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Analysis of DNA restriction fragments and polymerase chain reaction products towards detection of the AIDS (HIV-1) virus in blood[a]

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ABSTRACT

A high-performance capillary electrophoresis system with a polysiloxane-coated capillary and polymeric buffer additives was investigated for the analysis of DNA restriction fragments and polymerase chain reaction (PCR) products. Mobility data and Ferguson plots of the DNA fragments at different polymer (hydroxypropylmethylcellulose) concentrations indicated that effective molecular sieving was obtained consistent with existing data of conventional gel electrophoresis and with recent HPCE data. The precision and peak efficiency were excellent and the system was applied to the analysis of specific co-amplified DNA sequences (HIV-1 and HLA-DQ-alpha). After PCR, ultrafiltration was used in the sample preparation step to desalt the sample and to remove superfluous PCR reaction products. Electrokinetic injection was used for sample introduction into the capillary. The addition of ethidium bromide to the buffer resulted in longer migration times of DNA fragments and better peak resolution. During HPCE, an artifact associated with dilute DNA solutions leading to the appearance of extra peaks in the electropherogram was found.

INTRODUCTION

Understanding of diseases caused by viruses has continued apace with advances in molecular virology. As this understanding has increased, it has become obvious that more sensitive techniques are necessary to detect the pathogens, especially in latent infections. Without the ability to identify and measure accurately the amount of pathogen, it is very difficult to design an effective disease treatment. Further, to treat quickly an individual once specific therapy is known and to prevent the spread of the virus to other hosts, it is important to be able to detect that virus rapidly. The human immunodeficiency virus (HIV) is a good case in point.

In clinical settings, viruses are usually detected indirectly by identification of

[a] The authors would like to dedicate this paper to the memory of Bob Brownlee who passed away in February, 1991.

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antibodies to the virus in a host or by cytopathic effects *in vitro*. In latent infections, however, antibodies are often undetectable and *in vitro* assays are problematic. Until recently, it was impractical to look for the virus itself. At present, the polymerase chain reaction (PCR) technique allows detection of actual nucleic acids that are part of the HIV-1 virus [1]. PCR may generate millions of copies of specific DNA or RNA [usually 100–1000 base pairs (bp)], and multiple virus-specific sequences can be searched for and amplified in one reaction mixture.

The process of PCR can be entirely automated [2]. However, detection and quantification of the final amplified product remains a stumbling block to a rapid fully automated system of HIV detection. Conventional means of detection involving hybridization with [^{32}P]ATP labeled probes followed by polyacrylamide gel electrophoresis (PAGE) and autoradiography is time consuming, potentially hazardous and not readily amenable to automation. Chromogenic assays [3] have shown increased rapidity and potential for quantification. Unfortunately, only one pathogen can be detected at a time. Recent work with high-performance liquid chromatography has also shown promise for PCR fragment analysis [2,4].

To overcome the above problems in the analysis of PCR products, we have explored the use of high-performance capillary electrophoresis (HPCE). Our early results have shown promise to achieve one of our objectives, *viz.*, a fully automated analyzer for PCR fragments associated with HIV-1 [5–7]. The HPCE method, which involved a buffer system containing the cationic surfactant cetyltrimethylammonium bromide (CTAB), was used to detect simultaneously multiple retroviral DNA sequences [6]. However, a lack of understanding of the separation mechanism and poor precision were drawbacks leading us to pursue other alternatives.

With HPCE, Hjertén *et al.* [8] and Zhu *et al.* [9] showed that linear hydrophilic polymers can be used as buffer additives to achieve a "molecular sieving" effect to separate proteins and DNA fragments. Similar results, under conditions of electroosmotic flow, were obtained with a commercially available buffer system [10]. Recent work by Heiger *et al.* [11] involving polymerization of linear acrylamide within the capillary permitted high-efficiency separations of DNA fragments up to 12 000 base pairs. The viscous character of these 0%C or low %C media varied from a liquid, at 3% T[a], to a gel, at 12–14% T. Recent presentations by Heiger *et al.* [12] and Guttman and Cooke [13] showed further progress in this area. The term "physical" gels [14] has been used to describe these media for HPCE. Earlier, with classical gel electrophoresis, Bode [15–18] and Tietz *et al.* [19] demonstrated that linear polyacrylamide at low concentrations provided a molecular sieving medium for proteins and nucleic acids. The theoretical foundation for the molecular sieving mechanism was provided by Ogston [20] and Ornstein [21]. Polymers other than polyacrylamide, *e.g.*, polyethylene glycol [9,18], can also be used to achieve sieving in electrophoresis.

As a continuation of our previous work [5–7] dealing with the detection of PCR derived HIV-1 by HPCE, the focus of this paper is on optimization of the sample preparation and on the HPCE methodology. Ultrafiltration was used to remove salt and superfluous reaction components from the PCR samples and was instrumental in achieving better resolution and detectability. An OV-17-coated capillary in conjunc-

[a] C = g N,N′-methylenebisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 ml of solution.

tion with a buffer containing a polymeric additive was shown to yield excellent precision and efficiency. Selected polymers were compared in terms of sieving efficiency and the HPCE system was applied to the analysis of PCR-derived HIV-1 sequences. Finally, factors affecting precision and quantification are discussed.

EXPERIMENTAL

Apparatus

A P/ACE System 2000 (Beckman, Palo Alto, CA, USA) automated capillary electrophoresis instrument was used in the reversed polarity mode (negative potential at the injection end of the capillary) for all DNA separations. Detection was accomplished on-line by UV absorption at 260 nm. Data were acquired at 2–20 Hz and stored to disk; post-run analysis of data was performed using Beckman System Gold software (Beckman, San Ramon, CA, USA).

Materials

Most analyses were performed using a surface-modified fused-silica OV-17 capillary (J & W Scientific, Folsom, CA, USA), although OV-1, OV-225 and Carbowax coatings were also evaluated. The coating thickness was 0.1 μm as provided by the manufacturer. The capillary dimensions were 27 or 57 cm × 100 μm I.D. A 2–4-mm segment of polyimide coating was carefully removed from the tubing about 6.9 cm from the end before installation in a capillary cartridge (Beckman) for on-column detection.

HPLC-grade water prepared with a Milli-Q water system (Millipore, Bedford, MA, USA) was used for all water-containing reagents. All salts and other chemicals were either of molecular biology grade or of the highest purity available (Sigma, St. Louis, MO, USA). The basic running buffer system was 89 m*M* Tris borate–2 m*M* EDTA (pH 8.5) (TBE). To this basic buffer were added selected polymers at various concentrations: (1) hydroxypropylmethylcellulose, 4000 cP at 25°C (HPMC-4000), at 0.1–0.7% (w/w); (2) hydroxypropylmethylcellulose, 100 cP at 25°C (HPMC-100), at 0.5–1.0% (w/w); (3) polyethylene glycol (PEG), molecular mass 35 000, at 5.0% (w/w). Ethidium bromide (EB) was added to the buffer in some runs to a concentration of 10 μM. In addition, 0.1 *M* sodium borate (pH 8.35) (Beckman) was used for electroosmotic flow experiments. All buffers were filtered to remove particulates and degassed by sonication.

A Hae III digest of ϕX 174 RF DNA was purchased from New England Biolabs (Beverly, MA, USA). Low-molecular-mass DNA size standards were purchased from Bio-Rad Labs. (Hercules, CA, USA). Oligonucleotides for PCR and detection were synthesized by the Biomolecular Resource Center, University of California (San Francisco, CA, USA).

DNA sample preparation and PCR

DNA was extracted from the positive HIV-1 control cell line, U1.1, which harbors one copy of the HIV-1 provirus per cell, as well as two copies of HLA DQ-α [5,6]. The negative control was the promonocyte U937, the cell line from which U1.1 was derived. Following extraction, DNA was serially diluted and PCR amplified for both HIV-1 (115 bp of the gag gene) and HLA DQ-α (242 bp), as described previously

[6]. The 100-μl PCR amplified samples were divided into two 50-μl aliquots. One aliquot was analyzed by hybridization with ^{32}P-labeled probes, gel electrophoresis and autoradiography [5,6]. The second aliquot was subjected to ultrafiltration: Centricon-30 or Centricon-100 filters (W.R. Grace, Amicon Division, Beverly, MA, USA) were used to desalt and concentrate PCR-amplified samples. Typically, a 50-μl volume was diluted with 2 ml of distilled water, and centrifuged for 30 min at 5000 *g* in a Model GP or GPR centrifuge (Beckman). This procedure was repeated one to three times for most experiments. Following ultrafiltration, the DNA was analyzed by capillary electrophoresis.

The DNA molecular mass markers were not desalted; instead, they were diluted with water prior to injection. The concentration of total DNA ranged from 0.38 to 25.0 μg/ml.

Capillary electrophoresis

The capillary cartridge was inserted into the P/ACE instrument and subsequently filled with the running buffer and allowed to equilibrate to a preset temperature. The temperature was set at 25°C for most experiments; HPCE runs at 20, 35 and 45°C were also investigated. Samples were loaded onto the autosampler to await automatic injection.

Both positive-pressure and electrokinetic sample injections were used. Pressure injections were performed at 3.44 MPa (0.5 psi) for 15–99 s. Electrokinetic injections at negative polarity setting of the HPCE instrument were performed at 35–175 V/cm for 1–40 s. Separations within the coated capillaries were performed at negative polarity under constant voltage for each run, ranging from 175 up to 350 V/cm. Typically, runs lasted from 15 to 45 min.

The coated capillary was rinsed with two capillary volumes of running buffer after each run, and was then ready for the next injection. The capillary was stored dry overnight in the P/ACE 2000 system, after initiating a programmed sequence which involved rinsing with water and drying with nitrogen.

Electroosmotic flow in untreated and coated capillaries was measured by introducing (via pressure injection) a neutral marker, benzyl alcohol, into the capillary. The zone velocity was determined from the migration time of the neutral marker and the length of the capillary.

RESULTS AND DISCUSSION

Role of capillary coating

In previous work [5] we used polyethylene glycol (Carbowax)-coated capillaries and a CTAB buffer to separate DNA restriction fragments. With this separation system, larger DNA fragments showed greater mobilities than smaller fragments. As a consequence of the high separation efficiency, this method appeared to offer very good sensitivity. This is an important consideration for clinical samples where low viral loads often are encountered. However, as we pointed out [5], the mechanism of separation is complex and not completely understood and the precision was relatively poor. In view of this, we decided to investigate an HPCE system similar to that of Zhu *et al.* [9], involving the use of linear polymers added to the buffer.

While the capillary used by Zhu *et al.* [9] involved a proprietary polyacrylamide

coating, we explored the use of various commercially available polysiloxane-coated capillaries. These 100 μm I.D. capillary columns were originally designed for use with supercritical fluid chromatography but have recently found use in HPCE also [5, 22, 23]. Encouraging preliminary results with a relatively polar phase, DB-WAX (polyethylene glycol) and a commercially available buffer system were recently published by our group [5,6]. Recently, we have found other non-polar and intermediate polarity polysiloxane coatings (*i.e.*, OV-17, OV-1, OV-225) to be preferable as these phases yielded better precision and efficiency. The OV-17 coating in conjunction with various polymeric buffer additives was used for all the experiments in this paper.

It is important to note that, in addition to its sieving effect, the polymer additive causes an additional dynamic coating on the capillary wall and, consequently, suppression of the electroosmotic flow (EOF). Using the OV-17-coated capillary and a 0.100 *M* borate buffer (without the polymeric additive), the mobility of the EOF was appreciable, *i.e.*, $5.6 \cdot 10^{-4}$ cm^2/V s. However, replacing the borate buffer with a buffer containing a cellulose derivative resulted in a 98.5% reduction in the EOF mobility. Returning to a borate buffer without the polymer additive, again a negligible EOF was determined. Evidently, the cellulose derivative had dynamically modified the wall of the capillary. This result is consistent with the work of Hjertén *et al.* [8] and Zhu *et al.* [9] on coated capillaries. Earlier, in work with isotachophoresis, Reijenga *et al.* [24] found that various polymeric additives (*e.g.*, cellulose derivatives) were very effective in reducing the EOF in untreated fused-silica capillaries. It seems therefore, that in our case the polysiloxane coating on the fused-silica wall compounds this effect, thereby providing an extra guard against EOF.

HPCE of DNA restriction fragments

An example of a separation of DNA restriction fragments (25 μg/ml) using an OV-17-coated capillary and 0.5% HPMC-4000 as buffer additive is shown in Fig. 1. The Hae III restriction fragments of ϕX 174 DNA provide a good reference for PCR samples because it covers the range of 72–1353 base pairs. Electrokinetic injection was used for the separation in Fig. 1A. Pressure injection was used in Fig. 1B. DNA fragments migrated with mobilities that decreased with increasing base pair number. This is an advantage in terms of analysis speed. If the reverse were the case, then the elution of the larger DNA fragments would occur before the peaks of interest (*i.e.*, in our case the 100–300 bp range) could be detected. In addition, the early peaks generally yield higher plate counts because their residence times in the column are shorter [25].

Note that the electrokinetic injection yields a more efficient separation than the pressure injection. The inset shows *ca.* $1.8 \cdot 10^6$ plates/m for the 118 bp fragment. With electrokinetic injection from a low ionic strength solution, predominantly sample components (*i.e.*, DNA fragments) migrate into the capillary where they are effectively stacked against the higher viscosity run buffer [21]. No sample bias, commonly found with electrokinetic injection [26] should take place as the DNA fragments have equal mobilities in free solution (*i.e.*, same mass-to-charge ratio). With the pressure injection on the other hand, a water plug, in addition to the DNA, is introduced into the capillary, causing less effective stacking and apparent peak broadening. Pressure injection is also limited in HPCE for highly viscous run buffers as the sample injection volume is inversely proportional to the viscosity of the buffer [27].

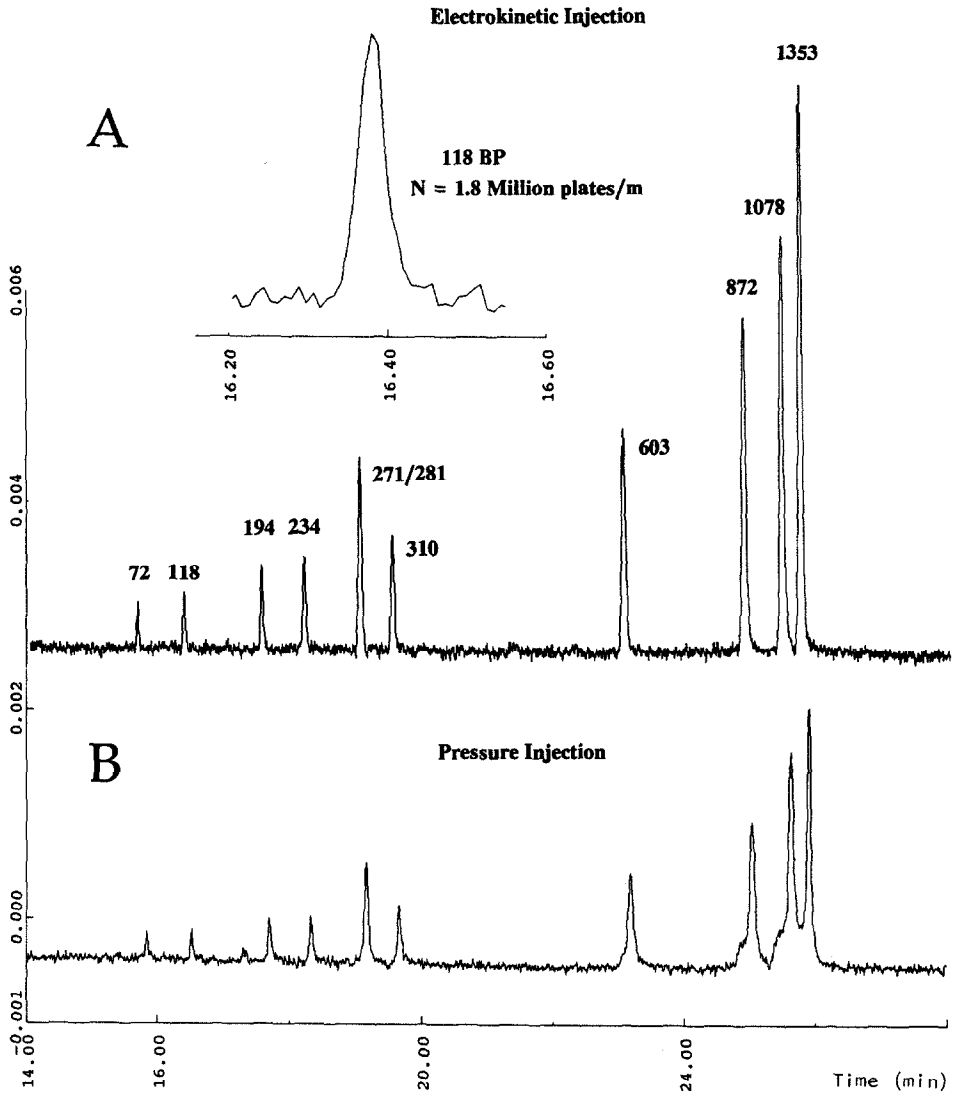

Fig. 1. Separation of a Hae III restriction digest of ϕX 174 DNA using (A) electrokinetic injection (5 s at 2 kV) and (B) positive pressure injection (60 s at 3.44 MPa). The inset shows an enlargement of the peak representing the 118 bp DNA fragment from (A) with a peak efficiency of $N = 1.8 \cdot 10^6$ plates/m. Buffer, 89 m*M* Tris borate–2 m*M* EDTA (pH 8.5)–0.5% HPMC-4000; column, OV-17-coated capillary, 57 cm × 100 μm I.D., 50 cm effective length; applied voltage, 10 kV; UV detection at 260 nm; temperature, 25°C; sample concentration, 25 μg/ml.

It also can be seen in Fig. 1 that, in spite of the high resolution, no separation between the 271 and 281 bp fragments was obtained. This was also the case with other HPMC formulations (*i.e.*, different concentrations and polymer molecular mass). However, baseline separation of this pair was recently achieved by Heiger *et al.* [11]

and Guttman and Cooke [13] with linear polyacrylamide-filled capillaries. Interactions between the DNA fragments and the HPMC polymer may account for the difference with the polyacrylamide system. In this regard, it is interesting that co-elution or even reversal of migration times between specific closely spaced DNA fragments was demonstrated in HPCE with linear polyacrylamide capillaries [11–14]. Electric field strength [11] or temperature-related effects [28] on the tertiary structure of DNA may be responsible for these phenomena. In our case, separations at capillary temperature settings other than 25°C (up to 45°C and down to 15°C) did not resolve the 271 and 281 bp fragments.

Role of intercalating agents

Intercalating agents such as ethidium bromide (EB) bind to DNA by inserting themselves between the base pairs of the double helix and have been used routinely in gel staining [29]. Fig. 2 shows the effect of adding 10 μM ethidium bromide (EB) to the buffer on the separation of ϕX 174 (note: EB was not added to the sample). It can

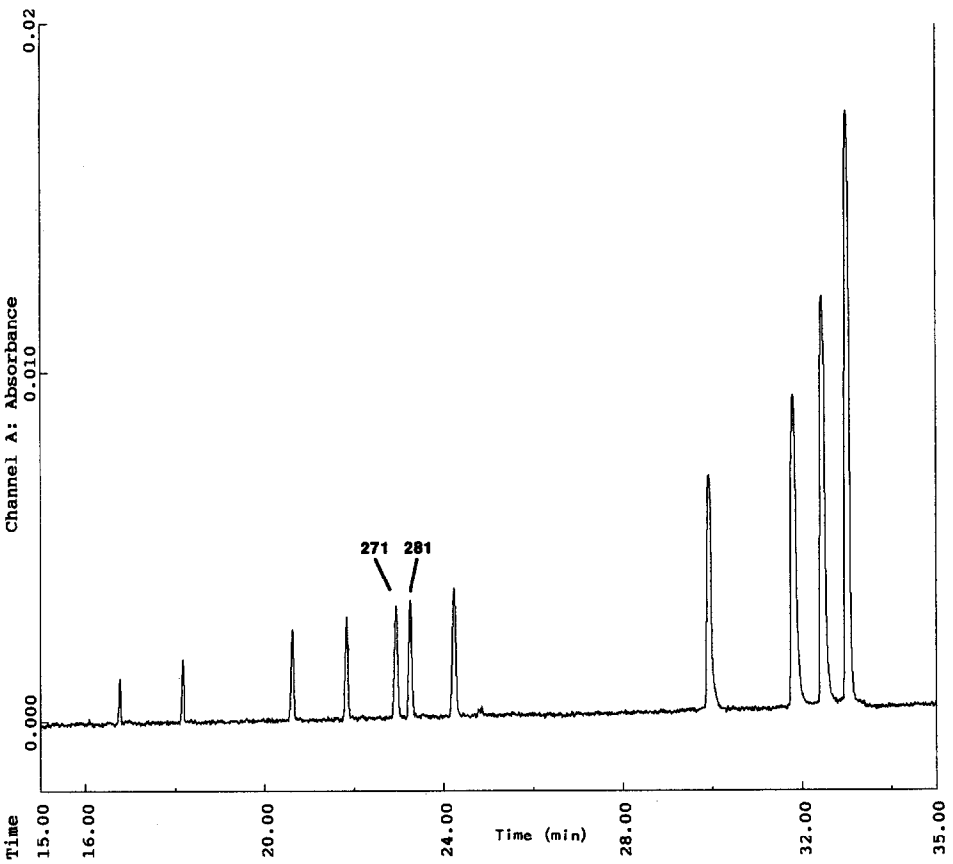

Fig. 2. Effect of ethidium bromide on DNA separation. Run buffer as in Fig. 1 with addition of 10 μM ethidium bromide. Sample concentration, 10 μg/ml. Electrokinetic injection at 2 kV for 10 s. All other conditions as in Fig. 1A. Note the separation of the 271 and 281 bp DNA fragments.

be seen that now, compared with Fig. 1, the 271 and 281 bp pair is completely baseline resolved while the migration times are longer. In addition, on average *ca.* 35% higher peak heights are obtained. The peak capacity is also greater with the EB separation: between the window of 118 and 194 bp, DNA fragments with a 3.0 bp difference are baseline resolved, as opposed to 5.3 bp in the separation without EB.

It is well known that EB alters the structure of DNA: the spacing of succesive base pairs is increased, the sugar phosphate backbone is distorted and the pitch of the double helix is decreased [29]. In addition, EB may alter the charge on the DNA during electrophoresis. These structure and charge-related effects may therefore alter the sieving behaviour of DNA fragments in a "physical" gel, as is evident in the longer migration times in Fig. 2 compared with Fig. 1. The structural changes in the DNA cause an apparent higher UV absorbance of the intercalated DNA. While these preliminary results with EB look promising, more work is necessary to determine its utility in PCR fragment analysis by HPCE. In particular, questions regarding quantification need to be addressed. For example, with HPCE, Kasper *et al.* [30] found the linear response range with UV detection to be dependent on the the EB concentration.

Detection limits

The HPCE system in Fig. 1 was used to determine the detection limits for specific DNA sequences by means of a dilution series. Electrokinetic injection was used. Successive dilutions with water were made from a stock solution containing 100 μg/ml total DNA, 10 m*M* Tris and 2 m*M* EDTA. Fig. 3 shows linear calibration

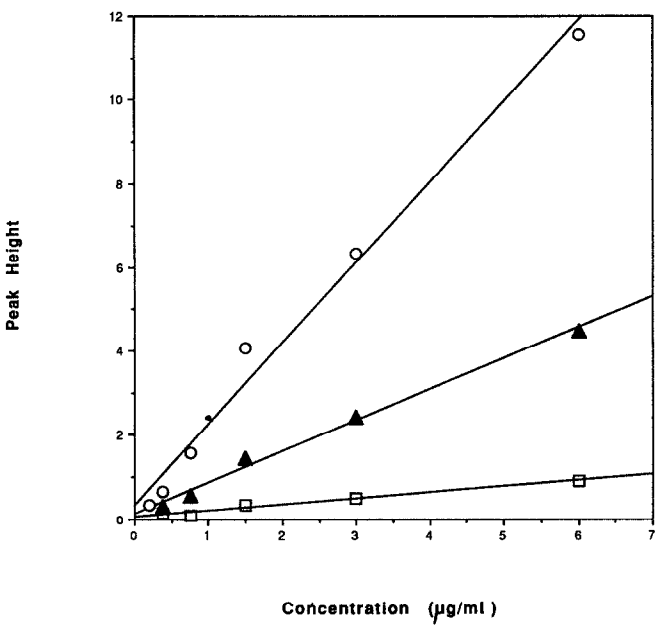

Fig. 3. Calibration graph (peak height *vs.* DNA concentration) for a dilution series in the relevant clinical range. Data were generated using the conditions in Fig. 1A. □ = 1353 bp; ▲ = 271/281 bp; ○ = 118 bp. The minimum detectable concentration for the 118 bp fragment is *ca.* 8 ng/ml.

graphs (peak height *vs.* concentration) for three selected peaks in the clinically relevant, low concentration range. At higher concentrations, the plots become non-linear owing to the increasing Tris salt concentration in the ϕX 174 sample, which, in turn, affects the amount of sample introduced into the capillary with electrokinetic injection [31]. Under optimum conditions, the minimum detectable concentration for the 118 bp restriction fragment is *ca.* 8 ng/ml at a 2:1 signal-to-noise ratio.

Molecular sieving

Next, the sieving efficacy of selected polymers was studied for the separation of DNA restriction fragments. Two cellulose-based polymers, HPMC-100 and HPMC-4000, were examined, in addition to polyethylene glycol (PEG) with an average molecular mass of 35 000. According to the manufacturer, the two hydroxypropylmethylcelluloses have average molecular masses of 26 000 and 90 000, respectively (the viscosities of 2% solutions at 25°C are 100 and 4000 cP, respectively). In addition, for comparison, data based on HPCE of DNA restriction fragments with low cross-linked polyacrylamide (3% T, 0.5% C) were included (data courtesy of Dr. Guttman, Beckman Instruments). In the latter instance, the polymerization was performed *in situ* (*i.e.*, within the capillary), as opposed to adding the polymer to the run buffer.

Molecular sieving with linear polyacrylamide was studied by Bode [15–18] and Tietz *et al.* [19]. It is now generally accepted that cross-linking, *per se*, is not required in order to achieve molecular sieving. The extent of sieving depends on the viscosity of the medium (through Stoke's law, when the viscosity is caused by a low-molecular-mass compound, *e.g.*, glycerol) and on the chain length of the polymer. The mechanism is thought to involve a random association of flexible, inert fibers in which molecules can migrate through dynamic pores. Similar molecular sieving may take place in HPCE with polyacrylamide, cellulose derivatives, PEGs and other polymers, as our data and those of others [9–14] indicate.

With cross-linked polyacrylamide gels, a plot of mobility *vs.* logarithm of molecular mass (or bp number) for nucleic acids should yield a straight line [15,16]. For HPMC-4000 and HPMC-100, mobility *vs.* bp plots (on a semi-logarithmic scale) are shown in Fig. 4A and B at different polymer concentrations. It can be seen that S-shaped curves, with a linear middle portion, are obtained. The shallowness of the slope of the curve is a measure of the molecular sieving power of the medium [16]. The steeper the slope of the curve, the less effective is the sieving. For example, it can be seen in Fig. 4A that at low concentration, *i.e.*, 0.1% (w/w), HPMC-4000 is not an effective sieving agent. The higher the polymer concentration, the better is the sieving, as found by others for linear and weakly cross-linked polyacrylamide [11,16,19]. The apparent deviation from linearity with the larger DNA fragments has also been observed by other workers [11,28]. The mobilities of these fragments are higher than expected and reportedly due to electric field effects [32]. As Heiger *et al.* [11] pointed out, field effects on mobility may occur in HPCE at much smaller fragment sizes than found in conventional gel electrophoresis because the field strength in HPCE is generally much higher.

It is interesting to compare the sieving of the DNA fragments with the HPMC-4000 and the HPMC-100. From Fig. 4A and B it can be seen that at the same polymer concentration [0.5% (w/w)] , greater mobilities are obtained with the low-

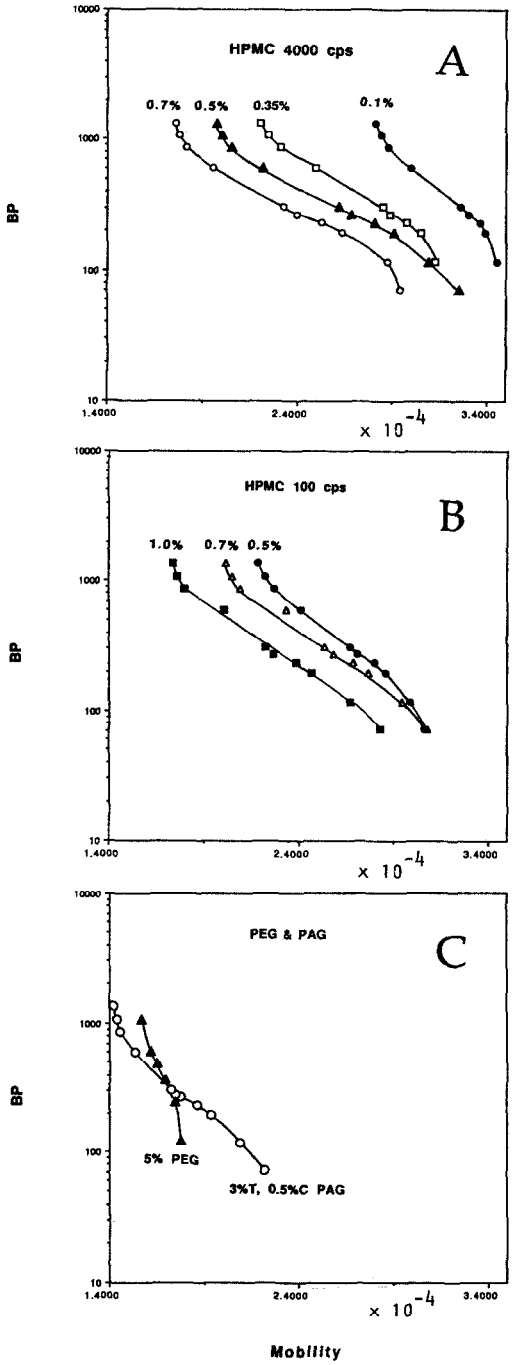

Fig. 4. Effect of different additives on molecular sieving. The plots (semi-logarithmic scale) show the dependence of mobility on the base pair number. DNA fragments from the Hae III restriction digest of ϕX were used as base pair markers. Polymeric additives are (A) HPMC-4000 at 0.1, 0.35, 0.5 and 0.7%; (B) HPMC-100 at 0.5, 0.7 and 1.0% (C) 5% PEG and polyacrylamide (3% T, 0.5% C).

molecular-mass HPMC. This is in agreement with the findings of Bode [16], who determined higher mobilities for nucleic acids in short-chain linear polyacrylamide compared with long-chain polyacrylamide. In another paper, Bode [18] demonstrated a similar chain length dependence for molecular sieving with PEG solutions.

Fig. 4C shows the data obtained with PEG (average molecular mass 35 000) and low-cross-linked polyacrylamide. Compared with the S-shaped polyacrylamide curve, the PEG plot is relatively steep. Hence the selected PEG is not an effective sieving agent for HPCE of DNA restriction fragments. It is not expected that other, lower molecular mass PEGs would provide an improvement in this respect [18]. Examination of Fig. 4A and C reveals that mobilities are lower with the polyacrylamide system than the HPMC-100 and HPMC-4000 systems. This is due to specific differences in the pore structure of these media under the specified conditions. It should also be noted that excellent peak efficiency was obtained with the polyacrylamide capillary [13]. As noted by Heiger *et al.* [11], *in situ* polymerization may have an advantage over a system where the polymer is dissolved in the buffer beforehand in that much higher polymer concentrations (and therefore higher viscosities) can be obtained in the capillary. For example, single-stranded oligonucleotides were separated with a 9% T, 0% C polyacrylamide and capillaries with up to 14% T were used [11]. Our approach of adding the polymer to the run buffer, although simple and reproducible, is limited in that it is more difficult to fill the capillary with viscous buffers. Therefore, only relatively non-viscous buffers can be used in our procedure.

Next, to characterize the sieving effect further, a plot of log mobility *vs.* HPMC-4000 concentration was made. Similar Ferguson plots (log mobility *vs.* %T) are routinely generated in PAGE. They are used to determine the size selectivity of migrating species and to calculate "free" mobilities. Fig. 5 shows the data obtained

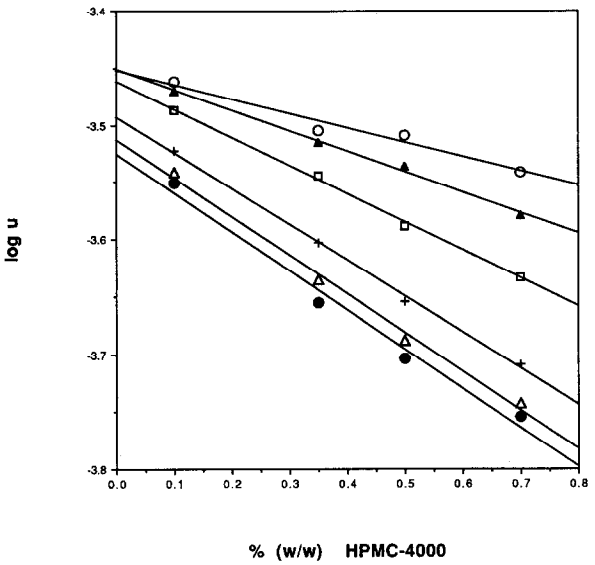

Fig. 5. Ferguson plot [log mobility *vs.* % (w/w) polymeric additive] for a buffer containing HPMC-4000 as the sieving component. Mobilities of selected ϕX 174 Hae III digest fragments were used to generate the plot. ○ = 118 bp; ▲ = 194 bp; □ = 310 bp; + = 603 bp; △ = 872 bp; ● = 1353 bp.

with linear regression analysis for selected DNA fragments. A similar plot was obtained for the data with the HPMC-100 (not shown). It can be seen that better sieving is obtained at higher polymer concentrations as the plots converge at low polymer concentrations. Better linearity, as evident from the correlation coefficients (0.995–0.998), is obtained for the 194, 310 and 603 bp fragments. Poorer linearity is obtained with either very small or larger DNA fragments (correlation coefficients 0.961 and 0.984, respectively).

Using a 0.089 *M* TBE buffer without a polymer additive, we determined the free solution mobility of all DNA fragments to be $3.87 \cdot 10^{-4}$ cm^2/V s ($\log u = -3.41$). This value is substantially higher than the free solution mobilities found from the intercepts of the Ferguson plots. Non-linearity of Ferguson plots at low polymer concentration has been observed with linear polyacrylamide by Tietz *et al.* [19] in a moving boundary electrophoresis system and recently by Heiger *et al.* [11] in HPCE.

Factors affecting precision

The precision results [expressed as relative standard deviation (R.S.D.) in migration time, peak area and peak height] obtained with DNA restriction fragments and an HPMC-4000 containing buffer system are summarized in Table I. Electrokinetic injection and the conditions in Fig. 1A were used. Two cases are distinguished. In the first, consecutive runs were performed while sampling each time from a different vial. In the second, runs were carried out by sampling from the same sample vial. It can be seen from Table I that in both cases excellent precision in migration times can be obtained (R.S.D. < 0.2%). Clearly, this is an improvement over our previous HPCE system involving CTAB buffers [5,6]. Moreover, the described HPCE system is very stable from day to day and we have used the capillary with the HPMC buffer on a daily basis for months without problems.

TABLE I

R.S.D. VALUES FOR FRAGMENTS OF DIFFERENT SIZE (BASE PAIR NUMBER)

Conditions as in Fig. 1A except for sample concentration, as indicated. MT = Migration time.

Base pairs	R.S.D. (%)								
	Vial-to-vial (10 μg/ml, $n=7$)			Vial-to-vial (5 μg/ml, $n=11$)			Within-vial (25 μg/ml, $n=9$)		
	MT	Height	Area	MT	Height	Area	MT	Height	Area
72	0.16	5.71	8.43	0.24	18.98	18.59	0.16	5.46	8.75
118	0.15	7.63	6.04	0.23	17.03	15.19	0.16	6.00	7.38
194	0.09	2.73	3.82	0.21	14.18	18.00	0.19	5.94	6.34
234	0.09	2.91	3.27	0.21	14.09	15.09	0.19	5.28	3.49
271/281	0.09	2.18	3.97	0.22	12.81	13.80	0.20	4.92	6.65
310	0.09	3.26	3.08	0.22	13.85	14.66	0.19	5.70	5.73
603	0.07	2.04	3.87	0.24	27.15	27.98	0.21	4.79	5.96
872	0.08	1.37	2.96	0.22	12.83	14.55	0.22	5.44	6.50
1078	0.08	1.06	1.91	0.22	12.22	13.79	0.22	5.22	5.32
1353	0.09	2.39	5.18	0.23	15.08	14.28	0.22	5.32	9.16

Peak-area precision was found to be concentration dependent. The results in Table I were obtained with a relatively low DNA concentration (10 μg/ml). Better peak-area precision for specific fragments was found at higher DNA concentrations. However, the lower concentration range is of more interest for our clinical application. It was also found that a relatively low injection voltage (*e.g.*, 2 kV) is preferable for the early-eluting 72 and 118 bp fragments, as at high injection voltages distorted peak shapes were obtained which, consequently, affected the precision (note that in Table I the peak-area precision for the 72 and 118 bp fragments is worse than for the other fragments). Possibly this may be due to localized (Joule) heating during the electrokinetic injection from a low-conductivity solution, which, in turn, could lead to (partial) denaturation of specific DNA fragments.

During the course of this work, we found that better peak-area precision was obtained by sampling from different vials (aliquoted samples). When sampling was done from the same vial, peak areas and heights for the first run were significantly larger than those obtained for subsequent runs. Consequently, the R.S.D. will be affected, as indeed is shown in Table I. This phenomenon has also been observed in our laboratory for oligonucleotide analysis with 3% T, 5% C gel-filled capillaries. In this instance electrokinetic injection is a requirement. Further work is in progress to elucidate fully the reason for the decreasing peak area with consecutive injections from the same vial.

Artifacts with HPCE

Another factor occasionally affecting peak-area and peak-height precision is an artifact related to low concentrations of total DNA in the sample. We have repeatedly found that certain DNA restriction fragments in the ϕX 174 sample, *e.g.*, the 603 bp fragment, at low total DNA concentration (*i.e.* < 10 μg/ml), yield proportionally lower peak areas when compared with other fragments. The effect is demonstrated in Fig. 6A for a sample with a 3 μg/ml total DNA concentration. The salt concentration in the sample was 0.03 *M* Tris. It can be seen that two "ghost" peaks (indicated by arrows) appear after elution of the last 1353 bp DNA fragment. At the same time, the peak height of the 603 bp fragment is considerably lower than expected, and peak shapes for the 72 and 118 bp fragments have deteriorated. When buffer salt (12.5 m*M* TBE) was added to the sample (Fig. 6B), less sample was injected and the peak efficiency decreased, as expected. However, the ghost peaks disappeared.

The ghost peaks were not present when samples at higher total DNA concentrations (*e.g.*, 25 μg/ml) were injected (results not shown). Conceivably, during the HPCE procedure, partial or complete denaturation of double-stranded DNA into single strands may have taken place, resulting in the ghost peaks in Fig. 6. As the mobility of single-stranded DNA is lower than that of corresponding double-stranded DNA, the peaks marked by arrows in Fig. 6 may represent denatured DNA originating from the 603 bp fragment. Other DNA restriction fragments samples (*e.g.*, a Bio-Rad Labs. DNA size standard) showed similar abnormalities when injected at low concentrations. However, it should be noted that the above phenomena were not always repeatable. The irreproducibility greatly affected the peak-area precision measurements at low concentrations. Hence it appears to be an artifact, possibly related to the presence of endonucleases, which denature DNA, or to the absence of certain double-helix-stabilizing cations.

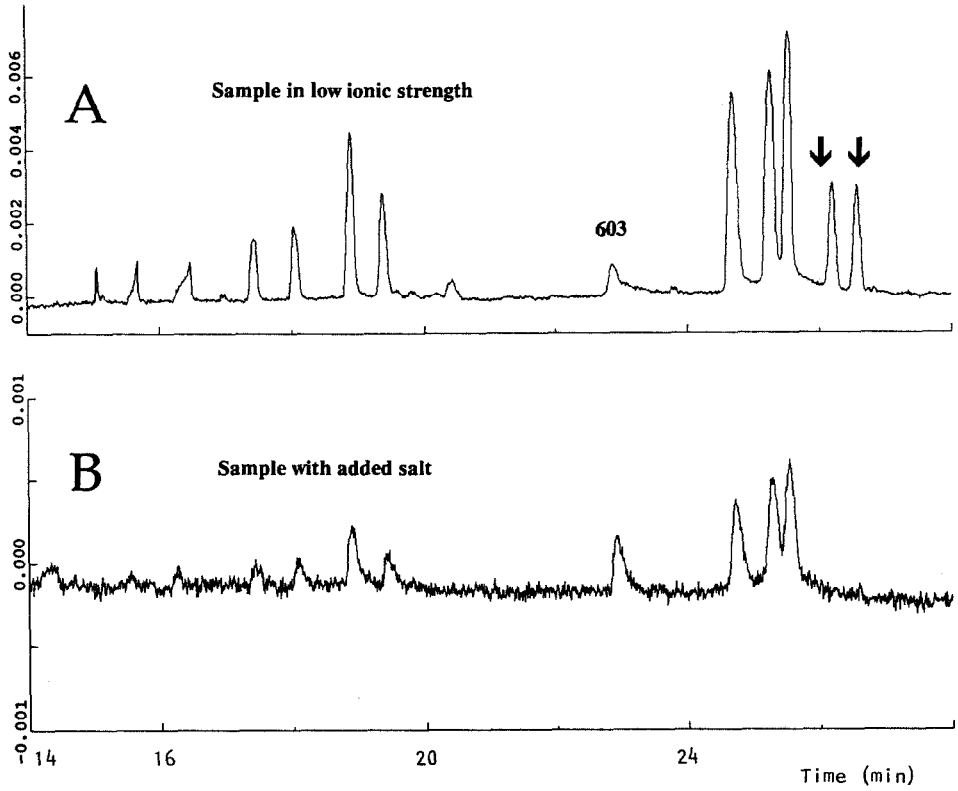

Fig. 6. Artifacts related to DNA and/or salt concentration. (A) Two "ghost" peaks (arrows) appear, while the 72 and 118 bp peaks are broadened. Sample concentration, 3 μg/ml with 0.03 m*M* Tris present. (B) Same sample with added buffer salt (12.5 m*M* TBE). Note the disappearance of the extra peaks. However, the other peak shapes have deteriorated owing to less efficient zone focusing. Conditions as in Fig. 1A.

Application to PCR amplified HIV-1

After establishing optimum separation conditions and good precision, the HPCE system was used to analyze PCR-amplified products. An HIV-1 positive control cell line, U1.1, was used for DNA extraction. This cell line harbors one copy of the HIV-1 provirus per cell in addition to two copies of HLA-DQ-α. HLA-DQ-α is present in all healthy cells and may therefore serve as an internal standard in clinical assays. Using specific primers [5], a 115 bp portion of the gag region of HIV-1 and a 242 bp sequence of HLA-DQ-α were co-amplified by means of 35 cycles of the PCR. Unfortunately, in our case, PCR reaction mixture components (*e.g.*, primers, primer–dimers, dNTPs), when electrophoresed with HPCE, co-migrate with the PCR products of interest. In addition, the PCR product sample has an appreciable ionic strength, which, as pointed out in the discussion in Fig. 6B, renders the electrokinetic injection less efficient with regard to peak shape.

To solve the above problem, we used ultrafiltration with the Centricon system (see Experimental and ref. 33) to concentrate and de-salt the sample. This resulted in removal of low-molecular-mass material and peak sharpening. Salt is removed by

dilution of the 50-μl volume PCR sample with 2 ml of glass-distilled water. After centrifugation with the Centricon system, the sample is reconstituted to its original volume of 50 μl, resulting in a theoretical 97.5% reduction in salt. An identical second Centricon procedure results in a 99.94% reduction in salt, a third procedure in a 99.99% reduction, etc. The effect of ultrafiltration with the Centricon system is shown in Fig. 7. The separations in Fig. 7 were obtained at 20 kV, resulting in less efficient separations but faster analysis times than for the 10-kV runs in Fig. 1. Faster analysis

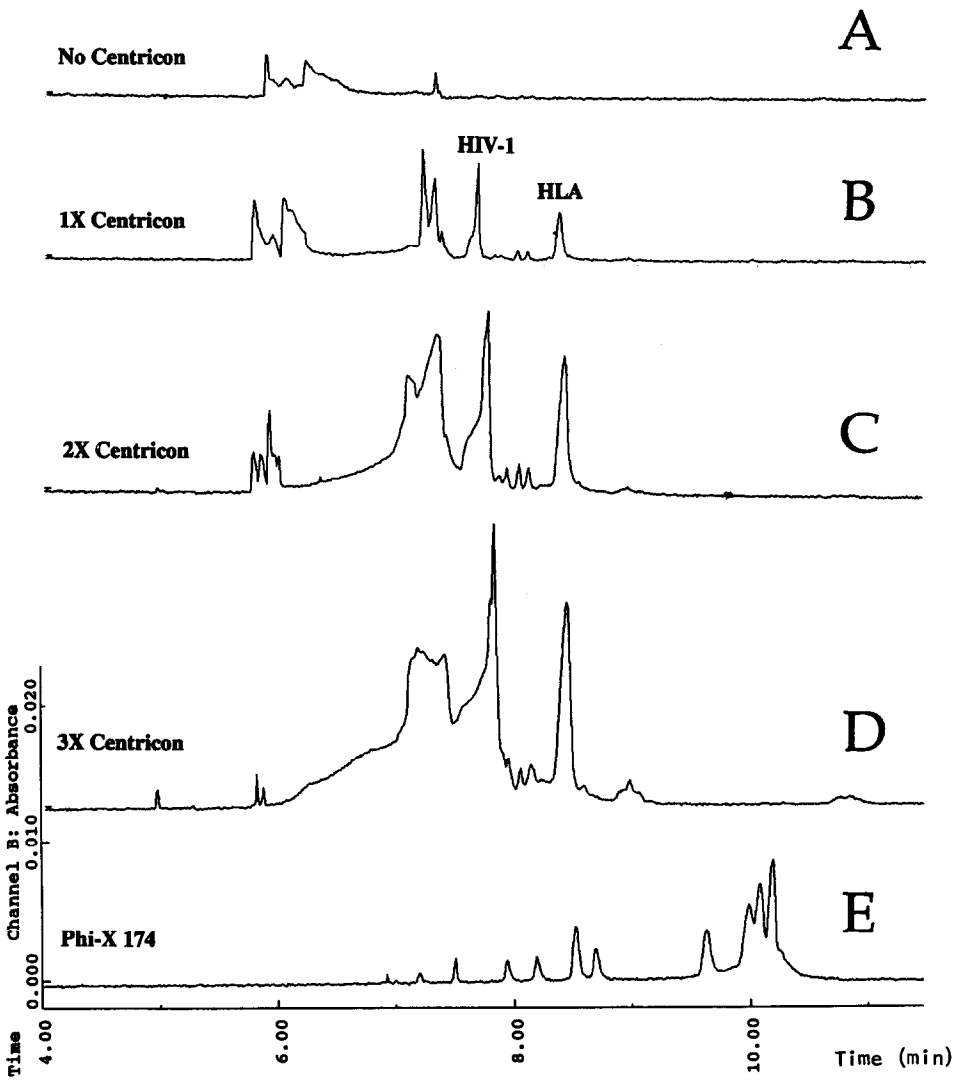

Fig. 7. Effect of ultrafiltration on the PCR-amplified DNA peaks. (A) Untreated sample (no ultrafiltration), a co-amplified HIV-1, HLA (115 and 242 bp, respectively) positive control. Concentration is achieved by increasing the number of desalting steps from (B) 1 × to (D) 3 × . (E) ϕX 174 DNA standard. Run voltage, 20 kV. Sample was injected electrokinetically at 10 kV for 10 s. All other conditions as in Fig. 1A.

times again at the expense of efficiency, can also be obtained by using shorter capillaries.

Fig. 7A shows the electropherogram of the PCR sample when no ultrafiltration was used. Fig. 7E shows the ϕX 174 DNA standard as reference. The peaks of interest are marked as HIV-1 and HLA. By ultrafiltration through the Centricon filters, primers and dNTPs are selectively removed, yielding prominent HIV-1 and HLA peaks (Fig. 7B). As pointed out above, the ultrafiltration procedure can be repeated to allow further concentration and desalting. This effect is shown in Fig. 7C and D. The nominal molecular mass cut-off of the membranes is the point at which more than 90% of the single- or double-stranded pieces of DNA will be retained [33]. For the Centricon-30 and Centricon-100 microconcentrators, the single-stranded DNA cut-off is 60 and 100 bases, respectively. For double-stranded DNA the cut-off is 50 and 125 bp, respectively. Consequently, for our application (HIV-1 sequence, 115 bp) the Centricon-30 is most suitable. The application of the above method to clinical samples is currently in progress.

CONCLUSIONS

We have achieved excellent separation efficiency and precision with sieving buffers for DNA restriction fragments. The HPCE system using a polysiloxane-coated capillary and polymeric buffer additives is relatively simple and straightforward. For clinical assays, precision is a key requirement and the current system, although less efficient in terms of plate count, is preferable compared to our previous HPCE system [5,6]. Our data indicate that certain linear polymers (*e.g.*, HPMC), when added to the buffer, provide effective molecular sieving of DNA fragments. The data are consistent with recent work in HPCE on polyacrylamide capillaries by Karger's group [11,12] and Ogston's model of molecular sieving [20]. Other polymers may work as well or better for different applications, *i.e.*, protein separations [9]. While our separation system offers the benefit of simplicity, the *in situ* polymerization system desribed by Heiger *et al.* [11] and Guttman and Cooke [13] offers interesting possibilities for a wide range of nucleic acids. In these systems, viscosity (%T) can be easily manipulated to optimize for a particular DNA analysis and, as in our case, a high separation efficiency is possible. Further work is necessary to study the role of ethidium bromide (and possibly other intercalating agents) in HPCE separations of DNA, in particular with regard to the quantification of real samples. Our ultimate goal is to achieve a sensitivity level that would enable us to detect a single copy of the target HIV-1 sequence in a blood sample.

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