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Ultrathin Slab Gel Separations of DNA Using a Single Capillary Sample Introduction System

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We demonstrate here a novel method for DNA separations which combines the parallel processing capabilities of slab gels with the advantages of sample introduction obtained with a single capillary. This sample introduction format allows rapid sequential separations or continuous analysis to be carried out on ultrathin slab gels with efficient heat dissipation. Ultrathin slab gels have been fabricated by using 57-μm spacers between quartz plates, and a single capillary has been used to introduce plugs of dsDNA fragments into the ultrathin gel. These fragment plugs were deposited along the entrance to the ultrathin gel at spatially discrete locations by micromanipulation of the capillary. Spatially resolved detection has been accomplished with an argon ion laser focused to a line for excitation and a CCD for collection of fluorescence. Double-stranded DNA separations are demonstrated in a plug injection format. This approach allows multiple unique samples to be rapidly deposited on the ultrathin slab gels for separation.

Molecular biologists have used the sized-based separation technique of gel electrophoresis for decades. It has proved useful for separations of DNA fragments in such varied means as pathogen detection,[1,2] restriction mapping[3,4] and sizing of large DNA fragments.[5,6] Increases in speed and resolution of the gel-based methods have extended their use to include separations of smaller-sized restriction fragments[7−12] and DNA sequencing reaction products.[13−20] These increases in speed and resolution have resulted from the implementation of analytical techniques such as capillary gel electrophoresis,[4,7−10,13] ultrathin slab gel electrophoresis,[14−18] and capillary array electrophoresis.[11,12,19,20] The fundamental advantage of these techniques lies in the increased heat dissipation that results when the surface area-to-volume ratio of the gel is increased, allowing higher potential fields to be used for the separation and, consequently, decreasing the separation time. Capillary gel electrophoresis in particular has received considerable attention due to the ease of sample introduction. On the other hand, the methods of ultrathin slab gel electrophoresis and capillary array electrophoresis allow multiple separations to be carried out in parallel, increasing the information capacity of a single run; however, the sample introduction methods for these high-throughput systems are currently rather complex.

The method described here combines the parallel processing capabilities of the ultrathin slab gels with the advantages of sample introduction generally found with a single capillary. This results in a similar throughput to the capillary array electrophoresis systems without the need to address a large array of capillary entrances. In this method, solutions of double-stranded (ds) DNA fragments are introduced into an ultrathin slab gel by way of a single capillary. The capillary is translated along the entrance to the ultrathin slab gel, depositing DNA fragments which are separated in different lanes on the gel. These lanes are not physical barriers designed into the gel but are apparent lanes, the result of migration of a DNA fragment plug in a straight line from the gel entrance to the detection region. The DNA fragments are electrokinetically transferred into the thin slab gel as opposed to being loaded into individual wells in the gel.

In this article, we describe the use of a novel system for sample introduction and separation of DNA fragments. The sample introduction system relies on the idea that DNA fragments have approximately the same mass-to-charge ratio regardless of size; therefore, all of the fragments from a restriction digest, for example, will migrate as a single plug through a free solution capillary. The system not only is used for the repeated transfer of the same sample to the gel but also can be used for transferring unique DNA samples to each separation lane in the gel.

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EXPERIMENTAL SECTION

Chemicals. Acrylamide stock solution, tris(hydroxymethyl)-aminomethane (tris), boric acid, hydrofluoric acid (HF), and [γ-(methacryloxy)propyl]trimethoxysilane were purchased from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic acid disodium salt (EDTA) was obtained from Fisher Scientific Co. (Fairlawn, NJ), and ammonium persulfate (APS) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

*Hae*III-digested ΦX174, PBR322, and PUC18 dsDNA were obtained from Sigma and were used without dilution. Ethidium bromide intercalating dye was obtained from Molecular Probes Inc. (Eugene, OR) and was used only in the separation buffer at a concentration of 0.500 mg/500 mL.

Ultrathin Slab Gel Construction. The channel structure in which the ultrathin slab gels were cast is similar to that used by Mesaros et al. for free solution continuous separations.[21,22] The channels were constructed of two pieces of polished quartz (16 cm long × 5 cm wide and $^1/_4$ in. thick) purchased from Technical Glass Products, Inc. (Mentor, OH). One end (5 cm) of each quartz plate was ground to an angle of approximately 45°. Thus, when the channel structure was formed, there was a beveled entrance that facilitated the alignment of the sampling capillary diameter with the gel entrance. Scotch tape ($^3/_4$ in. wide, 3M, St. Paul, MN) was used as a spacer to define the channel thickness. A piece of tape 16 cm long and trimmed to 0.5 cm wide was placed along each edge of one quartz plate. When the second plate was placed on top of the first plate, a rectangular opening was formed between the two, with an internal height of 57 μm.

Prior to casting the ultrathin slab gels, the quartz plates were immersed in a 0.5%(v/v) [γ-(methacryloxy)propyl]trimethoxysilane solution in 1:1 acetic acid/H_2O for at least 15 min so that the polyacrylamide would cross-link to the quartz plates and prevent migration of the gel out of the channel. The plates were then rinsed with water and/or 95% ethanol and dried. Special care was taken so that any residue on the plates was wiped clean and there were no streaks.

A 6% solution of acrylamide was prepared from a 40% acrylamide/bisacrylamide stock solution, 100 mM tris−borate−EDTA (TBE) buffer, and deionized water. This gel solution was then degassed for 10 min prior to the addition of the polymerization initiators, 10% APS and TEMED. This solution was poured immediately onto the quartz plate with the tape on the sides, and the second quartz plate was placed on top of the first, causing any excess gel solution to spill out of the channel. Immediately after pouring, the two plates were held together with $^3/_4$-in. Acco binders along the taped edges. The edges were then sealed with Devcon Corp. (Danvers, MA) 5-min epoxy. The solution was allowed to polymerize for 1 h, during which time the epoxy cured, allowing the removal of the Acco binders. During the polymerization period, the entrance and exit ends of the ultrathin slab gel were saturated with buffer solution, which appeared to decrease gel shrinkage. Several methods of filling the rectangular channel with gel solution after it was constructed were attempted but resulted in multiple bubbles, premature cross-linking, or extreme difficulty in taking the channel apart. The method used was very simple and reproducibly produced 57-μm-thick gels which were virtually bubble free.

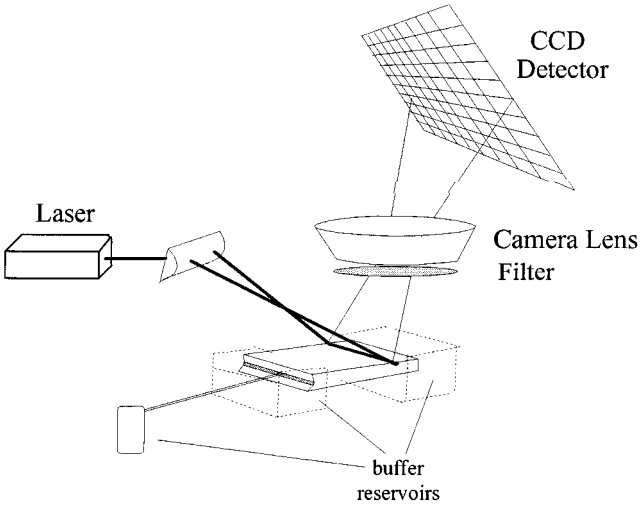

Figure 1. Schematic diagram of system setup. See text for details.

After complete polymerization, the ultrathin slab gel was suspended across two buffer reservoirs filled with 100 mM TBE buffer with ethidium bromide added, as shown in Figure 1. The buffer reservoirs were constructed from quartz (used for rigidity) in a similar configuration as was previously described.[21,22] The channel was suspended across the two buffer reservoirs, and Dow Corning high-vacuum grease (Midland, MI) was used both to secure the channel in place and to ensure isolation of the two buffer reservoirs. A platinum electrode was epoxied into each buffer reservoir, and a Bertan high-voltage power supply was used to provide the separation voltage. The gel was preelectrophoresed for 30 min at 2.0 kV (125 V/cm), the same voltage that was used during the experiments.

Capillary Transfer. The fused silica transfer capillary (50-μm i.d., 362-μm o.d.) was purchased from Scientific Resources, Inc. (Eatontown, NJ). This sulfonic acid-coated capillary was used for efficient transfer of all DNA fragments in a single plug. The exit end of this capillary was etched in HF to an o.d. of 70−110 μm as previously described,[23] and this etched tip was then easily positioned in the beveled entrance to the ultrathin slab gel.

Prior to experiments, the capillary was filled with 100 mM TBE buffer, and a potential field of −3 to −15 kV was applied across the length of the capillary. This potential field was applied with a Spellman high-voltage power supply (Plainview, NY) with the ground end at the capillary/gel juncture. In this way, the transferring potential was controlled independently of the separation field. Transfer of DNA plugs through the capillary was accomplished through electromigration. To make a plug injection, the entrance end of the capillary was moved to a vial of dsDNA, and a potential was applied across the length of the capillary for 3 s, followed by a 3−6-min plug of buffer to act as a spacer between consecutive plugs of DNA.

The transfer capillary was translated along the entrance of the slab gel by use of a Hurst (Princeton, NJ) stepper motor, which was controlled as previously described[21,22] via a Gateway 2000 computer (Sioux Falls, SD) with in-house-written software. The timing of the capillary movement was correlated with the expected

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elution time of the consecutive DNA plugs. The capillary was either moved at a rate of 20 ms/step with each step 25.4 μm at a time when buffer alone was eluting from the capillary or was continuously moved at a rate of 2500 ms/step, which corresponds to approximately 2 mm every 3 min, allowing a 2-mm spacing between each plug.

Detection System. The detection system used here was similar to that described by Smith et al.[16] and met the requirement of maintaining spatial integrity of the data across the width of the gel. The main components were a high-powered argon ion laser (6 W, Coherent, Santa Clara, CA) and a cryogenically cooled charge-coupled device (CCD) detector (Photometrics, Tucson, AZ). For the experiments shown here, the laser was used in the single-wavelength (514 nm) mode. The Photometrics CCD has a rectangular detection array of 1024 × 256, with each detection element 27 × 27 μm^2. Each detection element was read out individually or binned with consecutive elements and read out as a group. For these experiments, binning was done in only one dimension.

The 514-nm line from the argon ion laser was expanded with a Newport Optics beam expander (20×, Irvine, CA). The beam was then directed with two mirrors (Newport Optics) through a cylindrical lens (50.8 mm × 50.8 mm, F = 150 mm, Newport Optics) that shaped the expanded beam into a line approximately 5 cm long and 500 μm in width. This reshaped laser line was focused onto the top quartz plate at Brewster's angle to ensure minimum loss of power due to reflection and maximum transmittance of light into the detection region of the ultrathin slab gel.

Fluorescence emitted by the excited DNA:dye complex as it migrated through the 500-μm detection region was collected at an angle of 90°. It passed through a 550-nm-cutoff filter to eliminate stray light, including reflected laser light, and was demagnified 1.1 times by a 60-mm Nikkor camera lens (Nikon, f2.8) before reaching the CCD camera.

Data Collection and Analysis. The CCD was controlled using a Photometrics AT200 card in a Micron Pentium 133-MHz computer (Micron Electronics, Inc., Nampa, ID) and CCD9000 software (Photometrics). The detection region of the thin slab gel was focused onto a 1024 × 10 pixel array of the CCD. A new image was taken every second, and this 1024 × 10 pixel array was binned to a 1024 × 1 array. The CCD9000 software allowed 4200 consecutive images of this size to be stored in a single file. This file was then analyzed using on a Micron Dual-Pentium 133 Powerserver using Transform 2D (Fortner Research LLC, Sterling, VA) software.

A signal-averaging background subtraction program was written in-house using the Labview programming language. This subtraction program was used only when a constantly high background was observed in specific regions of the channel. This usually indicated that the channel plates were either smudged or scratched, causing more reflection than usual at these points. However, this apparently did not inhibit detection of fragments at this location, because removal of the higher background at these points resulted in visualization of masked DNA bands.

Safety Concerns. Care was taken when handling acrylamide monomers and the ethidium bromide dye, as they are toxic. In addition, investigators were removed from contact with the high-voltage power supply.

RESULTS AND DISCUSSION

Overview of Capillary Transfer System. A schematic of the entire system is shown in Figure 1 and is broken down into three main components: the transfer capillary, the gel separation system, and the detection system. The transfer capillary is used to introduce analyte solutions, in this case the dsDNA solutions, into the slab gel. This can be done via either pressure (data not shown) or electromigration. In the case of electromigration, the transferring potential is controlled independently of the separation field. The beveled edges of the channel ensure accurate alignment of the capillary/slab gel bores. By translating the capillary along the entrance to the ultrathin slab gel, the analyte solution can be introduced to the slab gel along its entire 4-cm width. If analyte solution is continuously flowing through the capillary, this translation results in continuous introduction of analyte into the gel. If, however, the analyte solution is injected as discrete plugs with buffer in between them, continuous translation of the capillary results in individual injection plugs of analyte solution being introduced into the thin slab gel.

The separation system is comprised of the ultrathin slab gel suspended across two buffer-filled reservoirs. The system is arranged so that the sample introduction end of the gel is held at ground and the negatively charged fragments of dsDNA migrate toward the positively charged anode. Thus, when a high-potential field is applied across the length of the ultrathin slab gel, electromigration of species is induced. The beveled entrance to the slab gel channel provides an optimal connection between the capillary and channel. DNA labeling by intercalation of ethidium bromide occurs in the separation system before the fragments pass through the detection zone.

As the separated DNA fragments migrate through the detection zone, the fluorescence from the intercalating dye is collected via a CCD, which maintains the spatial integrity of the fragments. Since the DNA solution is deposited sequentially across the entrance to the thin slab gel, the actual separation start time for each DNA fragment set is dependent on the time that the capillary is at the analogous position along the channel entrance.

Capillary-to-Gel DNA Transfer. The most significant aspect of this work is the capillary transfer and the relative ease with which it is accomplished. Many different parameters were considered in trying to understand the phenomena of the DNA plugs going from free solution migration in a capillary into the gel matrix. The most obvious, however, is the comparison of the matching of the capillary and gel potential fields. One would expect that this could be the single most important factor concerning lane width in the gel and/or sample loss.

An advantage of this system is that the length to the detector in the slab gel is variable and so, with a slight modification to the system, direct monitoring of the capillary-to-gel transfer region is possible. By focusing the laser line directly on the gel entrance, a rectangular region encompassing the capillary tip, the free solution transfer region, and a portion of the gel entrance was monitored with the CCD. Using this configuration, the potential field of the capillary has been varied across a wide range while the separation potential remained constant. There was no significant difference in either the apparent lane width on the gel (1 mm) or in the transfer of the DNA. A 1-mm-wide injection plug produced a 1-mm-wide plug at the detector. Lower capillary potential fields appear to result only in longer capillary transfer times. For this reason, a potential of −15 kV (250−300 V/cm)

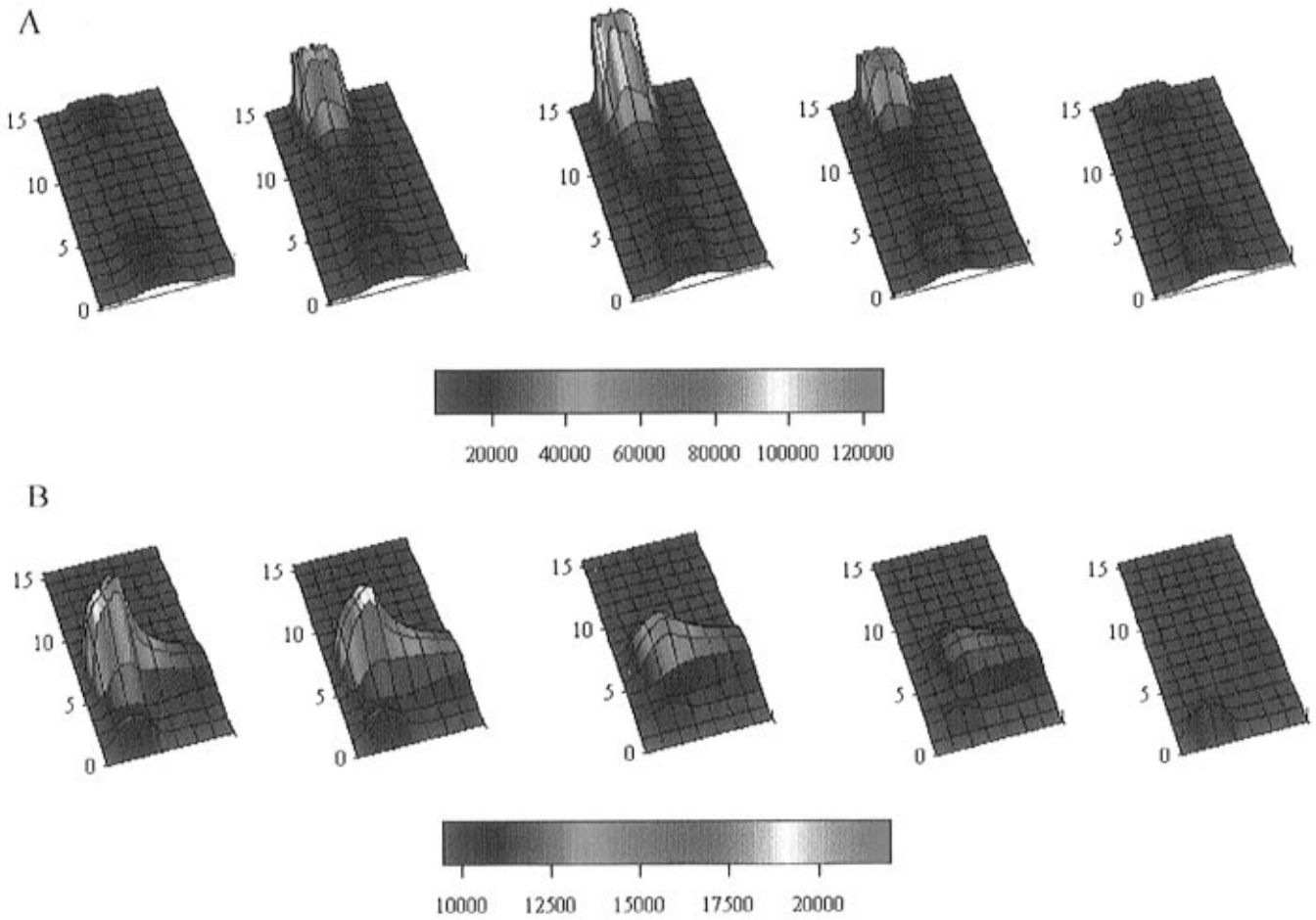

Figure 2. Surface plots of the capillary-to-gel DNA transfer region: width = 115 pixels; depth = 160 pixels. The capillary is at the lower/front portion of each image and the slab gel slab gel entrance at the top/back. V_{gel} = 125 V/cm; V_{cap} = 80 V/cm. The images were taken at ~25-s intervals, showing transfer of fluorescent DNA fragments from the capillary to the gel or solution, depending on the structural integrity of the capillary tip. (A) Capillary-to-gel alignment was correct, so the DNA migrates from the capillary directly into the gel entrance. In the last image, the DNA successfully entered the gel and was no longer visible in the viewing region. (B) Capillary tip was cracked, so the DNA exited the capillary tip and dissipated out into solution instead of migrating into the slab gel.

has been used in the capillary for all remaining experiments.

The capillary to gel monitoring did, however, highlight the importance of capillary alignment and integrity. Figure 2A shows five consecutive computer-reconstructed "snapshot" views of sample transfer from an intact capillary to the slab gel. In this case, the capillary has a flat tip and is accurately aligned with the slab gel entrance. In the first image, the capillary is just visible toward the front of the image. In the next three images, the labeled DNA fragments elute from the capillary and enter the slab gel at the back of the images. There is a brief period as the DNA fragments appear to be delayed in the gel entrance before migrating into the bulk gel. The final image in Figure 2A is taken after the DNA has actually entered the slab gel and migrated out of the gel entrance.

Figure 2B shows a similar reconstructed experiment where the capillary tip is cracked. In this case, the DNA is observed to dissipate into the solution rather than entering the slab gel. The system is optimized for detection of fragments within the ultrathin slab gel. This is quite apparent by a comparison of the scale bars in parts A and B of Figure 2. In Figure 2B, the DNA is within the beveled quartz entrance of the system, and the fluorescence does not transmit through this region optimally. Imaging the sample transfer between the capillary and the gel demonstrates that a poorly etched capillary can result in major sample loss at the interface between the capillary and the gel. Perhaps more importantly, these etched capillary experiments demonstrate that, with a well-etched capillary, sample transfer between the capillary and the gel can be highly efficient.

Injections of Discrete Plugs of dsDNA across the Slab Gel Entrance. Multiple injections of the same sample have been used to demonstrate the usefulness of this system and to study variability across the gel. In the experiments shown, six 3-s plugs of ΦX174 dsDNA (about 6 ng each) have been deposited 2 mm apart with approximately 3 min between consecutive plugs. The results of these injections are shown in Figure 3. This is a reconstructed top view of time versus CCD pixel number, showing evidence of six individual plugs which have been injected along the entrance to the thin slab gel. The individual bands on the plot are similar in appearance to the radiographic bands obtained in standard slab gel electrophoresis. The plot has not been corrected for the 3-min injection time bias; therefore, the data appear staggered (see next section). In this case, the capillary is started at the end of the channel, corresponding to pixel 1 in the detection zone, and translated toward pixel 400. It is quite apparent from the plug data that the DNA fragments migrate in a straight path from the injection to detection end of the channel, as expected. This demonstrates that structural barriers are not necessary between separation lanes and that multiple separations

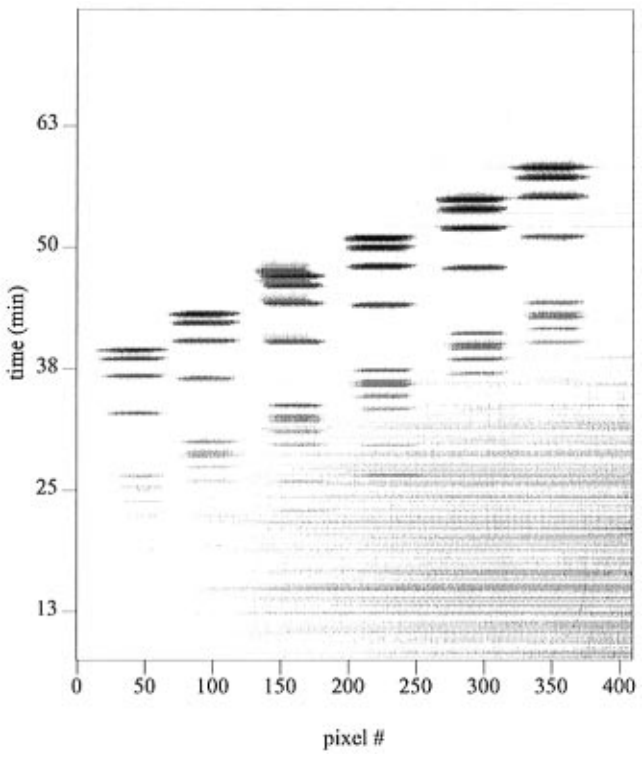

Figure 3. Plug separations of *Hae*III-digested ΦX174 DNA. The 6% polyacrylamide gel was approximately 57 μm thick, with a total gel length of 16 cm (11.5 cm to detector). The separation potential was 125 V/cm. The capillary was 42 cm long, with a tip of 100 μm; V_{cap} = 15 kV. Injections: 3 s at 15 kV every 3 min.

of dsDNA can be carried out on ultrathin slab gels with our sample acquisition and transfer method.

Normalization of Separations for Injection Time and Curvature along the Gel. Sequential injections along the entrance to the gel result in parallel separations that are offset in the initial time by the time it requires to translate the injection capillary. This can be corrected with appropriate software. In addition, we have also observed a small curvature of bands, "gel smiling", across our gel. The smiling phenomenon is an effect of nonuniform heating of the gel causing DNA fragments to travel faster in the warmer zones of the gel.[24] Although this smiling effect is not easily detected in Figure 3, it becomes more apparent when the migration times are corrected for injection time. To account for this variation, the data, after accounting for the staggered injection times, have been normalized to fragment 281 in injection 1. The plots of migration time versus band number shown in Figure 4 demonstrate the results of such normalization. In Figure 4A, the same fragments from sequential injections elute at a later time, in accordance with the injection time; hence, the 1353 fragment elutes 18.7 min later for the sixth injection than for the first injection. Normalization, as described above, eliminates this offset (Figure 4B). As an indicator of the normalization efficiency, a comparison of the bases in the fifth and sixth injections has been carried out. Regression analysis has been used to evaluate the precision of the fragments eluting between these injections. Based on the slopes of the regression lines of the normalized data and the time per base pair between fragments 1078 and 1353, the 1353 fragments in the two separations can only be determined to within 34 bp. However, using the same analysis

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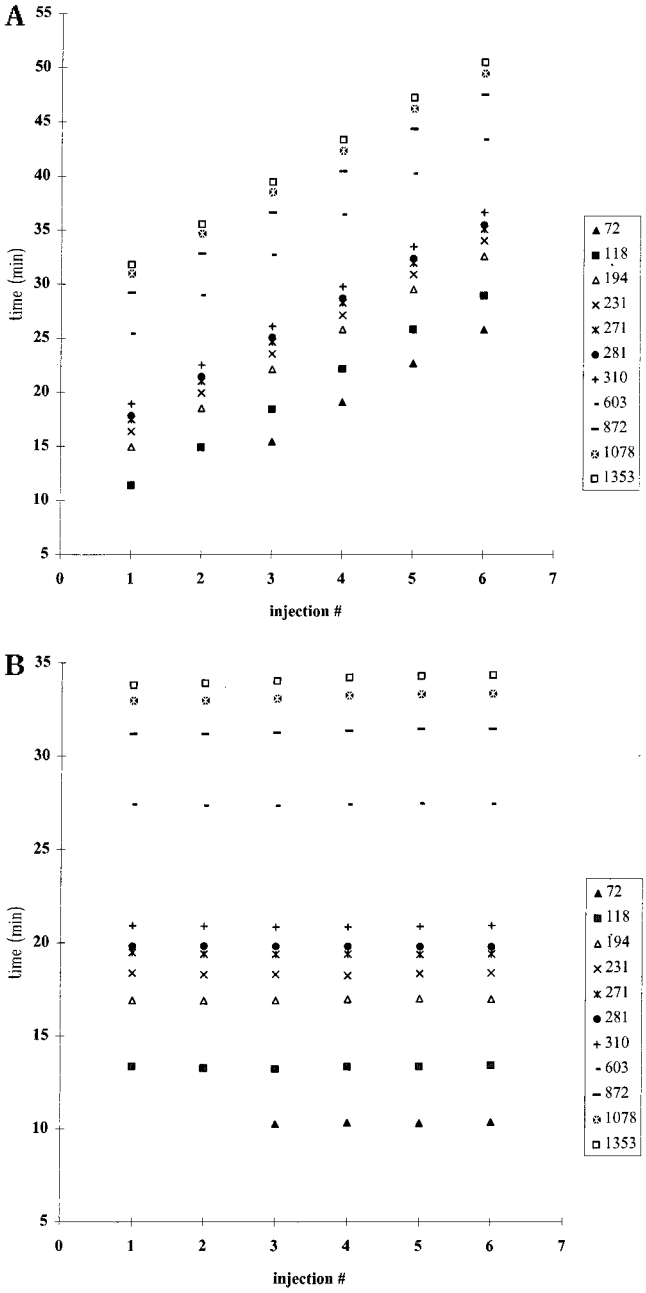

Figure 4. Plot of retention time versus injection number at the peaks of ΦX174 DNA. (A) Raw data. (B) Data normalized for injection time.

at the level of the 72 and 118 fragments, discrimination is better than 1 bp.

Although the normalization procedure described above works well for correction of data obtained on a single day, the day-to-day reproducibility is much more complicated. The distance from the capillary bore to the thin slab gel entrance can vary from day to day. This changes the amount of time before the DNA fragments enter the gel, causing an extra injection bias. In addition, slight variations in the gel composition and gel temperature cause differences in the migration rate of the DNA fragments which are difficult to compensate for. However, it is generally not necessary to correct for this type of day-to-day normalization, as sizing ladders are used as internal standards to account for these variations. This is the approach that will be used in the future for day-to-day normalization on our system.

Injection of Multiple Unique Samples. Using the electromigration injection procedure, it is just as easy to place different

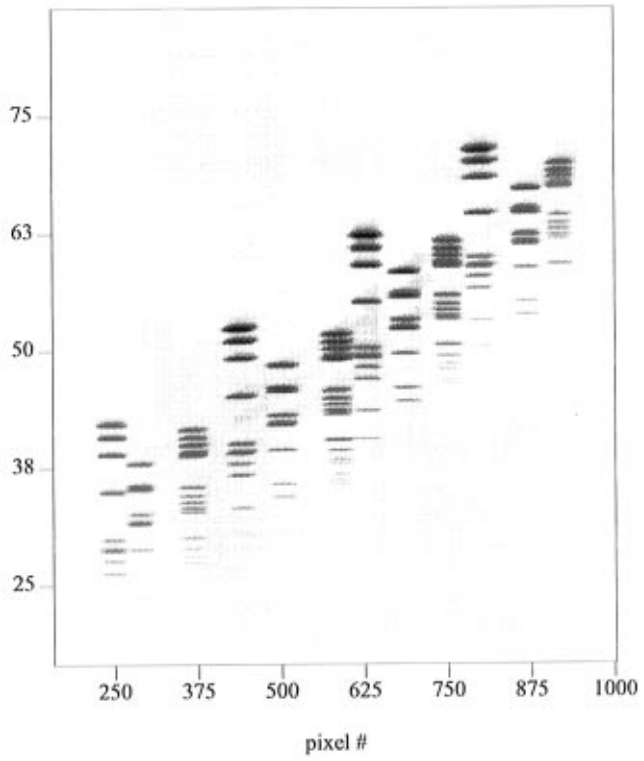

Figure 5. Twelve plug separations of *Hae*III-digested ΦX174, pUC18, and pBR322 DNA. Gel and separation conditions were the same as in Figure 3. The capillary was 50.3 cm long, with a tip of 90 μm; V_{cap} = 15 kV. Injections: 3 s at 15 kV every 3 min.

samples in each lane as it is to deposit the same one repetitively. In Figure 5, we demonstrate 12 unique injections of three different DNA restriction digests ranging in size from 8 to 1353 bp. Injections of *Hae*III-digested ΦX174, PUC18, and PBR322 have been made at intervals of 3 min. Once again, the capillary is continuously moved along the gel entrance at a rate of one 25.4 μm step every 2500 ms. The space between the lanes, therefore, is defined by the buffer spacing between the DNA samples. This separation demonstrates the ease with which different samples can be acquired and transferred onto the gel for electrophoresis. This process is a significant improvement over the attempted use of small sample wells when ultrathin slab gels are used. With the use of the capillary transfer method, it should be possible to acquire and transfer samples with apparent volumes below 1 pL.[23]

Figure 6 shows standard electropherograms extracted from 3D electropherograms similar to those shown in Figure 5. The 2D electropherograms are single-pixel observations, giving a better indication of the separation quality and the intensity of the individual peaks. In Figure 6, the corresponding restriction fragments of the ΦX174, PUC18, and PBR322 DNA are labeled. In all cases, the data have been corrected for the staggered injection time into the capillary. The time axis represents the time for the fragments to travel through the entire system, not just the separation time. The separation times in the channel alone are between 20 and 30 min at the potential fields employed. In addition, the resolution of the fragments is at the level expected for dsDNA in a 6% polyacrylamide gel. The 10-base differences are nearly baseline resolved for fragments 271 and 281 in Figure 6A and marginally resolved for fragments 257 and 267 in Figure 6B.

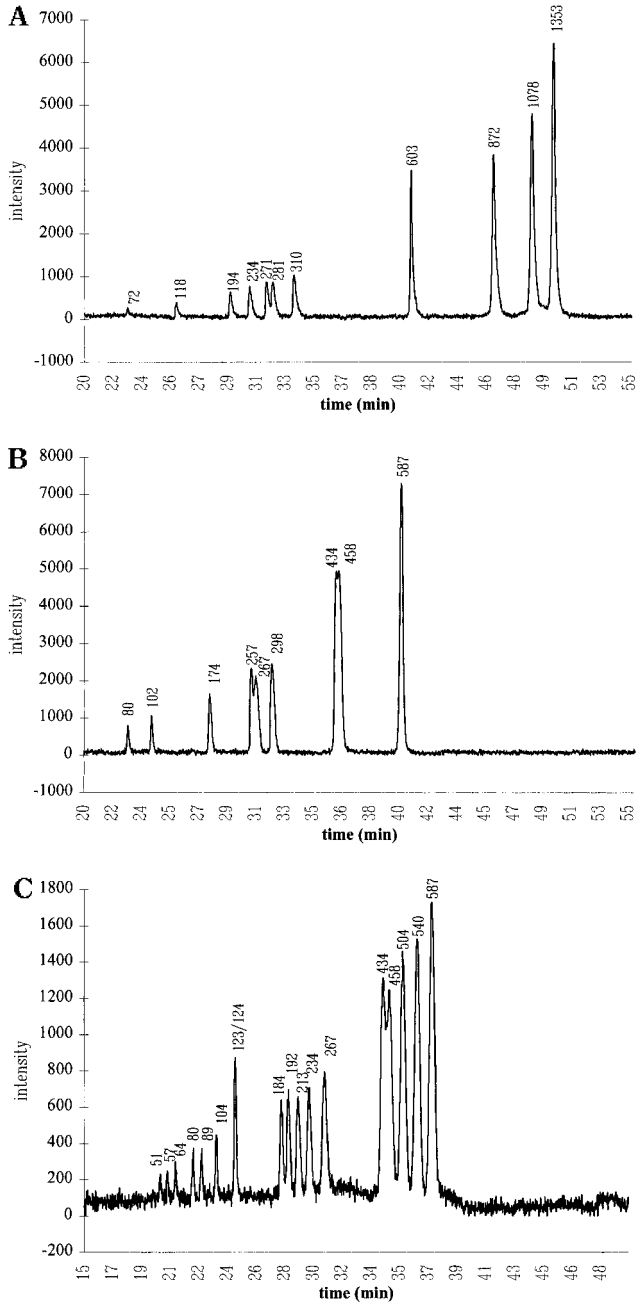

Figure 6. Signal versus time response at single pixels from data similar to those shown in Figure 5. Peaks have been labeled according to the number of DNA basepairs corresponding to that fragment. (A) *Hae*III-digested ΦX174. (B) *Hae*III-digested pUC18, showing nine of the 11 fragments. Fragments 11 and 18 were not visible above the baseline. (C) *Hae*III-digested pBR322, with 18 of the 22 fragments labeled. The smaller fragments (8−21) were not detected.

CONCLUSIONS

Continuous sample introduction and separation of dsDNA:dye complexes has been demonstrated in ultrathin slab gels. DNA ranging in size from 50 to 1400 bp has been separated with 6 ng of total DNA injected. Plug injections of DNA show that the lanes do not need to have a structural basis, as the DNA fragments travel linearly from injection to detection end. In addition, the lack of carryover between subsequent injections attests to the usefulness of the capillary injection system with multiple unique DNA samples.

The most significant advantage of our system over previously published ultrathin slab gel systems is the ability to repeatedly

introduce small-volume samples into the gel. Previous work with ultrathin slab gels has used small wells in the gel for sample introduction. This is problematic for gels less than 100 μm thick. The system described here is demonstrated with 57-μm-thick gels and offers the possibility to extend these separations into even thinner gels than those used here. The system design is relatively straightforward, and the sequential transferring of analytes allows for continual high throughput without the hurry-up and wait phenomenon typical of most parallel separations. This sample introduction system should be adaptable to many types of DNA separations and should be particularly useful as a means of transferring analytes onto the chip format.

In addition, this method circumvents the problems associated with casting sample wells in ultrathin gels. Gels as thin as 20 μm have been cast using a slightly different channel construction procedure, and separation strategies for these gels are underway. Eventually, the total number of lanes will be increased, with different DNA fragment sets separated in each lane. Based on the lane width of 1 mm obtained in these experiments and assuming optimum sample placement, 40 samples across the 4-cm-wide gel should be achievable. Although this is similar to the number of sample lanes used in conventional sequencers, the rapid and on-line sample introduction coupled to the ultrathin slab gels described here should eventually provide significant advantages in speed and automation.

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