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Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips

(microchemical analysis/microfabrication/confocal fluorescence detection/allelic fragment sizing/DNA sequencing)

ADAM T. WOOLLEY AND RICHARD A. MATHIES*

Department of Chemistry, University of California, Berkeley, CA 94720

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ABSTRACT **Capillary electrophoresis arrays have been fabricated on planar glass substrates by photolithographic masking and chemical etching techniques. The photolithographically defined channel patterns were etched in a glass substrate, and then capillaries were formed by thermally bonding the etched substrate to a second glass slide. High-resolution electrophoretic separations of ϕX174 *Hae* III DNA restriction fragments have been performed with these chips using a hydroxyethyl cellulose sieving matrix in the channels. DNA fragments were fluorescently labeled with dye in the running buffer and detected with a laser-excited, confocal fluorescence system. The effects of variations in the electric field, procedures for injection, and sizes of separation and injection channels (ranging from 30 to 120 μm) have been explored. By use of channels with an effective length of only 3.5 cm, separations of ϕX174 *Hae* III DNA fragments from $\approx$70 to 1000 bp are complete in only 120 sec. We have also demonstrated high-speed sizing of PCR-amplified HLA-DQα alleles. This work establishes methods for high-speed, high-throughput DNA separations on capillary array electrophoresis chips.**

Capillary electrophoresis (CE) is a powerful method for DNA sequencing, forensic analysis, PCR product analysis, and restriction fragment sizing (1, 2). CE provides faster and higher-resolution separations than slab gel electrophoresis because higher electric fields can be applied. However, unlike slab gel electrophoresis, conventional CE allows analysis of only one sample at a time. Mathies and Huang (3) have introduced capillary array electrophoresis, in which separations are performed on an array of parallel silica capillaries, and demonstrated that it can be used to perform high-speed, high-throughput DNA sequencing (4, 5) and DNA fragment sizing (6). This method combines the fast electrophoresis times of CE with the ability to analyze multiple samples in parallel. The underlying concept behind the approach was to increase the information density in electrophoresis by miniaturizing the "lane" dimension to $\approx$100 μm. The further miniaturization of electrophoretic separations to increase the number of lanes, the speed, and the throughput would be valuable in helping to meet the needs of the Human Genome Project (7, 8).

The electronics industry routinely uses microfabrication to make circuits with features < 1 μm in size. Microfabrication would allow the production of higher density capillary arrays, whose current density is limited by the capillary outside diameter (4–6). In addition, microfabrication of capillaries on a chip should make it feasible to produce physical assemblies not possible with glass fibers and to link capillaries directly to other devices on the chip. However, few devices for chemical separations have been made by microfabrication technology. A gas chromatograph (9) and a liquid chromatograph (10) have been fabricated on silicon chips, but these devices have not been widely used. Recently, several groups have fabricated individual CE devices on chips and performed capillary zone electrophoresis separations of fluorescent dyes (11, 12) and fluorescently labeled amino acids (13–15). However, it is not known whether high-resolution separations of DNA can be performed with these devices or whether multiple separation channels can be fabricated in a single chip.

We were therefore interested in microfabricating CE channels on planar glass substrates and exploring their use for DNA separations. We show here that photolithography and chemical etching can be used to make large numbers of CE separation channels on glass substrates. Procedures have been developed to fill these channels with hydroxyethyl cellulose (HEC) separation matrices, and we have been able to separate DNA restriction fragment digests on these chips in <2 min with excellent resolution. We have also characterized the injection techniques, the dependence of the separation on channel geometry, and the reproducibility of separations. The demonstration that high-speed DNA separations can be performed on microfabricated CE channel arrays establishes the feasibility of integrated devices for electrophoretic DNA analysis.

MATERIALS AND METHODS

Electrophoresis Chip Fabrication. Electrophoresis chips were made by bonding a chemically etched glass bottom substrate to a drilled glass top substrate to form capillaries. The etched pieces were produced by coating a glass substrate with a photoresist film and then transferring the channel pattern to the film by exposure to UV radiation through a patterning mask. The exposed portions of the film were dissolved, and the remaining film was hardened by heating. The exposed glass was chemically etched, and then the etched substrate was thermally bonded to the top glass plate, which had access holes drilled in it.

Fig. 1*A* shows the dimensions and layout of the separation chips. Fifteen CE devices were fabricated on each chip with all possible combinations of 30-, 50-, and 70-μm-wide separation channels and 30-, 70-, and 120-μm cross channels. The separation channels connect reservoirs 2 and 4, while the cross channels connect reservoirs 1 and 3. Precleaned microscope slides (75 × 50 × 1 mm, catalogue no. 12-550C; Fisher Scientific) made of soda lime glass were used for the top and bottom pieces. Four rows of 15 access holes 0.8 mm in diameter were drilled in the top pieces with a diamond-core drill. The glass pieces were first cleaned by spraying with H_2O, submerging in a bath of hot H_2SO_4/H_2O_2 for 10 min, and then thoroughly rinsing with H_2O. The bottom pieces were dried in a furnace at 150°C for 10 min, exposed to hexameth-

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Abbreviations: CE, capillary electrophoresis; HEC, hydroxyethyl cellulose; TO, thiazole orange; TO6, (*N,N'*-tetramethylpropanediamino)propylthiazole orange.

*To whom reprint requests should be addressed.

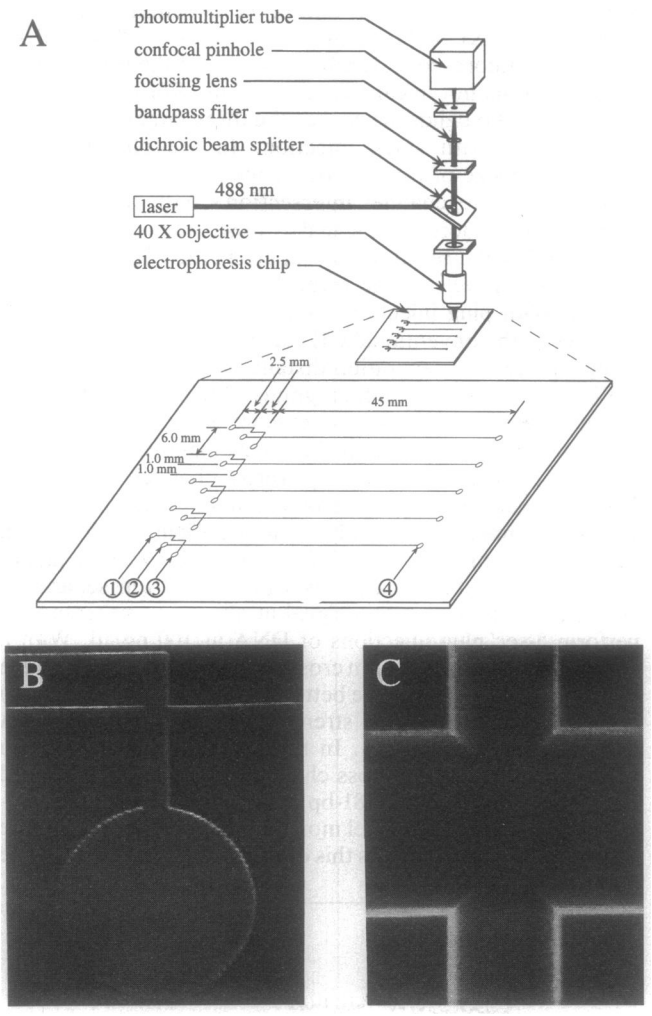

FIG. 1. (*A*) Schematic of the CE chip and the laser-excited, confocal fluorescence detection system. The size of the features in the channel intersection area is exaggerated, and only every third channel on the chip is shown. (*B*) Low-magnification (×25) electron micrograph of a 70-μm separation channel intersected by a 120-μm cross channel and a buffer reservoir (type 3). (*C*) High-magnification (×500) electron micrograph of the intersection of a 50-μm separation channel with a 30-μm cross channel.

yldisilazane vapor for 5 min, coated with a layer of Microposit S1400-31 positive photoresist (Shipley, Newton, MA) on a Headway photoresist spinner (6000 rpm), and then soft baked at 90°C for 25 min.

The photomask was designed with the computer-assisted design system KIC on a Sun SPARC 1 workstation and fabricated by Berkeley Microfabrication Laboratory staff with a GCA 3600F pattern generator and an APT chrome mask developer. The mask pattern was transferred to the substrate by exposing the photoresist to UV radiation through the mask in a Kasper contact mask aligner. The photoresist was developed in Microposit developer concentrate (Shipley)/H_2O, 1:1. The substrate was hard baked at 150°C for 60 min and then etched for 15 min in a 1:1 mixture of two aqueous NH_4F/HF etchants (BOE 5:1 and BOE 10:1, J. T. Baker, Phillipsburg, NJ). Etch depth was profiled with an Alphastep profilometer (Tencor, Mountain View, CA) and was controlled by monitoring the etch time.

After the bottom piece was etched, the film of photoresist was removed by immersing the slide in a mixture of hot H_2SO_4/H_2O_2 for 10 min. Prior to thermal bonding, the drilled top slide and etched bottom slide were again submerged in hot H_2SO_4/H_2O_2 for 10 min, rinsed thoroughly with H_2O, dried with N_2 gas, and then aligned. The slides were thermally bonded in a model 6-525 programmable furnace (J. M. Ney Co., Yucaipa, CA) using the following temperature program: ramp 5°C/min to 500°C and hold for 30 min, ramp 5°C/min to 550°C and hold for 30 min, ramp 5°C/min to 600°C and hold for 2 hr, ramp −5°C/min to 550°C and hold for 1 hr, ramp −5°C/min to 500°C and hold for 30 min, and finally, cool to room temperature.

Electrophoresis Procedures. Channel surfaces were coated by a modified version of the Hjerten coating protocol (16). Surfaces were derivatized by pumping a 0.4% (vol/vol) solution of γ-methacryloxypropyltrimethoxysilane in H_2O (pH adjusted to 3.5 with acetic acid) through the channels for 1 hr, rinsing with H_2O, allowing an aqueous solution of 4% (wt/vol) acrylamide to polymerize for 5 min to coat the channel surfaces, and then rinsing with H_2O. Following refs. 6 and 17, the separation matrix consisted of TAE buffer (40 mM Tris/40 mM acetate/1 mM EDTA, pH 8.2), 0.75% (wt/vol) HEC (M_n, 438,000; Aquilon, Hopewell, VA), and either 1 μM thiazole orange (TO) or 0.1 μM (*N,N'*-tetramethylpropanediamino)propylthiazole orange (TO6, ref. 18). HEC was added to TAE buffer and stirred overnight at room temperature. The dye was added to the HEC buffer, which was degassed under vacuum for 20 min, centrifuged in a tabletop centrifuge for 30 min, aliquoted, and then centrifuged for 5 min (12,000 rpm) in a microcentrifuge. Buffer reservoirs were formed by inserting micropipette tips into the drilled holes; electrical contact was made by inserting small Pt wires into the buffer reservoirs.

DNA restriction digest samples (ϕX174 *Hae* III fragments; New England Biolabs) were diluted in 1 mM Tris/0.1 mM EDTA, pH 8.2. PCR amplification was done on a hypervariable region in the second exon of the HLA-DQα locus (19) that can be encompassed by a single 242-bp PCR amplification fragment (20). A DNA sample (HLA-DQα genotype 1.2/3) was PCR-amplified with an AmpliType HLA-DQα forensic DNA amplification and typing kit (Perkin–Elmer) and provided by George Sensabaugh of the School of Public Health, University of California, Berkeley. The sample was precipitated with ethanol and then resuspended in 1 mM Tris/0.1 mM EDTA, pH 8.2, prior to injection.

The sieving matrix was vacuumed into the separation channel via reservoir 4. The cross channel, and the separation channel between reservoir 2 and the cross channel, was filled with TAE buffer lacking HEC. The channels were preelectrophoresed for 10 min at 180 V/cm. Samples were introduced into the cross channel by rinsing and filling reservoir 3 first with 1 mM Tris/0.1 mM EDTA, pH 8.2; applying vacuum to reservoir 1; rinsing and filling reservoir 3 with sample; and then applying vacuum to reservoir 1. Samples were injected by either a "stack" (21) or a "plug" (Fig. 2) injection method. The stack injection involved applying a field of 180 V/cm between reservoirs 3 and 4, with reservoir 3 at ground and reservoirs 1 and 2 floating. For the plug injections, a field of 170 V/cm was applied between reservoirs 1 and 3, with reservoir 3 at ground and reservoirs 2 and 4 floating. Electrophoresis was at 180 V/cm, except where otherwise noted.

Fluorescence Detection Apparatus. The detection apparatus was similar to that described earlier (6, 17). An excitation beam (1 mW, 488 nm) from an air-cooled Ar ion laser was passed into a confocal microscope (Axioplan, Zeiss) and reflected with a dichroic beam splitter (FT 510, Carl Zeiss) to a 40 × 0.60 n.a. objective (LD Epiplan, Carl Zeiss), which focused the beam to an ≈10-μm spot within the channel, ≈3.5 cm from the intersection of the separation channel with the injection channel. Fluorescence was collected by the objective, passed through the dichroic beam splitter, filtered by a bandpass filter (530DF30, Omega Optical, Brattleboro, VT), and focused on a 400-μm confocal pinhole followed by

photomultiplier detection. Photoelectron pulses were amplified and discriminated by a photon counter (model 1106, Princeton Applied Research) and counted with a PCA II data acquisition card (The Nucleus, Oak Ridge, TN) in a 486 personal computer.

RESULTS

To characterize the capillary electrophoresis chips, electron micrographs of the channel features were obtained prior to bonding. Fig. 1*B* shows a low-magnification electron micrograph of the intersection of a 70-μm separation channel with a 120-μm cross channel, as well as an injection reservoir. Fig. 1*C* shows a higher-magnification electron micrograph of the intersection of a 50-μm separation channel with a 30-μm cross channel. The sloping sidewalls and flat bottoms of the channels, as well as the quality of the etch, can be seen clearly. Channel depth was 8 μm for a 15-min etch time; channel top widths were measured as 27, 47, 66, and 118 μm for mask line widths of 10, 30, 50, and 100 μm, respectively. With etch times of 15 min, features of this size were made reliably and uniformly. Deeper, 16-μm etches were obtained by increasing the etch time to 30 min, but with nonuniform undercutting of the photoresist.

The development of a protocol for reliable injection of samples is critical. The electropherograms in Fig. 2 compare stack and plug injections using a φX174 *Hae* III DNA sizing standard. In the stack injection, DNA is continuously stacked into the separation channel during the injection time. Separation of the φX174 *Hae* III bands occurs in <120 sec at 180 V/cm. The fluorescent signal is strong, but the resolution is not as good as in typical CE separations. For example, the 271- and 281-bp bands, as well as the three largest bands, are not resolved with stack injection. In the plug injection method, the size of the injection zone is determined by the geometry of the channel intersection. With a 1-sec plug injection, the fluorescent signal is lower than for the stack injection, but the resolution is superior; the 271- and 281-bp fragments, as well as the three largest fragments, are resolved. With plug injections, separations as good as those obtained with conventional CE can be completed in <2 min, using an effective separation distance of only 3.5 cm!

Fig. 3 presents electropherograms obtained with four different channel geometries to explore their effects on the injection and separation. In the electropherogram obtained with a 30-μm separation and 30-μm cross channel, the 271- and 281-bp fragments are not resolved, nor are the 1078- and 1353-bp fragments. Generally, it was difficult to fill 30-μm separation channels with the HEC solution by vacuum and to obtain reproducible separations with any 30-μm separation channels. Thus, to see any signal at all, it was necessary to perform 5-sec plug injections of DNA at 100 ng/μl. With a 50-μm separation and 30-μm cross channel, all fragments were observed and resolved. The better performance allowed us to achieve satisfactory signal strength with 10 times less DNA and only a 1-sec injection. In the separation with a 50-μm separation and 120-μm cross channel, all peaks are resolved except for the 271- and 281-bp fragments. Separations performed with a cross channel more than twice the width of the separation channel (such as this one) did not give reproducible

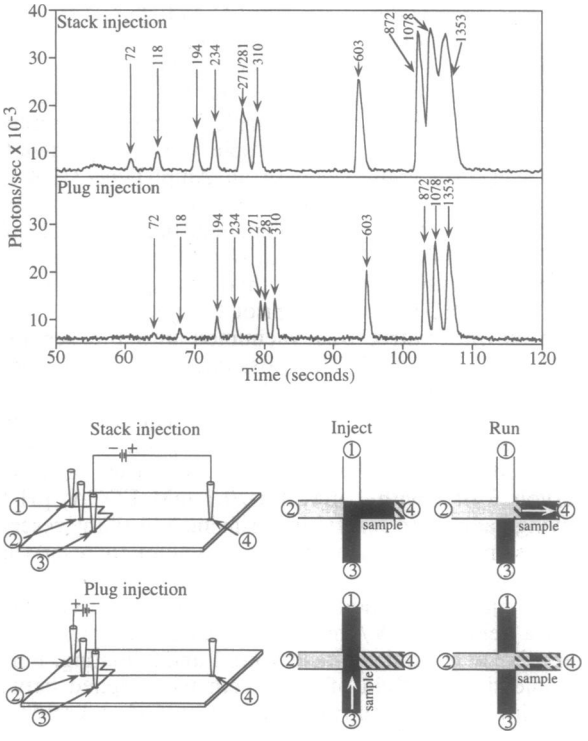

FIG. 2. (*Top*) Electropherograms comparing the stack and plug injection methods. A sample containing φX174 *Hae* III fragments at 10 ng/μl was injected for 1 sec in each experiment. The buffer consisted of the standard TAE/HEC sieving medium with 1 μM TO. These experiments were performed with a 50-μm separation channel and a 30-μm cross channel. (*Middle*) Schematic diagram of stack injection method. (*Bottom*) Schematic diagram of plug injection method. The diagonal lines indicate the HEC in the separation channel. A signal of 8000 photons per second over background corresponds to 100 pg of DNA per μl in the separation channel. [Our on-column detection limit (1000 photons per second over background) is ≈2 fg of DNA for a typical band (1-sec width, 100-sec migration time), corresponding to a sample limit of detection of 100 pg/μl for plug injections and 50 pg/μl for stack injections.]

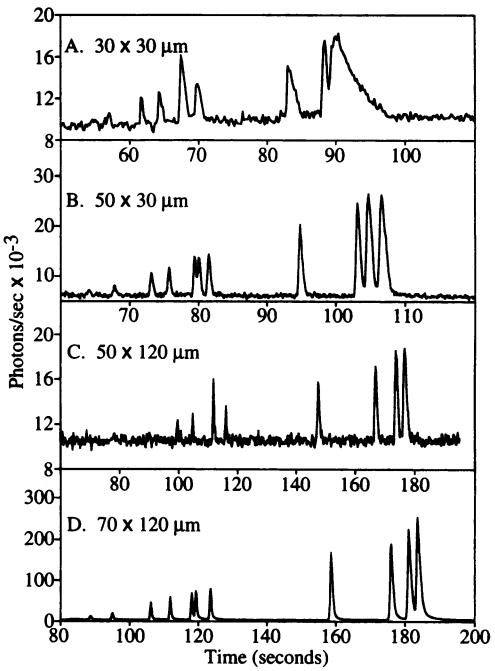

FIG. 3. Electropherograms obtained with various separation and cross channel sizes. Samples were *Hae* III digests of φX174 phage DNA. (*A*) Separation channel, 30 μm; cross channel, 30 μm; sample (plug injected for 5 sec), 100 ng/μl; separation in the presence of 1 μM TO. (*B*) Separation channel, 50 μm; cross channel, 30 μm; sample (plug injected for 1 sec), 10 ng/μl; separation in the presence of 1 μM TO. (*C*) Separation channel, 50 μm; cross channel, 120 μm; sample (stack injected for 1 sec), 10 ng/μl; separation in the presence of 0.1 μM TO6. (*D*) Separation channel, 70 μm; cross channel, 120 μm; sample (plug injected for 1 sec), 10 ng/μl; separation in the presence of 0.1 μM TO6. Sensitivities of DNA detection with TO and TO6 are comparable at the concentrations used.

migration times, and the channel current decreased with each successive run. We attribute this to the dilution of the ionic strength and the HEC in the separation channel by the lower-ionic-strength solution in the cross channel, which produced longer separation times and lowered the efficiency of the electrokinetic injection. The bottom electropherogram, obtained with a 70-μm separation and 120-μm cross channel, has all the bands well-resolved. The high signal strength in this electropherogram can be attributed to the fact that this channel's surface was coated just prior to use, which minimized the loss of dye and DNA by adsorption to the surfaces of the channels (17).

Fig. 4 illustrates the effect of the electric field on CE chip separations. The electropherograms obtained at 100 and 140 V/cm exhibit baseline resolution of the 1078- and 1353-bp fragments, while the 180-V/cm separation exhibits nearly baseline resolution of those fragments. The resolution of the 271- and 281-bp fragments is little affected by the electric field. In summary, the resolution of the φX174 *Hae* III fragments is not affected significantly by the electric field for these field strengths, but the separation is much faster at 180 V/cm.

To examine the reproducibility and stability of the channels, multiple runs were performed on the same channel. Fig. 5 shows the first three and the last three in a series of eight consecutive separations of φX174 *Hae* III fragments. All runs were performed in a 50-μm separation channel with a 30-μm cross channel. The same HEC in the separation channel continued to separate the DNA fragments reproducibly, even after eight runs. The 271- and 281-bp fragments were partially resolved in all experiments, with the resolution improving when the sampling rate was increased from 5 Hz (runs 1–3) to 10 Hz (runs 6–8). Although the migration times and the fluorescent signal of the fragments varied slightly from run to run, the reproducibility of the separations was excellent. The small variations in mobilities (2–3% relative standard deviation) are typical for multiple capillary runs (6) and are attributed to the ionic strength and HEC dilution effects mentioned above. We have performed up to 75 separations in a channel with a single HEC filling.

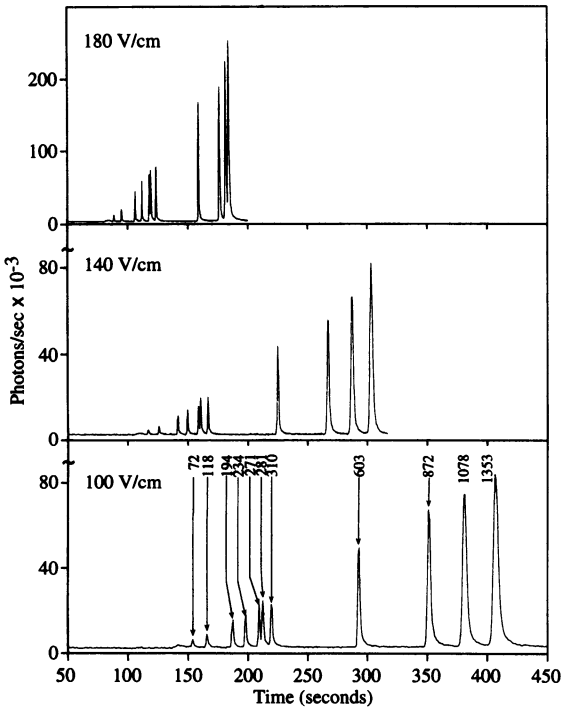

FIG. 4. Effect of electric field on CE chip separations. Samples contained φX174 *Hae* III fragments at 10 ng/μl; the running buffer consisted of the standard TAE/HEC with 0.1 μM TO6. Experiments were performed with a 70-μm separation channel and a 120-μm cross channel.

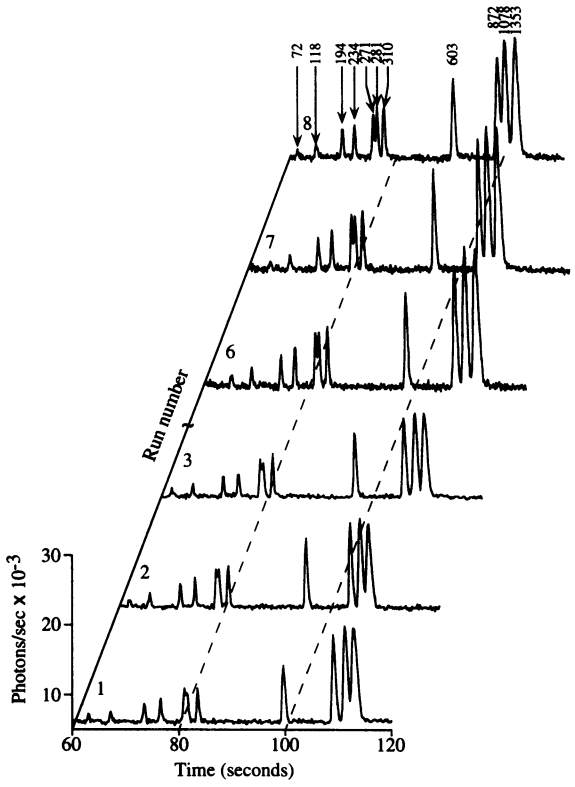

FIG. 5. The first three and the last three in a series of eight consecutive separations obtained with the same channel. The separations were performed with a 50-μm separation channel and a 30-μm cross channel, with 1.0 μM TO in the TAE/HEC running buffer; the sample was a φX174 *Hae* III digest at 10 ng/μl. Data points were collected at a sampling rate of 5 Hz for runs 1–3 and at 10 Hz for runs 6–8.

DISCUSSION

We have demonstrated that high-speed DNA fragment separations can be performed with capillary arrays microfabricated on glass chips. Electrophoresis of a restriction fragment digest on a 3.5-cm microfabricated channel exhibits resolution as good as that obtainable with fiber capillaries that are 10 times longer. Electrophoretic separations from 72 to 1353 bp are complete in only 120 sec, ≈10 times faster than with typical CE. We have also characterized two different injection methods, the effects of channel geometry and electric field, and selected conditions and parameters that lead to reliable devices. Sizing with CE chips is as fast as fluorescence burst sizing by flow cytometry (22) but is also applicable to DNA fragments much smaller than the current lower limit of ≈1000 bp with the fluorescence burst methodology.

In our analysis of the effects of channel geometry, we found it easier to fill wide (>50 μm) separation channels with the TAE/HEC sieving buffer. When the cross channel was more than about twice as wide as the separation channel, the devices generally had short usable lifetimes, irreproducible mobilities, and lower signal strengths. These effects are most likely caused by dilution of the buffer in the separation channel by the lower-ionic-strength solution in the cross channel. Wide (50 and 70 μm) separation channels combined with narrow (30 μm) cross channels gave the most reproducible separations over the longest periods of time.

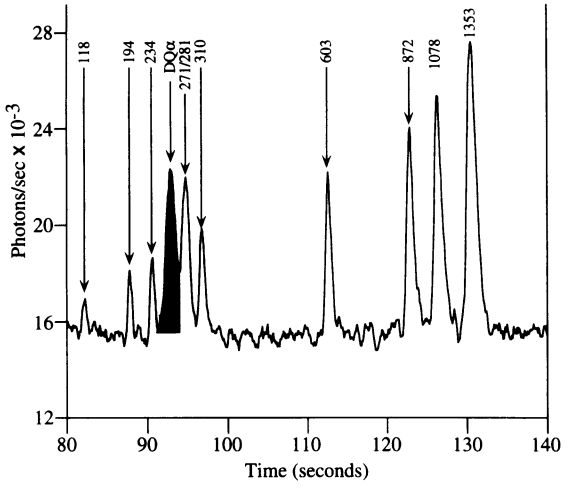

FIG. 6. High-speed sizing of PCR-amplified DNA fragments on a capillary array electrophoresis chip. The electropherogram shows the separation of a mixture of standard ϕX174 *Hae* III fragments spiked with PCR-amplified HLA-DQα DNA. The standard TAE/HEC buffer, containing 0.1 μM TO6, was used as the sieving matrix.

A comparison of the stack and the plug injection methods shows the advantages and disadvantages of each method. The stack method gives more signal than the plug injection method because more sample is electrokinetically loaded into the separation channel. With a 1-sec stack injection, the number of theoretical plates obtained for the 234-bp fragment was 6.0×10^4, corresponding to a plate height of 0.58 μm. With a 1-sec plug injection, the number of theoretical plates for the 234-bp fragment was calculated to be 1.3×10^5, corresponding to a plate height of 0.27 μm. With published values for diffusion coefficients of DNA in gels (23), the widths of the initial injection plugs were estimated (15) as 480 μm for the 1-sec stack injection and 330 μm for the 1-sec plug injection. These widths are consistent with previous work (15, 24, 25). Under our conditions, the injection plug width is the limiting factor for the number of theoretical plates. The width of the injection plug can be decreased by controlling the potentials at all the channels in the junction (26). The optimum method of sample injection, accordingly, will depend on whether signal or resolution is more important. For example, to perform chip-based separations requiring extremely high resolution, such as DNA sequencing, minimizing the length of the injection plug will be critical. Alternatively, the highest sensitivity (50-pg/μl sample limit of detection) is achieved with stack injection.

Now that high-speed DNA separations have been demonstrated on capillary array electrophoresis chips, a variety of extensions of this technique can be envisioned. It is feasible to fabricate up to $\approx$80 independent separation and loading channels on a single chip with our current channel geometry and lengths. This number is primarily limited by the placement and size of the access holes. If methods can be developed for facile loading of multiple samples, even higher densities of channels should be feasible. Capillary arrays on chips should be useful for rapid, parallel sizing of PCR products for genetic analysis and forensic identification. For example, Fig. 6 shows a separation of a mixture of a ϕX174 *Hae* III standard and a solution containing the HLA-DQα PCR product. The PCR product (shaded) was detected at about 90 sec and estimated to be 256 bp by using the ϕX174 *Hae* III fragment mobilities. This establishes the feasibility of performing rapid DNA typing of, for example, the HUMTHO1 locus, with our capillary array electrophoresis chips (27). Microfabrication should also allow the construction of integrated devices that incorporate DNA preparation, amplification, and analysis on a single chip. Coupling of our technology with recent developments in photolithographic DNA synthesis (28) and microfabricated cell analysis devices (29) should lead to powerful microchemical DNA analysis systems.

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