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Intrinsic isotachophoretic preconcentration in capillary gel electrophoresis of DNA restriction fragments

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Abstract

Reproducible migration times are required for accurate base-pair assignment of polymerase chain reaction (PCR) products in capillary gel electrophoresis (CGE). However, migration times are influenced by injection plug length and ionic strength of the sample. In this paper we introduce a new isotachophoresis (ITP)–CGE system where the transition from ITP to CGE is achieved by the mobility shift of DNA from free solution (ITP) to sieving gel buffer (CGE). With intrinsic isotachophoretic preconcentration in capillary gel electrophoresis (IICGE) large volume injections (up to 700 nl) are possible with accurate migration times in CGE independent of injection plug length or sample ionic strength.

1. Introduction

Although in capillary zone electrophoresis (CZE) only very small amounts of sample are required, the rather high concentration detection limits are a severe drawback. In order to be able to detect low sample concentrations, large sample volumes have to be injected, and sample analytes have to be concentrated to maintain a high resolution. A popular solution can be sample stacking whereby analytes are introduced at a very low ionic strength compared to that of the background electrolyte (BGE) [1]. Due to the relatively high local electric field, analytes migrate very quickly out of the sample zone and stack down in the BGE.

Field-amplified injection techniques for DNA analyses in capillary gel electrophoresis (CGE) have already been described. For example, a presample injection of a water plug creates a large field strength at the beginning of the capillary. This enhances the sample introduction into the capillary [2,3].

Another method is the introduction of an isotachophoretic (ITP) preconcentration step followed by separation in zone electrophoresis. In ITP–CZE the sample analytes are initially stacked between leading and terminating electrolytes. The concentration of the sample components are adapted to the concentration of the leading ion according to the Kohlrausch regulation function [4]. After the preconcentration ITP step the terminating ions pass the analyte ions and the analysis changes into zone electrophoresis. Several ITP–CZE combinations have been described. One possibility is the column coupling system described by Mikkers [5] and Everaerts et al. [6]. However, column coupling systems require rather complicated equipment. On-line versions of ITP–CZE can be performed in a

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single capillary on a commercial CE instrument. Foret et al. [7] described the possibility of on-column transient preconcentration in CZE. Schwer and Lottspeich [8] presented presample injections of high ionic strength buffer followed by the sample plug. Recently, van der Schans et al. [9] showed roughly the same method to be applicable for the analysis of DNA restriction fragments. However, migration times of equal-sized DNA molecules are not constant because the length of the gel decreases for increasing injection plug lengths. Moreover, non-homogeneous electric field strength distribution induced by sample stacking or ITP preconcentration steps causes migration-time shifts. These shifts lead to inaccurate base-pair assignment of polymerase chain reaction (PCR) products. In this paper we will discuss an ITP–CZE procedure whereby the transition from ITP to CZE is achieved by the mobility shift from free solution to gel buffer. Constant reproducible migration times in the CGE are obtained by creating a constant length of the gel.

2. Experimental

All experiments were performed on a P/ACE 2200 from Beckman Instruments (Fullerton, CA, USA). The instrument was modified to enable pressure injections at 1.8 p.s.i. instead of the instrument's own 0.5 p.s.i. (3447 Pa). Detection was performed by UV absorbance at 254 nm.

Running temperature was kept at 20°C. The length of the polyacrylamide-coated capillary was 47 cm. The effective length to the detector was 40 cm. The voltage was 9.4 kV (200 V/cm) applied in the reversed mode (cathode on injection side) for the zone electrophoresis experiments. For the intrinsic isotachophoretic preconcentration in capillary gel electrophoresis (IICGE) experiments a constant current of 18 μA was established.

2.1. Chemicals

Tris(hydroxymethyl)aminomethane (Tris), acetic acid, boric acid, benzoic acid and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Vinylmethoxysilane oligomer was purchased from ABCR (Karlsruhe, Germany). Butyric acid, propionic acid, acrylamide, ammonium persulphate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Sigma Chemicals (Bornem, Belgium). ΦX174/*Hae*III was purchased from Beckman as supplied in the Beckman dsDNA 1000 kit. The PCR product of 118 bp was kindly donated by A.W.H.M. Kuypers (Nijmegen Academic Hospital, Nijmegen, Netherlands)

2.2. Coating procedure

Capillaries with an I.D. of 100 μm from Scientific Glass Engineering (Milton Keynes, UK) were rinsed with 1 *M* KOH (3 h), water (0.5 h) and methanol (0.5 h). The capillary was rinsed for 2 h with a mixture of 4 ml methanol, 200 μl acetic acid and 200 μl vinylmethoxysiloxane oligomer. During this treatment vinyl groups were attached to the capillary surface, after which the capillary was rinsed again with methanol and water. Then the capillary was filled with 0.1 *M* Tris–borate buffer (pH 8.3) containing 5% acrylamide, 0.1% TEMED and 0.1% ammonium persulphate. After 24 h the gel was pushed out of the capillary with water using a gas-tight syringe. The capillary was mounted in a capillary cartridge and rinsed with buffer. This procedure was based on the method described by Hjertèn [10]. However, methoxyvinylsiloxane oligomer was used instead of 3-methacryloylpropyltrimethoxysilane. The coating using 3-methacryloylpropyltrimethoxysilane seems to be less stable because of the presence of the weak ester bond [11]. Our experiences are that the coating using methoxyvinylsiloxane oligomer is more stable.

2.3. Gel buffer

Linear polyacrylamide gel buffers were prepared by polymerisation of 8% acrylamide dissolved in 0.1 *M* Tris buffer containing 0.5% TEMED and 0.08% ammonium persulphate for 48 h at 4°C [12]. The Tris buffer was adjusted to

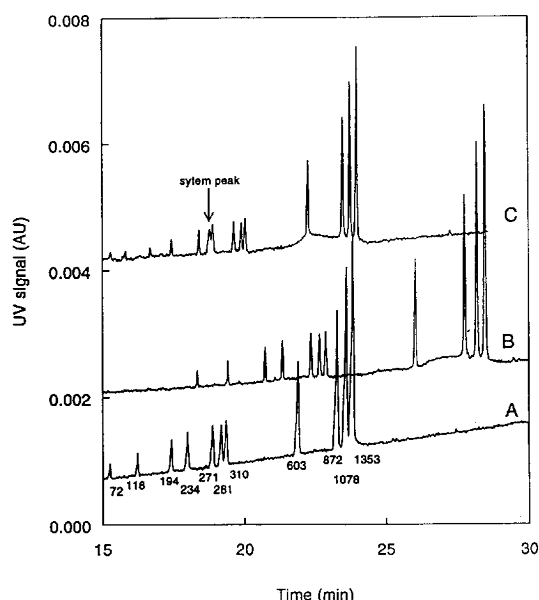

Fig. 1. Electropherogram of the separation of ΦX174/*Hae*III. Buffer: 3% polyacrylamide in 50 m*M* Tris–borate at pH 8.3. Voltage: 9.4 kV. (A) 4-s pressure injection 100 μg DNA/ml. (B) 80-s pressure injection 5 μg DNA/ml. (C) 80-s pressure injection 5 μg DNA/ml in 50 m*M* KCl, 10 m*M* Tris–HCl, 1.5 m*M* $MgCl_2$. Numbers refer to the number of base pairs.

pH 8.3 with hydrochloric acid, boric acid or benzoic acid. After polymerisation the gel was diluted to 3% polyacrylamide with water and buffer to achieve the appropriate concentration. The use of replaceable gels avoids sample carry over and sample-induced gel damage [13].

3. Results and discussion

3.1. DNA analysis in capillary zone electrophoresis

In Fig. 1 the electropherograms are given for the separations of the ΦX174/*Hae*III digest where the sample was introduced at different injection times and different ionic strengths in a 3% polyacrylamide buffer (50 m*M* Tris–borate at pH 8.3). The viscosity of the gel buffer was 23 cP. A 4-s pressure injection results in a plug length of 2 mm and a 80-s injection in a plug length of 27 mm. Plug lengths and viscosity were determined according to formulas given by Bello et al. [14]. Fig. 1A refers to the 4-s pressure injection. Good resolution is achieved, where all peaks are baseline resolved throughout the range of 72 to 1353 base pairs. Fig. 1B refers to a 80-s pressure injection of ΦX174/*Hae*III. The sample component concentration was 20 times lower than in the electropherogram representing the 4-s injection. Good resolution is still achieved due to the induced sample stacking conditions, because the ionic strength of the sample is low

Table 1
Migration times in minutes of DNA fragments analysed in zone electrophoresis in 50 m*M* Tris–borate buffer at pH 8.3

Injection time (s)	4	80	80	160	160
DNA conc. (μg/ml)	100	5	5	2.5	2.5
Matrix	Water	Water	Salt	Water	Salt
Basepairs					
72	15.3	18.3	15.8	20.2	17.7
118	16.2	19.4	16.7	21.5	18.2
194	17.4	20.8	17.4	23.1	18.9
234	18.0	21.4	18.4	23.8	19.3
271	18.9	22.4	19.6	25.0	19.9
281	19.2	22.7	19.9	25.3	20.1
310	19.4	22.9	20.0	25.6	20.2
603	21.9	26.0	22.3	29.5	22.0
872	23.3	27.8	23.5	31.7	23.1
1078	23.6	28.2	23.7	32.3	23.3
1353	23.9	28.5	24.0	32.7	23.5

and the voltage drop over the sample plug is high. The field strength in the rest of the capillary decreases, resulting in a lower velocity and longer migration times. However, real DNA samples do not have low ionic strengths. In particular, PCR products are delivered in electrolytes containing significant amounts of chloride [15]. Fig. 1C shows the result of 80-s pressure injection of ΦX174/*Hae*III dissolved in 63 m*M* chloride. A system peak appears between 234 and 271 base pairs when large amounts of salt matrix are injected. Again the electropherogram shows good resolution, but this is not achieved by sample stacking. The high ionic strength sample induces a low electric field in the sample plug, but the excess of chloride acts as a leading ion and creates temporary ITP conditions resulting in rather sharp zones [16]. However, migration times of the small base-pair strands (<600 bp) are shifted because the excess of chloride must migrate away first. In Table 1 the migration times of the DNA fragments for different injection times and ionic strengths are given. It can be concluded that migration times are strongly affected by gel length and sample ionic strength.

3.2. Intrinsic isotachophoretic preconcentration capillary gel electrophoresis

Migration times are influenced by the length of the gel and field strength distribution. The solution to this problem therefore must be found in a constant length of the gel and constant electric field distribution during the DNA separation. A new approach to this is the intrinsic isotachophoretic preconcentration in capillary gel electrophoresis (IICGE). The outline of such a system is shown in Fig. 2. First the capillary is filled with gel buffer. Then a plug of free solution buffer with the same electrolyte composition (pre-sample injection) is injected, followed by the sample. The outlet is placed in the leading electrolyte, and the inlet is placed in a terminating electrolyte. The total length of the sample plug and free solution buffer is held constant to maintain a constant length of the gel. Since the polyacrylamide in the buffer is uncharged and the electric osmotic flow is suppressed, the polyacrylamide stays in this position. The pre-sample injection is necessary to create an ITP section, where the sample analytes are concentrated and separated from the excess of chloride. DNA fragments are not separated since they have equal mobility because of a constant charge to mass ratio in free solution [17]. If the ITP stack reaches the gel, the

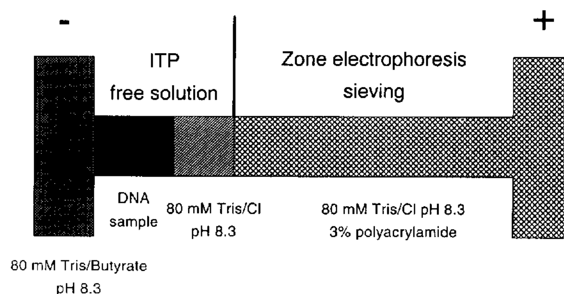

Fig. 2. Outline of the IICGE method. Preconcentration takes place in free solution. Separation takes place in sieving part of the capillary. For further information see text.

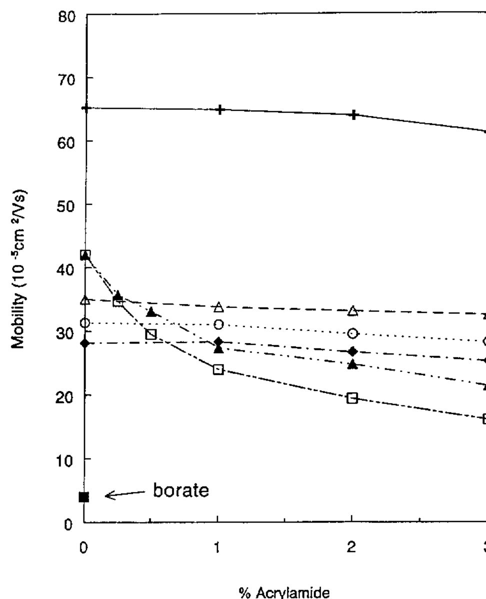

Fig. 3. Mobilities of different ions and DNA versus gel concentration. Mobility of ions are measured in indirect UV mode at 230 nm using a 10 m*M* Tris–benzoate buffer at pH 8.3. Symbols: + = chloride; △ = acetate; ○ = propionate; ◆ = butyrate; ▲ = 72 bp DNA; □ = 603 bp DNA; ■ = borate.

mobility of the DNA strongly decreases because it is size and gel-concentration dependent in gel [18]. If a suitable terminating ion is chosen, the DNA fragments migrate further in zone electrophoresis. A comparable system has already been described by Ornstein [19] and Davis [20] for proteins, where the transition from ITP to CZE is also achieved by a pH shift (see also Hjertèn et al. [21]).

In order to choose a suitable terminating ion, we measured the effective mobilities of several ions in free solution and gel buffer. In Fig. 3 the mobilities of different ions and dsDNA fragments are given for different polyacrylamide concentrations. The mobility could be calculated using field strength, migration time and capillary length. The ions were analysed in a gel buffer using indirect UV detection at 230 nm. The buffer contained 10 m*M* of Tris adjusted to pH 8.3 with benzoic acid. Sulphate ions from the initiator ammonium persulphate were also present in the sieving buffer. These co-ions can lead to artifacts and system peaks in the electropherogram when indirect UV detection is used and make the electropherogram difficult to interpret [22]. Therefore, the capillary was filled with gel buffer containing Tris–benzoate, whereafter the capillary ends were placed in free solution buffer. A 10-kV voltage was applied for 7 min allowing the sulphate ions to migrate out of the capillary. Then the sample ions were injected and analysed.

Ions like chloride, acetate and propionate are only slightly retarded in gel because of their small size. Chloride is always faster than DNA and can act as leading ion. Acetate, propionate and butyrate have lower mobility in free solution but higher than DNA in a 3% polyacrylamide gel. This means that in free solution these ions will act as a terminator, but they will overtake DNA as DNA will migrate further in a zone electrophoretic way. It is clear that borate does not fulfil this requirement. Borate has a lower mobility than DNA in free solution. The mobility of borate in gel buffer will be lower than the mobility of DNA, too. The injection of sample between chloride and borate would result in a isotachopherogram where no separation of the DNA takes place at all, because DNA remains stacked between chloride and borate (Fig. 4).

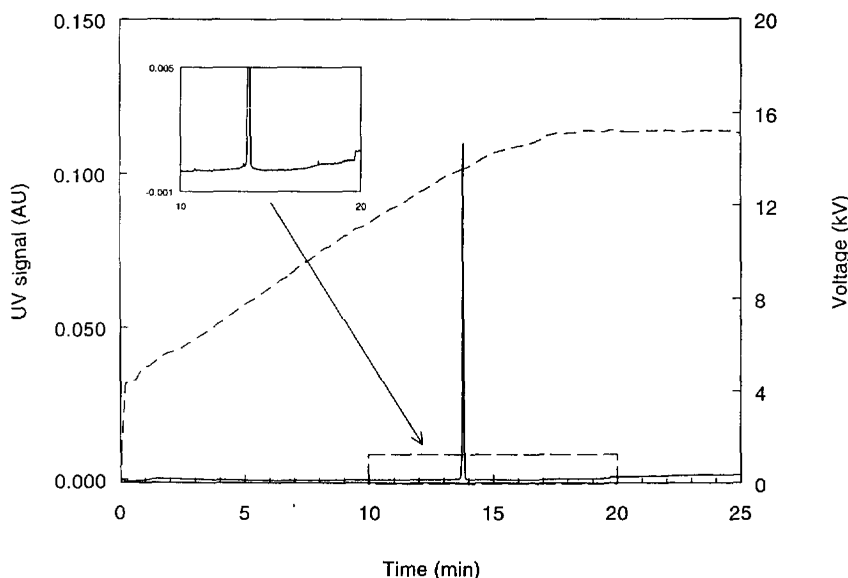

Fig. 4. Electropherogram for the separation of ΦX174/*Hae*III applying borate as terminator. Capillary and outlet vial: 3% polyacrylamide in 80 m*M* Tris–HCl at pH 8.3. Inlet vial: 100 m*M* Tris–borate at pH 8.3. Pre-sample injection: 300 s by pressure 80 m*M* Tris–HCl at pH 8.3. Sample injection: 10 s by pressure, sample as in Fig. 1A. Current: 18 μA. Solid line = UV signal, dashed line = voltage.

Butyrate was chosen as terminator because of the large difference in mobility between DNA and butyrate in free solution. A large mobility difference will have a more effective zone-sharpening effect.

3.3. DNA analysis using intrinsic isotachophoretic preconcentration in CGE

Fig. 5 shows an electropherogram of ΦX174/*Hae*III using IICGE conditions. Tris–chloride buffer was injected for 300 s followed by 100 s injection of sample (sample plug length = 52 mm; total ITP length = 182 mm). The voltage over the capillary is plotted on the right axis. Butyrate ions enter the capillary and must have the same velocity as the chloride ions to fulfil the isotachophoretic condition. This means that the voltage over the capillary increases during the run because of the lower mobility of the terminator. The field strength in the butyrate zone will be constant and therefore also the velocity of the DNA during the separation. The voltage curve shows a smaller slope at the beginning. The system regulates the concentration of the chloride according to the Kohlrausch regulation function. The chloride concentration in the sample was 63 m*M*, while the chloride concentration in the buffer was 32 m*M*. (Dissociation degree of Tris at pH 8.3 is 0.40. Total concentration Tris is 80 m*M*, so the concentration chloride is 32 m*M*.) The voltage becomes constant when the last chloride ions leave the capillary and the capillary is totally filled with butyrate. An extra peak is observed when the last chloride ions pass the detector. This peak is caused by impurities in the butyrate electrolyte which are concentrated to very sharp zones and are not retarded by the sieving buffer. It acts as a nice reference peak for the leading–terminator transition and can be used to correct for the amount of chloride introduced in the capillary by the sample. Fig. 6 shows the electropherogram of 50 μg/ml sample injected for 12 s and a 5 μg/ml sample injected for 100 s. With the longer injection time also a long high ionic strength sample plug is introduced, resulting in different migration times for the equal-sized DNA fragments. However, the

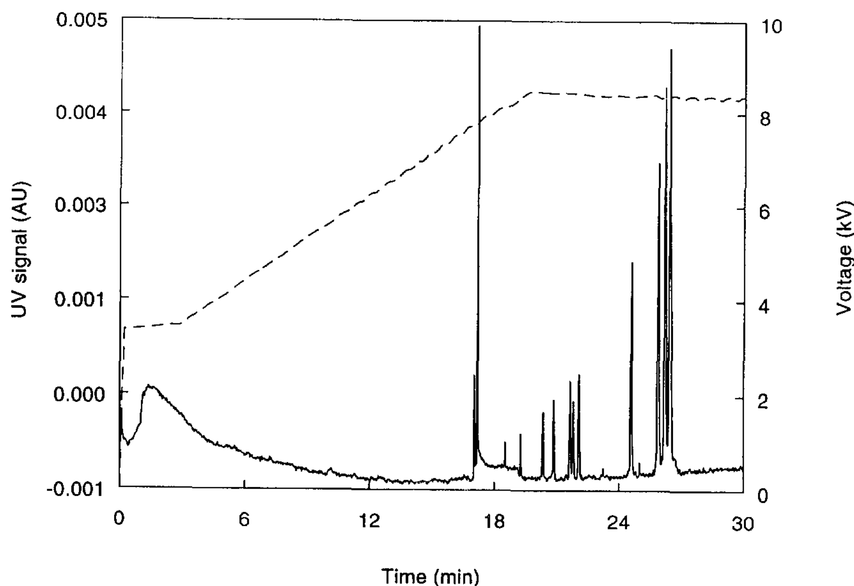

Fig. 5. Electropherogram for the separation of ΦX174/*Hae*III applying butyrate as terminator. Capillary and outlet vial: 3% polyacrylamide in 80 m*M* Tris–HCl at pH 8.3. Inlet vial: 100 m*M* Tris–butyrate at pH 8.3. Pre-sample injection: 300-s 80 m*M* Tris–HCl at pH 8.3 by pressure. Sample injection: 100 s by pressure, sample as in Fig. 1C. Current: 18 μA. Solid line = UV signal, dashed line = voltage.

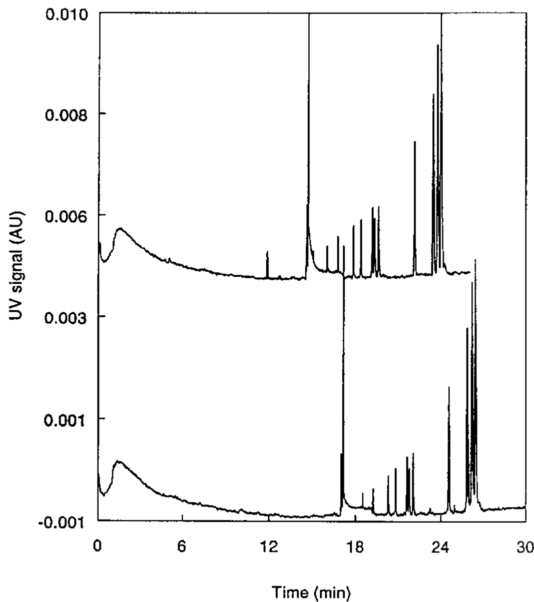

Fig. 6. Electropherograms for the separation of ΦX174/*Hae*III at different injection times. Upper electropherogram: 388-s pre-sample pressure injection of 80 m*M* Tris–HCl at pH 8.3; 12-s pressure injection of sample 50 μg/ml ΦX174/*Hae*III. Lower electropherogram: 300-s pre-sample pressure injection of 80 m*M* Tris–HCl at pH 8.3; 100 s pressure injection as in Fig. 1C. Further conditions as in Fig. 5

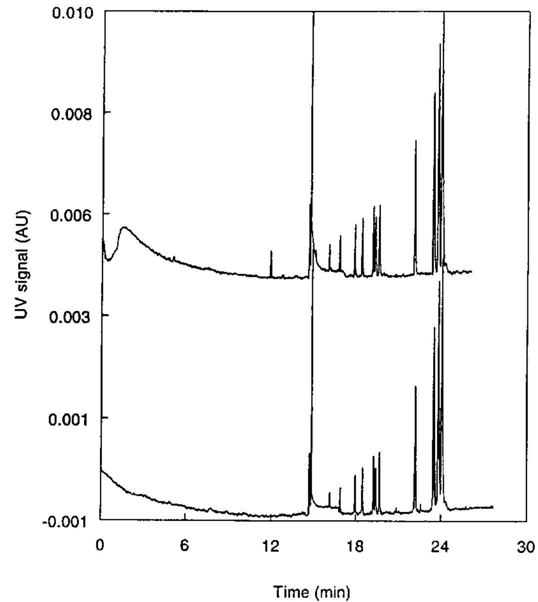

Fig. 7. Overlay of electropherograms with matched ITP peaks. Further conditions as in Fig. 6

reduced migration times are constant when the two electropherograms are overlaid and the ITP peaks are matched (Fig. 7). Table 2 shows the results of the migration times at different sample injection times. The migration times can be corrected for chloride amount by subtracting the time of the ITP peak from the DNA migration times. The migration times referring to the 210-s pressure injection in water matrix are an exception. A large part of the capillary was filled with very low ionic strength sample, and the ITP steady-state was never reached.

Fig. 8 shows the separation of an ΦX174/*Hae*III and a 118-bp PCR product in a Tris–borate zone electrophoretic system. PCR product and ΦX174/*Hae*III were injected in different runs for 100 s under pressure. Base-pair assignment of the PCR product fails because of differing ionic strengths of the sample. The PCR product has 118 base pairs but migrates like 200 bp compared with the standard. Fig. 9 shows the same analysis but under IICGE conditions. When the ITP peaks are overlaid, it is clear that the PCR peak of 118 bp migrates exactly like the 118-bp peak of the ΦX174/*Hae*III.

4. Conclusion

Large sample volume injection in CGE leads to inaccurate migration times for equal-sized DNA molecules injected at different plug lengths and ionic strength. Also, stacking and ITP preconcentration techniques induce heterogeneous field strength distribution, resulting in different migration times. IICGE is a method that makes use of the mobility shift of DNA from free solution to sieving buffer to achieve the transition from ITP to CZE. This can only be realised by the choice of a correct terminator (in this case butyrate), which must be slower than DNA in free solution but faster in the sieving buffer. This method enables the injection of a large sample volume (up to 700 nl) in a capillary of 47 cm. Constant reduced migration times are achieved

Table 2
Migration times and reduced migration times of DNA fragments as achieved in IICGE (reduced migration times are printed bold)

Injection time (s)	12	12	100	100	100	100	210	210	210	210
DNA conc. (μg/ml)	50	50	5	5	5	5	2.5	2.5	2.5	2.5
Matrix	Water	Water	Water	Water	Salt	Salt	Water	Water	Salt	Salt
Basepairs										
ITP	14.6	**0.00**	13.3	**0.00**	17.0	**0.00**	14.2	**0.00**	19.0	**0.00**
72	16.0	**1.47**	14.8	**1.46**	18.5	**1.50**	15.7	**1.54**	20.6	**1.54**
118	16.8	**2.21**	15.6	**2.23**	19.3	**2.25**	16.5	**2.32**	21.4	**2.29**
194	17.8	**3.29**	16.6	**3.31**	20.3	**3.32**	17.7	**3.47**	22.4	**3.37**
234	18.4	**3.81**	17.2	**3.85**	20.9	**3.84**	18.2	**4.00**	23.0	**3.90**
271	19.2	**4.60**	18.0	**4.64**	21.6	**4.61**	19.1	**4.83**	23.8	**4.69**
281	19.3	**4.76**	18.1	**4.80**	21.8	**4.77**	19.2	**5.00**	23.9	**4.84**
310	19.6	**5.04**	18.4	**5.08**	22.1	**5.05**	19.5	**5.30**	24.2	**5.12**
603	22.1	**7.55**	20.9	**7.62**	24.8	**7.56**	22.2	**7.97**	26.7	**7.63**
872	23.4	**8.85**	22.3	**8.95**	25.9	**8.85**	23.6	**9.39**	28.0	**8.95**
1078	23.7	**9.17**	22.6	**9.26**	26.2	**9.16**	23.9	**9.71**	28.3	**9.27**
1353	24.0.	**9.41**	22.8	**9.59**	26.4	**9.39**	24.2	**9.98**	28.6	**9.51**

because the gel length is kept constant, and migration-time shifts caused by different ionic strengths can be easily corrected. This method makes CE more applicable for real sample analysis with a minimum of sample preparation for PCR products.

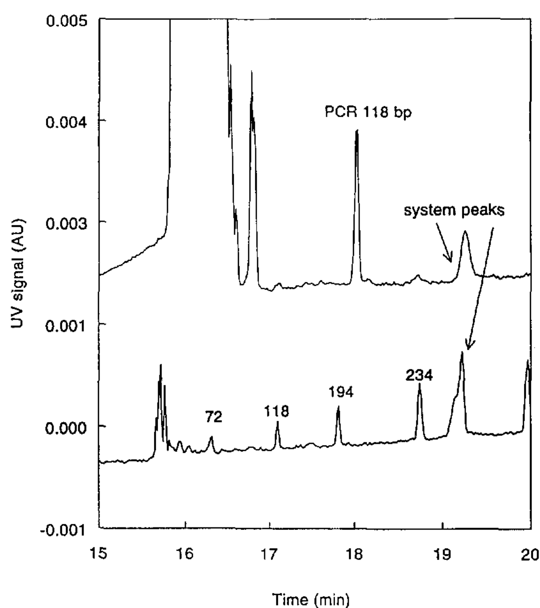

Fig. 8. Base-pair assignment of PCR products fails in CGE using Tris–borate buffer. Upper electropherogram: PCR 118 bp injected for 100 s. Lower electropherogram: sample as in Fig. 1C injected for 100 s. Further conditions as in Fig. 1

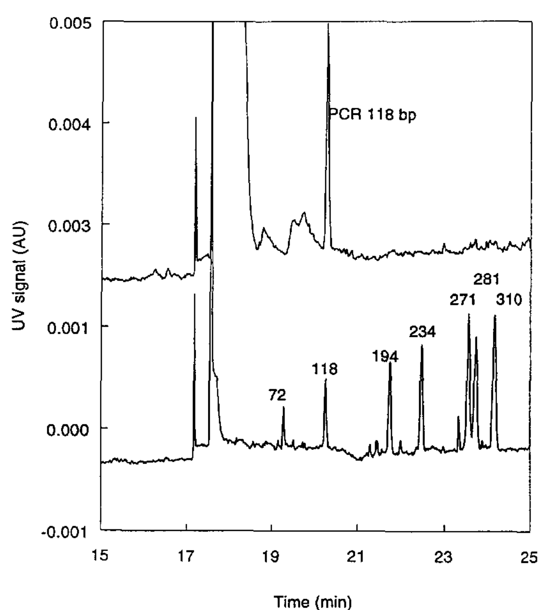

Fig. 9. Correct base-pair assignment of PCR product in IICGE. Upper electropherogram: PCR 118 bp injected for 100 s. Lower electropherogram: sample as in Fig. 1C injected for 100 s under pressure. Further conditions as in Fig. 5

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