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Analysis of DNA restriction fragments and polymerase chain reaction products by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) is a new, high-resolution tool for the analysis of DNA restriction fragments and DNA amplified by the polymerase chain reaction (PCR). By combining many of the principles of traditional slab gel methods in a capillary format, it is possible to perform molecular size determinations of human and plant PCR amplification products and DNA restriction fragments. DNA restriction fragments and PCR products were analyzed by dynamic sieving electrophoresis (DSE) and capillary gel electrophoresis (CGE). As part of this study, sample preparation procedures, injection modes, and the use of molecular mass markers were evaluated. Optimum separations were performed using the uPage-3 (3% T, 3% C) CGE columns with UV detection at 260 nm. Membrane dialysis and ultrafiltration/centrifugation proved to be nearly equivalent methods of sample preparation. Reproducibility studies demonstrated that blunt-ended, non-phosphorylated markers (specifically allele generated markers) provide the most accurate calibration for PCR product analysis. This study demonstrates that CE offers a high-speed, high-resolution analytical method for accurately determining molecular size and/or allelic type as compared with traditional methodologies.

1. Introduction

DNA, which is found in virtually every cell in the human body, has recently become recognized as a source of identification for individuals in criminal cases and unidentified human remains. The technology that forms the basis for DNA testing has been advancing rapidly over the last decade. The current, widely used procedure for DNA typing is based upon restriction fragment length polymorphism (RFLP) [1]. As originally conceived, variation in the length of the target DNA restriction fragments is based upon differences in the presence or absence of restriction sites. Two drawbacks often associated with the detection of RFLP markers are the necessity of a relatively large amount of non-degraded DNA (20–100 ng) and detection based on radioactive isotopes. In humans, RFLP loci with as many as 80 different alleles have been reported [2]. Alleles at these loci are visualized via Southern hybridization. Theoretically, hundreds of alleles varying in length from 9 to 30 base pairs (bp) can be identified at such a locus. Hybridizing bands on a Southern blot varying in length by only a few core sequences are extremely difficult to differentiate. This difficulty, com-

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bined with the possibility of band shifting, has caused concern with the accuracy of RFLP human DNA fingerprinting [3].

RFLP analysis will likely be replaced by more accurate and faster polymerase chain reaction (PCR) procedures [4,5] which require orders of magnitude less DNA than RFLP and can work on DNA which is degraded. The use of PCR products allows more exact determination of allelic profiles [6–8]. Currently, there are several PCR-based procedures under development: amplified fragment length polymorphism (AmpFLP) [9], short tandem repeats (STRs) [10–12], variable number tandem repeats (VNTRs) [13], randomly amplified polymorphic DNA (RAPD) [14] and simple sequence repeats (SSRs) [15]. RFLP and the PCR procedures listed above are based on length differences of the DNA restriction fragments or amplification products.

DNA restriction fragments and PCR products have traditionally been separated by conventional slab gel electrophoresis, but the fragments have similar charge-to-mass ratios, therefore the separation mechanism is based on length-induced drag and the porosity of the polymer used to produce the gel. Traditional slab gel electrophoresis is time consuming, labor intensive, and difficult to quantitate. Capillary electrophoresis (CE) is a high-resolution tool which provides an alternative to traditional slab gel methods for the analysis of DNA restriction fragments and PCR products. CE employs many of the principles of traditional slab gel methodology for DNA sizing, but overcomes many of its disadvantages. Analysis is faster due to the use of thin-walled fused-silica capillaries which rapidly dissipate heat and allow higher run voltages. CE also provides the capability for real-time detection and accurate quantitation by using on-line optical detection systems. CE is well suited for high-resolution analysis of DNA in very small sample volumes and many of the separations typically performed in slab gels can be easily transferred to CE resulting in enhanced separations with extremely high efficiencies.

As with any separation technique, sample preparation, calibration, mode of injection, and separation chemistry all play an important role in a successful analysis. PCR reactions and restriction digests can have chloride concentrations high enough to inhibit the analysis by CE. Calibration standards for the size determination of DNA products that are typically analyzed by slab gel do not always work for CE separations. CE separations, like HPLC separations from fifteen years ago, are evolving and can be optimized with respect to time, selectivity and efficiency. Recent developments in buffer systems and capillary columns represents important technical advances in analytical CE. Two approaches have been taken for the analysis of DNA by CE: dynamic sieving electrophoresis (DSE) and capillary gel electrophoresis (CGE). DSE is based on the use of entangled polymer solutions or linear non-cross-linked polymers. Analysis times for DNA restriction fragments and PCR products are in minutes as compared to traditional slab gel methods or CGE. Resolution in DSE is controlled by the size and concentration of the polymer, where large polymers result in large pores; shorter polymers result in small pores [16]. The higher the concentration of polymer used, the greater the resolution in the separation. DSE has been found to have some significant limitations. Cellulose-based polymer systems, such as hydroxyethylcellulose, show significant lot-to-lot variability which can result in dramatic changes in resolution and reproducibility. The variability in molecular mass distribution of the cellulosic polymers and subsequent changes in the electrophoretic separation, precludes them from use in forensic DNA analysis [17]. Synthetic polymer systems currently under development may overcome this problem and provide a reliable sieving system in the future.

CGE, which uses covalently bonded, cross-linked linear polyacrylamide as the sieving mechanism, has been found to provide the highest level of resolution for DNA analysis of PCR products on a routine basis. By varying the concentration of the monomer and the degree of cross-linking, it is possible to create stable gels with a discrete range of pore sizes. The high resolution and reproducibility of CGE give this

technique the potential to perform routine forensic analysis. CGE has been successfully used for the analysis of three STR systems; the mitochondrial dinucleotide repeat, the tetrameric STR HUMTHO1 and the SSRs ATT1–ATT5 [18,19]. CGE combines the resolution required to size these PCR products, which can contain alleles a single repeat apart (2 and 4 bp, respectively) with the reproducibility required for forensic DNA typing.

The availability of commercially produced capillary columns and polymer systems greatly simplifies the routine analysis of DNA restriction fragments and PCR products. However, questions remain for many researchers on which are the most practical methods of sample cleanup, which mode of injection to use, which calibration marker to use for determining the size of the restriction fragment or PCR product, and which separation chemistry to choose to perform the analysis. The following report describes our study of four sample preparation methods for DNA cleanup, an evaluation of pressure and electrokinetic injections, the evaluation of four DNA calibration markers, and a comparison of three commercially available separation chemistries.

2. Materials and methods

2.1. DNA calibration markers

All PCR products used in this study were prepared by Ms. Rhonda Roby and Ms. Demris Lee (Armed Forces DNA Identification Laboratory, AFIP, Washington, DC, USA) according to the standard amplification procedures described by Roche Molecular Systems (Alameda, CA, USA). DNA restriction digests (ΦX174/HinfI, ΦX174/HaeIII) were obtained from Gibco BRL (Gaithersburg, MD, USA). DNA marker XI was obtained from Boehringer Mannheim (Indianapolis, IN, USA) and the HUMTHO1 allelic ladder was obtained from Promega (Madison, WI, USA).

PCR products and standards were prepared for analysis by membrane dialysis to remove salts. CGE was performed using the Dionex CES1A CE system (Dionex, Sunnyvale, CA, USA) with reverse polarity and UV absorbance set at 260 nm. Separations were performed using the uPage-3 (3% T, 3% C)[1] polyacrylamide gel columns supplied by J & W Scientific (Folsom, CA, USA) with the μPage buffer at ambient temperature. Ethidium bromide (10 μM) was added immediately before the analysis. Capillary effective length was 40 cm × 75 μm I.D. Data collection and analysis were performed using the Dionex AI 450 data system. Samples were injected electrokinetically and separated with an applied voltage of 225 V/cm.

2.2. Sample preparation of DNA restriction fragments and PCR products

Two methods for DNA restriction fragment and PCR product cleanup and desalting were evaluated in this study. Membrane dialysis, the first method, was performed by floating a VS 0.025-μm membrane (Millipore, Bedford, MA, USA) on water. PCR products were applied to the surface of the dialysis membrane as a single drop and dialysis was allowed to occur for 20 min (Fig. 1). The PCR product was then analyzed for chloride content by ion chromatography (IC). The second method, ultrafiltration/centrifugation, was performed using the Millipore Ultra-Free 30 filter (Millipore). A 250-μl volume of restriction digest or PCR product was placed in each of the filtration units and the volume brought to 300 μl with water. The units were centrifuged for 5 min at 2000 *g*. An additional 250 μl of water were added to each unit and centrifugation repeated two additional times. At each step the filtrate was removed from the collection cups and saved for chloride analysis by IC. The final concentrated PCR product and restriction digest was also measured for chloride content by IC and then analyzed by CE.

IC was performed on a Dionex DX-300 ion chromatograph using a AS4A anion-exchange

[1] T = [g acrylamide + g N,N'-methylenebisacrylamide (Bis)]/100 ml solution; C = g Bis/%T.

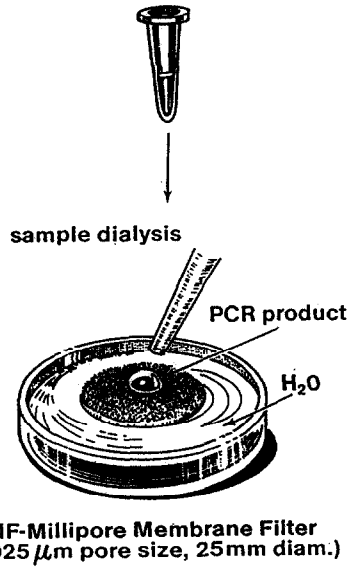

Fig. 1. Sample preparation of DNA by membrane dialysis.

column with chemically suppressed conductivity detection. The flow-rate was 2 ml/min using a 1.8 m*M* sodium carbonate–1.7 m*M* sodium hydrogencarbonate buffer system. Data collection and analysis were performed using the Dionex AI450 data system.

Fluorescent derivatization was performed using thiazole orange ($2.24 \cdot 10^{-1}$ m*M*), mixed 1:10 with the ΦX174/HaeIII restriction digest. Intercalation was allowed to proceed for 15 min prior to analysis. Analysis was performed using the ATI-Unicam (Madison, WI, USA) CE module and a GTI/Spectrovision fluorescent detector which has been retrofitted with an Ar^+ ion laser. The separation was performed using the ABI DNA fragment analysis reagent and buffer (Applied Biosystems, Foster City, CA, USA). Capillary effective length was 50 cm × 75 μm I.D. Samples were injected electrokinetically and separated with an applied voltage of 210 V/cm. Data collection and analysis were performed with the Dionex AI-450 data system.

2.3. Modes of injection

The injection study was performed using the 123-bp DNA ladder (Gibco BRL). Analysis was performed on the Applied Biosystems 270A-HT CE system using a 0.1 *M* phosphate buffer, pH 8.0, 0.5% hydroxyethylcellulose (HEC) and 10 μ*M* ethidium bromide. The electrokinetic injection was performed for 3 s at 100 V/cm. The vacuum injection was performed for 5 s. The separation was performed using a 100 μm I.D. DB-17 surface-modified fused-silica capillary (50 cm length, 30 cm effective length) and 50% phenylmethyl silicon stationary phase. Capillaries were flushed with the sieving buffer for 5 min prior to the first run. Analysis was performed using reverse polarity (−120 V/cm). The temperature was set at 30°C and UV detection at 260 nm.

2.4. Separation chemistries

Three separation chemistries were evaluated for the analysis of DNA restriction fragments and PCR products: The J & W uPage-3, the Dionex Nucleophor sieving buffer and capillary, and the Applied Biosystems DNA fragment analysis reagent and buffer.Separations of the HaeIII restriction digest of ΦX174 and the STR HUMTHO1 using the uPage-3 were performed on the Dionex CES1A system. Electrophoresis was performed using reverse polarity and the uPage buffer with ethidium bromide (10 μ*M*). Temperature was ambient and UV absorbance was set at 260 nm. All injections were performed electrokinetically at −7 KV for 5 s. The capillary had an effective length of 40 cm, with run voltages of 210 V/cm.

Separations of the HaeIII restriction digest and HUMTHO1 using the Nucleophor and ABI DNA fragment analysis sieving chemistries were performed on the ABI 270A-HT CE system. The Nucleophor sieving buffer uses a derivatized capillary of a proprietary nature. Prior to analysis the capillary is rinsed with sieving buffer for nine 2-min cycles. Electrophoresis was performed using reverse polarity. Temperature was set at 30°C and UV absorbance at 260 nm. All injections were performed electrokinetically at −7 kV for 5 s. The capillary had an effective length of 50 cm, with run voltages of 210 V/cm. The Applied Biosystems DNA fragment analysis

reagent uses a bare fused-silica capillary. Prior to analysis the capillary is flushed with 0.3 *M* NaOH, deionized water, 5 *M* HCl, and deionized water for 1 min each. The capillary was then rinsed with sieving polymer for 8 min. Electrophoresis was performed as described above. Temperature was set at 30°C. Following the initial analysis of the HaeIII digest and the HUMTHO1 samples, the DNA fragment analysis reagent was modified by the addition of urea (20%) and ethidium bromide (10 μM).

3. Results and discussion

3.1. DNA calibration markers

Restriction digests have traditionally been used as calibration markers for gel electrophoresis, and are used today to size PCR-generated products run on slabs. However, digest fragments can have overhanging bases and/or phosphorylated 5′ ends. PCR products are blunt ended and non-phosphorylated. CE, due to its high efficiency and resolution, has the ability to differentiate between DNA fragments with very little difference in charge/mass ratio. We were also interested in determining whether a restriction digest could be effectively used as a calibration standard for PCR analysis. In doing so, we constructed a model, assuming evenly distributed nucleotides to calculate the charge/mass ratio. Based on this model, we made the hypothesis that the charge/mass ratio for the restriction fragments would be slightly greater than the PCR products of the same base-pair number.

The determination of the size of a DNA restriction fragment or a PCR product can be accomplished by two methods. In the first, the DNA ladder and sample are spiked with a mobility standard (bromophenol blue). Following electrophoresis, a comparison of the R_F values of the standard DNA ladder and the sample can then be made and a size assigned to the unknown sample. Increased precision of R_F measurements is facilitated by increased resolution, close control over the separation conditions, and by repetitive measurements of the R_F values.

In the second method, an accurate measurement of the sizes of DNA restriction fragments and PCR products is made based on the production of an exact calibration plot. As DNA fragments are of a known discrete size, a simple peak-position calibration method can be utilized (Fig. 2). In this case, a series of DNA markers of a known size are separated using CE. The calibration curve is generated by plotting the size of the DNA marker versus the peak retention time. The peak position method is limited by the appropriate size standards.

The HaeIII and HinfI restriction digest of ΦX174 have been used as size reference standards for PCR products and other restriction digests. Unfortunately, the use of these ladders as standards should be limited to DNA restriction fragments cut with the same restriction enzyme. The HaeIII cleavage generates blunt ended fragments which are phosphorylated at the 5′ ends. HinfI cleavage results in a cohesive 5′ overhang of 4 bp (ANTC), which also has a terminal phosphate at the 5′ end. The terminal configuration of the restriction fragment plays an important role in the restriction fragments migration toward the cathode; the 5′ phosphates and terminal overhangs affecting the charge/mass ratio. In the model, the charge/mass ratio of all DNA fragments was calculated based on a 20-bp number. The molecular mass of the DNA fragments were determined by multiplying the number of base-pairs by their molecular mass (M_r = 618). The charge of each molecule was then calculated, assuming that there is no protonation of the molecule and no change in pH. The calculated charge/mass ratio is then determined for each marker (Fig. 3).

The PCR-like ladder (DNA marker XI) does not have the terminal 5′ phosphate. Therefore, there is no additional charge and the mobility in the capillary gel is less than either the HaeIII or HinfI markers. A calibration curve for these ladders can be constructed, and the base-pair number of the PCR-amplified DNA in question can be determined (Fig. 4; Table 1). At this time the Boehringer Mannheim DNA marker XI

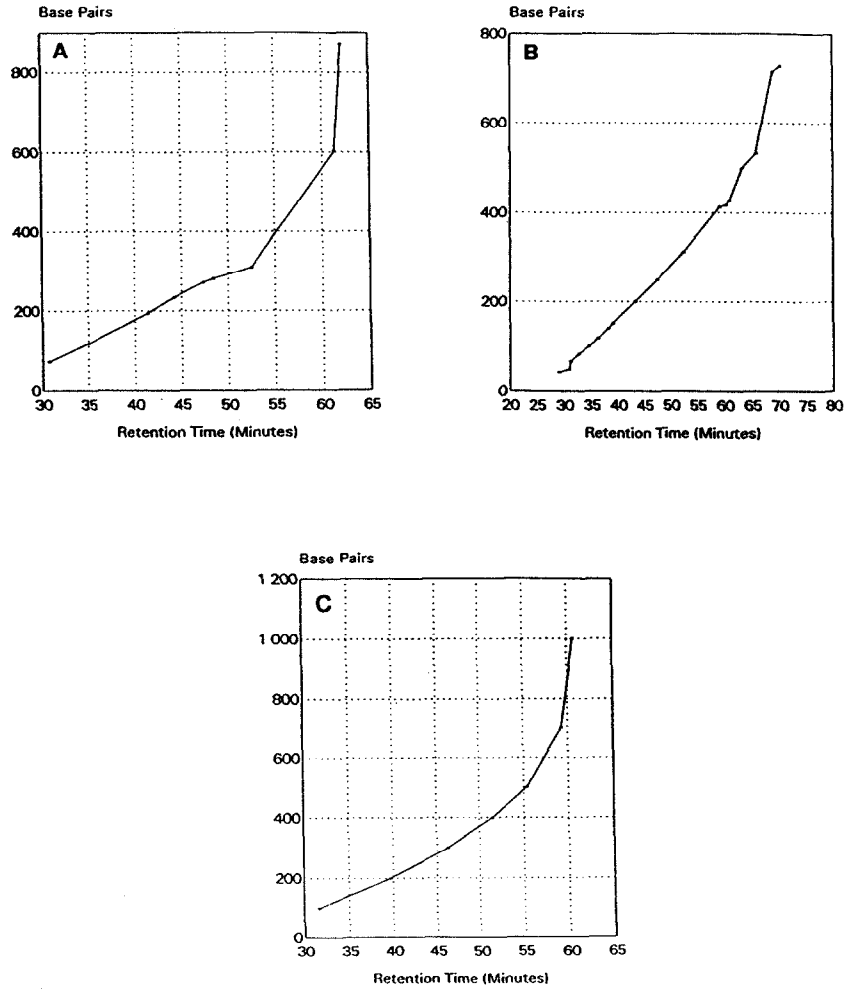

Fig. 2. "Peak position calibration plots" for (A) ΦX174/HaeIII restriction digest, (B) ΦX174/HinfI restriction digest and (C) Boehringer Mannheim DNA marker XI.

provides us with a more reliable reference marker for PCR analysis, while the restriction fragments show greater variability about our points of interest in the calibration curve. The Boehringer Mannheim DNA marker XI showed the mean value for the mitochondrial dinucleotide repeat: R-136 to be exactly as predicted to the actual value of 136, as determined by DNA sequencing.

In the case of the human STR HUMTHO1, the Boehringer Mannheim DNA marker XI did not provide an accurate base-pair number from the calibration curve. In this case, the values generated from the calibration curve were consistent for alleles 5 and 6, but the values were smaller than the true value for the larger alleles by as much as 4 bp (Table 2). The HUMTHO1 allelic ladder was used for this DNA typing system to accurately assign base-pair number and allelic designation (Fig. 5). The Boehringer Mannheim DNA marker XI was also found to be incorrect when determining the base pair number of the soybean SSRs, ATT1 and ATT5. In this case, the calibration was consistently larger

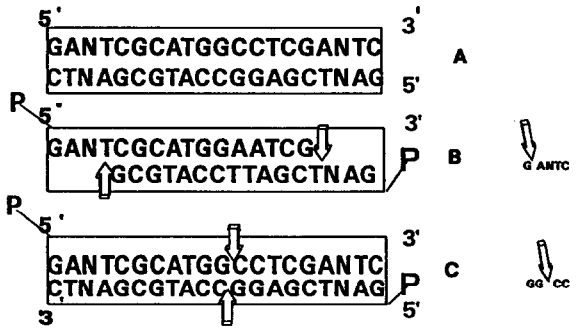

Fig. 3. This model demonstrates the difference between the three DNA markers and their relative charge/mass ratios. This model assumes an even distribution of nucleotides, at constant pH and temperature to prevent protonation. The DNA length is 20 bp for all three markers. The calculated charge/mass ratios were determined by multiplying the mass of the base pair (618) by the number of base-pairs divided by the total charge. This model demonstrates the 5′ overhang of 4 bp of the HinfI restriction fragment and the blunt end of the HaeIII restriction fragment. (A) PCR product, M_r 12 360, charge/mass ratio 0.0029; (B) ΦX174/HinfI, M_r 9888, charge/mass ratio 0.00303; (C) ΦX174/HaeIII, M_r 12 360, charge/mass ratio 0.00307.

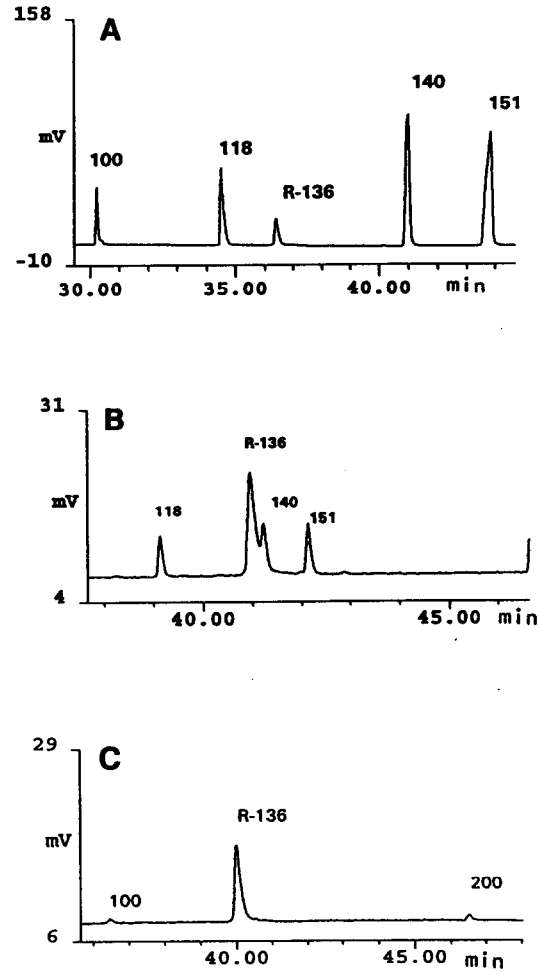

Fig. 4. Analysis of the human mitochondrial dinucleotide repeat, R-136, using the three calibration markers: (A) ΦX174/HaeIII, calculated base-pair number = 141 bp; (B) ΦX174/HinfI, calculated base-pair number = 137 bp; (C) Boehringer Mannheim DNA marker XI, calculated base-pair number = 136 bp.

by 6–7 bp (Table 3). However, when the allelic ladders were used, accurate genotyping was possible (Fig. 6).

The evaluation of the markers demonstrated that some PCR-generated products can be calibrated precisely when the reference marker is a PCR-generated or PCR-like ladder. The model provides us with charge/mass ratios that correspond to the actual data. The HinfI and HaeIII restriction digests show a variability of 2–5 bp away from the actual size of the mitochondrial dinucleotide repeats, while the PCR-like ladder provided us with the best calibration system. For PCR products which are larger than the mitochondrial dinucleotide repeat, or have an unusual sequence, standard, commercially available markers may not provide accurate calibration plots. Therefore, it is important to use markers generated from the alleles themselves, as in the case of HUMTHO1.

It is also important to remember that accurate base-pair numbers are obtained only when the unknown fragments and the separating systems are identical for the standard and the unknown. If ethidium bromide is used in the buffer system when the calibration curve was constructed, it must be used in the buffer system for the unknown. In addition, sieving polymers used on both standards and unknowns must be the same, they must be run at the same concentration and ionic strength, and the separations must be performed at the same voltage. A new calibration curve should be constructed for each day's analysis.

Table 1
Determination of base-pair number of the human mitochondrial dinucleotide repeat, R-136, using the three DNA calibration markers: Boehringer Mannheim DNA marker XI, ΦX174/HinfI and ΦX174/HaeIII

	XI ($n = 25$)		HinfI ($n = 19$)		HaeIII ($n = 12$)
	136.72	136.79	137.53	137.15	144.84
	136.8	137.48	137.92	137.04	145.11
	136.84	137.73	137.61	137.35	144.91
	136.67	135.06	136.35	138.38	139.13
	136.74	137.34	137.8	138.46	139.16
	136.8	137.23	137.44	138.47	139.13
	136.67	136.80	138.01		140.32
	136.92	137.23	137.52		145.66
	136.8	137.35	138.67		139.56
	136.78	137.14	138.42		139.62
	137.12	136.25	138.39		139.72
	136.89	136.33	138.62		139.64
	137.18		137.81		
Mean	136.86		137.81		141.40
S.D.	0.50		0.62		2.78
R.S.D. (%)	0.003		0.004		1.38

Actual base-pair number = 136.

3.2. Modes of injection

The option of using pressure or vacuum injections is only available when using sieving polymers. The application of high pressure to a cross-linked gel will disrupt the polymer and result in the formation of a void. When performing DSE, the method of injection used for the analysis of DNA will have an effect on the separation efficiency. Vacuum or positive pressure injections

Table 2
Determination of HUMTHO1 allele size using the Boehringer Mannheim DNA marker XI

	Allele						
	5 (179 bp)	6 (183 bp)	7 (187 bp)	8 (191 bp)	9 (195 bp)	10 (199 bp)	11 (203 bp)
	179.51	183.4	186.12	189.23	192.74	195.85	199.48
	179.25	183.4	186.12	189.23	192.74	196.24	198.96
	179.41	183.45	186.23	188.89	192.93	195.96	199.24
	179.44	183.55	185.99	189.08	192.80	195.63	198.84
	179.12	183.24	186.34	189.04	192.78	195.88	198.45
	178.94	183.16	185.92	188.81	192.63	195.39	198.55
	178.94	183.16	185.92	188.81	192.63	195.79	198.68
Mean	179.23	183.34	186.09	189.01	192.75	195.82	198.89
S.D.	0.24	0.15	0.16	0.18	0.10	0.27	0.37
R.S.D. (%)	0.13	0.08	0.09	0.10	0.05	0.14	0.10

Values in the table are base-pair numbers determined from the calibration curve.

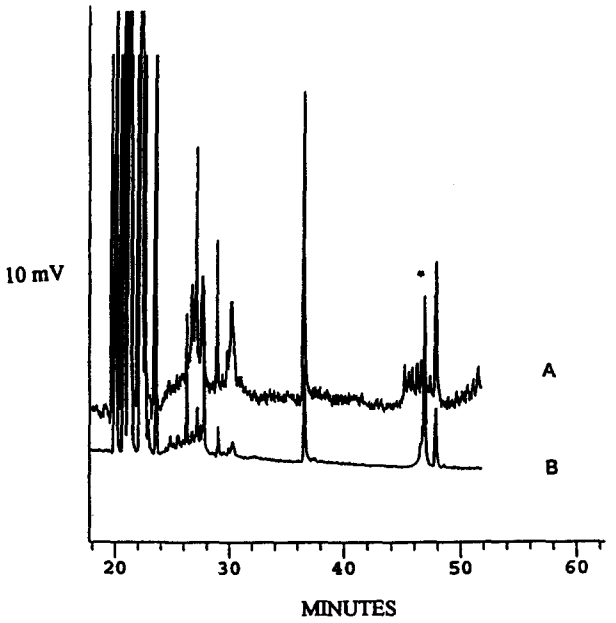

Fig. 5. Analysis of the HUMTHO1 using the Boehringer Mannheim DNA marker XI and HUMTHO1 allelic ladder. The base-pair number, as determined by the calibration plot using DNA marker XI is incorrect, however, the allelic determination is correct when calibrated against the allelic ladder. In this case, the individual is a homozygote for allele 10 (*).

Table 3
SSR fragment size determination for F2 generation of Williams and Jackson soybean genotypes at locus ATT5 using the Boehringer Mannheim DNA marker XI

Sample	SSR (base-pairs)	
	Williams (142 bp)	Jackson (151 bp)
Williams ATT5	136.33	
Jackson ATT5		144.76
J × W F1 hybrid	136.73	145.31
17ATT5-F2		145.14
18ATT5-F2		145.19
19ATT5-F2	136.99	145.53
20ATT5-F2	136.59	145.12
21ATT5-F2		144.30
22ATT5-F2	136.60	145.10
23ATT5-F2	136.08	144.94
24ATT5-F2	136.59	145.12
25ATT5-F2	136.09	144.38
26ATT5-F2	136.12	
Mean	136.46	144.99
S.D.	0.34	0.37
Range	0.91	1.23
Standard error	0.11	0.11

can be used; however, this type of injection can result in significant band broadening which reduces the overall efficiency and resolution of the separation (Fig. 7A). The loading mechanism for pressure or vacuum injection results in a volume of the DNA suspension being drawn into the capillary (Fig. 8). In most cases, the DNA is suspended in either water, or a buffer which is a different ionic strength or pH than the electrophoresis buffer. This results in the establishment of a boundary layer between the sample buffer and the electrophoresis buffer and zones of differing conductivity. When the voltage is applied to the capillary, the DNA in this boundary region has a higher mobility than the DNA in the remaining sample buffer. This results in the observed band-broadening or doublets. Since the presence of doublets can also be attributed to artifacts of the amplification process, the use of electrokinetic injection is recommended (Fig.

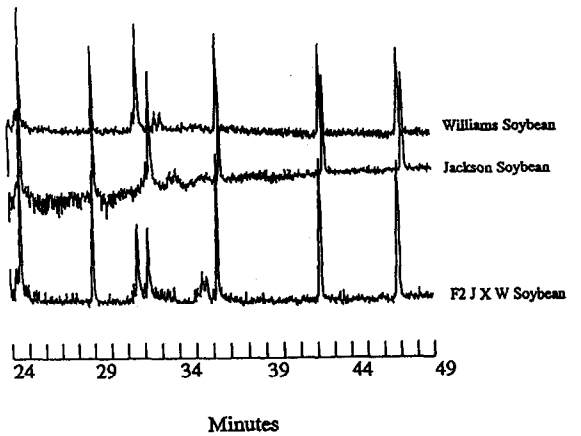

Fig. 6. Analysis of Williams × Jackson (J × W) F2 soybean samples using the P1 alleles and the Boehringer Mannheim DNA marker XI. The base-pair number, as determined by the calibration plot using DNA marker XI is incorrect, however, the allelic determination is correct when calibrated against the P1 alleles.

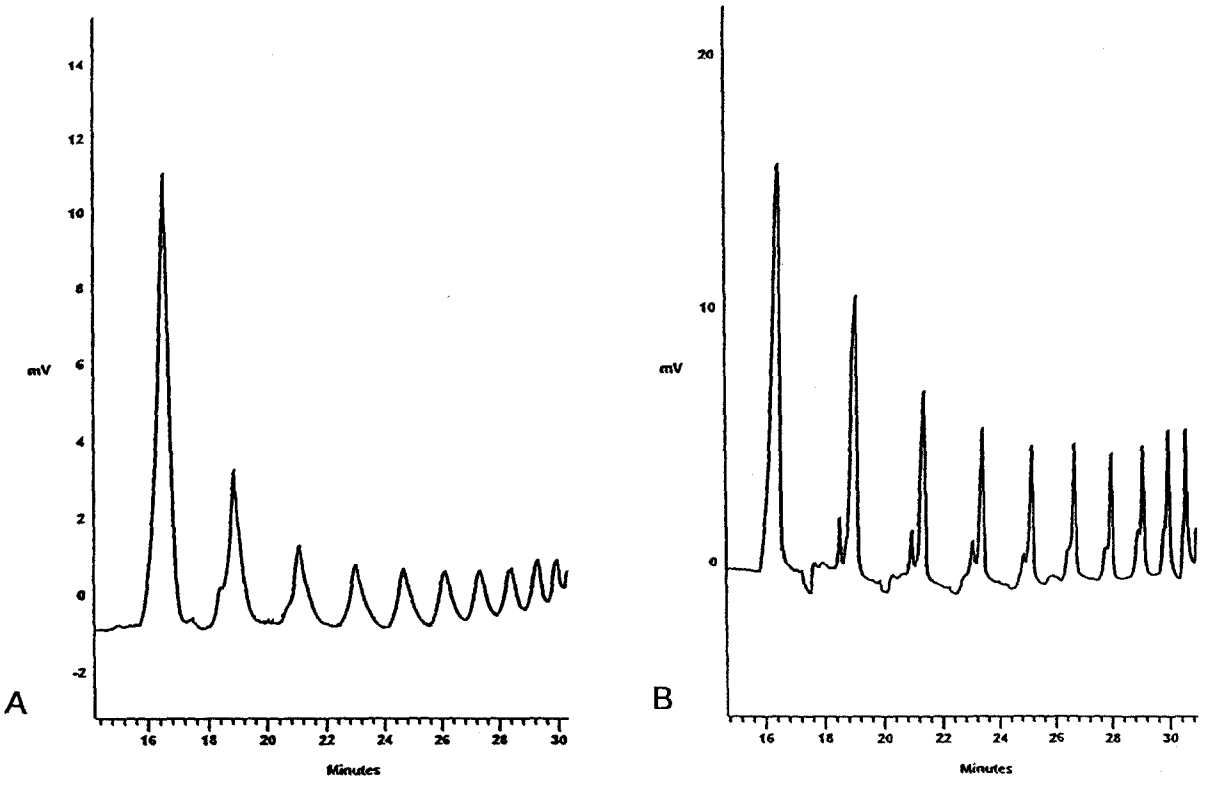

Fig. 7. Effect of injection mode of the separation of the 123-bp DNA ladder. (A) The 123-bp ladder using vacuum injection (5 s at 0.17 kg/cm^2); buffer, 0.1 *M* phosphate, pH 8.0, 0.5% HEC, 10 μM ethidium bromide; capillary, DB-17 coated capillary, 100 μm I.D., 30 cm effective length; 30°C; detection at 260 nm; sample concentration, 0.025 mg/ml. (B) The 123-bp ladder using electrokinetic injection (3 s at 100 V/cm). Separation as described above. As shown above vacuum injection results in peak fronting and band broadening, which is clearly evident throughout the separation.

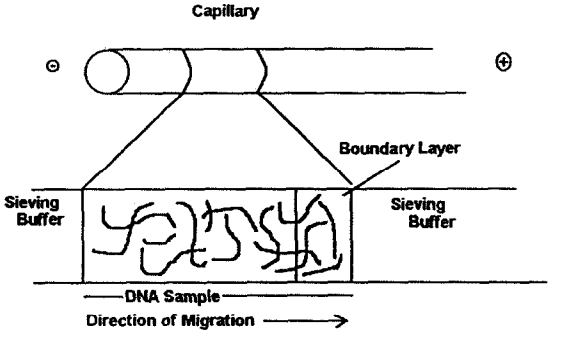

Fig. 8. Schematic diagram of a pressure/vacuum injection, which results in the formation of a boundary layer between the sample buffer and the electrophoresis sieving buffer. This establishes regions of different conductivity, the sample having a lower conductivity than the sieving buffer, and results in differential migration of the sample when the voltage is applied. Band broadening and peak fronting are commonly observed as a result.

7B). In addition, no sample injection bias should be observed because all DNA fragments have the same electrophoretic mobility in free solution.

3.3. Sample preparation

The sample preparation of any DNA sample actually begins with the extraction of the DNA from the sample. This can be quite easy, as in the case of DNA from blood or bacterial cultures, to quite difficult, as in the case of bone or degraded tissues. In any case, the quality of the extraction will have a significant effect on the subsequent digestion or amplification. The extraction procedure chosen should be one that provides the highest yield possible, while still

maintaining as low levels of inhibitors or other contaminants as possible. If the next step in the preparation of the DNA for analysis is restriction enzyme digestion, sample preparation for CE is usually a matter of cleaning up excess salts from the sample. This can easily be done by membrane dialysis. Amplification products may require pre-concentration and the removal of chloride prior to analysis.

If PCR procedures have been optimized to routinely produce enough product for analysis by CE, membrane dialysis is an effective method of sample preparation. Over 80% of the chloride present in the PCR reaction can be removed by a single-step dialysis (Table 4; Fig. 9). If the PCR reaction has not been optimized and the reactions must be concentrated, ultrafiltration/centrifugation can yield a more concentrated prod-

Table 4
Removal of chloride from PCR products by membrane dialysis

Sample	Chloride in PCR products (μg/ml)	Chloride removed (%)
1	262.4	84.2
2	308.3	70.8
3	281.1	85
4	316	87.4
5	278.5	83.3
6	280.5	81
7	283.9	84.3
8	280.9	79
9	279	94.5
10	282.5	76.8
Mean	285.31	82.63
S.D.	14.66	6.03
Cost/analysis (US$)	1.01	

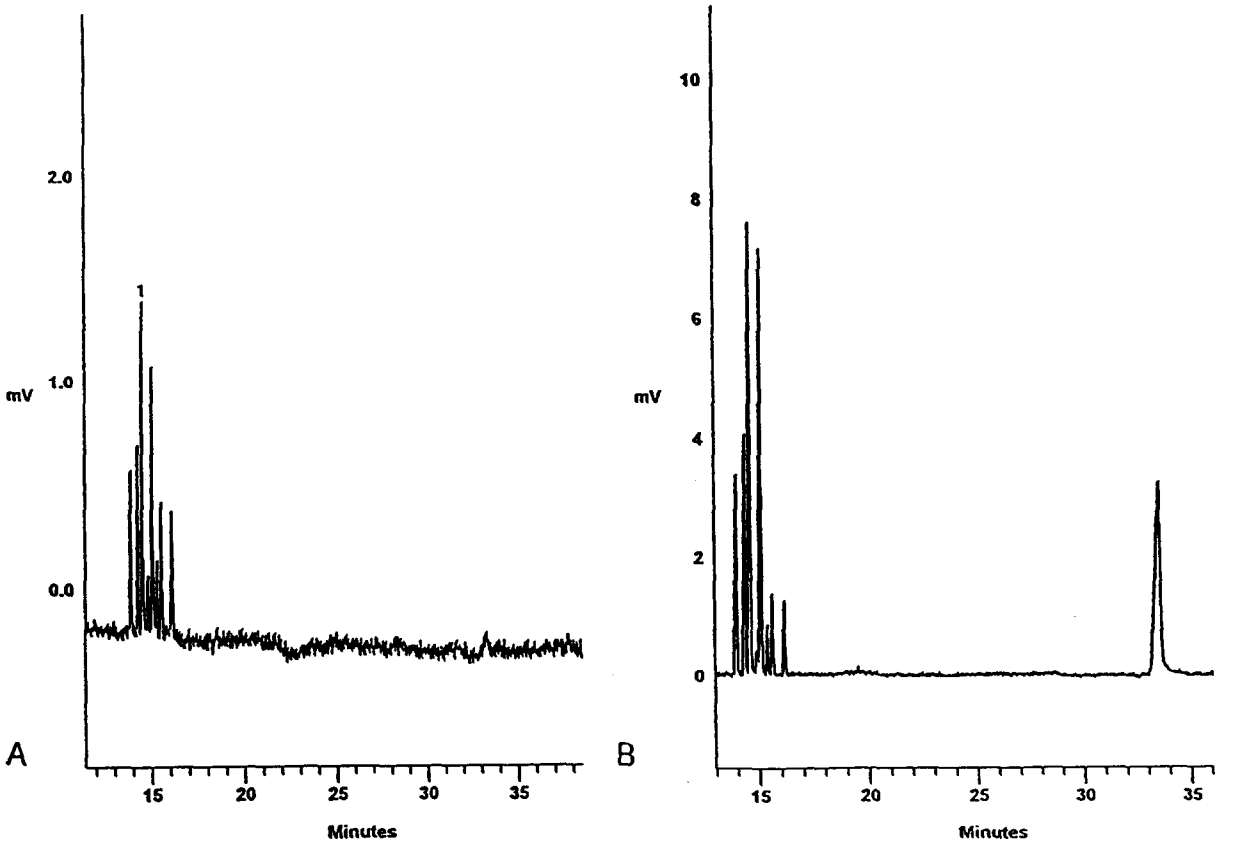

Fig. 9. (A) Sample analysis of the crude PCR product without sample preparation to remove excess chloride. (B) Analysis of the same PCR product following a 20-min dialysis on the Millipore MF membrane filter (0.025 μm pore size, 25 mm diameter).

uct that is up to 90% chloride free and easily detected by CE (Table 5; Fig. 10).

An alternative approach to the concentration of the PCR product is fluorescent derivatization. In this approach, as is the case with HPLC, fluorescence is used merely to enhance the detection limits of the sample, without having a major impact on the analytical chemistry. Intercalators, such as thiazole orange, are used to simply increase the detector response of the DNA in the PCR product or restriction digest (Fig. 11). The use of intercalator chemistry and laser-induced fluorescence can overcome many of the problems of non-optimized PCR procedures; however, intercalation chemistry should not be substituted in place of proper sample cleanup and the removal of salts. Intercalators reduce the charge/mass ratio of the DNA as they are incorporated into the helical structure and add rigidity to the molecule. This can cause a reduction in separation efficiency and resolution if it is not carefully controlled (Fig. 11B). If intercalation chemistry is combined with poor sample preparation, there can be a dramatic loss of resolution.

Table 5
Removal of chloride from PCR products by ultrafiltration/centrifugation

Sample ($n = 5$)	Chloride in PCR products (μg/ml)	Chloride removed (%)
1	537	95.5
2	537	95.0
3	537	98.2
Cost/analysis (US$)	2.20	

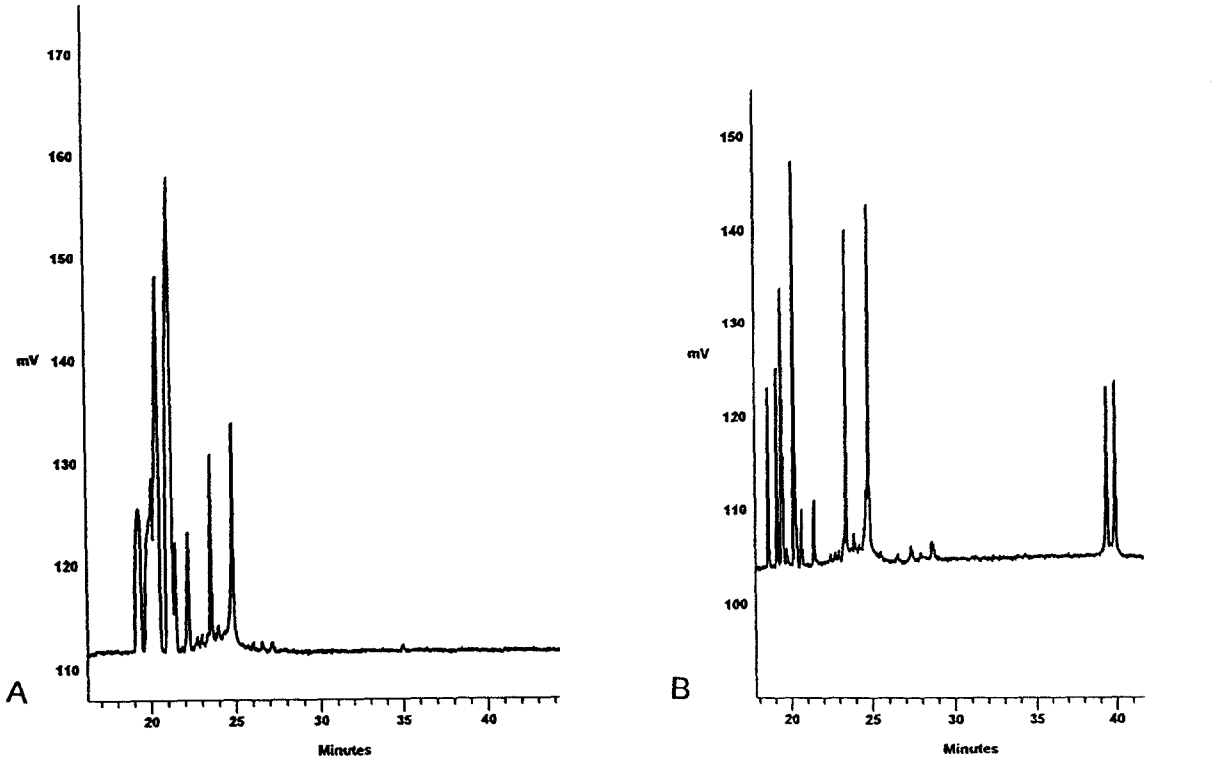

Fig. 10. (A) Sample analysis of a crude PCR product (HUMTHO1) without sample preparation to concentrate the sample and remove excess chloride from the reaction product. (B) Analysis of the same sample following ultrafiltration/centrifugation using the Millipore UF filter unit.

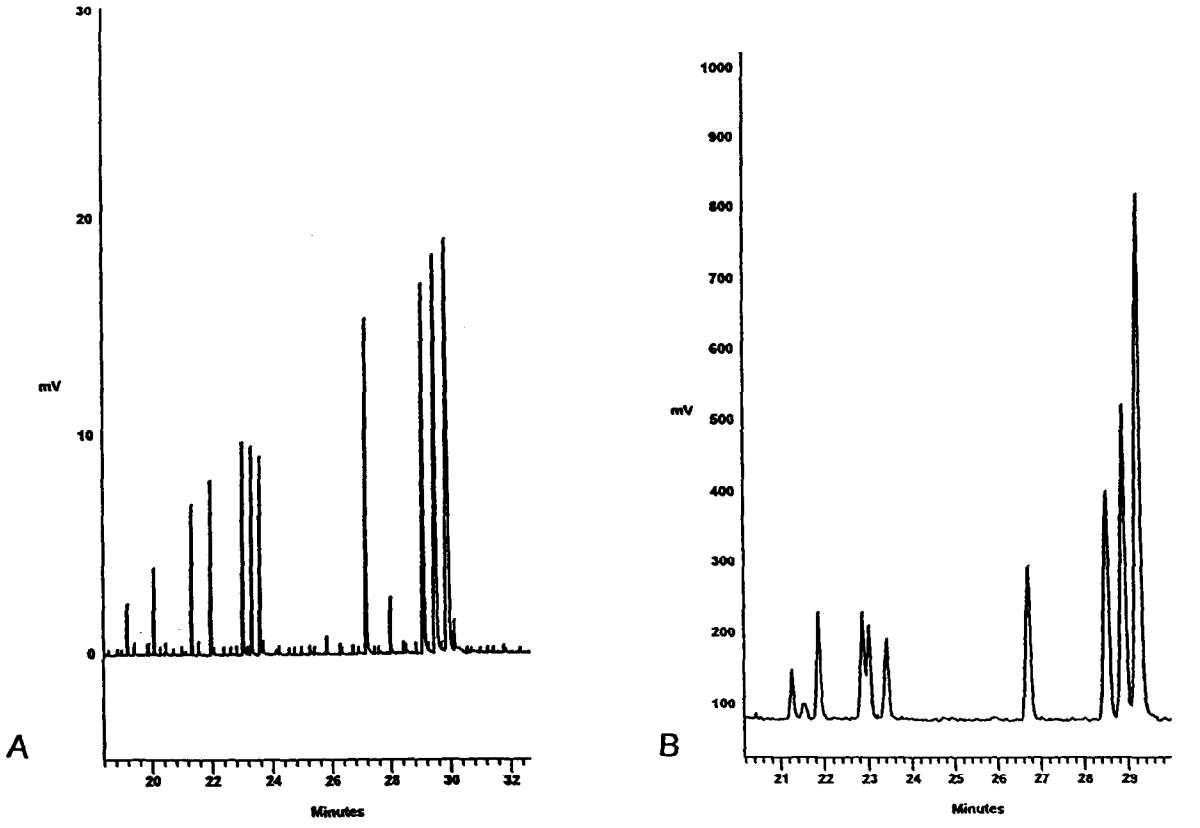

Fig. 11. (A) Analysis of the ΦX174/HaeIII restriction digest using UV detection at 260 nm. Fragment sizes from left to right are: 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 bp. (B) Analysis of the ΦX174/HaeIII restriction digest using laser-induced detection and thiazole orange intercalation chemistry. This results in a 30 × increase in the signal, however, the intercalation resulted in a loss of resolution.

3.4. Separation chemistry

Separations based on the J & W 3% polyacrylamide gel capillary columns have traditionally provided the best separations for dsDNA less than 500 bp in size. However, these capillaries can be difficult to work with because the 3% T, 3% C gel matrix and the buffer system are strong UV absorbers, the dimensions of the capillary are not optimum for the optical design of all commercial systems, and the capillaries can be difficult to install. In spite of these limitations, this capillary is the standard by which other separation chemistries are judged.In this study, we evaluated the performance of three separation chemistries on two types of samples, the HaeIII restriction digest of ΦX174 and the HUMTHO1 PCR product. The HaeIII digest contains restriction fragments from 72 to 1353 bp in size, while the HUMTHO1 sample contains two alleles, 183 and 191 bp in size. The J & W gel capillaries provided good resolution of the smaller fragments of the HaeIII digest. The resolution for the 271–281 bp fragments was 1.88. However, this chemistry was unable to resolve the fragments over 600 bp in size (Fig. 12). This clearly demonstrates the limitations of the separation chemistry. In the separation of the HUMTHO1 alleles, the J & W chemistry provided a high-resolution separation, with resolution of 2.83 between the alleles (Fig. 13). It is in the separation of dsDNA fragments and PCR products between 100 and 400 bp in size that the J & W chemistry excels.The separation of the

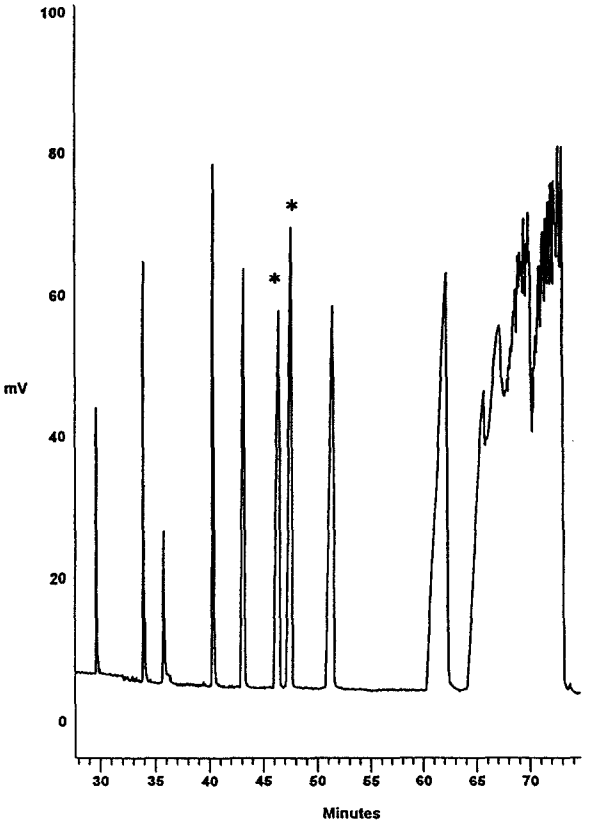

Fig. 12. Analysis of the ΦX174/HaeIII restriction digest using the uPage-3 gel capillary column. The resolution of the 271–281 bp fragments (*,*) was 1.88.

HaeIII digest with the Nucleophor chemistry from Dionex was satisfactory. Resolution of the 271–281 bp fragments was 1.62 (Fig. 14). This chemistry was able to baseline resolve all of the fragments of the restriction digest must faster than the J & W chemistry. However, this chemistry was unsatisfactory for the analysis of the HUMTHO1 alleles in that the alleles were not baseline resolved. Resolution measured 0.97 (Fig. 15). At this time the Dionex chemistry has not demonstrated the resolving power to perform high-resolution analysis of PCR products or restriction fragments less than 10 bp in size.

The DNA fragment analysis reagent and buffer from Applied Biosystems gave a satisfactory separation of the HaeIII digest. Resolution of the 271–281 bp fragments was 5.29. All restric-

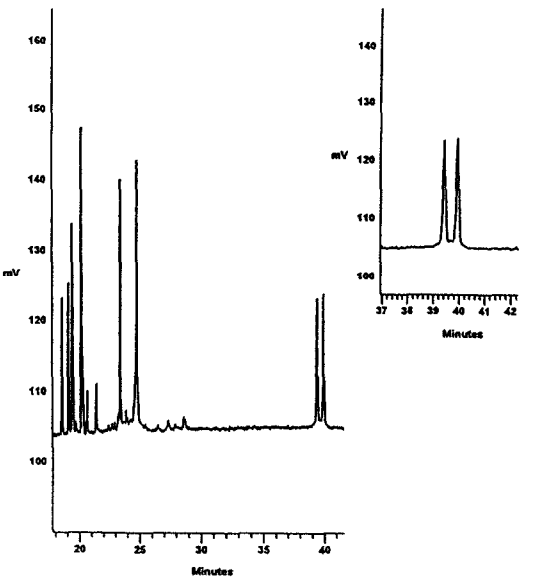

Fig. 13. Analysis of the HUMTHO1 PCR product using the uPage-3 GCE column. The resolution of the 183 and 191 bp alleles was 2.83.

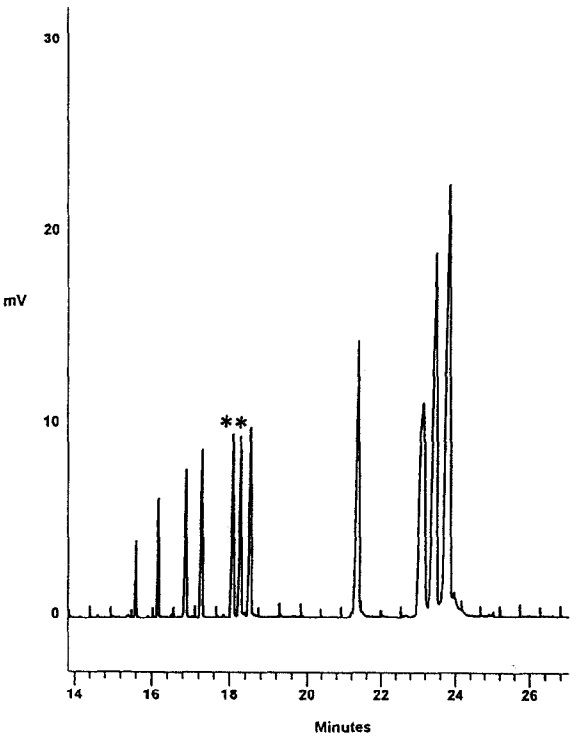

Fig. 14. Analysis of the ΦX174/HaeIII restriction digest using the Dionex Nucleophor sieving buffer and capillary column. The resolution of the 271–281 bp fragments (*,*) was 1.62.

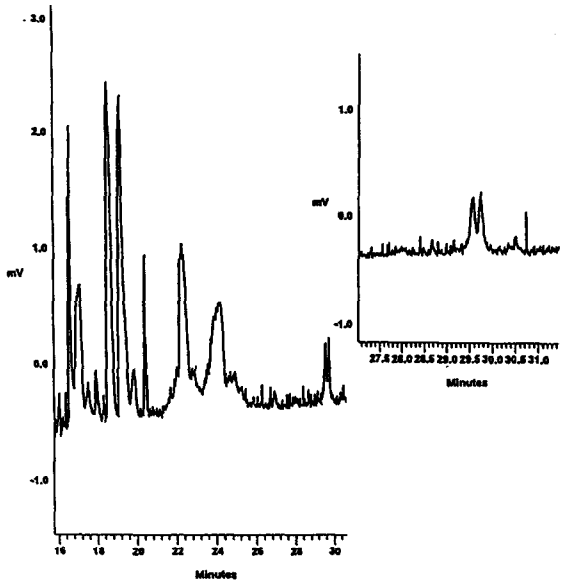

Fig. 15. Analysis of the HUMTHO1 PCR product using the Dionex Nucleophor sieving buffer and capillary column. The resolution of the 183 and 191 bp alleles was 0.97.

tion fragments were baseline resolved and the separation was only slightly longer than the Dionex chemistry, while still being half as long as the J & W chemistry (Fig. 16). In the analysis of the HUMTHO1, this chemistry provided baseline resolution between the alleles, $R_s = 1.97$ (Fig. 17). When this chemistry was modified to include urea and ethidium bromide the resolution of the alleles increased to 2.35 and the separation was nearly equivalent to that of the J & W gels (Fig. 18). With minor modifications to this separation chemistry, it should be possible to do accurate and reproducible allelic typing of individuals using HUMTHO1 with a separation chemistry that runs in half the time as the J & W chemistry, and with the same resolution and reproducibility. Short-term projects in our laboratory will focus on this particular issue, as well as the application of this chemistry to other allelic systems.In this study we have evaluated the analysis of DNA by CE from a variety of perspectives; calibration, sample preparation, mode of injection, and separation chemistry. It is clear that sample preparation can be easily performed by either membrane dialysis or ultrafiltration/centrifugation depending on the concentration of DNA in the PCR reaction. The calibration of capillary systems for DNA sizing is best done using the specific allelic ladder of the DNA being studied. The current ladders and restriction digest being used for CE analysis may not have the sequence compatibility or terminal configuration necessary to construct an accurate calibration curve. The use of electrokinetic injection eliminates the potential of band broadening and loss of resolution associated with pressure injections. This is clearly the way to initiate a high-resolution analysis. Separation chemistries are evolving and the development of synthetic sieving polymers show great promise. These new

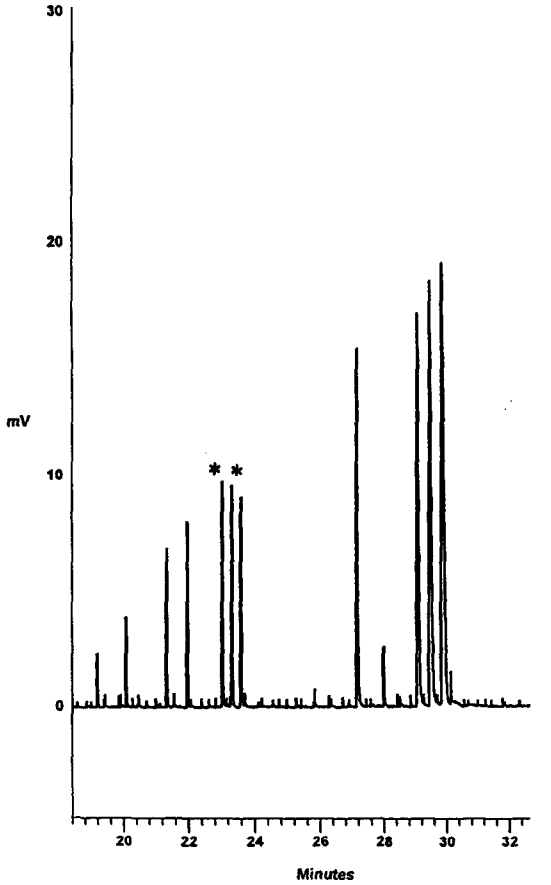

Fig. 16. Analysis of the ΦX174/HaeIII restriction digest using the Applied Biosystems DNA fragment analysis reagent and buffer. The resolution of the 271–281 bp fragments (*,*) was 5.29.

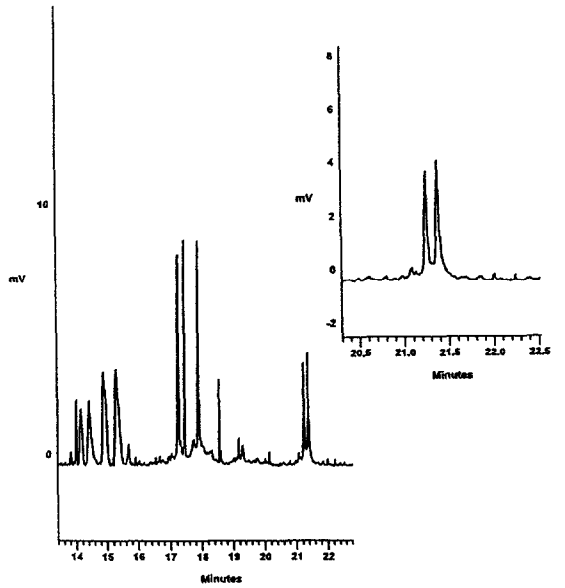

Fig. 17. Analysis of the HUMTHO1 PCR product using the Applied Biosystems DNA fragment analysis reagent and buffer. The resolution of the 183 and 191 bp alleles was 1.37.

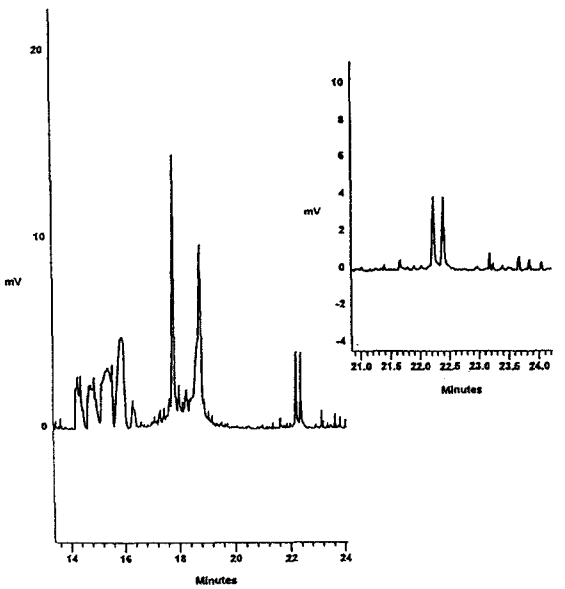

Fig. 18. Analysis of the HUMTHO1 PCR product using the modified Applied Biosystems DNA fragment analysis reagent and buffer. The buffer was modified by the addition of urea and ethidium bromide. The resolution of the 183 and 191 bp alleles was 2.35.

chemistries will reduce the time and cost of DNA analysis and help to make large-scale DNA typing systems a reality.

Disclaimer

The opinions or assertions herein are those of the authors and do not necessarily reflect the views of Department of the Army or of the Department of Defense.

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