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Effects of sample matrix and injection plug on dsDNA migration in capillary gel electrophoresis

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Abstract

Reproducible DNA migration times are required for accurate basepair assignment in restriction fragment mapping and polymerase chain reaction product identification. Our data shows DNA migration time shifts with changes in sample ionic strength. Secondly, loss of resolution with replaceable polyacrylamide gels was observed when increasing the length of the sample plug with pressure injection. An easy way to correct for the migration time shifts is to incorporate an internal DNA standard directly into the separation process by consecutively injecting the DNA sample and the DNA standard. This allows for compensation of any possible migration time variation caused by high ionic strength sample matrices. Also high-resolution separations can be maintained with large injection volumes (long injection plug) by using consecutive injections of 0.1 M Tris–acetate buffer and the DNA sample.

1. Introduction

Capillary gel electrophoresis (CGE) has been used recently for the analysis of double-stranded deoxyribonucleic acid (dsDNA) molecules such as DNA restriction fragments and polymerase chain reaction (PCR)[2] products [1]. Advantages of using CGE for dsDNA separations over slab gel methods are direct quantitation, higher resolution and fast single sample analyses using nanoliter sample volumes. CGE offers the option of employing cross-linked and non-cross-linked gels [2]. The use of non-cross-linked gels allows the replacement of the sieving buffer in the capillary after each run, eliminating any

sample-induced gel damage or sample carryover [3,4].

Injection in CGE can be performed either electrophoretically or by pressure. An electromigration injection typically requires sample desalting to reduce the ionic strength of the sample allowing sufficient amounts of DNA to enter the gel-filled capillary. It should be mentioned that desalting may change the DNA concentration of the sample due to incomplete sample recovery. However, it is important to note that with cross-linked gels electromigration is the only possible injection method. Further, electromigration offers better peak efficiency than a pressure injection for low ionic strength samples, since DNA molecules are stacked against the gel buffer while they migrate into the capillary resulting in sharper peaks [5,6]. With the use of replaceable sieving matrices (non-cross-linked or physical gels) pressure injection is

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possible with no sample preparation requirement and therefore no sample loss.

This paper describes some peculiar characteristics observed with pressure injection of dsDNA samples using replaceable polyacrylamide gels. Specifically DNA-size-dependent shifts in migration times and peak splitting were observed with long injection times and/or high-ionic-strength samples. This effect can be alleviated by means of stacking techniques which allows long injection times when using high ionic strength samples.

2. Materials and methods

The capillary electrophoresis system used in these experiments was a P/ACE 2100 from Beckman Instruments (Fullerton, CA, USA) which was equipped with both UV absorbance and Laser-Induced Fluorescence (LIF) Detector. Capillary gel running buffer (i.e. replaceable sieving buffer) and capillaries used in these experiments were from the eCAP dsDNA 1000 kit (Beckman Instruments). Analyses using the LIF detector were performed with the LIFluor dsDNA 1000 kit (Beckman Instruments). The ΦX-174 HaeIII restriction digest used throughout the study was obtained from two sources: Promega, (Madison, WI, USA) and New England Biolabs. (Beverly, MA, USA). No difference between the two samples was observed. Aliquots were made of the original restriction digest in water and frozen. Frozen samples were brought to ambient temperature prior to injection. PCR samples used in this study were kindly donated by Dr. Edward Rossomando, University of Connecticut Health Center.

Gel buffer was filtered through a 0.45-μm pore size cellulose acetate filter (Schleicher & Schuell, Keene, NH, USA). Capillary length was 30 cm to the detector, 37 cm total and a constant running voltage of 7.4 kV (200 V/cm) was used (cathode on the injector end). Detection was either UV absorbance at 254 nm or LIF using a 488-nm air-cooled Argon Ion laser as the excitation source. An emission band-pass filter of 530 nm was used during LIF detection. The capillary

cartridge temperature was maintained at 20°C. The capillary was rinsed before each run for 3 min at 20 p.s.i. (1 p.s.i. = 6894.76 Pa) with gel buffer. Pressure injections were performed at 0.5 p.s.i. The length of the injection plug was 1.3 mm for a 10-s pressure injection into the capillary.

3. Results and discussion

A typical electropherogram of ΦX-174 HaeIII digest using the gel buffer (non-cross-linked gel) is shown in Fig. 1a using a 10-s pressure injection. Good resolution is achieved without using intercalators, excessively long capillaries or volt-
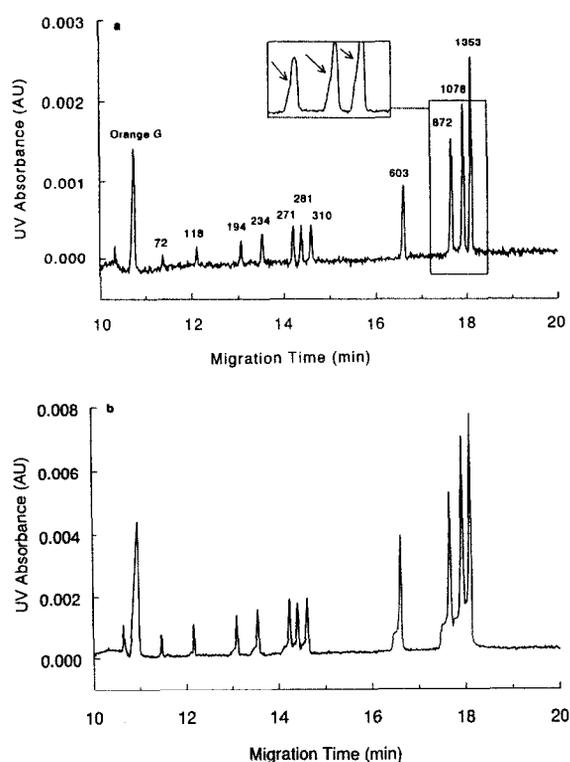
Fig. 1. (a) Electropherogram of a 100 μg/ml ΦX-174 RF DNA HaeIII. Capillary and buffer: as supplied by Beckman eCAP dsDNA 1000 kit. Separation voltage 7.4 kV, cathode (−) on injection end. Pressure injection of 10 s. Detection: UV absorbance at 254 nm. Peaks are labeled according to the number of DNA basepairs corresponding to that fragment. Orange G is a reference marker. (b) Same conditions and peak assignments as in (a) except injection time was 30 s.

age programming. Fig. 1b represents identical conditions except a longer 30 s pressure injection was performed. Migration time shifts from a 10-s pressure injection are negligible compared to a 30-s pressure injection. However, the fronting shoulders become more pronounced by increasing the size i.e. length of the injection plug for 30 s (see Fig. 1b, inset 1a). The shoulders are less apparent for the 10-s pressure injection, but the inset in Fig. 1a still shows the appearance of shoulders. Although selectivity has not changed, the peak shoulders represent a significant loss in efficiency resulting in decreased resolution throughout the basepair range shown.

Resolution loss and migration time shifts are more pronounced when the ΦX-174 HaeIII sample is dissolved in a salt matrix which is a typical matrix for restriction fragments and PCR products. Low salt concentrations such as 20 mM NaCl in the ΦX-174 HaeIII sample, caused significant changes in peak shape evidenced by the fronting shoulders on all dsDNA fragments (Fig. 2a).

A possible explanation for the fronting shoulder development with dsDNA in water is the relatively high electric field in the sample zone. The resulting high velocity of dsDNA in the sample zone will lead to a stacking of dsDNA molecules at the water/gel interface. This results in asymmetric peaks with diffused fronts and sharp ends. The amount of dsDNA diffusing/migrating into the gel thus partially escaping the ongoing stacking at the water–gel interface, will determine the extent of fronting shoulder.

An explanation of the observed fronting shoulders/peak splits in a salt matrix, might be found in both electrophoretic and isotachophoretic effects occuring simultaneously. Beckers and Everaerts [7] showed that an excess of chloride in the sample can lead to irregulated electrophetic migration behaviour. Mikkers et al. [8] noted irregular behaviour may cause peak shoulders or split peaks. In addition to these effects, stacking can also occur when an aqueous solution is injected into a sieving gel matrix [9].

A double injection technique, where a plug of 0.1 M Tris–acetate (pH 8.3) is placed in front of the dsDNA sample, corrects this shoulder prob-
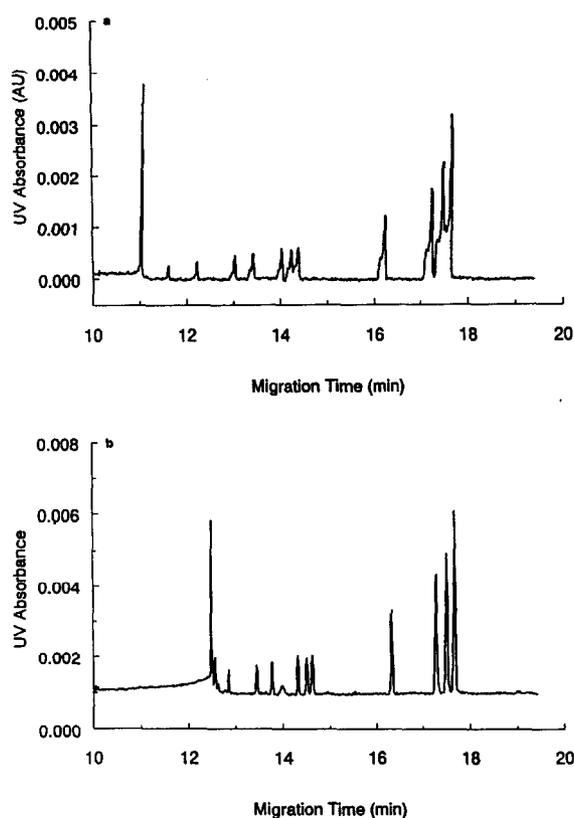

Fig. 2. (a) Electropherogram of a 50 μg/ml ΦX-174 RF DNA HaeIII dissolved in 20 mM NaCl. Conditions and peak assignments as in Fig. 1a. (b) Influence of presample injection of 0.1 M Tris–acetate pH 8.3. Injection procedure: first injection: 10-s pressure injection of Tris–acetate; second injection: 20-s pressure injection of 50 μg/ml ΦX-174 HaeIII sample in 20 mM NaCl.

lem (see Fig. 2b). The relative low resistance of the Tris–acetate, compared to resistance of gel buffer and sample, induces a localized lower electric field than in the sample. This causes electrophoretic stacking of the dsDNA in the Tris–acetate (see Fig. 3). This double injection method allows sample loadability of two or three times the normal amount with no significant loss of resolution.

The salt matrix also induces migration time shifts for the smaller dsDNA molecules (see Fig. 4). Obviously, shifts in migration time cause erroneous DNA basepair assignments when using values based on calibration curves with dsDNA from a different sample matrix. The

a

$E_1 > E_2$

b

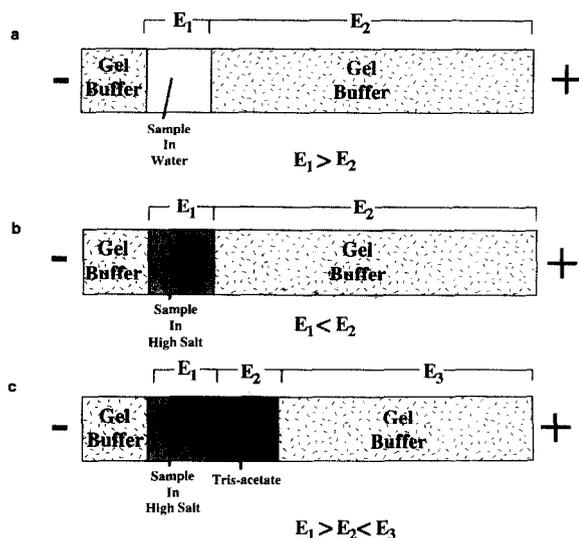$E_1 < E_2$

c

$E_1 > E_2 < E_3$

Fig. 3. Axial electric field profiles for (a) injection of sample in water, (b) injection of sample in high salt and (c) double injection technique as described in text.

presence of salt in the sample causes a lower potential drop in the beginning of the separation resulting in migration time shifts since the mobility of different size dsDNA molecules is field dependent [10,11]. The migration time shifts are dependent on the mobility of the different dsDNA molecules. The shift for the smaller dsDNA is greater because they are
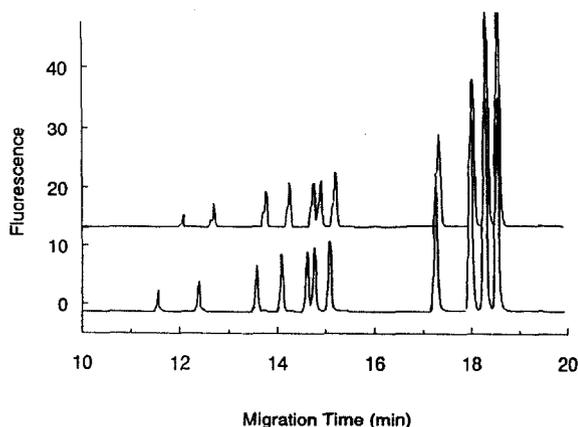

Fig. 4. High ionic strength samples cause a migration time shift. Upper electropherogram: 50 μg/ml ΦX-174 HaeIII in 20 mM NaCl. Lower electropherogram: 50 μg/ml ΦX-174 HaeIII in water. Conditions and peak assignments as in Fig. 1a; detection: LIF.

exposed longer to the lower electric field induced by the salt matrix.

Careful selection of an internal standard to correct for variations in sample matrix needs to be considered. The most accurate internal standard for correcting dsDNA migration times would be to use other well characterized DNA fragments of a similar size to the DNA of interest yet different enough to allow resolution of all fragments. The procedure to compensate for changes in salt matrix is to inject a separate, well characterized dsDNA with an identical matrix to the sample PCR product or restriction fragment of interest. However this approach is not useful in practice since obtaining identical ionic strengths for both sample and standards would be difficult. A simple method exists to compensate for migration time shifts using a dsDNA internal standard. An injection of PCR sample immediately followed by a DNA standard, (double injection method) can be used to correct for migration time shifts due to high ionic strength dsDNA samples. The majority of PCR and restriction fragment digests have sample matrices of high ionic strength. As shown in Fig. 5a (PCR, ΦX-174 HaeIII) the high ionic strength of the PCR sample acts in a similar way to the Tris–acetate pre-sample plug. In this case, the PCR sample is injected first followed by ΦX-174 HaeIII digest dissolved in Milli-Q water, which acts as the dsDNA internal standard. The result is that the ΦX-174 HaeIII fragments, experience a higher electric field and therefore migrate into the high salt sample matrix. This is an easy and effective way of introducing a well characterized dsDNA standard into an unknown PCR or restriction fragment digest sample while also compensating for shifts in migration times and eliminating dsDNA peak shoulders. However, Fig. 5a shows the PCR product still has a shoulder, since it does not experience a lower electric field stacking. Further, it may be possible to determine the concentration of the PCR product itself if the dsDNA standard concentration is known. An additional advantage of the double injection technique is that the original sample of interest is not contaminated with internal standard dsDNA. Fig. 5b shows the
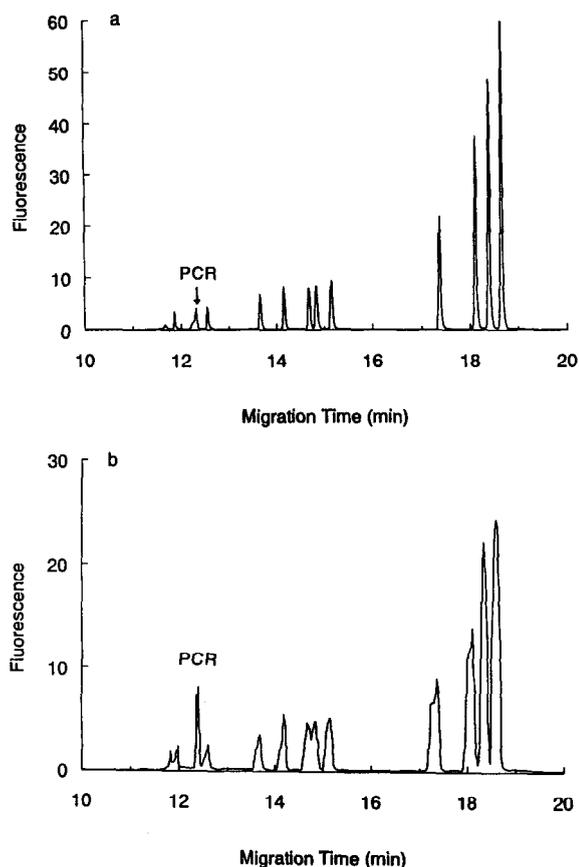
Fig. 5. (a) Electropherogram of PCR sample and DNA standard. First injection: PCR sample (Sabin 3, 97 bp); second injection: ΦX-174 HaeIII 10 μg/ml. (b) Electropherogram of DNA standard and PCR sample. First injection: ΦX-174 HaeIII 10 μg/ml; second injection: PCR sample (Sabin 3, 97 bp). Peak assignment and conditions as in Fig. 4; PCR = peak of PCR product.

reversed situation wherein the DNA standard is injected prior to the PCR product. The observed band broadening is due to the salt matrix of the PCR sample migrating into the ΦX-174 HaeIII water plug. As a result, the back end of the ΦX-174 HaeIII zone will migrate at a slower velocity relative to the front since it experiences a lower electric field due to the salt matrix, leading to excessive band broadening.

The electropherograms shown in Figs. 4 and 5 were analyzed employing the LIF detector. There are several advantages in using the LIF

detector for dsDNA detection. Since the PCR samples were analyzed directly from the thermal cycler, without sample preparation, many of the reactants e.g. dNTPs, polymerase were still present and would be detected using UV detection. By adding a fluorescent intercalator, to the running buffer, a complex is formed between the dsDNA and the intercalator thus the PCR reactants are not detected. Also, the lower detection limits possible with the LIF allow sample dilution which decreases the salt matrix effect. One final advantage of using LIF is the resolution of the separation is better when an intercalator is used [12]. It should be noted that the intercalator used had no effect on the sample induced migration time shifts or fronting shoulders.

4. Conclusions

Migration behaviour of dsDNA is influenced by the salt concentration in the sample matrix. Potentially this might be a problem in basepair assignments. A simple method to obtain a reliable basepair assignment is a double injection technique in which PCR sample and DNA standard are injected sequentially. Another possible analysis problem occurred when long injection times were used resulting in a loss of resolution. A presample injection of Tris–acetate makes sample loadability of two or three times the usual amount possible without significant loss in resolution.

The double injection technique of PCR sample, followed by an injection of a DNA standard in water, corrects for migration time shifts caused by differenes in ionic strength. However the injection order seems to be critical, since a reversed order of injection causes peak shoulders. These two injection techniques combined with LIF detection offer a more sensitive and accurate method for the analysis of PCR products and restriction fragments.

References

[1] K.J. Ulfelder, H.E. Schwartz, J.M. Hall and F.J. Sunzeri, Anal. Biochem., 200 (1992) 260.

[2] D.N. Heiger, A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 516 (1990) 33.

[3] Y.F. Pariat, J. Berka, D.N. Heiger, T. Schmitt, M. Vilenchik, A.S. Cohen, F. Foret and B.L. Karger, *J. Chromatogr. A*, 652 (1993) 57.

[4] M. Gilges, M.H. Kleemiss and G. Schomburg, presented at the *5th International Symposium on High Performance capillary Electrophoresis, Orlando, FL, 25–28 January, 1993*, Poster.

[5] H.E. Schwartz, K.L. Ulfelder, F.J. Sunzeri, M.P. Busch and R.G. Brownlee, *J. Chromatogr.*, 559 (1991) 267.

[6] L. Ornstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.

[7] J.L. Beckers and F.M. Everaerts, *J. Chromatogr.*, 508 (1990) 19

[8] F.E.P. Mikkers, F.M. Everaerts, Th.P.E.M. Verheggen, *J. Chromatogr.*, 169 (1979) 1.

[9] J. Chrambach, *The Practice of Quantitative Gel Electrophoresis*, VCH, Deerfield Beach, FL, 1981.

[10] A. Guttman, *Appl. Theor. Electrophoresis*, 3 (1992) 91.

[11] A. Guttman, B.J. Wanders and N. Cooke, *Anal. Chem.*, 64 (1992) 2348.

[12] A. Guttman and N. Cooke, *Anal. Chem.*, 63 (1991) 2038.