

Wall Coating for DNA Sequencing and Fragment Analysis by Capillary Electrophoresis

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Abstract: In capillary electrophoresis, covering the inner capillary surface with a coating is an efficient way to minimize both the electroosmotic flow and sorption of analytes on the capillary wall. We modified the procedure by Cobb et al. [*Anal. Chem.* **62**, 2478 (1990)] for preparing wall coating to permit large-scale production. Specifically, we use a positive pressure to fill the capillary with both thionyl chloride and later vinylmagnesium bromide solution. This enables large-scale production of the coating by treating 100 m capillary pieces at a time. We found that no extensive flushing with either organic solvents or sodium hydroxide is needed before the reactions are performed. Application of liquid thionyl chloride with positive pressure scavenges residual humidity on the capillary surface and eliminates a need for extensive drying of the capillary. In the polymerization step, elimination of TEMED from the polymerization mixture and incubation at 70°C enables a homogeneous coating to be prepared in capillaries as long as 100 m. The prepared wall coating is stable for approximately 110 runs of DNA sequencing in a denaturing matrix and over 300 runs of DNA fragment analysis under nondenaturing conditions. © 1998 John Wiley & Sons, Inc. *J Micro Sep* 10: 175–184, 1998

Key words: capillary wall coating; DNA sequencing; DNA fragment analysis; DNA profiling; genotyping; thionyl chloride; vinylmagnesium bromide; acrylamide, hydroxyethyl cellulose

INTRODUCTION

In capillary electrophoresis, a permanent wall coating has often been used to eliminate or reduce substantially electroosmotic flow and interactions between analytes and the capillary wall [1–22]. Electroosmotic flow can be completely eliminated by attachment of a polymer layer to the wall as demonstrated in 1985 by Hjertén [1], who attached polyacrylamide to the silica surface by means of γ -methacryloxypropyltrimethoxysilane. Unfortunately, this coating has a limited lifetime at alkali pH due to alkali hydrolysis of the $-\text{Si}-\text{O}-\text{Si}-$ bond. Other chemistries have since been applied to attach a polymer layer to the inner capillary wall [3, 8, 21] A wall coating significantly more stable than that of Hjertén was obtained when silica surface was first

chlorinated and then reacted with vinylmagnesium bromide followed by acrylamide polymerization [3, 16, 17].

DNA sequencing and fragment analysis are important applications of capillary electrophoresis [23–44]. The low electrophoretic mobility of large DNA molecules makes these assays extremely sensitive to inhomogeneities in the wall coating. This is due to “eddy migrations” which result from a residual ζ -potential. Fung and Yeung have published DNA sequencing separations in uncoated capillaries. [45]. However, no resolution data were shown for fragments of 108–420 bp, and the capillaries required time-consuming treatment between runs.

This article describes a method for the large-scale production of a stable wall coating which is suitable for DNA sequencing and DNA fragment analysis under both denaturing and nondenaturing conditions. The wall coating permits high-resolution separation of DNA sequencing ladders to over 650

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bp or nondenaturing fragment up to 40 kbp to be performed.

EXPERIMENTAL

Materials. Chloroform, acetone, thionyl chloride, 1 M vinylmagnesium bromide, formamide, acrylamide, and ammonium persulfate were purchased from Aldrich (Milwaukee, WI). Anhydrous tetrahydrofuran was obtained from Fluka (Buchs, Switzerland), hydroxyethyl cellulose (HEC) from Polysciences (Warrington, PA), urea and TBE buffer [89 mM Tris, 89 mM boric acid, 1 mM ethylenediaminetetraacetic acid (EDTA)] from Amersham Life Sciences (Chicago, IL), polyethylene oxide from Union Carbide (Houston, TX), Vistra Green from Amersham Life Sciences (Chicago, IL), 20 and 100 bp ladders from (GenSura, Inc., Del Mar, CA), and 9-amino acridine from Sigma (St. Louis, MO). Fused silica capillaries (200 μm o.d., 75 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ).

Preparation of samples. DNA sequencing fragment ladders (T track) were prepared by the following procedure: 2 μL of 1.5×10^{-7} M primer (F10F) [46] and 2 μL of T reagent from the Thermo-SequenaseTM labeled primer cycle sequencing kit (Amersham Life Sciences, Chicago, IL) were mixed in 0.2-mL tubes with 10 μL of solution containing 0.2 μg of M13mp18 single-stranded DNA (ssDNA). The tubes were briefly vortexed, centrifuged, and then placed in a Peltier thermal cycler, model PTC-225 (MJ Research, Inc., Watertown, MA), for 20 cycles (30 s at 95°C, 10 s at 60°C). After thermal cycling, a 35- μL aliquot of 100% ethanol was added to precipitate the DNA. The mixture was incubated at -20°C for approximately 45 min, and samples were centrifuged at 3100 g for 30 min to pellet DNA. The supernatant was removed and the samples were vacuum dried for 20 min under simultaneous mild heating and then stored at -20°C . DNA samples were dissolved in 5 μL of formamide each. Prior to electrophoresis, samples were denatured by heating to 90°C for 3 min.

Instrumentation. A 48-capillary prototype system [47] was used to separate fluorescently labeled DNA sequencing ladders. The instrument optical design was based on a system described by Mathies and Huang [48]. Light from a 20-mW argon-ion laser (488 nm) passes through a microscope objective that sequentially focuses on each capillary as it moves at a scan rate of 1 Hz. Confocal fluorescence imaging is used in the system, in which the exciting light is focused on the capillary by a microscope objective and the emitted light is collected by the same objec-

ive. After passing through a dichroic beamsplitter, which rejects the exciting laser light, the emitted light is focused on a pin hole. Only light passing through the pinhole and a 530-nm interference filter reaches the photomultiplier.

Method. Electrophoresis was performed at room temperature in capillaries (200 μm o.d., 75 μm i.d.) with a total length of 65 cm and effective length of 40 cm with a coating made as described below and assembled into arrays of 16 capillaries. For DNA sequencing and denaturing fragment analysis, the separation matrix contained 2% hydroxyethyl cellulose (MW 90,000–105,000), 6 M urea, 10% formamide and 1X TBE, pH 8.1 [49]. The nondenaturing matrix for fragment analysis contained 1% polyethylene oxide (MW 10^6), 1X TBE, 20 μM 9-amino acridine and 2 nM Vistra Green, pH 8.1. The capillaries were filled before each run with the separation matrix from the anode pressure manifold using nitrogen at a pressure of 2.8 MPa (400 psi). Between runs, capillaries were flushed with deionized water. Samples were injected electrokinetically for 20–60 s at 6 kV from a cathode manifold that had the footprint of a half microwell plate. The separation was performed at 12 kV for ca. 70 min. Data was analyzed using ArrayQuantTM software (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Wall coating preparation. A 100-m length of fused silica capillary (200 μm o.d., 75 μm i.d.) was placed in a 70°C water bath and flushed with thionyl chloride from a chamber pressurized with dry nitrogen to 13.8 MPa (2000 psi). After 100 μL of thionyl chloride passed through the distal end of the capillary, the pressure was reduced to 1.4 MPa (200 psi) and the capillary was flushed with thionyl chloride for at least 12 h. When approximately 1 mL total of thionyl chloride had passed through the capillary, a 2.5-mL gastight Hamilton syringe was filled with 1 M vinylmagnesium bromide and attached to the capillary inside the pressure chamber. The chamber was then pressurized to 13.8 MPa (2000 psi). The capillary was voided of thionyl chloride (i.e., ca. 500 μL of it passed through the capillary), and when vinylmagnesium bromide appeared at the capillary end, the pressure was reduced to 1.4 MPa (200 psi). The flushing with vinylmagnesium bromide was continued for an additional 6 h. The syringe was then replaced with another 2.5-mL Hamilton syringe filled with anhydrous tetrahydrofuran (THF). The capillary was rinsed with 2.5 mL THF at 13.8 MPa, followed by a ca. 3 mL of deionized water delivered

by a high-pressure pump (41 MPa, i.e., 6000 psi) and with nitrogen (13.8 MPa) to get water out of the capillary. A polymerizing mixture was prepared by mixing a 2 mL of 3% acrylamide and 160 μ L of freshly prepared 1 M ammonium persulfate. After brief degassing of the acrylamide solution by evacuation, the capillary was filled with the polymerizing mixture and placed in a water bath (70°C) for 30 min. After 30 ± 0.5 min, the capillary was removed from the bath and flushed with approximately 12 mL of deionized water using a high-pressure pump (41 MPa).

The previous version of this procedure [3] was suitable for preparation of approximately 1 m of coated capillary and required careful flushing of the capillary with sodium hydroxide and water [1]. Hjertén and Kubo [10] subsequently published another procedure containing additional flushing steps (including chloroform, acetone, and HCl). During our process development, no improvements in resolution, durability, or quality of the wall coating were observed for inclusion of such steps prior the reaction with thionyl chloride. The capillaries which were treated with thionyl chloride directly without any previous rinsing steps performed equally well for analysis of both DNA sequencing ladders and double-stranded DNA (dsDNA) fragments. Both variations in the fused silica itself may account for the discrepancies between reports.

The first step of the coating process requires drying the capillary. Previously, this was done by flushing the capillary with dry nitrogen at 120°C. Iller [50], however, notes that residual water remains in silica even after an extensive drying at high temperatures. Since thionyl chloride is highly reactive with water, we used it as a scavenger for residual water on the silica surface and flushed the capillary directly with thionyl chloride.

In the original procedure [3], the capillary rinsing and the reactions with thionyl chloride and vinylmagnesium bromide were performed under reduced pressure, an approach that is impractical on a large scale. The maximum pressure difference that can be achieved between the ends of capillary using a negative flushing pressure equals atmospheric pressure only and is insufficient to flush 100 m of capillary at a time. The process scale-up therefore required the use of positive pressure.

One of our objectives was to reduce the number of steps in the procedure. The undiluted 1 M solution of vinyl magnesium bromide as purchased (Aldrich) has been successfully used for the reaction without any capillary clogging. However, the 1.7 M vinylmagnesium chloride as purchased (Fluka, Buchs,

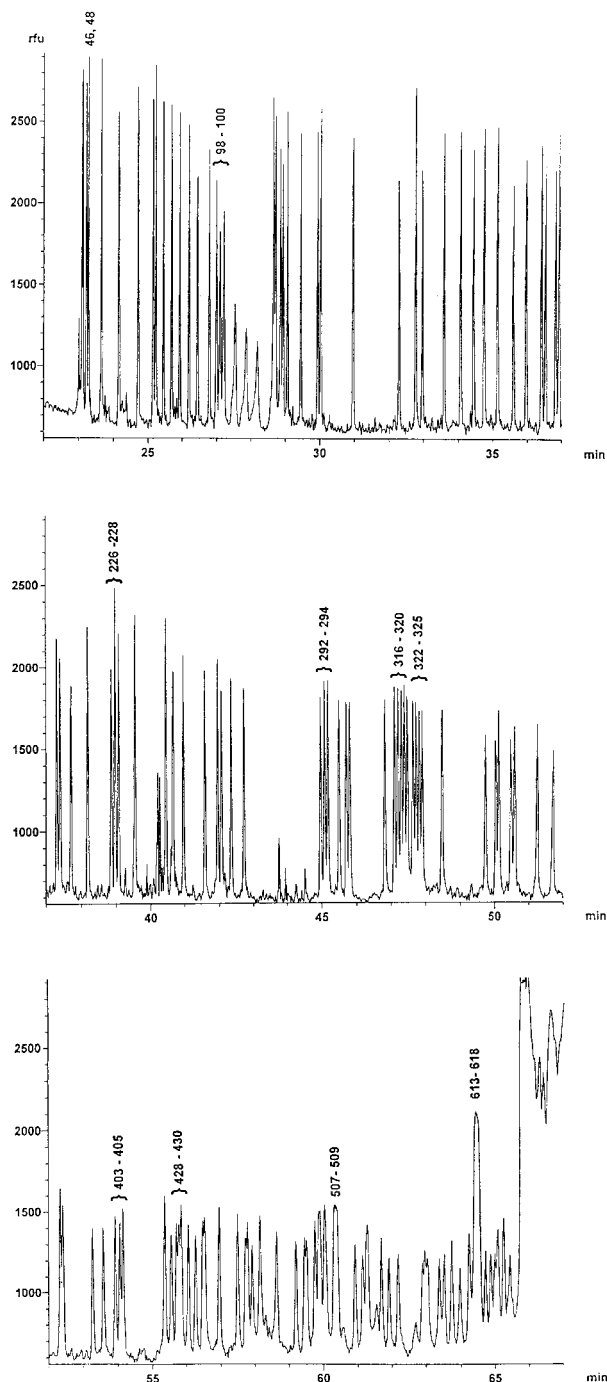


Figure 1. Electropherogram of FAM-10-FAM labeled primer sequencing reaction products from M13mp18 terminated with dideoxythymidine triphosphate, run 48. Experimental conditions: capillary (200 μ m o.d., 75 μ m i.d.): 65 cm total length, 40 cm effective length; sieving matrix: 2% hydroxyethyl cellulose (MW 90,000–105,000), 6 M urea, 10% formamide, 1X TBE. Voltage 12 kV.

Switzerland) proved unsuccessful and quickly resulted in clogged capillaries. No negative impact on the quality of the wall coating was observed if thionyl chloride and vinylmagnesium bromide were not separated by a plug of an inert liquid such as anhydrous

THF to prevent the reaction at their interface. THF is very hygroscopic, and its use increases a risk of contamination of capillary with water and thus hydrolysis of chlorinated silica prior to the reaction with vinylmagnesium bromide.

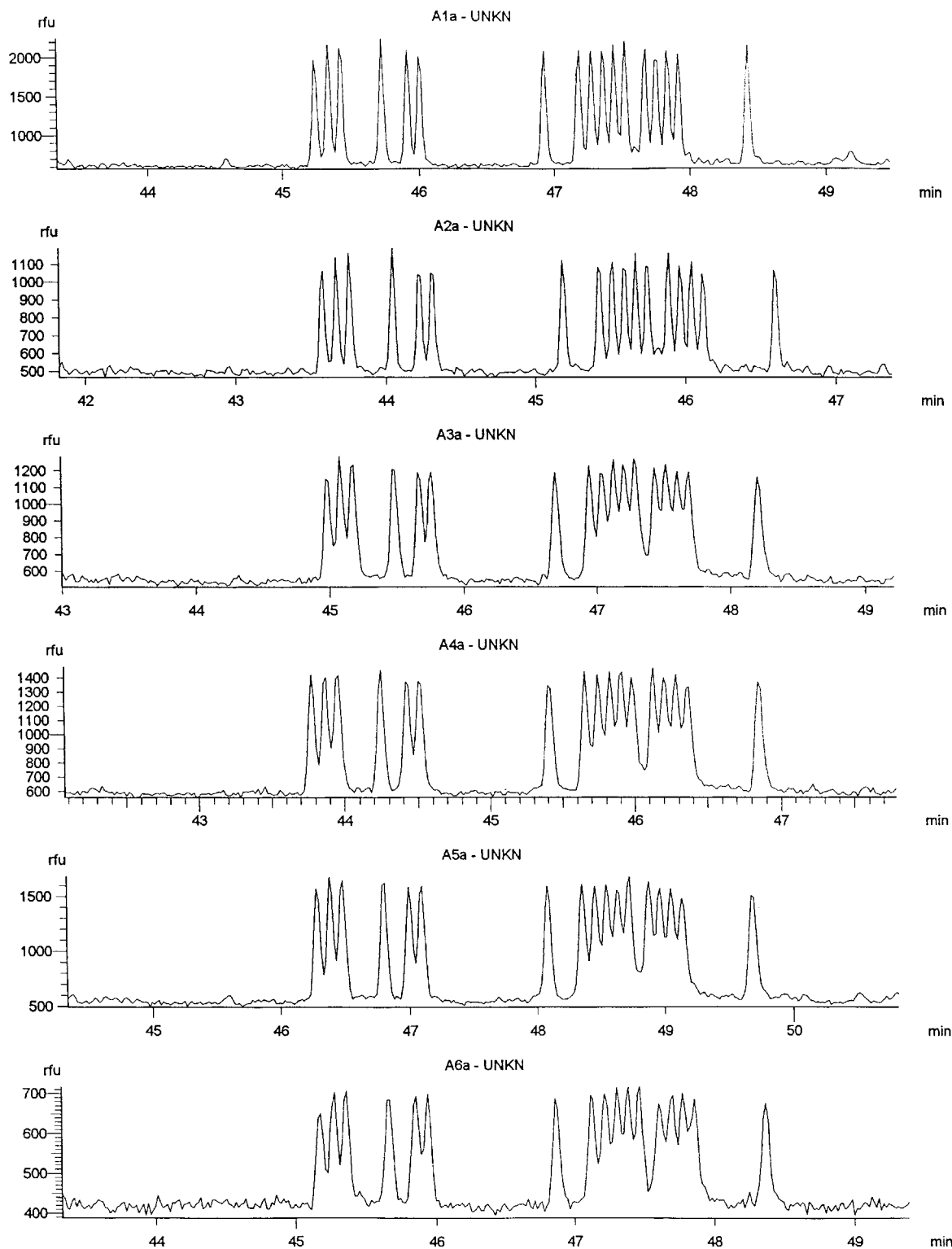


Figure 2. Detail of electropherograms in the first six capillaries, run 48. For experimental conditions, see Figure 2.

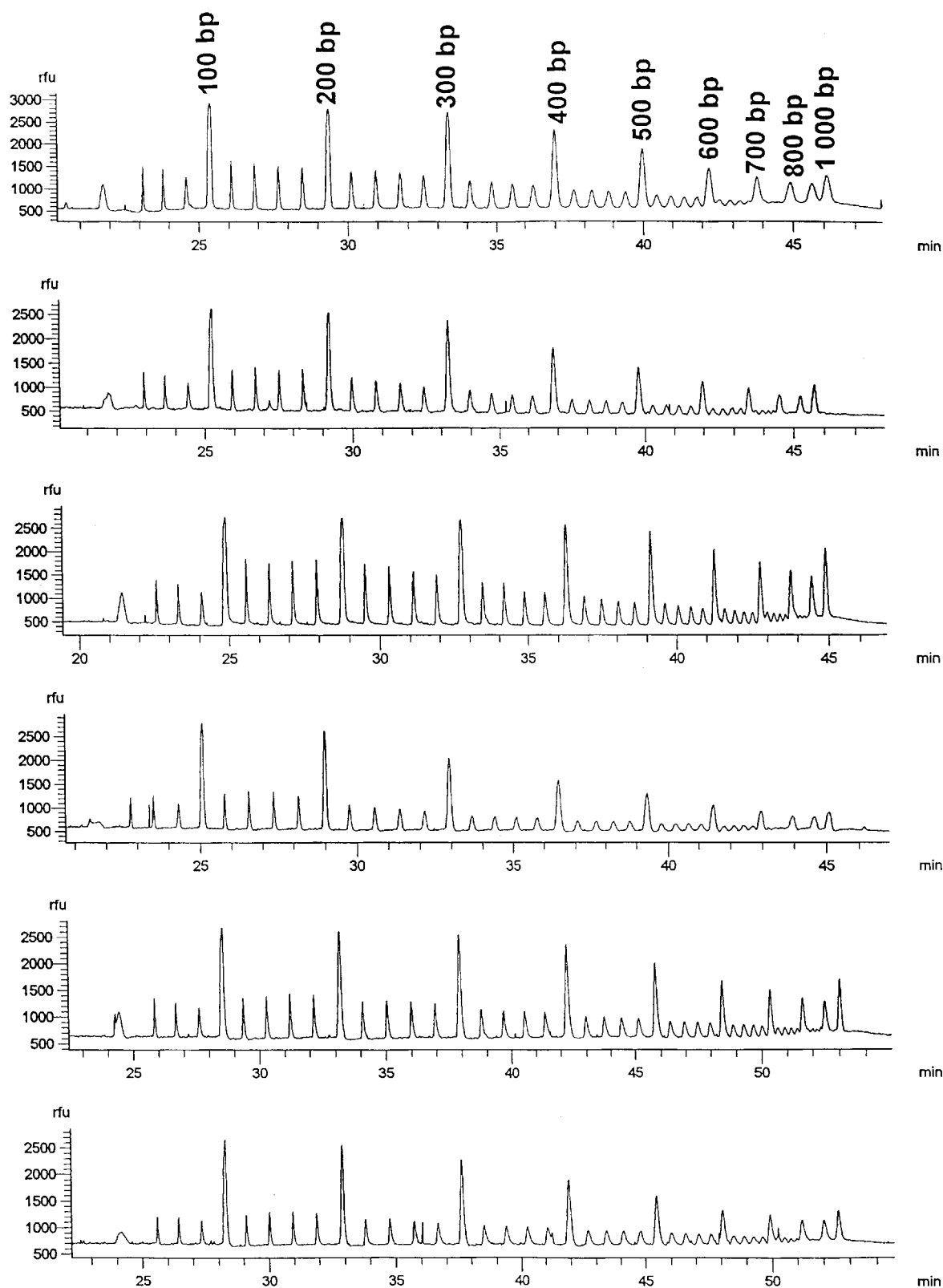


Figure 3. Electropherograms of a 1:2 mixture of 20 and 100-bp dsDNA ladders. Experimental conditions: capillary (200 μm o.d., 75 μm i.d.): 65 cm total length, 40 cm effective length; sieving matrix: 1% polyethylene oxide (MW 10^6), 1X TBE, 20 μM 9-amino acridine, 2 nM Vista Green. Voltage 12 kV.

Polymerization of acrylamide into a uniform linear polyacrylamide layer on the capillary surface can be difficult to control. Large-scale production of coating requires longer pieces of capillary to be treated. This is difficult, because the time needed to fill 100 m capillary is comparable with the time of acrylamide polymerization under normal conditions. In principle, polymerization should start after the whole length of capillary is filled with the polymerization mixture to ensure a homogenous coating. Photopolymerization initiated with methylene blue [51] proved unsuccessful because residual methylene blue remained in the wall coating even after extensive flushing, causing an unacceptable high fluorescence background. A reaction at increased temperature in the presence of ammonium persulfate but without TEMED eventually proved the easiest and most reliable procedure. To operate all reactions in the same water bath at a common temperature, the reaction time and the concentration of acrylamide and ammonium persulfate were optimized to perform the polymerization at 70°C. At the end of the polymerization time, the viscosity of the polyacrylamide solution must be sufficiently low that the capillary can be flushed with water. The optimum reaction time was found to be 30 min. Shorter times do not guarantee sufficient or uniform coating. At 40 min or longer polymerization, the viscosity of the polyacrylamide solution was so high that it was sometimes difficult or even impossible to flush the polyacrylamide solution out of the capillary. After 90 min the polyacrylamide forms a complete gel.

The susceptibility of polyacrylamide to hydrolysis still remains a potential problem. Replacement of acrylamide with a hydrolytically more stable derivative such as acryloylaminoethoxyethanol [52], acryloylaminoethoxyethylglucopyranose [53], acryloylaminoethoxyethylglucopyranose [53], acryloylaminoethoxyethylglucopyranose [53], acryloylaminoethoxyethylglucopyranose [53] or another monomer should further improve the lifetimes of the wall coating.

DNA sequencing. Figure 1 shows T-terminated fragments from a M13mp18 reaction. The electropherogram shows a partially resolved triplet at 507–509 bases as well as a characteristic pattern at fragments 313–325, containing a quintuplet and a quadruplet. Figure 2 shows detail of this section for the first six capillaries of a typical capillary array during our test procedure to evaluate fragment resolution.

DNA fragment analysis. Frequently, DNA fragment separations are performed under nondenaturing conditions, i.e., in the absence of urea or formamide. A typical electropherogram separating mixture of a 20-bp ladder and a 100-bp ladder is shown in Figure 3. With the nondenaturing matrix, the degradation of the coating is obviously slower

and over 300 runs have been performed without a loss of resolution.

Evaluation of the coating quality. To evaluate the quality of the wall coating and its degradation over time, a simple measurement of migration times was not sufficient. The differences in migration time of selected standards do not necessarily reflect differences in residual electroosmotic flow. For example, variation in cooling efficiency between the individual capillaries in the installed array are more pronounced since electrophoretic mobility depends on temperature. Eventually, a separation of T-terminated sequencing fragments of plasmid M13mp18 was chosen to test wall coating performance. Although the quality of the separation matrix and the reaction samples influences the evaluation, the method has proven to be a useful measure of the wall coating quality. To express the quality of separation of DNA sequencing fragments quantitatively, we measured relative valley depth (RVD) for the peak quadruplet 322–325 in the T-track of plasmid M13mp18. The peak quadruplet together with the adjacent peak quintuplet is easy to recognize in the electropherogram. Relative valley depth is calculated using the formula

$$\begin{aligned} \text{RVD (\%)} &= \frac{\frac{1}{4}(P_1 + P_2 + P_3 + P_4) - \frac{1}{3}(V_1 + V_2 + V_3)}{\frac{1}{4}(P_1 + P_2 + P_3 + P_4) - \frac{1}{2}(B_1 + B_2)} \\ &\times 100 \end{aligned}$$

where P_1 – P_4 are the fluorescence signals at the peaks, V_1 – V_3 are fluorescence signals at the valleys, and B_1 , B_2 are two values of the baseline fluorescence (Figure 4). Therefore, for the baseline resolution, i.e., when all three valleys between the peaks

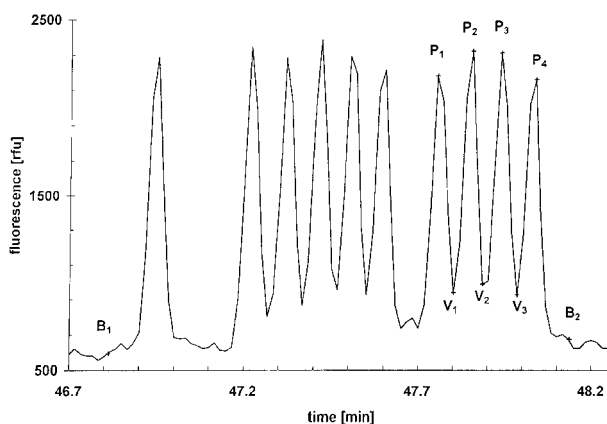


Figure 4. Values of fluorescence signal taken to calculate relative valley depth.

reach the baseline, RVD equals 100, whereas when no peaks and valleys can be distinguished in the quadruplet, RDV equals 0. A value of RVD over 33% is defined as a good wall coating for purposes

of DNA sequence calling, while arrays with values below 25% are rejected.

Ageing of the wall coating. Deterioration of the wall coating results in a loss of resolution and in a

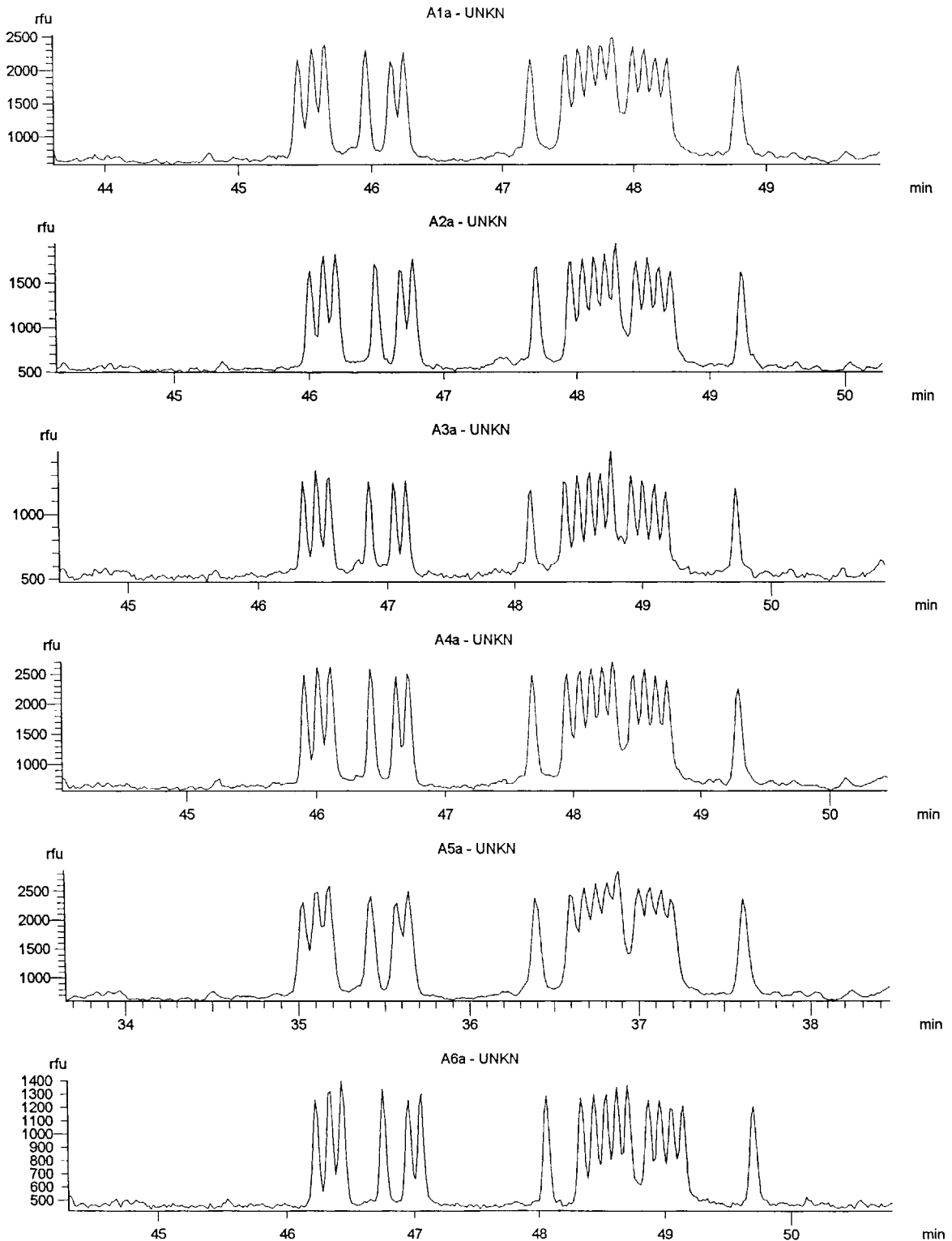


Figure 5a. Effect of capillary aging on the electrophoretic patterns: (a) run 109; (b) run 118. For experimental conditions, see Figure 1.

drop of the RVD value. It may be caused by different factors: alkali hydrolysis, shearing of the polymer layer, or adsorption of ionizable compounds and particulates. Figures 5(a) and 5(b) show details of

electropherograms in the first six capillaries in runs 109 and 118, respectively. Whereas run 109 exhibits very good resolution, there is a drop of resolution in run 118, although some capillaries still exhibit satis-

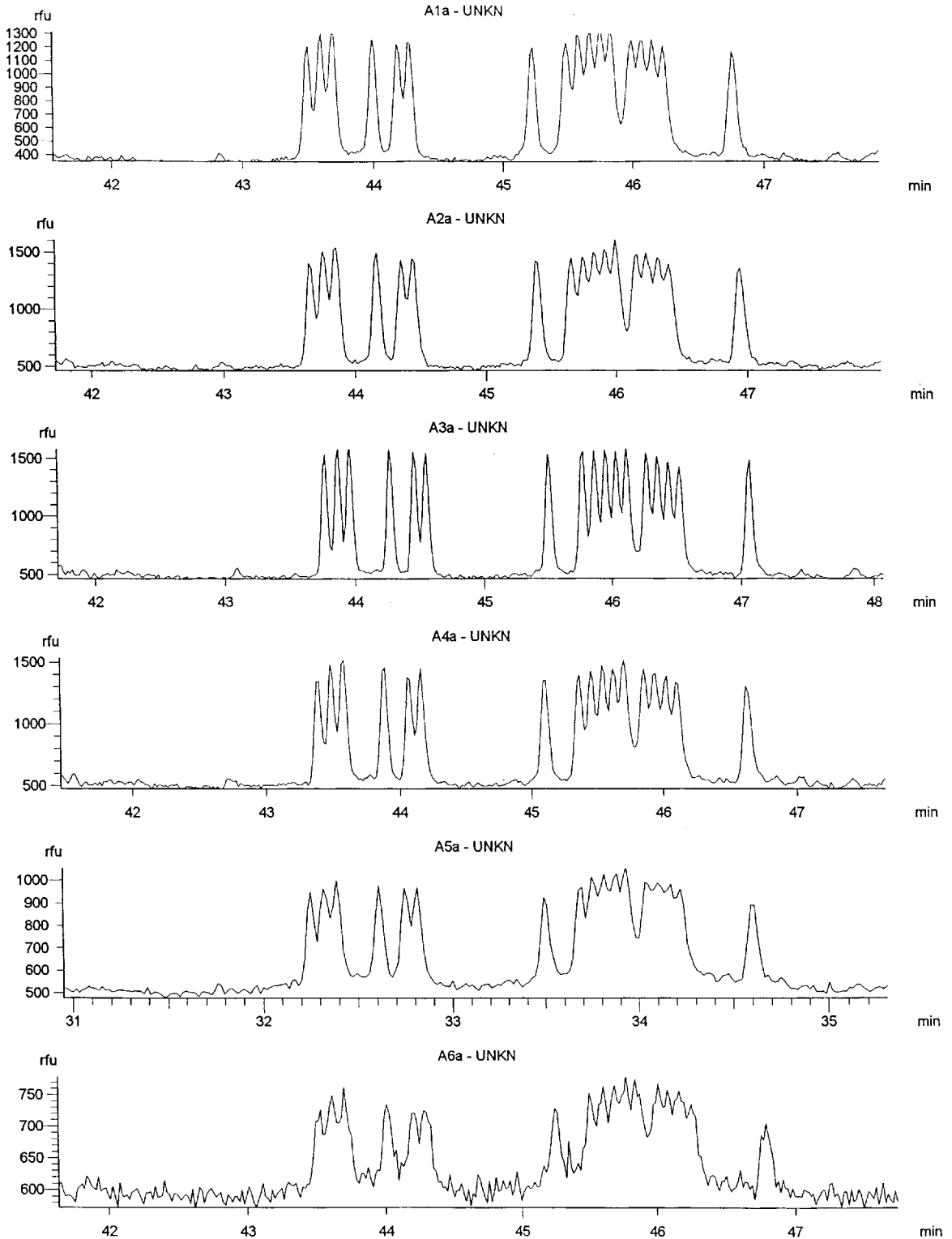


Figure 5b. Continued

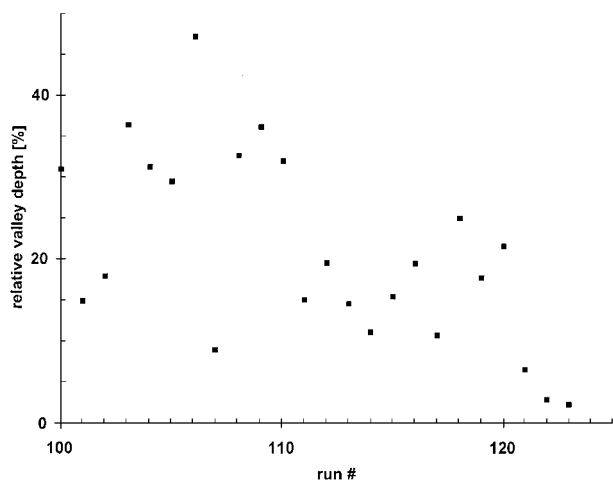


Figure 6. Dependence of average relative valley depth on the number of runs.

factory performance. Figure 6 shows the effect of the number of runs on the relative valley depth. For this array, the resolution has deteriorated after run 110 as reflected by the low value of the average RVD. Figure 6 demonstrates that RVD measurement is not a robust method for quality control. For example, the low values of RVD for runs 101, 102, and 107 are apparently caused by factors other than quality of capillary wall coating (aged gel matrix or samples, injection irregularities). However, it is the best method available. If one keeps in mind that a failure of separation need not be caused by a poor wall coating exclusively, the method can be used to evaluate the wall coating quality.

REFERENCES

- S. Hjertén, *J. Chromatogr.* **347**, 191 (1985).
- R.M. McCormick, *Anal. Chem.* **60**, 2322 (1988).
- K.A. Cobb, V. Dolník, and M. Novotny, *Anal. Chem.* **62**, 2478 (1990).
- J.A. Lux, H. Yin, and G. Schomburg, *J. High Resolut. Chromatogr.* **13**, 145 (1990).
- J.K. Towns and F.E. Regnier, *J. Chromatogr.* **516**, 69 (1990).
- B. P. Salmanowicz, *Chromatographia* **41**, 99 (1995).
- J.K. Towns, J. Bao, and F.E. Regnier, *J. Chromatogr.* **599**, 227 (1992).
- M. Huang, W.P. Vorkink, and M.L. Lee, *J. Microcol. Sep.* **4**, 233 (1992).
- M. Huang, W.P. Vorkink, and M.L. Lee, *J. Microcol. Sep.* **4**, 135 (1995).
- S. Hjertén and K. Kubo, *Electrophoresis* **14**, 390 (1993).
- M.A. Strege and A.L. Lagu, *J. Chromatogr.* **630**, 337 (1993).
- Z.X. Zhao, A. Malik, and M.L. Lee, *Anal. Chem.* **65**, 2747 (1993).
- D. Schmalzing, C.A. Piggee, F. Foret, E. Carrilho, and B.L. Karger, *J. Chromatogr. A* **652**, 149 (1993).
- A. Fridstrom, L. Lundell, L. Nyholm, and K. E. Markides, *J. Microcol. Sep.* **9**, 73 (1997).
- C.L. Ng, H.K. Lee, and S.F.Y. Li, *J. Chromatogr. A* **659**, 427 (1994).
- M. Nakatani, A. Shibukawa, and T. Nakagawa, *J. Chromatogr. A* **672**, 213 (1994).
- M. Nakatani, A. Shibukawa, and T. Nakagawa, *Electrophoresis* **16**, 1451 (1995).
- M. Huang, M. Bigelow, and M. Byers, *Am. Lab.* **128**, 32 (1996).
- A.E. Barron, W.M. Sunada, and H.W. Blanch, *Electrophoresis* **16**, 64 (1995).
- M.H.A. Busch, J.C. Kraak, and H. Poppe, *J. Chromatogr. A* **695**, 287 (1995).
- M. Chiari, M. Nesi, J.E. Sandoval, and J.J. Pesek, *J. Chromatogr. A* **717**, 1 (1995).
- M. Huang, J. Plocek, and M.V. Novotny, *Electrophoresis* **16**, 396 (1995).
- Y. Baba, R. Tomisaki, C. Sumita, M. Tshako, T. Miki, and T. Oghihara, *Biomed. Chromatogr.* **8**, 291 (1994).
- A.E. Barron, H.W. Blanch, and D.S. Soane, *Electrophoresis* **15**, 597 (1994).
- N. Best, E. Arriga, D.Y. Chen, and N.J. Dovichi, *Anal. Chem.* **66**, 4063 (1994).
- N. Bianchi, C. Mischiata, G. Feriotto, and R. Gambari, *Nucleic Acids Res.* **21**, 3595 (1993).
- S. Carson, A.S. Cohen, A. Belenkii, M.C. Ruiz-Martinez, J. Berka, and B.L. Karger, *Anal. Chem.* **65**, 3219 (1993).
- D.Y. Chen, H.P. Swerdlow, H.R. Harke, J.Z. Zhang, and N.J. Dovichi, *J. Chromatogr.* **559**, 237 (1991).
- J. Cheng, T. Kasuga, K.R. Mitchelson, E.R.T. Lightly, N.D. Watson, W.J. Martin, and D. Atkinson, *J. Chromatogr. A* **677**, 169 (1994).
- M. Chiari, M. Nesi, and P.G. Righetti, *Electrophoresis* **15**, 616 (1994).
- S.M. Clark and R.A. Mathies, *Anal. Biochem.* **215**, 163 (1993).
- C. Gelfi, P.G. Righetti, V. Brancolini, L. Cremonesi, and M. Ferrari, *Clin. Chem.* **40**, 1603 (1994).
- C. Gelfi, P.G. Righetti, L. Cremonesi, and M. Ferrari, *Electrophoresis* **15**, 1506 (1994).
- M. Gilges, M.H. Kleemiss, and G. Schomburg, *Anal. Chem.* **66**, 2038 (1994).
- D.N. Heiger, A.S. Cohen, and B.L. Karger, *J. Chromatogr.* **516**, 33 (1990).
- B.R. McCord, D.L. McClure, and J.M. Jung, *J. Chromatogr. A* **652**, 75 (1993).
- M. Nesi, P.G. Righetti, M.C. Patrosso, A. Ferlini, and M. Chiari, *Electrophoresis* **15**, 644 (1994).
- 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**, 57 (1993).

39. A. Paulus and D. Husken, *Electrophoresis* **14**, 27 (1993).
40. M.C. Ruiz-Martinez, J. Berka, A. Belenkii, F. Foret, A.W. Miller, and B.L. Karger, *Anal. Chem.* **65**, 2851 (1993).
41. D. Figeys and N.J. Dovichi, *J. Chromatogr. A* **717**, 113 (1995).
42. J. Sudor and M.V. Novotny, *Anal. Chem.* **66**, 2446 (1994).
43. H. Swerdlow and R. Gesteland, *Nucleic Acids Res.* **18**, 1415 (1990).
44. K. Ueno and E.S. Yeung, *Anal. Chem.* **66**, 1424 (1994).
45. E.N. Fung and E.S. Yeung, *Anal. Chem.* **67**, 1913 (1995).
46. J. Ju, A.N. Glazer, and R.A. Mathies, *Nature Med.* **2**, 246 (1996).
47. J. Bashkin, D. Roach, J. Leong, D. Barker, and R. Johnston, *J. Capillary Electrophoresis* **3**, 60 (1996).
48. R.A. Mathies and X.C. Huang, *Nature* **359**, 167 (1992).
49. J. Bashkin, M. Marsch, D. Barker, and R. Johnston, *Appl. Theor. Electrophoresis* **6**, 23 (1996).
50. R.K. Iller, *The Chemistry of Silica* (Wiley, New York, 1979).
51. T. Lyubimova, S. Caglio, C. Gelfi, P.G. Righetti, and T. Rabilloud, *Electrophoresis* **14**, 40 (1993).
52. M. Chiari, C. Micheletti, M. Nesi, M. Fazio, and P.G. Righetti, *Electrophoresis* **15**, 177 (1994).
53. M. Chiari, N. Dell'Orto, and A. Gelain, *Anal. Chem.* **68**, 2731 (1996).
54. E. Simò-Alfonso, C. Gelfi, R. Sebastiano, A. Citterio, and P.G. Righetti, *Electrophoresis* **17**, 723 (1996).