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Capillary electrophoresis of polymerase chain reaction-amplified DNA using fluorescence detection with an intercalating dye

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ABSTRACT

The application of the fluorescent intercalating dye, YO-PRO-1 to the analysis of polymerase chain reaction-generated DNA fragments from 120 to 400 base pairs in size was investigated. Analysis of samples was performed using a non-gel sieving buffer containing Tris–borate, hydroxyethyl cellulose, and the intercalating dye. Minimum detectable concentrations were less than 500 pg/ml DNA. For samples requiring resolution of four base pairs or less, it was necessary to add a second intercalating agent, ethidium bromide to the buffer. Using this procedure, a number of loci of interest in genetic typing were examined.

INTRODUCTION

The polymerase chain reaction (PCR) enables the amplification of specific fragments of DNA that are present in trace amounts of biological material [1–3]. Capillary electrophoresis (CE), with its high throughput and resolving power, has great potential for application to the analysis of such samples. Generally these types of analyses have been performed using gel-filled capillaries due to the high resolution required [4–8].

Recently, CE separation of DNA fragments has been developed using non-gel sieving buffers [8–12]. These buffers can be flushed out of the capillary following each run. Analyses carried out using buffers containing hydroxyethyl cellulose (HEC) have provided impressive separation efficiencies [12]. We have used similar buffer systems to obtain 4 base pairs (bp) resolution of a mixture of alleles in the 200–300 bp size range with UV detection [13].

The goal of this work has been to develop the application of laser induced fluorescence to the analysis of PCR-amplified DNA. The DNA fragments used in this study are a series of PCR-amplified alleles resulting from variable number tandem repeats (VNTRs) with repeat units 4 bp in length. The resolution of DNA fragments with differences in size this small presents a particularly challenging separation.

The use of laser fluorescence has the potential to improve the sensitivity and selectivity of the analysis. The PCR process can generally produce sufficient material for analysis by UV detection, (μg/ml quantities). However, due to the sample extraction and amplification process, occasionally an insufficient sample may be presented for analysis. Also, residual salts in the PCR mixture can inhibit the electrokinetic injection process, reducing sensitivity. These factors make the improved sensitivity obtainable though fluorescence attractive.

Since DNA has little native fluorescence, PCR products must either be tagged using fluorescent primers, or stained using a fluorescent dye [14,15]. The use of fluorescent dyes to stain or intercalate the DNA is inherently more sensitive

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than tagging via the PCR as the maximum number of fluorescent tags per PCR product is only two, one for each primer. Fluorescent intercalating dyes, however, can bind at multiple sites on the DNA molecule. For example, thiazole orange was found in one experiment to bind to the DNA fragment at a ratio of one dye molecule per every two bp of DNA [6].

We began this study with an investigation of the concentration dependence of YO-PRO-1, {1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole) - 2-methyidene]-quinolinium)-3-trimethylammonium propane diiodide}, when used as a visualizing and intercalating agent for the laser-induced fluorescence detection of double-stranded DNA fragments. In our earlier work with UV detection, we had used ethidium bromide in the buffer to improve the resolution by intercalating the DNA [13]. Our goal was to replace the ethidium bromide in the buffer with YO-PRO-1, the YO-PRO-1 providing florescence detection as well as resolution enhancement through intercalation. The YO-PRO-1 dye has a strong binding constant to DNA and little intrinsic fluoresence unless bound to DNA [17].

Experiments were performed to determine if the resolution obtained would permit the analysis of the small differences in size of DNA fragments used in genetic typing. The studies revealed that while high sensitivity can be obtained with this procedure, for resolution better than 6 bp, the addition of ethidium bromide was still necessary.

MATERIALS AND METHODS

Sample preparation

The pBR322 *Hae*III digested DNA (Sigma, St. Louis, MO, USA) was diluted to 0.005 μg/ml with deionized water. Biological samples were prepared by extracting the DNA [18] and amplifying via the PCR [1]. Samples containing mixtures of alleles were prepared by amplification and recovery of specific alleles present in blood samples obtained from specific donors. The amplified DNA was diluted to 2 ml with deionized water and desalted using dialysis via centrifugation at 1000 *g* through a Centricon 100 ultrafiltration device (Amicon, Boston, MA, USA). Following ultrafiltration, the sample was dialyzed further by pipeting it onto a 0.1-μm VSWP membrane filter (Millipore, Bedford, MA, USA) which was floating in a petri dish filled with deionized water. Dialysis was carried out on the floating filter for 30 min [19]. The dual filtration process was developed to remove salts and excess primers which can interfere with sample injection by preferentially migrating into the capillary during electrokinetic injection [13]. Following the filtration steps, the samples were pretreated with YO-PRO-1 (Molecular Probes, Eugene, OR, USA) by diluting 1:10 with distilled water containing 50 ng/ml of the dye.

Capillary electrophoresis

A P/ACE 2050 capillary electrophoresis instrument (Beckman, Palo Alto, CA, USA) was used with a Laser Module 488 argon ion laser (Beckman). The instrument was operated in the constant current mode at 38 μA (approximately −13 kV) and 25°C. The separation buffer was prepared using a modification of a procedure published by Nathakarnkitkool *et al.* [12]. The column was 60 cm effective length × 0.1 μm I.D. DB-17 (J&W, Folsom, CA, USA) with 0.1 μm phase thickness. The buffer was prepared in the following manner: 0.1 m*M* EDTA (Sigma) was added to 100 m*M* Trizma (Tris) base (Sigma) and 100 m*M* boric acid (Sigma) pH adjusted to 8.7 with cesium hydroxide (Sigma). HEC with a viscosity range of 86–113 cP for a 2% solution (Aldrich, Milwaukee, WI, USA) was dissolved in this buffer at a concentration of 1.0% (w/v), and the solution was filtered through a 0.45-μm cellulose acetate disposable filter (Corning 25943; Corning Glass Works, Corning, NY, USA). Prior to analysis ethidium bromide (Sigma) was added to the run buffer to a concentration of 1.27 μ*M* and YO-PRO-1 dye was added to a concentration of 50 ng/ml. In the analysis of pBR322 *Hae*III digested DNA, ethidium bromide was not added and the YO-PRO-1 concentration was 5 ng/ml.

When fresh capillaries were prepared, the new columns were hand-flushed by attaching a syringe to the end of the capillary column and forcing buffer through the length of the capillary. Each day, the capillary column was rinsed for 20

min with HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ, USA) and then for 20 min with buffer. Prior to each individual run, the columns were rinsed for 2 min with methanol and 6 min with buffer [13]. Following the methanol and buffer wash, the samples were injected electrodynamically at 5 kV for 10 s. Typical run times were 35 min.

RESULTS AND DISCUSSION

The YO-PRO-1 dye was selected for these studies due to its compatibility with the argon-ion laser excitation 488 line, good photostability, and a partition coefficient between DNA and a 10% ethanol solution of $8 \cdot 10^6$ [17]. While there is also a dimer available with an even higher partition coefficient of $6 \cdot 10^8$, (YOYO-1), we did not pick this dye for our initial experiments due to concern about irreversible binding to the column walls [20]. Another advantage to YO-PRO-1 is that it has virtually no fluorescence unless bound to DNA [17]. This property was especially significant given that our intention was to add the dye directly to the sieving buffer. Unbound fluorescent dye without this property could significantly reduce sensitivity and enhance the background signal.

Initial experiments were carried out in order to determine the proper ratio of dye to DNA. Work carried out by Schwartz and Ulfelder [14] on thiazole orange (a dye similar in structure to YO-PRO-1) suggested that the ratio of dye to DNA would be critical in maintaining separation efficiency. Ethidium bromide did not produce such dramatic effects, although there is a rela-

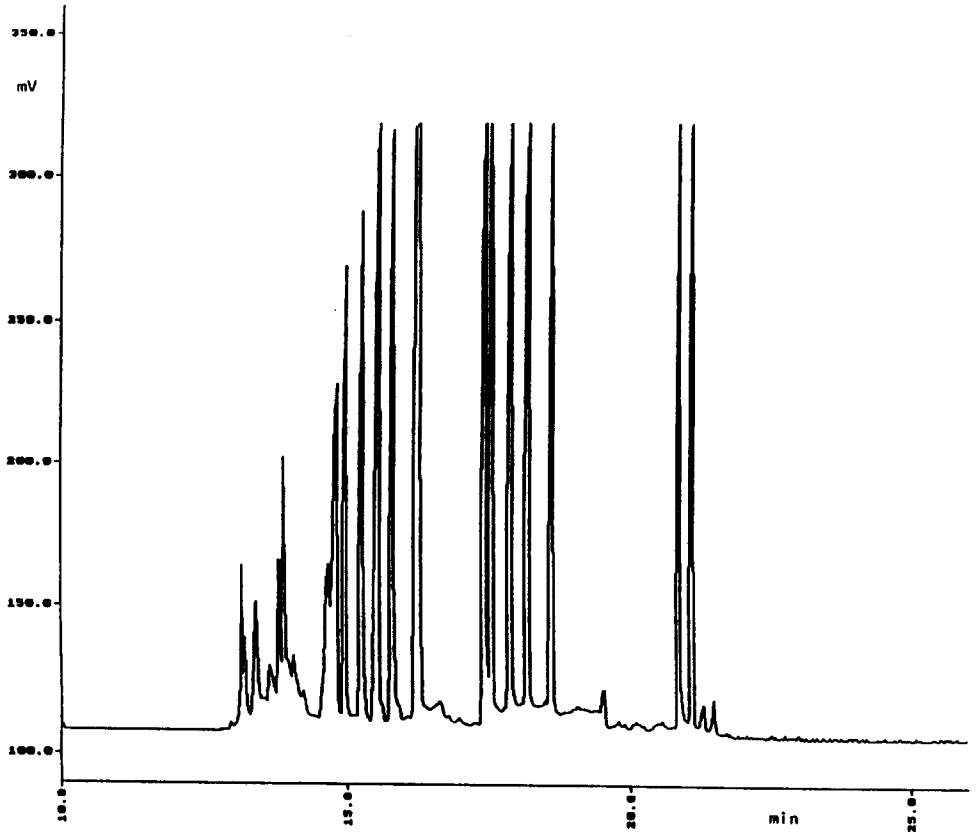

Fig. 1. pBR322 *Hae*III digested DNA (50 μg/ml) showing effect of depletion of intercalating dye in the buffer. Note the reduced fluorescence of the late eluting peaks. Buffer: 100 m*M* Tris–borate, pH adjusted to 8.7 with CsOH, 0.75% HEC, 1.27 μ*M* ethidium bromide, 50 ng/ml YO-PRO-1.

tionship between efficiency and the concentration of this dye in the buffer [13,21].

It is our opinion that studies of this nature must be carried out with extreme care, as the dynamic interactions between column surface and buffer can have an overwhelming effect on the quality of the separation, obscuring secondary effects such as small differences in dye concentration. Different capillary columns can produce dramatically different separations and dye molecules and buffer components can irreversibly bind to the walls of these columns.

Initially we examined 50 μg/ml of the pBR322 sample using 50 ng/ml YO-PRO-1 dye in the presence of 1.27 μg/ml ethidium bromide (Fig. 1). This figure shows an effect which we ascribe to the depletion of the YO-PRO-1 dye as the DNA moves through the buffer. Note how the three late-eluting peaks show minimal fluorescence. Presumably, dye throughout the column has been depleted before these peaks have had a chance to come in contact with it. This effect illustrates the high binding constant of YO-PRO-1 with DNA. We also saw effects resulting from adsorption of the dye to the walls. Even after removing both ethidium bromide and YO-PRO-1, and washing out the column for 30 min with methanol and dye-free running buffer, significant fluorescence was seen for early eluting peaks in a subsequent analysis.

Further work revealed that when ethidium bromide was not present, the optimum concentration of the YO-PRO-1 dye was 5 ng/ml with a DNA concentration of 500 ng/ml. At dye concentrations higher than this, the DNA peaks of 434 bp and above broadened and became poorly resolved. However, even at a concentration of 100 ng/ml the resolution of peaks from 80 to 267 bp was not greatly diminished. Fig. 2 shows the effect of YO-PRO-1 concentration on the selectivity of the method. Note that only at a YO-PRO-1 concentration of 5 ng/ml were the peaks of 434 bp and 458 bp separated.

The effect of changing the concentration of the pBR322 DNA standard from 500 to 5 ng/ml, with a YO-PRO-1 concentration of 5 ng/ml, was minimal. In fact, with DNA:dye ratios from 100:1 to 1:10, the number of theoretical plates determined for the 267 bp peak varied only from

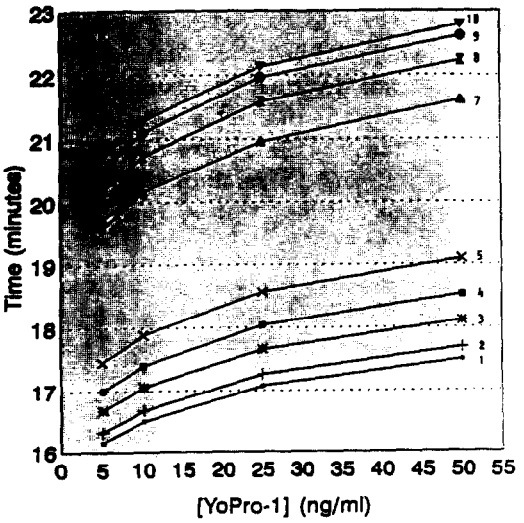

Fig. 2. Selectivity effects of increasing YO-PRO-1 dye concentration for 5 ng/ml pBR322 *Hae*III digested DNA. 1 = Peak 184; 2 = peak 192; 3 = peak 213; 4 = peak 234; 5 = peak 267; 6 = peak 434; 7 = peak 458; 8 = peak 504; 9 = peak 540; 10 = peak 587.

$4 \cdot 10^5$ to $7 \cdot 10^5$, and resolution ranged from 6 to 9 bp. These experimental data do not suggest a major effect on efficiency due to DNA:dye ratio.

In general for the pBR322 *Hae*III digested DNA we obtained excellent results with DNA concentration ranges from 5 to 500 ng/ml using 5 ng/ml of YO-PRO-1 dye in the buffer. Fig. 3 gives an example of the pBR322 standard at the 5 ng/ml concentration. The minimum detectable concentration at which the last 11 major peaks could be determined was 500 pg/ml of pBR322 *Hae*III digested DNA using a 30-s injection at 5 kV. This high sensitivity is due to the dye's near-zero fluorescence unless bound to DNA [17].

Analysis of samples

In this laboratory, we are currently exploring a number of genetic markers for application to forensic analysis. By combining all the major alleles present in a sample population into a single reference standard, the ability of the CE method to resolve any given pair of alleles can be experimentally verified. Three loci containing VNTRs with repeat units of 4 bp were selected for this study to determine if a viable technique

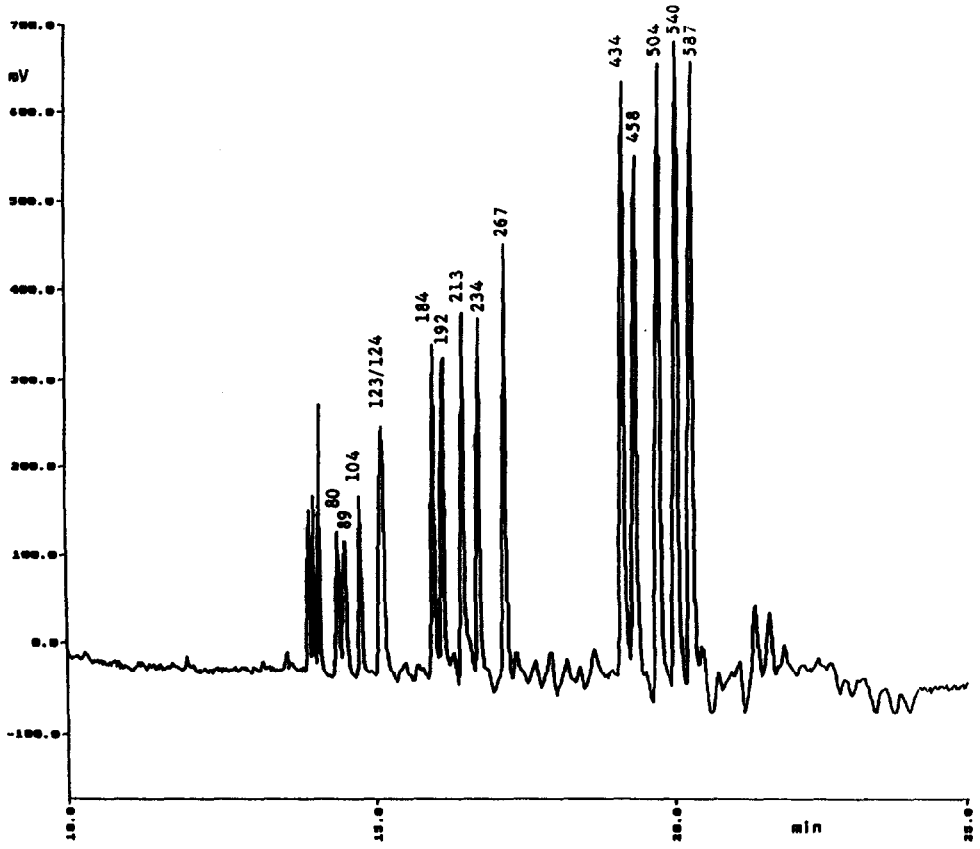

Fig. 3. pBR322 *Hae*III digested DNA (5 ng/ml). Buffer as in Fig. 1 except no ethidium bromide in buffer and YO-PRO-1 concentration of 5 ng/ml.

for the analysis of such samples could be developed using CE with laser fluorescence and non-gel sieving buffers.

Each of these samples contained a series of alleles which consist of 4 bp repeats. The first locus examined was HUMTHO1 (TC-11) located on chromosome 11. The alleles used in this sample contained 7 DNA fragments ranging from 183 to 207 bp [15,22]. One problem was immediately apparent. The resolution obtained in preliminary experiments with pBR322 DNA and a YO-PRO-1 concentration of 5 ng/ml was not adequate to resolve these samples. Resolution of 4 bp or better was needed. Fig. 4 shows that even with the concentration of HEC increased to 1%, it is not possible to resolve a mixture of alleles from the TC-11 genetic locus.

In a further attempt to improve resolution, ethidium bromide was added to a concentration of 1.27 μM. The results yielded resolution of nearly 4 bp, but sensitivity was reduced more than 10-fold. This concomitant loss of sensitivity may be the result of two factors: the higher background fluorescence of ethidium bromide, and a competition between ethidium bromide and YO-PRO-1 for active sites on the DNA molecules.

To help improve the sensitivity, the concentration of YO-PRO-1 in the buffer was increased to 50 ng/ml and the PCR samples were diluted 10:1 with distilled water containing 50 ng/ml YO-PRO-1 dye prior to analysis. Fig. 5 shows the result using a buffer containing 1.27 μM ethidium bromide and 50 ng/ml YO-PRO-1. Peak intensity is equivalent to that shown in Fig. 4, with all 7 alleles clearly distinguished. Thus, the addition of ethidium bromide is necessary for improved efficiency even when YO-PRO-1 dye is

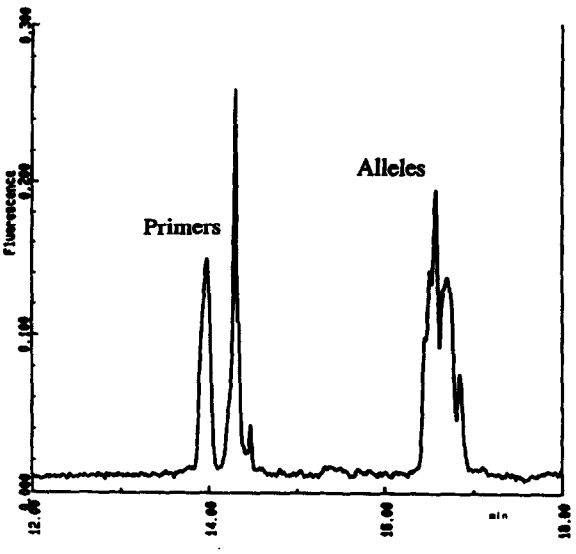

Fig. 4. Mixture of 7 HUMTHO1 alleles amplified via the PCR with each allele 4 bp apart. Buffer: 100 m*M* Tris–borate, pH adjusted to 8.7 with CsOH, 1.0% HEC, 5 ng/ml YO-PRO-1.

present. Increasing the HEC polymer concentration to 1% further enhanced the column efficiency.

The improved resolution of the sample peaks

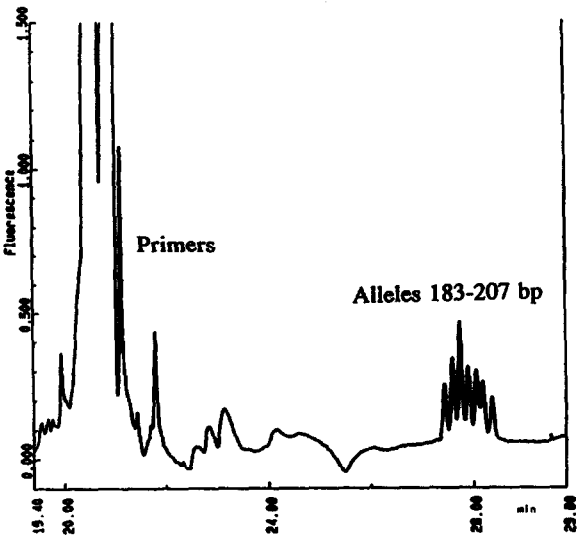

Fig. 5. Mixture of 7 HUMTHO1 alleles amplified via the PCR with each allele 4 bp apart. Buffer: 100 m*M* Tris–borate, pH adjusted to 8.7 with CsOH, 1.0% HEC, 5 ng/ml YO-PRO-1, 1.27 μM ethidium bromide. Sample diluted 1:10 with 50 ng/ml YO-PRO-1.

may result from a specific effect of ethidium bromide. In addition to being usable at a higher concentration than YO-PRO-1, ethidium bromide has a single positive charge and a nonpolar end. The ethidium bromide may reduce wall effects of the capillary by ion pairing with residual silanol groups with have been missed in the column coating process. Subsequently, the exposed non-polar end may lower electroosmotic flow and increase overall resolution. The higher ethidium bromide concentration in the buffer may also play a role in the resolution enhancement, as the YO-PRO-1 dye concentration may not be sufficient to completely intercalate the DNA.

While the major improvement in sensitivity came as a result of the increase in YO-PRO-1 concentration, pretreatment of the sample with the YO-PRO-1 dye did appear to improve sensitivity as well. Further experimentation is required to determine the actual rates of exchange between bound and unbound dye in the presence of ethidium bromide and other buffer components. It has been shown that similar dye–DNA complexes dissociate under gel electrophoresis conditions in the absence of free dye [23]. As shown above (Fig. 1), in the presence of excess DNA the amount of YO-PRO-1 in the run buffer can be locally depleted, resulting in variable fluorescence intensity. For this reason pretreatment of unknown quantities of DNA with YO-PRO-1 is desirable.

The second genetic marker analyzed was a 4 bp repeat located on chromosome 12 within intron 40 of the Von Willenbrand Factor gene [24]. This locus has been given the name vWA. The sample contained a mixture of 7 alleles ranging in size from 138 to 162 bp. The concentration of this PCR product was not as high as that for the TC-11 system. For this reason, the sample was diluted 1:1 with 50 ng/ml YO-PRO-1 and the injection time was increased to 20 s. Fig. 6 shows this sample. The results reveal excellent resolution of all 7 alleles.

The last genetic marker examined was a pair of VNTRs located 5′ to the human myelin basic protein gene on chromosome 18g22-qter [25]. This locus, identified as MBP, contains two VNTRs, each with a 4 bp repeat. The first

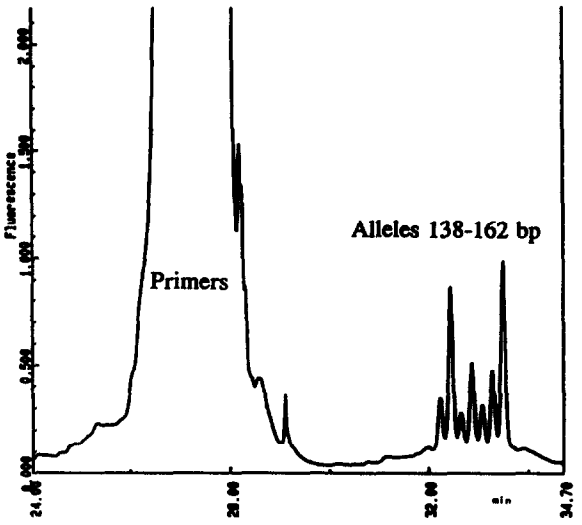

Fig. 6. Mixture of 7 vWA alleles amplified via the PCR with each allele 4 bp apart. Buffer as in Fig. 5. Sample diluted 1:1 with 50 ng/ml YO-PRO-1.

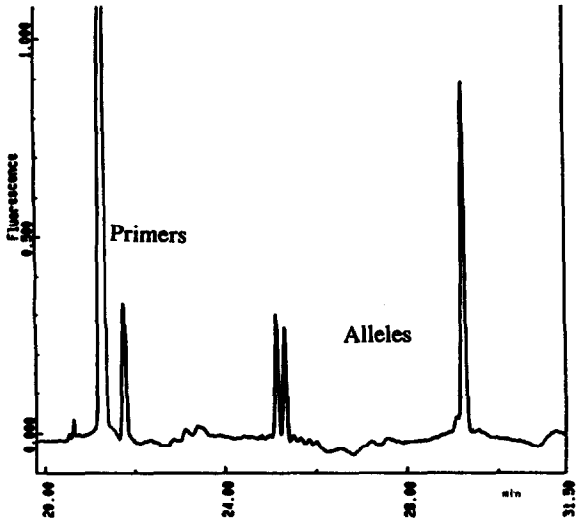

Fig. 8. Single set of MBP alleles extracted from liquid blood sample and amplified via the PCR. Buffer as in Fig. 5.

VNTR contains alleles from 122 to 142 bp in length while the second VNTR contains alleles from 208 to 232 bp in length.

Fig. 7 shows a mixture of alleles from this locus. The 3′ PCR primer for the MBP system can attach at two different locations, both before and after the second VNTR in the region, allowing the two VNTRs to be amplified at the same time. The size of the second set of alleles is a function of the length of both VNTRs. The CE buffer system easily separates both sets of alleles simultaneously.

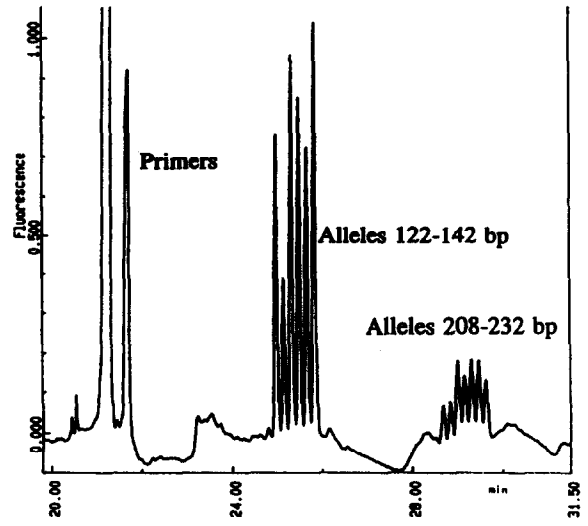

Fig. 7. Mixture of 13 MBP alleles amplified via the PCR. Note that this is a binary system. Buffer as in Fig. 5.

Fig. 8 shows the analysis of a sample of DNA extracted and amplified at the MBP locus. The lower-molecular-mass VNTR shows two alleles while a single peak is seen from the higher-molecular-mass VNTR, thus both of the DNA fragments obtained from the second VNTR have the same apparent molecular mass.

CONCLUSIONS

The results presented here show that CE has great potential in the analysis of PCR-amplified DNA of forensic interest. By using non-gel sieving buffers coupled with fluorescence detection, sensitive and specific detection of PCR-amplified DNA can be achieved. The intercalating dye YO-PRO-1 has been shown to facilitate sensitive detection of DNA fragments and to allow a wide range of concentration ratios of DNA/dye with little degradation in performance. For the analysis of samples requiring high separation efficiency, it is necessary to add

ethidium bromide to the buffer as well as YO-PRO-1. Using this procedure, alleles from a number of VNTRs of interest in genetic typing were investigated. Further work will be performed to examine the role ethidium bromide plays in improving column performance and to develop internal standards for the precise determination of sample retention times.

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