μSPE devices were determined to be 191 cm^2 for a device packed with 200 μg of Merck G-60 resin and 84 cm^2 for a device packed with 210 μg of Hyperprep material. Therefore, only ~20% of the total surface area of the silica resin is accessible to ssDNA. However, the remaining surface area is accessible to low-molecular-mass impurities, such as heme, a well-known PCR inhibitor. These additional binding sites are important for the overall performance of the device because the smaller impurities that are adsorbed inside the pores would otherwise compete with DNA for binding sites.

A larger DNA-accessible surface area in the μSPE device can be created using smaller particles that are more effectively packed, but a high packing density in the μSPE device might create problems with back-pressure as well as clogging with crude samples, e.g., plasma. In addition, the particle size must not be too small, to avoid leakage through the glass fiber frit which could contribute to the inhibition of biological reactions downstream, e.g., PCR. In fact, we repeatedly observed that very tightly packed columns were more irreproducible in their binding and elution behavior, which was largely due to the difficulties in maintaining homogenous and constant flow rates throughout the device at 20 psi. The use of resins with a relatively small particle distribution (e.g., Hyperprep) or the use of irregular-shaped particles is favorable in the packing of microdevices, whereas larger particles (e.g., Merck G-60) are difficult to pack efficiently into small devices. This problem is exacerbated with an increase in particle size and particle heterogeneity. Consequently, the choice of particle size is limited in this respect to particles that allow device fabrication and operation below the pressure rating of the microdevice. Consequently, when considering the binding capacity of a μSPE device, there must be a tradeoff between the large surface area of the device efficiently packed with small particles (e.g., 5- to 15-μm particles) and the potential clogging problems associated with crude biological mixtures in an efficiently packed device.

Physicochemical Aspects of Silica That Impact DNA Adsorption and Elution

Although our data show that the efficiency of the μSPE device is a function of the experimental device parameters discussed above, the silica surface chemistry and the adsorption properties of the silica resins for ssDNA contribute to the binding capacity and DNA recovery of the device. One parameter that must be considered is the acid-base character of the surface silanol groups, which will determine the selectivity of silica toward ssDNA. Silica particles can be categorized as "Type B" silica which contains more geminal silanol groups, whereas "Type A" silica contains more free and vicinal silanol groups (53). Hydrogen-bond acceptor solutes (basic solutes) are adsorbed preferentially on acidic silanols (i.e., free silanol and vicinal silanol groups; Type A silica), while solutes with hydroxyl groups are adsorbed preferentially on associated silanols (i.e., geminal silanol groups; Type B silica). The silica particles chosen for the experiments discussed in this report provide a variety of surface active groups as well as encompass a broad range of silica particle and pore sizes. Theoretically, a fully hydroxylated silica surface should have a $pK_a = 7.1 \pm 0.5$, but the actual pH of silica particles may be in the range of 3.5–9.5, depending on the manufacturing methods (53). Although the pH value of the silica aqueous suspension is not directly correlated with the surface structure, it correlates well with the retention behavior of polar analytes. The acidic silica particles are more efficient at binding proton acceptor analytes, whereas the basic silica particles are more efficient at binding proton donor analytes (53, 54). The lower the pH of the silica (i.e., the higher concentration of free and vicinal silanol groups), the stronger the adsorption of negatively charged molecules (proton acceptor), indicating that

TABLE 2

Capacities and Recoveries of Human Genomic DNA Using Different Silica Particles as the Solid-Phase Extraction Materials[a]

Silica particles	DNA loading capacity[b]	Sample loaded	Surface area (cm^2)[c]	Recovery in fractions 9–10 (~3 μl)	Recovery in fractions 7–13
Supelco (irregular-shaped, pH 7.7)	24.7 ± 3.4 ng/mg (50.6 ± 6.4%)	Purified DNA	N.A.[d]	60 ± 22% (1.78 ± 0.64 ng)	99 ± 18% (2.97 ± 0.54 ng)
		White blood cell	N.A.	34 ± 4% (1.36 ± 0.15 ng)	68 ± 8% (2.75 ± 0.32 ng)
Hyperprep (30–40 μm, 200 m^2/g, 120 Å, pH 8.1)	6.77 ± 2.46 ng/mg (14.4 ± 5.5%)	Purified DNA	413 ± 23	50 ± 30% (1.50 ± 0.91 ng)	80 ± 32% (2.43 ± 0.91 ng)
		White blood cell	413 ± 11	26 ± 4% (1.06 ± 0.19 ng)	69 ± 7% (2.81 ± 0.34 ng)
Merck G-60 (40–63 μm, 550 m^2/g, 60 Å, pH 7.5)	28.2 ± 5.6 ng/mg (60.9 ± 12.2%)	Purified DNA	1045 ± 110	49 ± 9% (1.47 ± 0.27 ng)	94 ± 10% (2.83 ± 0.29 ng)
		White blood cell	1100 ± 0	40 ± 7% (1.62 ± 0.30 ng)	77 ± 6% (2.98 ± 0.18 ng)

[a] All the data are the average of three experiments with one standard deviation.
[b] Adsorbed DNA (ng) divided by the amount of silica particles (mg).
[c] Total surface area of silica particles used.
[d] Not available (N.A.).

DNA should be more effectively bound to the surface of Type A silica.

To establish the acid/base properties of the resins used in our experiments, we prepared a 10% (w/v) slurry of the silica resins in distilled water and, once adequately equilibrated, measured the pH of these solutions. The pH values of the G-60 silica, irregular-shaped silica, and Hyperprep silica slurries were 7.5, 7.7, and 8.1, respectively. These pH values indicate that the Hyperprep silica displays Type B silica properties and DNA should bind less effectively to these silica particles. In addition, G-60 silica should be a better silica material for purifying DNA than these. The percentage of DNA recovered from G-60 silica indicates that the DNA binding properties of these silica particles are, indeed, slightly better than the Hyperprep silica particles. These observations corroborate the theoretical considerations of adsorption behavior of DNA on silica surfaces discussed above, where increased protonation of the silanol groups reduces electrostatic repulsion and enhances DNA binding.

Table 2 provides details on the quantitative aspects of DNA recovered in fractions 7–12 for the various silica particles tested. All silica particles exhibit a high binding capacity with a DNA recovery of >80%. This is significant, since maximizing the mass of DNA recovered per unit volume of eluent will be of major importance in a miniaturized format. Irregular-shaped silica particles show the highest recovery of DNA in a small fraction (~3 μl) of the total volume, with more than 50% of the DNA recovered in only a small percentage of the applied volume—this translates to an enrichment factor of ~1.7 for the retained DNA. Based on the equilibrium experiments, it is important to note that with devices having a bed volume of ~500 nl (~200 μg of silica), 3 ng DNA does not overload the μSPE device. Results from equilibrium experiments indicate that the tendency for DNA to preferentially adsorb on Type A silica can be corroborated with these capacity experiments (shown in Table 2). A quantitative evaluation of the binding capacity under our experimental conditions reveals that most of the DNA loaded on the device was bound to the surface of the silica particles, as indicated by the absence of peaks in fractions 1–7, which constitute the loading and washing steps of the procedure.

Extraction of Human Genomic DNA from White Blood Cells

The extraction of genomic DNA from a crude biological matrix imposes a much larger demand on the binding capacity of the solid phase material than the retention of prepurified DNA. In addition to DNA, biological matrices contain a complex mixture of components (RNA, proteins, lipids, drug metabolites, and inorganic ions), some of which may compete with DNA for the binding sites on the surface of the silica, reducing the overall binding capacity of the solid phase device. Using human WBCs as a DNA source, we sought to determine whether genomic DNA directly released from cell nuclei into loading buffer could be retained on the μSPE silica surface, the PCR inhibitors removed during the wash step, and the eluted DNA successfully amplified. A human WBC suspension containing ~630 cells [~4.0 ng of DNA (23)] was mixed with loading

buffer containing 1% Triton X-100. This solution was loaded onto the μSPE device packed with irregular-shaped silica. Device loading was accomplished using the injection capabilities of a CE instrument with a positive pressure of 20 psi. The device was washed as in previous experiments, with the bound DNA eluted and collected for PCR amplification. The amplification of the 650-bp fragment of the β-globin was, again, used to indicate the presence of DNA template in the elution fraction after the μSPE process. As shown in Fig. 3D, the presence of the PCR-amplified β-globin gene product verifies that sufficient DNA from WBCs was extracted for amplification. These results contrast those shown in Fig. 3E where, as a negative control, 10 μl of a cellular suspension with roughly the same number of cells was lysed using Triton X-100 (1% final concentration in the lysate), directly added into the PCR mixture, and used as a template for PCR. The distinct absence of a peak in the migration time window where the β-globin gene PCR product is seen in Fig. 3E indicates the presence of PCR inhibitors in the WBCs. This is not surprising in light of a recent report by Hämäläinen *et al.* (24) indicating that neurotoxin ribonuclease in eosinophils acts as a potent reverse transcription inhibitor, emphasizing the importance of DNA or RNA purification prior to PCR or RT-PCR. This experiment clearly demonstrated that the μSPE step not only retained genomic DNA but also removed PCR inhibitors. However, the generally lower fluorescence signal intensities of the PCR product in Fig. 3D in comparison with the control in Fig. 3A indicate that either less DNA template was available after extraction of DNA from the WBCs or that PCR inhibitors were not completely removed in the wash step during μSPE. Consequently, a more quantitative method was used to measure the exact amount of DNA eluted under these conditions.

Quantitative Evaluation of Different Silica-Based Resins for μSPE of DNA from Human White Blood Cells

Selective DNA binding and elution. The results described above demonstrate that prepurified human genomic DNA can be effectively adsorbed to a solid phase, survive a wash procedure with 80% isopropanol, and be efficiently eluted with 10 mM TE—a binding capacity of ~10–30 μg DNA per gram of silica was observed. However, one must be cognizant that these conditions differ substantially from those associated with biological matrices which contain a variety of components that can compete with DNA for the binding sites on the silica surface. The qualitative aspects of the DNA extraction of such devices are largely controlled by the ability of the silica resins to selectively bind DNA under the experimental conditions chosen (but not other analytes), so that DNA can be separated from the accompanying impurities. Typically, 1 μl of blood contains approximately 5000 leukocytes that, in turn, contain a total of ~35 ng of DNA (23). The lysate of white blood cells contains over 300 times more proteinaceous material by weight than DNA (data obtained from the following experiment). Therefore, in order to obtain highly purified DNA, specific binding of DNA to the silica surface in the presence of excess impurities is essential. Consequently, the quantitative aspect of μSPE of DNA from a biological matrix was evaluated, again, using WBCs as the DNA source.

WBCs were lysed as before by Triton X-100 and mixed with the loading buffer. In each experiment, 10 μl of lysed WBC solution (~600 cells) was loaded on a μSPE device constructed, in triplicate, with one of the three types of silica. Using the same approach and DNA fluorescence for quantitation, the DNA profiles obtained from the μSPE of DNA directly from WBCs are given in Fig. 5 with additional quantitative details provided in Table 2. The overall recovery of DNA (fractions 7–13) from WBCs using the three different silica particles was relatively high, averaging greater than 70%, indicating that ~30% of the WBC DNA was either not eluted or lost during the loading and washing steps. As shown in Fig. 5, the DNA profiles associated with extraction of DNA from a WBC lysate are distinct from those obtained with prepurified DNA. As seen with prepurified DNA, the profiles obtained with the irregular-shaped silica yielded a bell-shaped curve with peak maximum at fraction 9 (Fig. 5A), while DNA profiles with the Hyperprep and G-60 silica displayed peaks that were not as well-defined, with peak maxima scattered throughout different fractions (i.e., fractions 8, 9, and 10; Figs. 5B and 5C). As a result of the differences in device efficiency with the different silicas, the relative recoveries of DNA in a small elution volume are different. This is seen by the recovery of DNA in fractions 9 and 10 which was ~40% with the irregular-shaped and G-60 silica, but only 26% with the Hyperprep silica.

It is obvious that less-efficient recovery of DNA from biological matrices would limit this method for the purification and enrichment of less abundant DNA, e.g., viral DNA in serum. The limited capacity and the loss of DNA in such small devices is, however, not a problem if the recovery of the DNA does not have to be quantitative. For example, only a limited amount of DNA template must be obtained to achieve the amplification of a specific DNA fragment by PCR in a subsequent analysis step. The effective removal of proteins is much more important in this case than the quantitative retention and/or the complete elution of all DNA fragments. On the other hand, the ineffective removal of nuclease or protease activity during the purification could render the amplification reaction worthless.

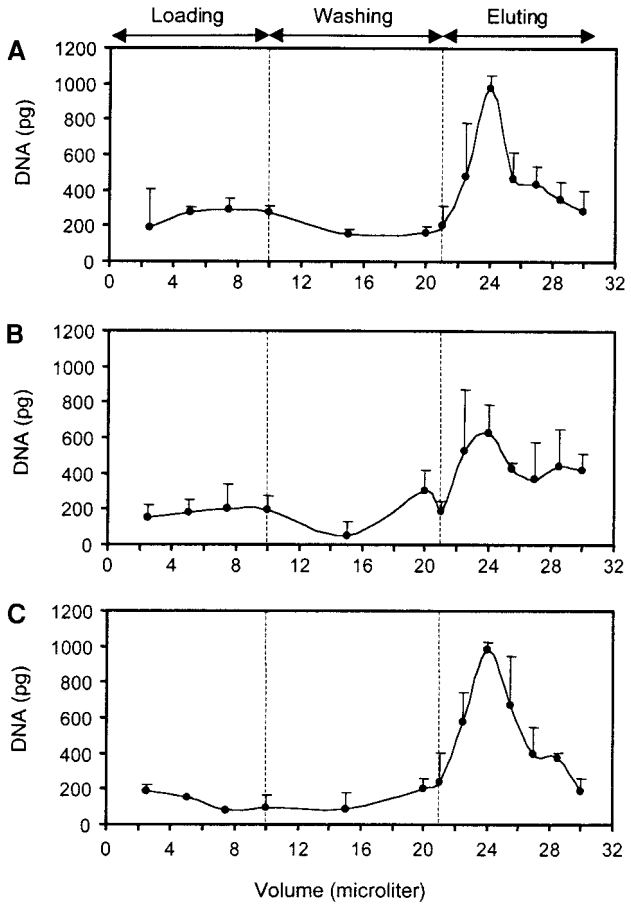

FIG. 5. DNA profiles in μSPE processes of purifying human genomic DNA from white blood cells. In all the μSPE processes, 10 μl lysed human white blood cell solutions in 6 M GuHCl (containing 1% Triton X-100, used for lysing the white blood cells, 63 cells/μl) was loaded, and 13 fractions were collected in 48 μl 10 mM TE for DNA quantification by PicoGreen assay. Fractions 1–4, loading step; fractions 5–7, washing step; fractions 8–13, elution step. (A) Irregular-shaped silica devices; (B) Hyperprep silica devices; (C) G-60 silica devices. Each point was the average (X) of three experiments with the sample standard deviation (s) as the error bar.

Selective protein binding and elution. It was not an unexpected finding that μSPE of DNA directly from a WBC lysate was associated with a poorer efficiency than observed with prepurified human genomic DNA. The decrease in the relative capacity of the silica for selectively binding DNA in a complicated matrix is clearly the result of competition for nonspecific binding sites by other endogenous components. Since the protein concentration in WBC lysates is relatively high, proteins are likely competitors for the binding sites on the silica surface and, thus, contribute to the poorer efficiency. The adsorption and elution of WBC proteins was evaluated mainly because hemoglobin, potentially present in the lysate due to a low-level contamination with red blood cells, is a well-known PCR inhibitor (22, 23). Avoiding the solid-phase retention of hemoglobin followed by its coelution with the DNA is an important facet in μSPE, particularly when the PCR-based analysis of DNA directly from whole blood is desired. Therefore, protein removal by μSPE was explored using the lysate resulting from rupture of intact WBCs (~600 cells). Collecting the fractions in 48 μl of a 100 mM borate buffer allowed for direct protein quantitation via the CBQCA (fluorescence) protein assay described under Materials and Methods. Since the buffers used for loading (GuHCl/Triton X-100), washing (isopropanol), and elution (Tris/EDTA) affected fluorescence in the protein assay (43, 44), independent calibration curves were developed for each of these solutions (data not shown). Under

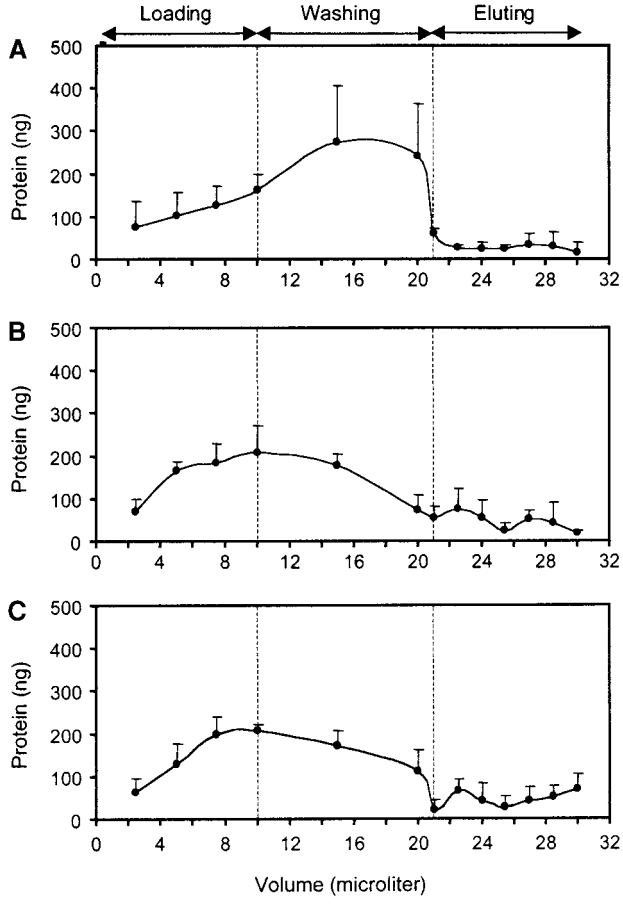

FIG. 6. Protein profiles in μSPE during purification of human genomic DNA from white blood cells. In all the μSPE experiments, 10 μl lysed human white blood cell solutions in 6 M GuHCl (containing 1% Triton X-100, used for lysing the white blood cells, 63 cells/μl) was loaded. In each μSPE experiment, 13 fractions were collected in 48 μl 0.1 M borate buffer for protein quantification by CBQCA assay. Fractions 1–4, loading step; fractions 5–7, washing step; fractions 8–13, elution step. (A) Irregular-shaped silica devices; (B) Hyperprep silica devices; (C) G-60 silica devices. Each point was the average (X) of three experiments with the sample standard deviation (s) as the error bar.

TABLE 3

Protein Removed during DNA Purification from Human White Blood Cells[a]

Silica particles	Surface area (cm^2)	Protein removed in fractions 1–4	Protein removed in fractions 5–7	Protein coeluted in fractions 9–10 (~3 μl)	Protein removed in fractions 1–7
Supelco (irregular-shaped, pH 7.7)	N.A.[b]	39 ± 16% (468 ± 186 ng)	48 ± 20% (573 ± 242 ng)	4.1 ± 1.5% (50 ± 18 ng)	87 ± 5% (1037 ± 60 ng)
Hyperprep (30–40 μm, 200 m^2/g, 120 Å, pH 8.1)	480 ± 50	52 ± 12% (625 ± 142 ng)	26 ± 2.4% (305 ± 31 ng)	6.5 ± 3.3% (78 ± 41 ng)	78 ± 10% (930 ± 114 ng)
Merck G-60 (40–63 μm, 550 m^2/g, 60 Å, pH 7.5)	1210 ± 110	50 ± 9.3% (603 ± 105 ng)	25 ± 8% (302 ± 99 ng)	5.7 ± 5.6% (68 ± 69 ng)	75 ± 5% (905 ± 61 ng)

[a] See Fig. 6 for protein profiles. In each case of loading white blood cells, the number of cells loaded is 600 and the total protein amount loaded is 1200 ng. All results are shown as the average with one standard deviation (s) (n = 3).
[b] Not available (N.A.).

our conditions, detection of protein as low as 100 ng/ml was possible, corresponding to a concentration detection limit of 100 ng/μl in each fraction. Figure 6 shows the protein profiles obtained when purifying DNA directly from WBC lysate via μSPE, while Table 3 provides details on the quantitative protein concentration results. In all the cases, 80–90% of the protein was removed by the μSPE process during the loading and washing steps. The protein results mirrored those with DNA, with the Supelco irregular-shaped silica providing the best results (87% removal of protein), with less efficient removal observed with Hyperprep and G-60 silica (~75%). Overlay of the results of Fig. 6A with those in Fig. 5A shows the excellent correlation between cleansing the solid phase of protein during the wash step (fractions 5–7) and desorption of DNA during the elution step (fractions 8–13). The almost flat protein profiles during the elution steps indicate that little or no protein coeluted with DNA during the desorption step. The lower protein concentrations in the wash step of the profiles for Hyperprep and G-60 silica may indicate that the bound proteins are more difficult to desorb from the surface. These results indicate that with use of the appropriate silica surface adsorption sites for effectively binding ssDNA, but not for the large excess of binding competitors, i.e., proteins, can be accomplished. It is possible that a more extended wash step could reduce the amount of protein coeluted with the DNA, but it will also increase the volume of solvents processed. In microfabricated devices with limited capacity for storage and handling of liquids, the remaining impurities in the DNA might be acceptable if the volume of liquid necessary for the entire DNA extraction procedure is kept at a minimum.

Direct μSPE of DNA from Cultured Cancer Cells

Having optimized the quantitative removal of protein and extraction of DNA from WBCs on the submicroliter scale, the universal nature of the μSPE process for the purification of PCR-suitable DNA needed to be demonstrated with other biological matrices. Moreover, if a μSPE process were to be an effective means of DNA extraction in a microminiaturized process, the method would not only have to be robust in nature and applicable to a wider range of sample matrices, but also yield PCR-suitable DNA for a wide spectrum of DNA targets. For this purpose, three sequences associated with mutations in the breast cancer susceptibility genes, BRCA1 and BRCA2 (11; Huschenbett-Hühmer, J., unpublished results), were utilized as new PCR amplification targets from four biological matrices—these included (1) a WBC lysate, (2) WBCs suspended in serum, (3) whole blood, and (4) cultured cancer cells (DU-145, MCF-7). Amplification of the BRCA1 sequence associated with mutations in exons 2 and 20 yielded 258- and 399-bp fragments while that with exon 11 of the BRCA2 gene resulted in amplification of a 201-bp fragment. These experiments were carried out on the microliter scale using a syringe pump to control the flow during the loading, washing, and elution steps with PCR amplification mediated by a multiplex PCR system as listed in Table 1 for mutation detection (11; Huschenbett-Hühmer, J., unpublished results). All samples were prepared for μSPE by mixing either a defined number of cells or a volume of the cellular suspension with the loading buffer (6 M GuHCl/1% Triton X-100) as described in the legend to Fig. 7. The DNA (which was eluted into 10 μl of 10 mM TE) was added directly to the PCR mixture (total volume of 25 μl) for the multiplex PCR amplification of BRCA1/BRCA2 fragments. Figure 7 shows the electropherograms resulting from CE-LIF analysis of the amplified

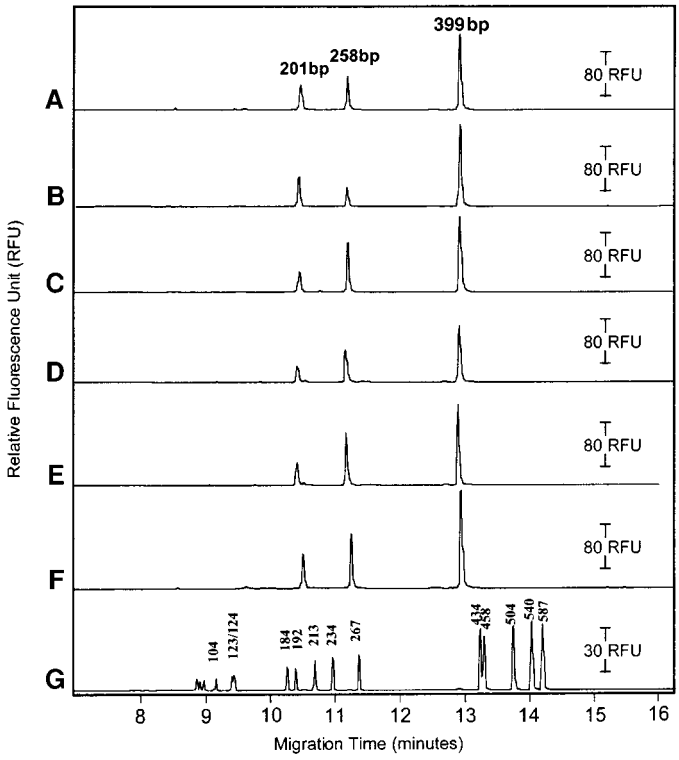

FIG. 7. PCR of μSPE-purified DNA from serum, whole blood, and cancer cells by silica μSPE for mutation screening of breast cancer susceptibility genes BRCA1 and BRCA2—The electropherograms from CE-LIF analysis of the multiplex PCR products (201, 258, and 399 bp). Hyperprep silica particles were used to make all the μSPE devices (0.25–0.30 mg, 5–7 mm $\times$ 0.38 mm). A syringe pump was used to control the flow. In each case, the sample was mixed with the loading buffer (6 M GuHCl containing 1% Triton X-100), 20 μl of 80% isopropanol was used to wash the device and 10 μl of 10 mM TE was used to elute the DNA. The eluent was added into the PCR mixture for amplifying 3 fragments of BRCA1 and BRCA2 genes. (A) 10 μl lysed white blood cell loading solution (~600 cells). (B) 10 μl lysed white blood cells in serum solution (~600 cells). (C) 10 μl whole blood loading solution (~0.2 μl thawed whole blood). (D) 10 μl lysed prostate cancer cells (DU-145, ~4000 cells). (E) 10 μl lysed breast cancer cells (MCF7, ~4000 cells). (F) Positive control, no μSPE, 16 ng purified human genomic DNA was used for PCR amplification. (G) DNA size markers (pBR 322 *Hae*III digest).

PCR products from WBCs (~600 cells, Fig. 7A), WBCs in serum (~600 cells; Fig. 7B), and WBCs in whole blood (0.15 μl whole blood; Fig. 7C) and the positive control (prepurified DNA; Fig. 7F). A cursory glance indicates that the purified DNA provided by μSPE clearly allowed for the specific PCR amplification of the 258-, 399-, and 201-bp fragments (compare peak migration times with the DNA standard migration times in Fig. 7G). Noting that the fluorescence intensity scale is identical in all electropherograms, there is a detectable but relatively small difference in the amplification of these fragments regardless of whether the DNA source is prepurified genomic DNA or WBCs in serum. Of particular interest is the fact that the amplification of these particular sequences is apparently unaffected when μSPE is applied directly to whole blood (Fig. 7C), where even small amounts of contaminating hemoglobin would obliterate the PCR process. These results demonstrate, in no uncertain terms, that potentially interfering protein components are removed and that sufficient DNA is eluted for PCR amplification. If hemoglobin, or other potentially interfering components (22–24), is present in the eluted fractions containing the DNA, these components must be present at concentrations low enough to render them ineffective with respect to DNA amplification.

As a final test of the robust and flexible nature of the μSPE process, the ability to successfully extract DNA from cultured prostate and breast cancer cells was evaluated. With these experiments, prostate and breast cancer cells in suspension (~4000 cells) were individually mixed with the loading buffer (6 M GuHCl/1% Triton X-100) and μSPE executed. The electropherograms in Fig. 7 illustrate the results of multiplex PCR amplification following μSPE of DNA from DU-145 prostate cancer and the MCF-7 breast cancer cells (Figs. 7D and 7E). The intensities of the PCR products are high, providing no evidence for partial inhibition of the PCR process. All were comparable to the amplification observed with prepurified genomic DNA (Fig. 7F).

Perhaps most importantly, our results show that DNA can be purified from various biological matrices in a very simple procedure with a resultant DNA quality that is suitable for a number of downstream applications. The high quality of the extracted DNA is a prerequisite for the successful combination of the extraction method with other relevant analysis steps in, for example, microfabricated devices or in high-throughput protocols.

CONCLUSIONS

The purpose of this study was to investigate the usefulness of a miniaturized solid-phase extraction method based on silica resins for the isolation of DNA in microliter volumes. Despite the fact that the quantitative recovery of DNA from a resin-based extraction device with microliter volumes is difficult, the simplicity of the procedure makes it ideal for a miniaturized DNA purification system. The system is operational with microliter volumes of three solutions that are easily pumped through the microdevice under low pressure. The μSPE DNA purification procedure is complete in several minutes (<10 min), making it much faster and simpler than the conventional methods, as well as allowing it to match the time frame of other miniaturized methods. Kilobase-pair-sized DNA fragments are efficiently retained and eluted under the optimized buffer conditions described and the DNA

recovered in the eluate is directly applicable to amplification by PCR. Prepurified DNA fractions are recovered with the efficiency greater than 80%, making the technique a method of choice for integrated purification and desalting steps in miniaturized procedures. A μSPE device exhibiting a large surface-area to volume ratio can be assembled from porous silica resins, but a device packed with very small particles is associated with poor reproducibility. Fully hydrated Type A silica resins should provide a better solid phase for DNA binding than Type B silica or other silica-type surfaces. Using a 500-nl silica bed volume, roughly 70% of the DNA from a WBC lysate can be recovered and over 80% protein can be removed. This can be improved with further optimization. With a binding capacity that is clearly adequate in the presence of a large excess of protein, PCR-suitable DNA can be obtained from white blood cells, cultured blood cells, and whole blood by μSPE. We have provided evidence that this miniaturized DNA purification is well suited for extracting DNA from various biological matrices, a prerequisite for the integration of μSPE into a microchip for clinical diagnostics.

ACKNOWLEDGMENTS

The authors acknowledge the grant support for this project from the National Cancer Institute (Grants AR-07591 and 1 R21 CA78865-01). The authors also express their gratitude to Professor Michael Hopkins in the Department of Chemistry (University of Chicago) for assistance in fluorescence-based assays; Dr. Saijun Fan (Long Island Jewish Medical Center, Albert Einstein College of Medicine, New York, NY) for providing the cancer cells; Rick Nestler (Molecular Diagnostics Laboratory, University of Pittsburgh Medical Center) for purifying blood samples; and Dr. Jefferey A. Kant and Dr. Jana Huschenbett-Hühmer for providing blood samples, PCR primer information, and thermocycling information as well as advice and encouragement on this project.

REFERENCES

1. Kheterpal, I., and Mathies, R. A. (1999) *Anal. Chem.* **71,** 31A–37A.
2. Gao, Q., Pang, H. M., and Yeung, E. S. (1999) *Electrophoresis* **20,** 1518–1526.
3. Wang, Y., Ju, J., Carpenter, J. M. A., Sensabaugh, G. F., and Mathies, R. A. (1995) *Anal. Chem.* **67,** 1197–1203.
4. Wang, Y., Wallin, J. M., Ju, J., Sensabaugh, G. F., and Mathies, R. A. (1996) *Electrophoresis* **17,** 1485–1490.
5. Hofgärtner, W. T., Hühmer, A. F. R., Landers, J. P., and Kant, J. A. (1999) *Clin. Chem.* **45,** 2120–2128.
6. Woolley, A. T., Sensabaugh, G. F., and Mathies, R. A. (1997) *Anal. Chem.* **69,** 2181–2186.
7. Liu, S., Shi, Y., Ja, W. W., and Mathies, R. A. (1999) *Anal. Chem.* **71,** 566–573.
8. Shi, Y., Simpson, P. C., Scherer, J. R., Wexler, D., Skibola, C., Smith, M. T., and Mathies, R. A. (1999) *Anal. Chem.* **71,** 5354–5361.
9. Simpson, P. C., Roach, D., Woolley, A. T., Thorsen, T., Johnston, R., Sensabaugh, G. F., and Mathies, R. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2256–2261.
10. Munro, N. J., Snow, K., Kant, J. A., and Landers, J. P. (1999) *Clin. Chem.* **45,** 1906–1917.
11. Tian, H., Jaquins-Gerstl, A., Munro, N. J., Trucco, M., Brody, L. C., and Landers, J. P. (2000) *Genomics* **63,** 25–34.
12. Tian, H., Brody, L. C., Munro, N. J., and Landers, J. P. (2000) Rapid detection of deletion, insertion and substitution mutations via heteroduplex analysis using capillary- and microchip-based electrophoresis, In press.
13. Elles, R. (1996) Molecular Diagnosis of Genetic Disease, pp 169–183, Humana Press, Totowa, NJ.
14. Oda, R. P., Strausbauch, M. A., Hühmer, A. F. R., Borson, N., Jurrens, S. R., Craighead, J., Wettstein, P. J., Eckloff, B., Kline, B., and Landers, J. P. (1998) *Anal. Chem.* **70,** 4361–4368.
15. Hühmer, A. F. R., and Landers, J. P. (2000) Submitted.
16. Woolley, A. T., Hadley, D., Landre, P., deMello, A. J., Mathies, R. A., and Northrup, M. A. (1996) *Anal. Chem.* **68,** 4081–4086.
17. Waters, L. C., Jacobson, S. C., Kroutchinina, N., Khandurinna, J., Foote, R. S., and Ramsey, J. M. (1998) *Anal. Chem.* **70,** 158–162.
18. Cheng, J., Waters, L. C., Fortina, P., Hvichia, G., Jacobson, S. C., Ramsey, J. M., Kricka, L. J., and Wilding, P. (1998) *Anal. Biochem.* **257,** 101–106.
19. Shoffner, M. A., Cheng, J., Hvichia, G. E., Kricka, L. J., and Wilding, P. (1996) *Nucleic Acids Res.* **24,** 375–379.
20. Cheng, J., Shoffner, M. A., Hvichia, G. E., Kricka, L. J., and Wilding, P. (1996) *Nucleic Acids Res.* **24,** 380–385.
21. Wilding, P., Kricka, L. J., Cheng, J., Hvichia, G., Shoffner, M. A., and Fortina, P. (1998) *Anal. Biochem.* **257,** 95–100.
22. Ochert, A. S., Slomka, M., Ellis, J., and Teo, C. (1994) *in* Methods in DNA Amplification (Rolfs, A., Weber-Rolfs, I., and Finckh, U., Eds.), pp. 47–53, Plenum, New York.
23. Rolfs, A., Schuller, I., Finckh, U., and Weber-Rolfs, I. (1992) PCR: Clinical Diagnostics and Research, pp. 1–18, pp. 51–58, pp. 79–88, Springer-Verlag, Berlin, Germany.
24. Hämäläinen, M. M., Eskola, J. U., Hellman, J., and Pulkki, K. (1999) *Clin. Chem.* **54,** 465–471.
25. Elles, R. (1996) Molecular Diagnosis of Genetic Diseases, pp. 1–98, Humana Press, Totowa, NJ.
26. Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-van Dillen, P. M. E., and van der Noordaa, J. (1990) *J. Clin. Microbiol.* **28,** 495–503.
27. Vogelstein, B., and Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 615–619.
28. Marko, M. A., Chipperfield, R., and Birnboim, H. C. (1982) *Anal. Biochem.* **121,** 382–387.
29. Matitashvili, E., and Zavizion, B. (1997) *Anal. Biochem.* **246,** 260–262.
30. Walsh, P. S., Metzger, D. A., and Higuchi, R. (1991) *BioTechniques* **10,** 506–513.
31. Wang, K., Gan, L., Boysen, C., and Hood, L. (1995) *Anal. Biochem.* **226,** 85–90.
32. Seeger, C., Batz, H. G., and Ørum, H. (1997) *BioTechniques* **23,** 512–517.
33. Gelsthorpe, A. R., Gelsthorope, K., and Sokol, R. J. (1997) *BioTechniques* **22,** 1080–1082.
34. Rudi, K., Kroken, M., Dahlberg, O. J., Deggerdal, A., Jakobsen, K. S., and Larsen, F. (1997) *BioTechniques* **22,** 506–511.
35. Deggerdal, A., and Larsen, F. (1997) *BioTechniques* **22,** 554–557.

36. Strausbauch, M. A., Madden, B. J., Wettstein, P. J., and Landers, J. P. (1995) *Electrophoresis* **16,** 541–548.
37. Strausbauch, M. A., Xu, S., Ferguson, J. E., Nunez, M. E., Machacek, D., Lawson, G. M., Wettstein, P. J., and Landers, J. P. (1995) *J. Chromatogr.* **717,** 279–291.
38. Strausbauch, M. A., Landers, J. P., and Wettstein, P. J. (1996) *Anal. Chem.* **68,** 306–314.
39. Dong, M., Oda, R. P., Strausbauch, M. A., Wettstein, P. J., and Landers, J. P. (1997) *Electrophoresis* **18,** 1767–1774.
40. Roche, M. E., Anderson, M. A., Oda, R. P., Riggs, B. L., Strausbauch, M. A., Okazaki, R., Wettstein, P. J., and Landers, J. P. (1998) *Anal. Biochem.* **258,** 87–95.
41. Fan, S., Wang, J. A., Yuan, R. Q., Ma, Y. X., Meng, Q., Erdos, M. R., Brody, L. C., Goldberg, I. D., and Rosen, E. M. (1998) *Oncogene* **16,** 3069–3082.
42. Singer, V. L., Jones, L. J., Yue, S. T., and Haugland, R. P. (1997) *Anal. Biochem.* **249,** 228–238.
43. Liu, J. P., Hsieh, Y. Z., Wiesler, D., and Novotny, M. (1991) *Anal. Chem.* **63,** 408–412.
44. Liu, J. P., Shirota, O., and Novotny, M. (1991) *Anal. Chem.* **63,** 413–417.
45. Buck, G. E., O'Hara, L. C., and Summergill, J. T. (1992) *J. Clin. Microbiol.* **30,** 1331–1334.
46. Poncin, J., Mulkens, J., Arends, J. W., and de Goeij, A. (1999) *Diagn. Mol. Pathol.* **8,** 11–19.
47. Product information of Promega Wizard PCR Preps DNA Purification System.
48. Melzak, K. A., Sherwood, C. S., Turner, R. F. B., and Haynes, C. A. (1996) *J. Colloid Interface Sci.* **181,** 635–644.
49. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
50. Information on the web site: //www.qiagen.com/catalog/chapter_13/chap13b1.asp (1999).
51. Dolan, J. W. (1998) *LC–GC* **16,** 350–354.
52. Unger, K., and Kern, R. (1976) *J. Chromatogr.* **122,** 345–354.
53. Poole, C. F., and Poole, S. K. (1991) Chromatography Today, pp. 311–325, Elsevier Science B.V., New York.
54. Forgacs, E., and Cserhati, T. (1997) Molecular Basis of Chromatographic Separation, pp. 116–132, CRC Press, Boca Raton, FL.