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Rapid, parallel separations of D1S80 alleles in a plastic microchannel chip

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Abstract

We have performed fast, parallel separations of alleles of the D1S80 locus in a plastic, multi-channel chip, replicated from a microfabricated master and laminated with a plastic film. The array of 16 channels was filled with a replaceable sieving polymer, and a size-dependent, electrophoretic separation of the DNA fragments was performed in all channels in less than 10 min, representing a 30-fold increase in throughput compared to that on a single-capillary instrument. To detect the fragments in all 16 channels in parallel during the run, we designed and built a scanning, confocal, laser-induced fluorescence system. The electropherograms were then used to determine the sample genotype. To demonstrate the use of multiplexed, microchannel arrays for real-life samples, we amplified D1S80 alleles from genomic DNA extracted from whole blood and separated these alleles by electrophoresis in the plastic chip. Evaluation of the electrophoretic data showed that, using a 300- and a 1000-base pair fragment as internal mobility markers, 83% of the alleles were assigned correctly, using the allele identification from a single capillary instrument as a reference. This work demonstrates that, with improvements in the microchannel electrophoresis system, it is feasible to perform rapid, parallel genotyping in mass-produced, inexpensive, disposable plastic devices for large-scale applications in medicine and the life sciences.

Keywords: Microchips; Microchannels; Instrumentation; DNA; D1S80 alleles

1. Introduction

The completion of the Human Genome Project (HGP) early in this millennium will provide a wealth of genetic information and is expected to cause paradigm shifts in the life sciences [1]. For example, the identification of all encoded proteins will advance the detailed knowledge of biochemical pathways, which will in turn further rationalize drug design. In addition, the availability of a human sequence information will allow determination of genotypes and markers such as single nucleotide polymorphism (SNPs), which are believed to occur in humans at a 1% level. Through understanding how a particular genotype interacts with a potential drug, the development of safer, and more effective, individualized medicine is conceivable. In order to administer a personalized drug, a detailed genetic profile of the individual must be obtained. Therefore, the development of personalized medicine will increase dramatically the demand for genetic tests. However, current methodologies, based largely on slab gel electrophoresis and to a smaller percentage on capillary electrophoresis (CE), are too cumbersome and labor intensive to be cost effective for large-scale genetic testing. The projected increase in

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genetic testing calls for alternative, high-throughput, cost-effective analytical procedures.

The development of integrated, multiplexed, miniaturized chemical analysis systems has received considerable attention for the past decade. This concept has been termed μ-TAS (micro-total analysis system) or ''lab-on-a-chip'' [2,3], and takes advantage of the integration of sample processing steps such as metering aliquots, mixing, reacting, separating and detecting in a miniaturized format. The μ-TAS format most often used is an interconnected channel structure on a planar substrate with electro-driven movement of fluids and/or sample components [4–6].

The inherent miniaturization of chip-based systems is of particular advantage for biochemical analysis. The small size of the devices, which are typically manufactured using 4-in. wafer technology, allows extremely rapid analysis (1 in.=2.54 cm). Because the effective separation length in electrophoretic separations is millimeters to a few centimeters, rather than at least 20 cm in commercial CE instrumentation or slab gels, the separation time is reduced to between seconds and a few minutes. For example, DNA fragment separations can be done in less than 60 s in a chip, whereas the conventional slab gel electrophoresis requires 30 min or longer [7]. Furthermore, the reduction in volume of the sample to low nanoliter volumes enables economical usage of expensive reagents. The small size and almost immediate response time of the planar chips makes them also suitable for portable chemical analysis systems, which could be used for environmental monitoring, space biology, clinical monitoring, or diagnostic point-of-care applications.

In order to meet the anticipated high throughput requirements for genetic analyses, improvements in speed will not be sufficient. In addition, multiplexing will be necessary. In CE, the introduction of a 96 capillary array in sequencing instrumentation [8,9] resulted in an order of magnitude higher throughput per instrument compared to traditional slab autosequencers, but posed considerable technical challenges with respect to the alignment of a capillary array. Overcoming these challenges added to the overall cost of the instrument. For genotyping, reproducibility from capillary to capillary is a concern because small variations in capillary diameter, effective capillary length (due to the alignment of the detection system) and surface properties could result in differences in migration time. It is also critical to design and manufacture a high-sensitivity detection system which operates sufficiently rapidly to detect in all channels simultaneously. Recently, Mathies and co-workers have shown parallel genotyping of up to 96 samples in a common device [10–12].

To manufacture the channel structures, fabrication methodology developed in the semiconductor industry is adapted to materials suitable for the application of high electric fields such as glass and a number of plastic materials [13]. In short, the microfabrication process begins with the design of a photomask with the desired channel structure and the the transfer of this pattern by illumination through the photomask to silicon or glass covered with a photoresist. Subsequently, chemical etching forms channels typically 10 to 50 μm deep and 10 to 150 μm wide [3].

At this time, the vast majority of μ-TAS applications have been demonstrated in planar glass devices. However, glass microdevices are costly because of the cost of high-quality glass substrates for microfabrication and the extensive labor and time required to bond and drill access holes. Therefore, to make the usage of glass microdevices economical, glass chips must be re-used and run multiple times. However, especially for clinical diagnostic purposes, a single-use device is preferred, because it will eliminate cross-contamination errors, improve assay throughput and reduce analysis cost. This cost reduction is a prerequisite for high-throughput applications such as population screening.

Because inexpensive reproduction methods, such as injection molding and hot embossing, are available for plastic materials, replicated plastic devices can be used to perform analyses as opposed to a directly microfabricated device [14–16]. Polymer resins and films also tend to be less expensive on a raw material basis compared to glass substrates. Depending on the type of polymer, continuous processing methods can replicate devices rapidly and at low cost-per-part in high volume. For example, injection molding produces plastic replicas of a master at an estimated 30 s/part under automated manufacturing conditions. Manufacturing yields and device reproducibility are also expected to be significantly higher for plastic materials than for glass.

Depending on the application, treatment of the channel surface might not be necessary for plastic devices, as opposed to glass devices. For example, the size-based separation of DNA fragments in a sieving polymer requires a surface with low electroosmotic flow (EOF). Glass surfaces are charged and typically need to be treated to reduce the EOF, whereas more hydrophobic and neutral plastic materials have been used for high-resolution DNA separations without modification of the surface [15,16].

Genotyping, in particular, large-scale genotyping, is a potential application for high-throughput analysis systems [10,11,17,18] Large-scale genotyping becomes practical only when typing is rapid and inexpensive. Several authors have already demonstrated rapid and/or parallel genotyping in glass electrophoresis chips [5,12,19–21]. Plastic, multichannel electrophoresis chips seem well suited to large-scale genotyping projects because DNA separations can be performed rapidly at high throughput in a multiplexed fashion and because the plastic devices can be manufactured inexpensively.

To demonstrate the feasibility of parallel genotyping in a plastic, replicated chip, we have typed the D1S80 alleles amplified from genomic DNA extracted from samples of whole blood [22–24]. Because the locus contains a large number of possible alleles, this locus has been well studied and is attractive for forensic casework, such as identity testing. The genotype can be determined by amplifying the alleles present in a sample, separating the amplified fragments by electrophoresis, and comparing the separation against that of a standard, typically an artificially constructed ladder containing common alleles. The D1S80 locus contains a variable number of tandem repeats (VNTR) with a 16-base pair (bp) repeat unit. More than 22 different alleles have been reported, having from 14 to more than 41 repeat units. The alleles range from 369 bp (allele 14) to more than 801 bp (allele 41). Therefore, the fragments differ in length by approximately 4% or less.

The separation of DNA fragments of similar length is only the first step in genotyping. A routine method must produce data that allows unambiguous assignment of the alleles with high accuracy. The accuracy of a CE procedure must be equivalent or superior to that of conventional slab electrophoresis, which can rely on visual inspection and comparison with a standard in an adjacent lane. Unlike slab gel electrophoresis, CE methods rely solely on migration times of the individual fragments. In CE methods using commercial, single-capillary electrophoresis instruments and sequential analysis of the samples, run-to-run variations in the migration times are significant enough to affect correct assignment.

Therefore, several authors have evaluated various methods to unambiguously assign the alleles of a sample. Unfortunately, a simple interpolation of migration times over the desired DNA fragment size range does not work because the migration behavior of the alleles is not linear over the specified size range. However, by spiking the alleles of a sample with a set of DNA size standards, Isenberg et al. have shown that CE can be used to reliably assign the alleles of the D1S80 locus [23]. Mitchell and Walsh reported recently 100% agreement between types determined by CE and by slab electrophoresis for over 79 samples [24].

We approached this work in three stages. In the first stage, we optimized the size separation conditions for the D1S80 allelic ladder using a replaceable sieving matrix in a commercial CE system for the purpose of serving as a reference method to type samples. Then, genomic DNA was isolated from samples of whole blood obtained from a blood center, the D1S80 alleles were amplified from each sample, and the sets of amplified alleles were separated sequentially in a single capillary instrument. The separations obtained were used to type the D1S80 locus for each sample. In the third stage, we applied the method developed in the second stage to multiple channels.

2. Experimental

2.1. Materials

Tris–borate–EDTA (TBE) buffer concentrate and ammonium persulfate were obtained from Amresco (Sohon, OH, USA), thiazole orange (TO) from Aldrich (Milwaukee, WI, USA), ethidium bromide (EtBr) from Gibco/BRL/Life Sciences (Rockville, MD, USA), and 5-carboxyfluorescein (5-FAM) from Molecular Probes (Eugene, OR, USA). The fluores-

cein-labeled 100-bp DNA ladder was purchased from Bio-Rad (Hercules, CA, USA), and the AmpliFLP D1S80 Allelic Ladder from PE Biosystems (Foster City, CA, USA). Dimethylacrylamide and diethylacrylamide were used as received from Monomer Polymer Dajac (Feasterville, PA, USA). Tetramethylethylenediamine (TEMED) was obtained from Pharmacia Biotech (Piscataway, NJ, USA). DNA fragments of 150, 300, and 1000 bp used as internal markers were obtained from Research Genetics (Huntsville, AL, USA).

The sieving polymer, non-crosslinked polydiethylacrylamide–co-dimethylacrylamide (poly-DEA/DMA), was polymerized in the laboratory from a mixture of diethylacryamide and dimethylacrylamide as described previously [25]. The polymerized reaction mixture was extensively dialyzed against distilled water and lyophilized prior to reconstitution in the electrophoretic buffer at the desired concentration. Solutions were prepared by weighing dry polymer into buffer and rotating the solution overnight. Before use, the polymer solution was degassed by a combination of sonication, stirring and application of a 85 kPa vacuum. If required, TO or EtBr was added to final concentration in an aliquot of solution, and the aliquot was centrifuged briefly to eliminate introduced bubbles.

2.2. Sample preparation from whole blood

The D1S80 locus was amplified from aliquots of whole blood obtained from Dr. Leslie Tobler at the Irwin Memorial Blood Center in San Francisco, CA, USA. Genomic DNA was extracted using the QIAamp Blood Kit (QIAGEN, Santa Clarita, CA, USA) from 200 μl of blood per sample. D1S80 alleles were amplified using the primers and given protocol in the AmpliFLP D1S80 polymerase chain reaction (PCR) amplification kit (PE Biosystems). The genomic DNA was diluted 10-fold in Milli-Q water (18.2 MΩ cm) prior to amplification. Extracted genomic DNA and the products of the PCR were kept at −20°C until use. Prior to electrophoresis on the chip, the products of the PCR were desalted by floating membrane dialysis against Milli-Q water for 45 min (0.025 μm, VSWP membrane, Millipore, Bedford, MA, USA) or by spin-column (Centrisep Spin Columns, Princeton Separations, Adelphia, NJ, USA).

2.3. Allele separations by capillary electrophoresis

Before the amplified alleles were run on the chip, each blood sample was typed using CE on an ABI 310 instrument (PE Biosystems). Electrophoresis was performed at 50 V/cm in 1% poly-DEA/DMA–1× TBE–0.4 μ*M* TO in fused-silica capillaries, 75 μm in diameter. The capillaries were pre-coated with polyacrylamide using a modified Hjertén procedure [26]. The signal was monitored in the fluorescein channel. Each sample analyzed consisted of 5 μl of the amplified alleles, 2 μl of the allelic ladder, 5 μl of the control double-stranded (ds) DNA (1 ng/ml) and 5 μl of Milli-Q purified water. The sample was injected at 2 kV for 2 s.

A Beckman P/ACE 5000 instrument was used to investigate the effect of various run conditions on the separation of the allelic ladder. Electrophoresis was performed at varying polymer concentrations with TO as intercalant and voltage gradients in fused-silica capillaries, 75 μm in diameter. To obtain a separation length closer to that on the plastic chips, the injection was performed from the usual outlet end of the capillary, and therefore, the effective separation length was as short as 6.8 cm.

2.4. Chip preparation and sealing

Single-channel and multi-channel chips were produced using the mold and process described previously [15,25]. For the multi-channel chips, the initial master was etched in glass instead of silicon. Briefly, the channel patterns were wet-etched in a 12.7-cm square piece of flat glass (Nanostructures, Santa Clara, CA, USA) using a chrome mask (Photosciences, Torrance, CA, USA). Next, a nickel electroform ''daughter'' was prepared as a negative of the channel pattern; i.e., the pattern of channels appears as ridges on the electroform. The electroform was then mounted on a mold insert. Using the mounted electroform, chips were made by injection molding with a polymethylmethacrylate resin. Approximately 1000 chips were created per run. Reservoirs and registration holes were drilled mechanically. Chips were sealed by lamination of a

polymethylmethacrylate film or a Mylar film coated with adhesive (Great Plains Model Distributors, Champaign, IL, USA).

2.5. Chip layout

The channel layouts for the single-channel and multi-channel chips appear in Fig. 1a and b, respectively. The cross-section of the channel is trapezoidal, approximately 50 μm wide at the base, 150 μm wide at the top, and 50 μm deep. The cross-sectional profile of the channel in the injection-molded, multi-channel chip is compared in Fig. 2 to that of the nickel electroform daughter. The multi-channel chip was designed to be general for a variety of applications, including enzyme assays. To make more efficient use of the chip footprint, the direction of migration of the injected sample alternates across the detection zone, which is therefore fixed along one axis of symmetry. The layout is divided into quadrants, each containing the injection cross for four separate channels. Three reference channels on the chip allow for proper focusing of the optical head of the scanning instrument. These channels were filled with 10 μ*M* 5-FAM. The channel layout was constrained by a number of variables, including the reservoir spacing and dimensions of the optical detection zone.

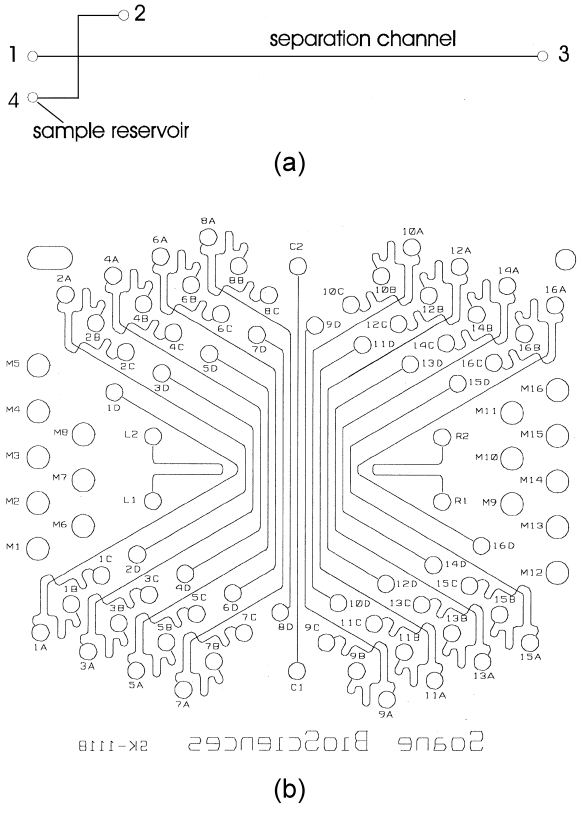

Fig. 1. (a) Layout of channels for the single-channel chip. (b) Layout of channels for the multi-channel chip. The left-most, center, and right-most channels are reference channels, which when filled with fluorescein, are used for proper alignment of the card under the beam. The remaining 16 channels are used for separations. The topology of each channel pattern is identical to that illustrated in (a). The chip is approximately 8.5 cm square.

2.6. Separation conditions in chips

Electrophoresis was performed in solutions of poly-DEA/DMA prepared in 1× TBE, pH 8.3. If the DNA was not labeled with fluorescein, the intercalating dye TO was included in the solution at 4 μ*M*.

All separations in chips were performed at ambient temperature, approximately 22°C. In previous work, we have demonstrated that there is a linear dependence of current on voltage gradient up to approximately 300 V/cm [15]. These results have been verified for the multichannel chips (data not shown), and based on this data, we do not expect adverse effects from Joule heating at the relatively low voltage gradient used for the separations reported in this work. Therefore, thermal control of the chip was not implemented.

To load the single-channel chips, reservoir 3 was filled with polymer solution, and a 85 kPa vacuum was applied at reservoir 1 to fill arm 3. After the separation matrix reached the channel intersection, reservoirs 1 and 2 were filled with polymer solution, and the vacuum was moved to reservoir 4. The vacuum was removed, and the sample was loaded into reservoir 4. The volume of each reservoir was approximately 10 μl.

To load the multiple-channel chips, an eight-channel vacuum manifold was used. Polymer solution was loaded into reservoirs 1, 2 and 4 of each channel pattern on one half of the chip, the chip was aligned on a stage, and the manifold was clamped into place. Again, a 85 kPa vacuum was used to fill the channels. The process was repeated on the opposite side of the chip. Then, the remaining reservoirs were

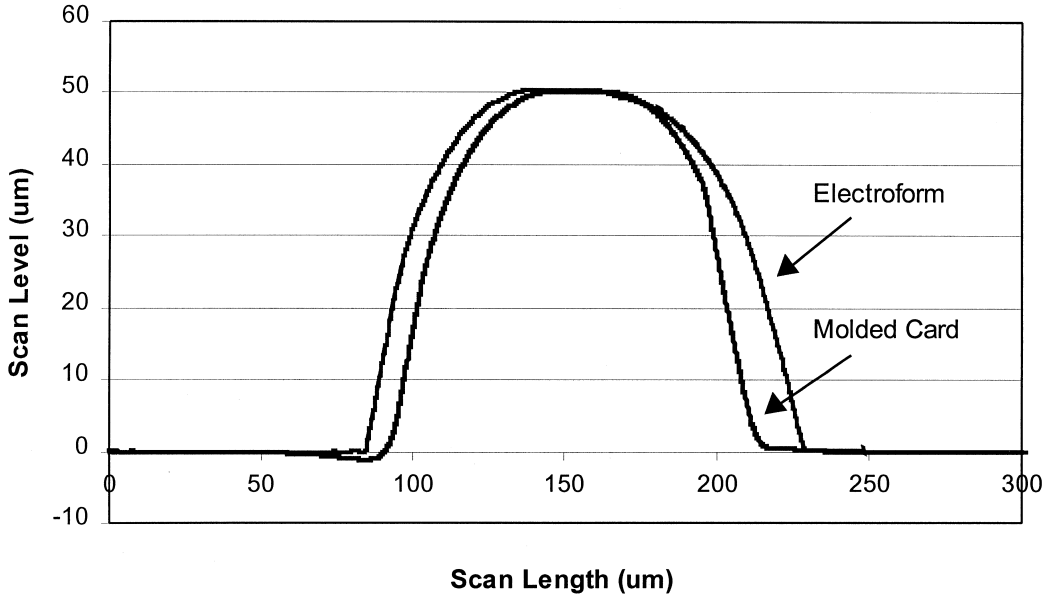

Fig. 2. Comparison of channel profile on multi-channel chip to that of the nickel electroform. The channel profile has been inverted for ease of comparison with that of the nickel electroform, which, being the mold, has ridges instead of depressions. The deviation between the channel profile and that of the mold is greatest in the width of the channel and virtually negligible in the depth of the channel.

filled with polymer solution, and reservoir 4 was replaced with sample.

Two sets of voltages were used for injection and separation of the fragments. The first set, the pull-across voltages, was applied to electrophorese the sample past the channel intersection (injection cross). The second set of voltages, the separation voltages, was applied to inject and separate the sample defined by the geometry of the channel intersection. The volume of the sample plug was approximately 0.5 nl. Voltages were determined by circuit modeling; it was assumed that the current in each arm was proportional to the length of each arm. The voltage gradient was calculated as that between reservoir 3 and the channel intersection.

2.7. Instrumentation

For single-channel separations, fluorescence was excited and collected using a confocal, laser-induced, epifluorescence system. The 488-nm line of a tunable argon-ion laser (543AP, Omnichrome, Carlsbad, CA, USA) was directed into an inverted epifluorescence microscope (Eclipse TE300; Nikon, Melville, NY, USA) through a bandpass filter (HQ480/40; Chroma Technology, Brattleboro, VT, USA). The beam was reflected off a 505 nm dichroic mirror (Q505LP; Chroma Technology) and was focused within the channel through a 10×, 0.45 NA objective (CFI Plan Apo 10X; Nikon). Fluorescence was collected through the same objective, passed through the dichroic and a second bandpass filter (HQ535/50; Chroma Technology) and focused through a 500 μm confocal aperture. The light passing through the aperture was detected by a photomultiplier tube (PMT) (R3896; Hamamatsu, Bridgewater, NJ, USA). The PMT output was amplified and filtered electronically by a low-noise current preamplifier (SR570; Stanford Research Systems, Sunnyvale, CA, USA), digitized, digitally filtered, and stored using a program written in LabView. Voltage was supplied by a four-channel, laboratory-constructed power supply capable of supplying 4.5 kV to each channel.

For multiplexed separations, fluorescence was detected using a confocal laser-induced fluorescence (LIF) scanner, as described previously [25]. Briefly,

the multichannel chip was aligned on a plate mounted upon a linear, motorized actuator that scanned the chip underneath the excitation beam at approximately 2 Hz during the application of the separation voltages. A top plate containing 96 platinum wire electrodes was then clamped in place and connected to the laboratory-constructed power supply. The excitation beam was provided by the 488-nm line of a 30-mW argon ion laser. The laser beam was transmitted through a single-mode fiber, reflected off a dichroic beamsplitter (505DRLP02; Omega Optical, Brattleboro, VT, USA) and passed through a 20×, 0.4 NA objective to the channel. Emitted fluorescence was collected through the same objective, passed through a bandpass filter (530DF30; Omega Optical) and focused through a confocal pinhole onto a PMT (HC120; Hamamatsu). The optics head could be focused to within 1 μm, and the depth of focus was approximately ±25 μm. The instrument was controlled from a personal computer using custom-developed software written in LabView (National Instruments, Austin, TX, USA). Data were recorded in the form of an EXCEL file. A conversion macro was written in EXCEL to generate text files for each of the 16 channels. Electropherograms were plotted and analyzed using these text files and Caesar software (Analytical Devices, Alameda, CA, USA).

3. Results and discussion

3.1. Method development in CE

We used the PE Biosystems ABI 310 CE instrument to examine the effects of the voltage gradient and polymer concentration in the sieving buffer on the separation of the D1S80 allelic ladder. One goal was to optimize the voltage gradient and sieving buffer composition to obtain the best speed and resolution in the separation. These conditions were used as the reference method on the ABI 310 to type the alleles in each sample. The genotype obtained from the chip separations was then compared to that obtained using the ABI 310.

Fig. 3 shows a representative separation of the D1S80 allelic ladder using the ABI 310. Electrophoresis was performed at 100 V/cm at 45°C in a non-coated, fused-silica capillary with 1% poly-DEA/DMA–1× TBE. We note that by using poly-DEA/DMA, a poly-alkyl-acrylamide, we did not need to modify the surface of the capillary, a time-

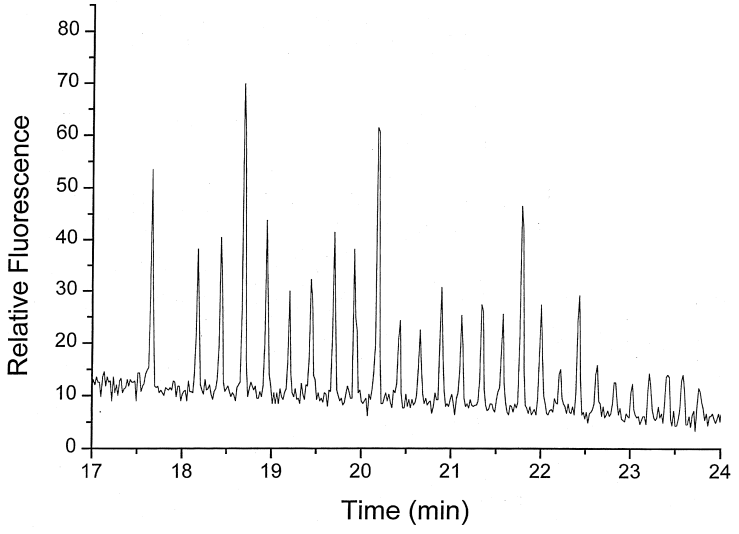

Fig. 3. Electropherogram of the D1S80 ladder in 1.0% poly-DEA/DMA–1× TBE–0.1 μg/ml ethidium bromide. Electrophoresis was performed using an ABI 310 in a 50 μm I.D., fused-silica capillary at 100 V/cm and 45°C. The effective separation length was 36 cm.

and labor-consuming activity which is necessary when using polyacrylamide as a sieving media. The effective separation length was 36 cm. EtBr was used at a concentration of 0.1 μg/ml, 10-fold less than that used by Gao et al. in their separations of the allelic ladder [27]. All alleles were baseline resolved within 24 min. An increase in the concentration of the sieving polymer to 2% improved resolution, but the separation time was twice that of the 1% polymer.

A second goal was to determine electrophoresis conditions transferable to the chip, where the separation length is much less than that in a commercial CE instrument used in standard fashion. The ABI 310 instrument, with a minimal effective capillary length of 30 cm, was not suitable for this experiment. Instead, we used a Beckman PACE 5000 CE instrument. By injecting from the outlet side, the effective separation length was 6.8 cm. Because Mathies et al. reported a higher signal-to-noise ratio for TO versus EtBr in separations of dsDNA in glass chips, we used 0.4 μ*M* TO as the intercalant. Fig. 4 shows the effect of voltage gradient (50, 100 and 150 V/cm) on the separation of the allelic ladder. The separations were performed in a 75 μm I.D. capillary filled with 1% polymer in 1× TBE. From Fig. 4, we concluded that using a short effective length, a smaller voltage gradient of 50 V/cm resolved the alleles better and was selected as the optimum. Separations of the ladder were performed in solutions containing 1% and 2% polymer. As expected, 2% polymer separated the fragments better than 1% polymer; however, the run-time was significantly longer. *S*/*N* ratios were excellent for all separations using 0.4 μ*M* TO in the fused-silica capillaries. Therefore, the conditions best suited for the chip were chosen to be 2% polymer, 0.4 μ*M* TO, and 50 V/cm.

Although the 27 D1S80 alleles are well separated using the 6.8 cm short distance on the Beckman P/ACE instrument, they were not resolved to baseline as in the ABI 310. We also observed better *S*/*N* with the ABI than with the Beckman instrument. Therefore, we used the ABI 310 to type the alleles amplified from whole blood samples. The amplified alleles were spiked with 5 μl of the allelic ladder as an absolute standard [27] and with fragments of 150, 300 and 1000 base pairs as a control. Fig. 5 shows a representative separation of the

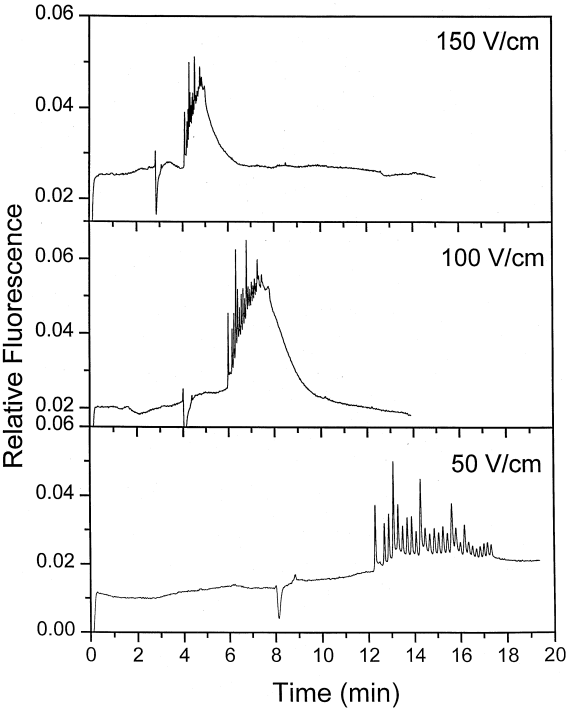

Fig. 4. Electropherograms of the D1S80 ladder at different field strengths with a separation distance of 6.8 cm. Electrophoresis was performed in 2.0% poly-DEA/DMA–1×TBE–0.4 μ*M* thiazole orange on a Beckman P/ACE 5000.

amplified alleles with the added absolute standard and control. Electrophoresis was performed in 1% poly-DEA/DMA at 50 V/cm.

As stated before, the unambiguous size assignment of the amplified alleles is the most difficult task in genotyping. Several methods for the typing of D1S80 have been discussed in the literature [23,24,27]. In sizing electrophoresis, it is commonly assumed that the migration distance or time depends linearly on the length of the fragment. For DNA sizing standards, this is typically true, and the length of the unknown fragment is inferred by interpolation from the sizing standard. However, in the case of D1S80 alleles, this procedure cannot be used. As shown in Fig. 6, using the data from Fig. 5, the migration time of the D1S80 alleles is higher then predicted from an interpolation of the dsDNA sizing standards. To complicate matters even further, the size versus migration time plot of the D1S80 standards is markedly curved and not linear, as would be

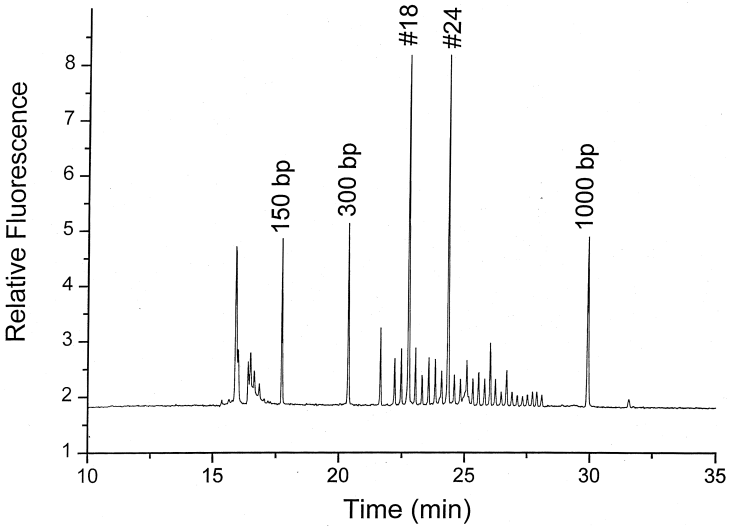

Fig. 5. Electropherogram of amplified alleles spiked with dsDNA standards and the D1S80 allelic ladder. Electrophoresis was performed on the ABI 310 at 100 V/cm in 1% poly-DEA/DMA–1× TBE–0.4 μ*M* TO. The sample consisted of 5 μl of amplified alleles from sample IH27, 2 μl of D1S80 ladder, and 5 μl of 150, 300 and 1000 bp DNA standards (1 ng/μl). The sample IH27 was heterozygous with alleles 18 and 24.

expected. For this reason, we investigated two methods recently reported for typing of D1S80 alleles from CE data.

In the method of Isenberg et al., the migration time for each peak is normalized to that of the 300 bp control fragment, and a run of the allelic ladder is performed frequently (i.e., every fourth run) to account for run-to-run variations in the electrophoretic conditions [23]. The alleles are typed by comparing the normalized migration time (NMT) for

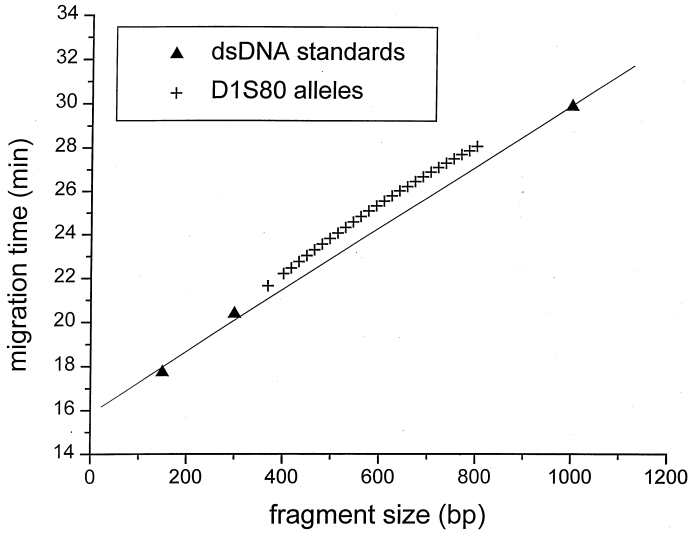

Fig. 6. Dependence of migration time on fragment length for D1S80 alleles. Experimental conditions as in Fig. 5. The alleles migrate slower than would be predicted from a linear best-fit of the migration time of the dsDNA standards.

a peak to those of the alleles in the ladder. When we used this method, run-to-run variations were still significant enough such that the D1S80 DNA control (18, 31) supplied by Perkin-Elmer was typed incorrectly as 18, 30.

In the method of Mitchell and Walsh, the allelic ladder is included as an internal standard, and increases in the peak intensity or area are used to type alleles [24]. This method should give less ambiguity. We normalized the area of each peak to that of allele 14, which is relatively uncommon. We also ran the allelic ladder separately and calculated the normalized peak areas for each allele. We then identified an amplified allele as that peak whose normalized peak area was greater than that if the ladder was run alone. The D1S80 DNA control was now typed correctly as 18, 31. In a real-world situation, this technique suffers the disadvantage that typing may be difficult if the amplification yields little product, but in our case, the amplifications yielded a significant amount of product.

3.2. Separation of allelic ladder in single channels of plastic microchips

As a first step towards performing genotyping in our multi-channel chip, we demonstrated that the D1S80 allelic ladder could be separated in the shorter separation distance of a chip. We started by using a plastic, single-channel chip [15] whose maximum separation distance is approximately 4.9 cm. Because of the pinched injection scheme [28], the width of the injection plug is much smaller in the chip (on the order of the channel width) than in conventional, automated CE. In general, a narrower injection plug means that separations can occur in shorter distances. For example, this phenomenon is used in standard slab protein electrophoresis by stacking the applied sample. Fig. 7 shows a separation of the allelic ladder in a single-channel plastic chip with an effective separation channel length of only 4 cm. Electrophoresis was performed in 2% polymer–1× TBE at 50 V/cm at ambient temperature. In other work, we found that the signal was more intense on our microscope instrumentation if we used 4 μM TO rather than 0.4 μM. While the fragments are not separated to baseline, each allele can be discerned.

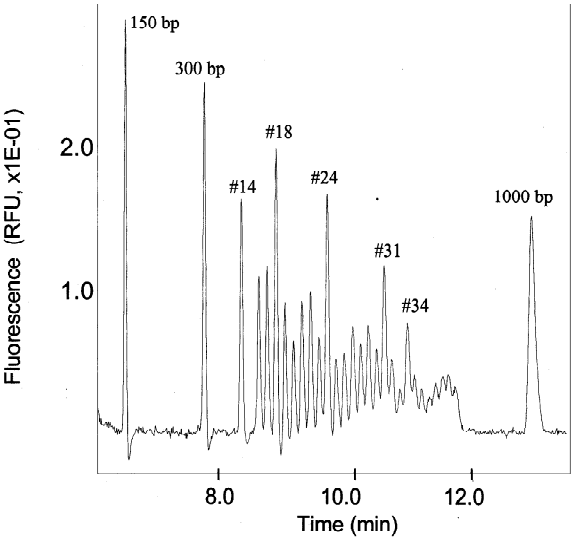

Fig. 7. Separation of the D1S80 allelic ladder in single-channel chip in 2% uncrosslinked polymer. All alleles in the ladder could be distinguished in a separation distance of only 4 cm.

Because the channels interdigitate in the multichannel chip, the effective separation distance is reduced to only 2.8 cm. Fig. 8 shows the separation of the allelic ladder in one of the 16 channels of the

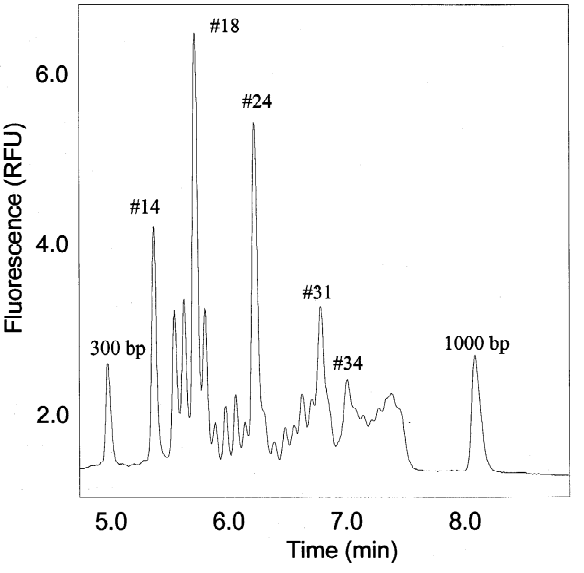

Fig. 8. Separation of the D1S80 allelic ladder in one channel of the multi-channel chip. Scanning detection was not employed. The separation distance was only 2.8 cm.

multichannel chip. Scanning detection was not employed. Electrophoresis was performed as before. Above allele 31 (641 bp), the peaks are difficult to distinguish. Therefore, we increased the polymer concentration to improve the peak-to-peak spacing. Use of up to 4% polymer resulted in no significant improvement in the separation of the higher alleles (>No. 31). There are at least two reasons that the separation in Fig. 8 is poorer than that of Fig. 7. The separation distance is 30% shorter, and, in the multichannel chip, there is a bend in between the injection cross and the detection point. In other work (not shown here), we have shown that the allelic ladder can be separated to baseline resolution within 4.7 cm in a straight channel. Furthermore, we could not use the eight-channel filling device to load solutions of more than 2% polymer without obtaining an air bubble in at least one of the channel intersections.

From typing the samples on the ABI 310, we knew that none contained alleles higher than No. 33. The most difficult separation is to separate adjacent alleles, which only one sample had. Acknowledging that the separation can be improved by changes in the chip layout and separation conditions, we proceeded to perform allele separations in the multichannel chip using 2% polymer.

3.3. Multichannel separations

Because of the poorer sensitivity of the scanning system, it was difficult to detect the allelic ladder when added to the amplified alleles. Therefore, we adapted the method of Mitchell and Walsh to the multichannel chip [24]. Instead of running the allelic ladder every fourth run as on a CE instrument, we loaded the standard in one lane per quadrant. By mixing samples, we constructed a simplified ladder, containing alleles 18, 24, 27, 31 and 33, which could all be resolved to baseline in 2.8 cm. To each sample or ladder, we added control fragments 150, 300 and 1000 bp in length. The control DNA fragments were loaded alone in one channel to unambiguously define their migration times. The remaining 11 channels were used to type alleles amplified from individual samples.

Before we performed the allele separations, we characterized the channel-to-channel variability of the migration time in the 16-channel chip. Allele 33, the highest in our samples, is approximately 2.4% different from alleles 32 or 34 in size. Therefore, to minimize ambiguousity, the migration time of a peak should vary by approximately 1.2% or less from channel to channel.

We performed 16 simultaneous injections of 5-FAM in a multi-channel chip filled with 2% polymer. Expressed as a percentage of the migration time, the standard deviation (SD) was 2.65% across all channels. However, the standard deviation was less than 1% if we calculated the standard deviation within groups of four channels that correspond to the four quadrants of the chip. The migration time and standard deviations are listed in Table 1. We are presently investigating the source of the quadrant dependency to reduce the channel-to-channel variability of migration time below 1%.

Fig. 9a–d are overlays of the electropherograms obtained from a run of the multichannel chip, as described above, overlaid by quadrant. Scanning detection was employed, and electrophoresis was performed at 50 V/cm. The separation was complete in less than 10 min, and the entire method (stage positioning, injection, separation, and creation of EXCEL worksheet) ran in less than 15 min. The standard deviation was 2.4% for the migration of the 300-bp fragment over all channels; this agrees with the results from injections of 5-FAM. We observed excellent signal-to-noise in each channel.

To assign the alleles, we used the NMT procedure, which was calculated for each peak by dividing the migration time of the peak by that of the 300-bp control fragment ran in the same channel. Table 2 summarizes the results of the assignment of the 11 sets of alleles. The NMT of each allele is listed, and the chip-determined D1S80 type is compared to the type determined on the ABI 310. To type the alleles, we aligned the electropherogram of the ''unknown'' alleles with that of the simplified ladder that had been run in the same quadrant as the unknown. The

Table 1
Summary of standard deviation (SD) of migration time for simultaneous injections of 5-FAM

	Q1	Q2	Q3	Q4	ALL 16
SD (%)	0.91	0.44	0.5	0.49	2.65

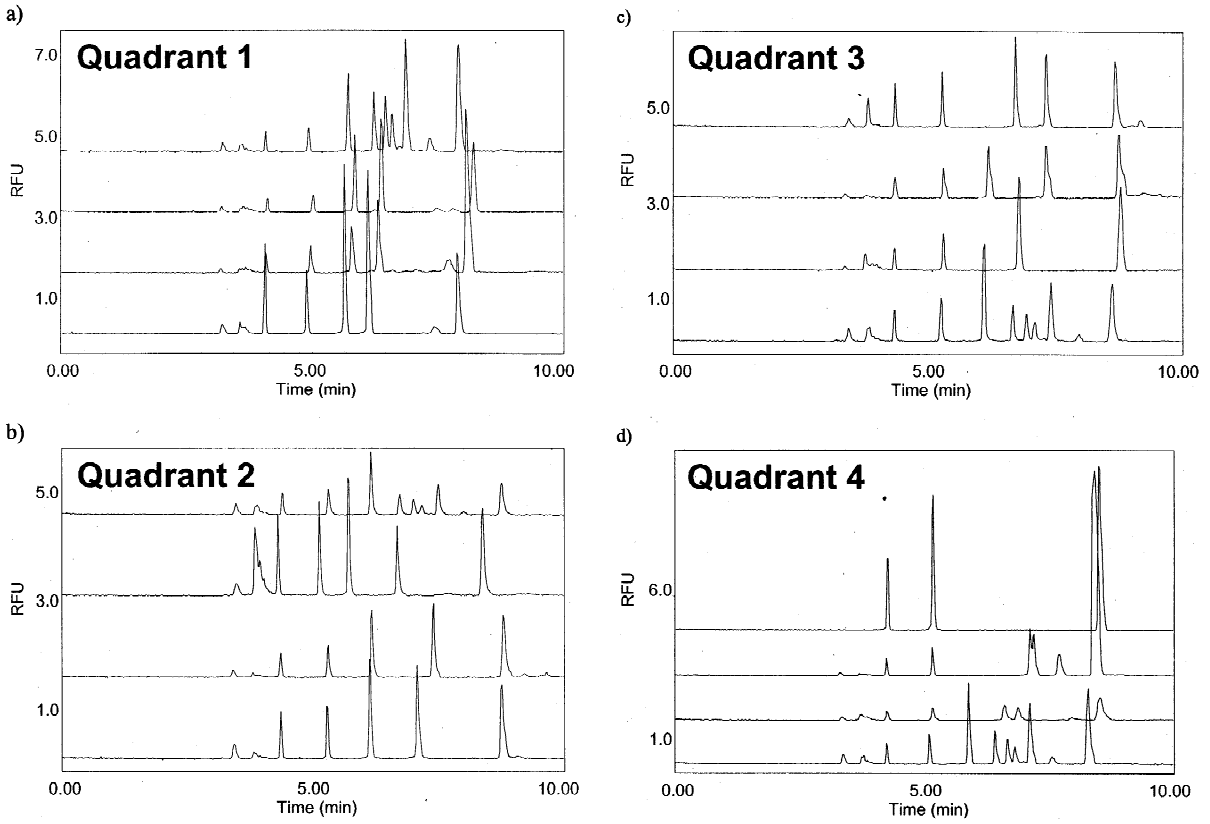

Fig. 9. Simultaneous separation of alleles in 16 channels, arranged by quadrant. The reference alleles were loaded in each quadrant (top electropherogram in quadrants 1 and 2, bottom electropherogram in quadrants 3 and 4). In quadrant 4, one lane contains only the control dsDNA as a reference.

two electropherograms were aligned in EXCEL using the 300 and 1000 bp peaks of the control DNA. Mitchell and Walsh reported that this technique resulted in 100% agreement of CE and slab gel typing [24]. To align the peaks, the electropherogram of the alleles was first shifted additively to align the 300-bp peak in sample and ladder. A sine-wave based correction factor was then applied to scale the time axis such that the 1000 bp peaks were also aligned. After alignment, the NMTs for the alleles were determined to four significant digits and typed by comparison to the NMTs for the allelic ladder. If the NMT for an allele fell exactly between NMTs for adjacent alleles, the type was considered ambiguous. The NMTs were inter- or extrapolated linearly for the alleles not present in the simplified ladder. Polynomial fitting of the NMT did not improve the typing accuracy. Of the 23 alleles in the 11 channels, 19 or 83% were assigned correctly in the chip using this method. We note that two samples had three peaks within the migration window of the allelic ladder, one peak of which is significantly less intense than the other two. The D1S80 locus occurs once on chromosome 1p, and therefore a homozygotic individual would be expected to have one allele, and a heterozygotic individual, two. Triallelism is a possible explanation but would be expected to be quite uncommon. Therefore, we cannot rule out possible cross-contamination in the PCR.

It has been shown previously that typing performed by CE can accurately match that performed by conventional slab electrophoresis. Through fur-

Table 2
Summary of genotyping results for multichannel chip

Allele	Base pairs		Quadrant 1				Quadrant 2			
		Sample:	Standard	IH-27	IH-40	IH-38	Standard	IH-14	IH-23	IH-8
		Type by CE:		18, 24	18, 24	18, 24		16, 27	18, 31	18, 28
		Type by chip:		18, 24	18, 24	18, 23		16, 27	18, 31	18, 28
			NMT							
			Channel 1	Channel 3	Channel 5	Channel 7	Channel 2	Channel 4	Channel 6	Channel 8
14	369		1.042				1.089			
16	401		1.110				1.125	1.117		
17	417		1.144				1.142			
18	433		1.161	1.156	1.159	1.152	1.160		1.161	1.159
19	449		1.178				1.178			
20	465		1.195				1.195			
21	481		1.212				1.213			
22	497		1.229				1.231			
23	513		1.246			1.246	1.248			
24	529		1.263	1.257	1.260		1.266			
25	545		1.279				1.284			
26	561		1.296				1.303			
27	577		1.312				1.321	1.315		
28	593		1.326				1.336			1.335
29	609		1.339				1.352			
30	625		1.353				1.367			
31	641		1.366				1.382		1.389	
32	657		1.380				1.398			
33	673		1.393				1.413			
34	689		1.407				1.428			
			Quadrant 3				Quadrant 4			
		Sample:	Standard	IH-33	IH-5	Control	Standard	IH-50	IH-37	IH-35
		Type by CE:		24, 27, 31	30, 31, 33			24	18, 30	24, 31
		Type by chip:		24/25, 28	32, 33, ?			24	18, 30	24, 31
			Channel 16	Channel 14	Channel 12	Channel 10	Channel 15	Channel 13	Channel 11	Channel 9
14	369		1.084				1.088			
16	401		1.119				1.124			
17	417		1.137				1.143			
18	433		1.154				1.161		1.166	
19	449		1.172				1.179			
20	465		1.189				1.198			
21	481		1.207				1.216			
22	497		1.224				1.234			
23	513		1.242				1.253			
24	529		1.259	1.267			1.271	1.270		1.275
25	545		1.275	1.267			1.288			
26	561		1.292				1.304			
27	577		1.308				1.321			
28	593		1.323	1.317			1.338			
29	609		1.337				1.354			
30	625		1.352				1.371		1.377	
31	641		1.367				1.387			1.388
32	657		1.381		1.379		1.401			
33	673		1.396		1.392		1.414			
34	689		1.411		1.495		1.428			

ther work, the agreement between D1S80 types determined by chip and capillary can be improved. For example, the inclusion of an allelic ladder in each lane will give the least ambiguous typing. Various strategies, including the use of two-color fluorescence detection, can be envisioned to improve the sensitivity of the system if the amplified alleles are dilute compared to the ladder. If an allelic ladder is not included in each lane, the variability in migration time can be improved. Re-design of the chip for longer channels and elimination of bends will facilitate improved resolution.

4. Conclusion

Our results demonstrate that it is feasible to perform high-throughput genotyping in injection-molded, plastic multi-channel chips. Given appropriate internal standards and optimization of chip design and separation conditions, we believe that the accuracy of typing can be improved to match that of the conventional slab-gel procedure. The results of these efforts will be the subject of a future report.

Here, we have used chips with only 16 channels, which were replicated reproducibly by injection molding and sealed by lamination of a thin film. Chips of similar size can accommodate at least 96 samples, as demonstrated by Shi et al. in glass [29]. Scanning, confocal, LIF detection was used successfully to detect the separations occurring simultaneously in all channels. Although the plastic has inherently higher autofluorescence than glass, we observed good signal-to-noise ratios with an intercalating dye for the allelic ladder at a concentration of 4 ng/μl, which corresponds to an average of 153 pg/μl per allele. Separations were fast, the highest allele reaching the detection point in less than 9 min. Compared to the 25 min required for typing by CE, we have already achieved a 30-fold increase in throughput. Using a higher channel density of 96 separation lanes, an increase in throughput of two-orders of magnitude is easily within reach. Combined with the relatively inexpensive injection molding process for manufacture of single-use plastic chips, cost-effective, large-scale genetic testing is therefore feasible.

In a separation length approximately 70% longer than that of the present multi-channel chip, we can separate the allelic ladder with near baseline resolution within only 7 min. In future designs, we plan to eliminate the bend between the channel intersection and the detection point to further improve the separation and to reduce the reservoir size to pack the channel patterns more closely. Optimization of injection conditions and of the molecular mass of the polymer in plastic chips separations is likely to reduce the separation time even further while either retaining or improving on the resolution of the D1S80 allelic ladder in conventional capillary electrophoresis.

This will allow us to improve the accuracy of typing on the chip by one or more of several techniques: inclusion of the allelic ladder in each channel, reduction of the channel-to-channel variation in migration, software alignment of separations in multiple channels, or two-color detection. Just as the accuracy of typing the D1S80 locus by CE has been improved to match that of conventional slab electrophoresis, we have now defined the parameters to allow for multiplexed typing on a single-use, plastic chip for large-scale genotyping.

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References

[1] E. Pennisi, Science 283 (1999) 1822.
[2] A. Manz, D.J. Harrison, E.M. Verpoorte, C.J. Fettinger, A. Paulus, H. Ludi, H.M. Widmer, J. Chromatogr. 593 (1992) 253.
[3] D.J. Harrison, A. Manz, Z. Fan, H. Luedi, H.M.W. Widmer, Anal. Chem. 64 (1992) 1926.
[4] S.C. Jacobson, R. Hergenroeder, L.B. Koutny, M.J. Ramsey, Anal. Chem. 66 (1994) 1114.
[5] A.T. Woolley, R.A. Mathies, Proc. Natl. Acad. Sci. USA 91 (1994) 11348.
[6] A.T. Woolley, R.A. Mathies, Anal. Chem. 67 (1995) 3676.
[7] A.P. Sassi, A. Paulus, (1999) manuscript in preparation.
[8] J.S. Bashkin, M. Bartosiewicz, D. Roach, J. Leong, D. Barker, R. Johnston, J. Cap. Electrophoresis 3 (1996) 61.
[9] M. Marsh, O. Tu, V. Dolnik, D. Roach, N. Solomon, K. Bechtol, P. Smietana, L. Wang, X. Li, P. Cartwright, A. Marks, D. Barker, D.J.B. Harris, J. Cap. Electrophoresis 4 (1997) 83.
[10] Y. Wang, J.M. Wallin, J. Ju, G.F. Sensabaugh, R.A. Mathies, Electrophoresis 17 (1996) 1485.
[11] Y. Wang, S.C. Hung, J.F. Linn, G. Steiner, A.N. Glazer, D. Sidransky, R.A. Mathies, Electrophoresis 18 (1997) 1742.
[12] P.C. Simpson, D. Roach, A.T. Woolley, T. Thorsen, R. Johnston, G.F. Sensabaugh, R.A. Mathies, Proc. Natl. Acad. Sci. USA 95 (1998) 2256.
[13] G.T.A. Kovacs, Micromachined Transducers Sourcebook, WCB McGraw-Hill, New York, 1998.
[14] M.A. Roberts, J.S. Rossier, P.H.G. Bercier, Anal. Chem. 69 (1997) 2035.
[15] R.M. McCormick, R.J. Nelson, M.G. Alonso-Amigo, D.J. Benvegnu, H.H. Hooper, Anal. Chem. 69 (1997) 2626.
[16] C.S. Effenhauser, G.J.M. Bruin, A. Paulus, E. Ehrat, Anal. Chem. 69 (1997) 3451.
[17] H. Wenz, J.M. Robertson, S. Menchen, F. Oaks, D.M. Demorest, D. Scheibler, B.B. Rosenblum, C. Wike, D.A.W.E. Gilbert, J. Genome Res. 8 (1998) 69.
[18] Y. Wang, J. Ju, B.A. Carpenter, J.M. Atherton, G.F. Sensabaugh, R.A. Mathies, Anal. Chem. 67 (1995) 1197.
[19] D. Schmalzing, L. Koutny, D. Chisholm, A. Adourian, P.D.E. Matsudaira, Anal. Biochem. 270 (1999) 148.
[20] D. Schmalzing, L. Koutny, A. Adourian, P. Belgrader, P.D.E. Matsudaira, Proc. Natl. Acad. Sci. USA 94 (1997) 10273.
[21] A.T. Woolley, G.F. Sensabaugh, R.A. Mathies, Anal. Chem. 69 (1997) 2181.
[22] M. Thymann, L.J. Nellemann, G. Masumba, L.N.M. Irgens-Moller, Forensic Sci. Int. 60 (1993) 47.
[23] A.R. Isenberg, B.R. McCord, B.W. Koons, B. Budowle, R.O. Allen, Electrophoresis 17 (1996) 1505.
[24] J.W. Mitchell, D. Walsh, Electrophoresis 19 (1998) 80.
[25] A. Paulus, S.J. Williams, A.P. Sassi, P. Kao, H. Tan, H.H. Hooper, SPIE Proc. 3515 (1998) 94.
[26] S. Hjertén, Chromatogr. Rev. 9 (1967) 122.
[27] Q. Gao, H.M. Pang, Y. Es, Electrophoresis 20 (1999) 1518.
[28] S.C. Jacobson, R. Hergenroeder, L.B. Koutny, R.J. Warmack, M.J. Ramsey, Anal. Chem. 66 (1994) 1107.
[29] Y. Shi, P. Simpson, J.R. Scherer, D. Wexler, C. Skibola, M.T. Smith, R.A. Mathies, Anal. Chem. 71 (1999) in press.