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Ultra-thin-layer agarose gel electrophoresis
I. Effect of the gel concentration and temperature on the separation of DNA fragments

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Abstract

A novel, rapid and efficient separation method is described for the analysis of double stranded (ds) DNA fragments in the form of horizontal ultra-thin-layer agarose gel electrophoresis. This separation technique combines the multilane, high-throughput separation format of agarose slab gel electrophoresis with the excellent performance of capillary electrophoresis. The electrophoretic separation of the fluorophore (Cy5)-labeled dsDNA molecules were imaged in real time by a scanning laser-induced fluorescence/avalanche photodiode detection system. Effects of the gel concentration (Ferguson plot) and separation temperature (Arrhenius plot) on the migration characteristics of the DNA fragments are discussed. An important genotyping application is also shown by characterizing the polymorphic region ($2\times$ or 4×48 base pair repeats) of the dopamine D_4 receptor gene (D_4DR, exon III region) for ten individuals, using PCR technology with Cy5-labeled primers and ultra-thin-layer agarose gel electrophoresis. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Agarose gel electrophoresis; Ultra-thin-layer agarose gel electrophoresis; DNA

1. Introduction

Agarose gel electrophoresis is a well established separation methodology for the analysis of double stranded (ds) DNA fragments [1]. Although, several attempts have been made to automate this technology [2], practically, it is still a manual process. Real automation of dsDNA fragment analysis appeared with the advent of capillary gel electrophoresis (CGE), which has emerged quickly during the last decade, to become an automated, fast and efficient separation tool for biopolymer analysis [3]. CGE has changed the gel thickness dimension of electrophoresis from the millimeter scale to the micrometer scale, enabling more efficient heat dissipation, and consequently higher separation performance [4].

Most of the important separation parameters in the gel electrophoretic analysis of DNA fragments, such as gel concentration and separation temperature are well characterized for both the agarose slab gels [5] and capillary gel electrophoresis [6,7]. However, the migration properties of the DNA fragments are somewhat different in those two separation formats. This is mainly caused by the significant difference between the applied electric field, which is, orders of magnitude larger in the latter instance. This signifi-

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cant increase in the separation field strength changes the migration behavior of DNA molecules through the sieving matrix. In conventional agarose slab gel electrophoresis, migration of fragments smaller than 500 base pairs (bp) can be described by the Ogston model [8]. In capillary gel electrophoresis, due to the orders of magnitude higher applied electric field (>100 V/cm), DNA fragments tend to migrate by reptation mode, or in extreme cases by biased reptation [9].

Ultra-thin-layer gel electrophoresis is a combination of the high throughput capability (multilane separation format) of conventional slab gel electrophoresis and the high-performance and fast capillary gel electrophoresis methods. Thin polyacrylamide slab gels have been proved as excellent separation platforms for high-throughput DNA sequencing applications [10,11]. Similar to capillary electrophoresis, the relatively high surface to volume ratio of the ultra-thin-layer electrophoresis platform improves heat dissipation during the electric field mediated separation, therefore, enabling the application of one order of magnitude higher electric field strengths (>40 V/cm) compared to conventional slab gel electrophoresis (1–4 V/cm). Automated injection of very small sample volumes (0.2–0.5 µl) is also possible in a multilane format by membrane-mediated sample loading [12].

In this paper we describe a novel, horizontal, ultra-thin-layer agarose gel electrophoresis method for rapid and efficient separation of dsDNA fragments and discuss the effects of gel concentration and separation temperature. As a practical example polymerase chain reaction (PCR) products were amplified from the polymorphic region of 48 base pair repeats of human dopamine D_4 receptor (D_4DR) exon III region and efficiently identified by ultra-thin-layer agarose gel electrophoresis.

2. Materials and methods

2.1. Instrumentation

The horizontal ultra-thin-layer agarose gel electrophoresis system with laser-induced fluorescence detection used in the experiments was described earlier [13]. Briefly, it employs a fiber bundle-mediated scanning detection system using a 640-nm red diode laser excitation source and avalanche photodiode detection (APD) with a 682 ± 22 nm wide band interference filter. A lens set scans across the multilane separation platform by means of a translation stage and collects the emitted fluorescent signal. The analog signal from the APD gets digitized by a micro-controller. The separation image is acquired and evaluated by a personal computer. The ultra-thin-layer separation platform is a 18×7.5 cm$\times190$ µm float glass cartridge with a 15-ml buffer reservoir at each end.

The injection of the Cy5-labeled dsDNA standards and PCR products onto the ultra-thin-layer agarose gel was accomplished by previously discussed membrane-mediated loading technology [14]. The injection side of the separation gel had a straight edge with no individual wells. Small volumes of samples (0.2–0.5 µl) were spotted onto the tips of the 24-tab membrane loader (Genetic BioSystems, San Diego, CA, USA) and carefully inserted in close proximity of the straight edge of the separation gel. A layer of high viscosity and low ionic strength well solution is used as an interface between the membrane and the separation matrix to assure the best possible sample transfer. After the insertion of the spotted loading membrane, the electric field was applied and the DNA fragments were quantitatively loaded into the separation matrix from the membrane. It is important to note that this technique enables rapid sample loading outside of the separation platform (benchtop), with the possibility of full utilization of various automated sample preparation and liquid handling (robotics) systems.

The agarose gel was replaced in the separation cartridge after each run, simply by briefly melting the used gel in the cassette in a microwave oven and pumping in the fresh melted one. Heating up the separation cassette in the microwave oven also prevented premature solidification of the freshly poured gel during the loading process.

2.2. Chemicals

In all the experiments low electroendosmosis (EEO) ($\mu_{EOF}=0.06\cdot10^{-4}$ cm^2/V s for 1.5%) [15]. Amresco's Agarose-III (Solon, OH, USA) was used in 45 m*M* Tris, 45 m*M* borate, 1 m*M* EDTA buffer

pH 8.3 (0.5×TBE). Tris, boric acid and EDTA were obtained from ICN (Costa Mesa, CA, USA), all in electrophoresis grade. Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) was used in the PCR reactions. The Cy5-labeled 50-bp sizing ladder (Pharmacia Biotech, Uppsala, Sweden) was diluted with deionized water to the required concentrations and was stored at −20°C. The sense (TGC TCT ACT GGG CCA CGT TC) and antisense (TGC GGG TCT GCG GTG GAG TCT) primers for the PCR reaction were synthesized and the sense primer was labeled at the 5′ end by the fluorescent dye Cy5 at Genset (La Jolla, CA, USA).

2.3. Procedures

An appropriate amount of agarose powder was suspended in 0.5× TBE buffer, boiled repetitively in a microwave oven until clear, and was kept at 60°C until use. This gel-buffer system exhibited acceptable separation performance up to 5 days storing at 60°C, then poor resolution and fuzzy bands prevailed. The preheated separation cassettes were filled with the melted agarose (60°C) and after several minutes of cooling/solidification, the gel-filled cartridge was ready to be used. The effective separation length of the agarose gel filled ultra-thin-layer cartridge was 6 cm. Agarose gel (2%) was used for the separation in 0.5× TBE (pH 8.3) buffer. The applied separation voltage was 750 V, generating 5–7 mA current. An aluminum heat-sink was employed to hold the separation cartridge and the separation temperature was regulated by a thermostated air bath with a precision of ±1°C.

PCR reaction: DNA was isolated from human blood and purified as described earlier by Nemeth et al. [16]. PCR amplification was done by using 50 ng of genomic DNA, 0.25 U Vent DNA polymerase, 400 μM of each dNTPs, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 20 mM Tris–HCl (pH 8.8 at 25°C), 2 mM MgCl$_2$, 0.1% Triton X-100 and 0.1 μM of each primer in a total volume of 20 μl. Thirty cycles were accomplished (1 min, 98°C; 1 min 70°C, 3.3 min 72°C) after 2 min of initial denaturation of the genomic DNA at 98°C in an EriComp thermal cycler (San Diego, CA, USA). The resulting PCR products were diluted 5-fold, then directly applied to the membrane sample loader and placed into the ultra-thin-layer agarose gel electrophoresis system.

3. Results and discussion

3.1. Theory

Similar to conventional agarose slab gel electrophoresis, in ultra-thin-layer agarose gel electrophoresis of DNA fragments, the size-dependent apparent mobility of the solute is a primary function of the concentration of the separation matrix ($P^{\%}$):

$$\mu = \mu_0 \exp(-K_R P^{\%}) \tag{1}$$

where μ is the apparent electrophoretic mobility, K_R is the retardation coefficient [17] and

$$\mu_0 = Q/f \tag{2}$$

is the free solution mobility of the solute. Q is the total charge and f is the translational friction coefficient of the migrating DNA molecule. Please note, that during electrophoresis, electroendosmosis (EEO)-induced bulk flow is present in most agaroses, therefore, DNA mobilities should be corrected for it [18].

As a first approximation, the electric field mediated force (F_e) on the sample molecules can be assumed to be constant per unit length, since each of the base pairs in the double stranded DNA molecule carries the same net negative charge at the separation pH of 8.3. Thus, the total charge of the migrating DNA molecule (Q) can be considered proportional to the number of base pairs (n) comprising the DNA molecule [19]. Therefore, the number of base pairs in the DNA molecule can be used for substituting the net charge:

$$F_e = EQ \sim En^k \tag{3}$$

Where the exponent k represents information about the apparent shape of the DNA molecule during its migration by the influence of the applied electric field (E) [20–22].

The translational friction coefficient (f) is mainly dependent on the viscosity of the separation matrix (η) which is influenced by the temperature of the agarose gel filled ultra-thin separation platform.

$$f = \text{const} \exp(E_a/RT) \tag{4}$$

where, E_a is the activation energy of the actual conformation of the viscous separation media, R is the universal gas constant and T is the absolute temperature.

By combining Eqs. (1)–(4), the electrophoretic mobility of the migrating dsDNA molecules in ultra-thin-layer agarose gel electrophoresis can be expressed as:

$$\mu = \text{const} \exp(-K_R P^\%) n^k \exp(-E_a/RT) \tag{5}$$

By taking the natural logarithm of Eq. (5) the following formula is derived:

$$\ln \mu = \ln \text{const} - K_R P^\% + k \ln n - E_a/RT \tag{6}$$

Effects of the individual members of this equation were evaluated during ultra-thin-layer agarose gel electrophoresis of Cy5-labeled dsDNA molecules.

3.2. Effect of the agarose concentration on the mobility of the dsDNA fragments

First, ultra-thin agarose layers were prepared with different gel concentrations, in order to obtain information about their molecular sieving capability on the Cy5-labeled dsDNA ladder (50–500 base pairs). The concentrations of the agarose gels were varied between 1 to 3% by 0.5% increments. Fig. 1 exhibits the natural logarithm of the electrophoretic mobilities as a function of the agarose gel concentration (Ferguson plots [23]). Please note that the observed electrophoretic mobilities were corrected with the EEO of the agarose matrix ($\mu_{EEO} = 0.06 \cdot 10^{-4}$ cm^2/ V s). As one can observe, non-linear convex plots were attained under ultra-thin-layer electrophoresis conditions, where the thickness of the gel was in the capillary dimension (<200 μm) and the applied electric field strength was one order of magnitude higher than in conventional agarose slab gel electrophoresis (42 V/cm vs. 1–4 V/cm). These plots suggest, that with decreasing gel concentration the mobility of the DNA fragments are less and less dependent on the sieving matrix concentration. Similar, non-linear, convex Ferguson plots were observed by Holmes and Stellwagen [24] using conventional agarose slab gels for the solute size range up to 500 base pairs. Tietz and Chrambach also noticed convex
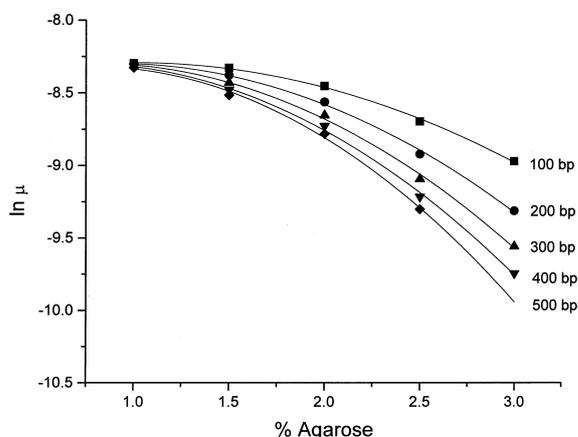
Fig. 1. Convex Ferguson plots of dsDNA fragments (Cy5-labeled 50-bp sizing ladder) in ultra-thin-layer agarose gel electrophoresis. Observed mobility values were corrected for EEO. Numbers on the plots correspond to the chain length of the individual DNA molecules.

Ferguson plots in agarose gel based protein separations, but concave plots in polyacrylamide based DNA separations [25].

Fig. 2 shows the natural logarithm of the relative mobility values (μ/μ_0, where the free solution mobility of DNA of the solute was defined from the y-axis interception of the extrapolated plots in Fig. 1, $\mu_0 = 2.61 \cdot 10^{-4}$ cm^2/V s) of the dsDNA fragments with various gel concentrations as a function of the
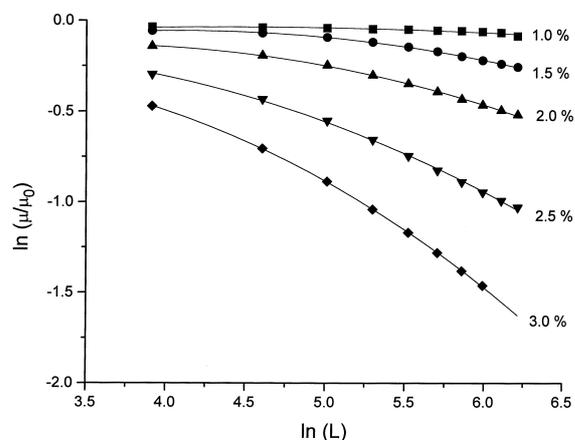
Fig. 2. Double logarithmic plot of the relative electrophoretic mobility and the chain length of the dsDNA molecules. Observed mobility values were corrected for EEO. Numbers on the plots correspond to the actual concentration of the agarose gel.

logarithm of the fragment lengths. The average slope values of the individual plots (k values in third term in the right side of Eq. (6)) are exhibiting increasing negative numbers with increasing gel concentration (Table 1). As one can see in Table 1, k is ranging from -0.0173 to -0.4848, the later corresponding to 3.0% agarose concentration. Please note that this value is still less than half of -1, which would be characteristic of reptation. This change in k suggests a slight shift from the Ogston regime towards the reptation regime, as higher gel concentrations are used [26]. However, under the discussed conditions, the primary separation mechanism of the Cy5-labeled DNA fragments in the 50–500 base pair range still appears to be molecular sieving.

3.3. Effect of the separation temperature on the mobility and activation energy

Fig. 3 delineates the Arrhenius plots for the different size Cy5-labeled DNA fragments. Effect of the separation temperature (20, 30, 40 and 50°C) on the electrophoretic mobility of the dsDNA fragments was evaluated. Please note that the observed electrophoretic mobility values were corrected with the temperature influenced viscosity change of the solvent (1.1% / K) [27], as well as with the temperature dependent EEO (20% / 10 K) [28]. As one would expect, the electrophoretic mobility values of the individual solute molecules increased with elevating temperature. It is also well known that the formation of a sieving structure of a separation medium, in this instance the agarose, requires a given certain activation energy (E_a) [29]. The average slope values of the individual plots in Fig. 3 multiplied by the universal gas constant are in fact these E_a values and are plotted as a function of the fragment lengths in Fig. 4. As one can see, activation energy values for

Table 1
The average slope values of the individual plots in Fig. 2

Gel concentration	k value
1.0%	-0.0173
1.5%	-0.0918
2.0%	-0.1728
2.5%	-0.3343
3.0%	-0.4848

k values are in the third term in the right side of Eq. (6).
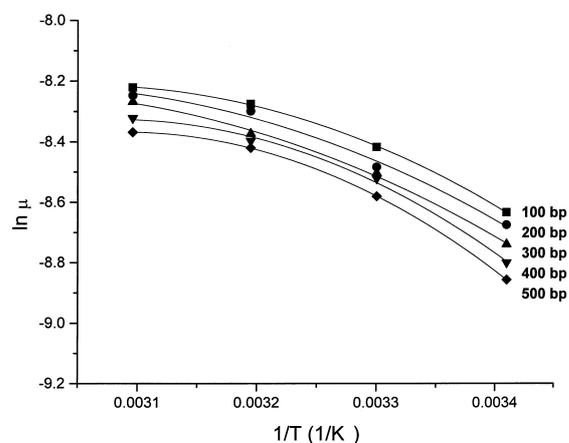
Fig. 3. Relationship between the logarithmic mobility and the reciprocal absolute temperature for the various size DNA fragments (Arrhenius plot) in ultra-thin-layer agarose gel electrophoresis. Observed mobility values were corrected for temperature-mediated viscosity and EEO changes. Numbers on the plots correspond to the chain length of the individual DNA molecules.

dsDNA fragments on ultra-thin-layer agarose gel electrophoresis exhibit an elevating function with increasing fragment length, probably due to the temperature initiated changes in the structure of the sieving matrix. Dovichi et al. [30] observed similar plots in capillary gel electrophoresis of DNA sequencing fragments using crosslinked polyacrylamide gels. This increasing function of the plot in Fig. 4 implies that the migration behavior of
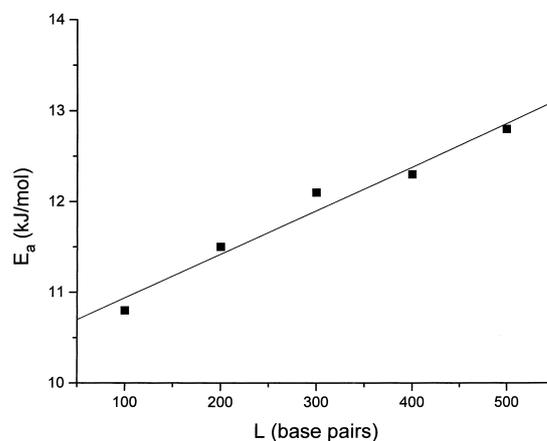
Fig. 4. Activation energy of the sieving matrix as a function of the fragment length of the solute.

dsDNA fragments is similar in agarose to that of DNA sequencing fragments in cross-linked polyacrylamide gels in capillary dimensions, suggesting that the electromigration process of the larger size dsDNA fragments require higher activation energy. Thus, they must probably distort through the pores of the polymer matrix harder, therefore, their electromigration process requires higher activation energy. This hypothesis is in good correlation with the assumption of sieving characteristics being the primary mechanism as we suggested above in the discussion of the plots in Fig. 2.

3.4. Detection sensitivity and application

The detection sensitivity of the ultra-thin-layer
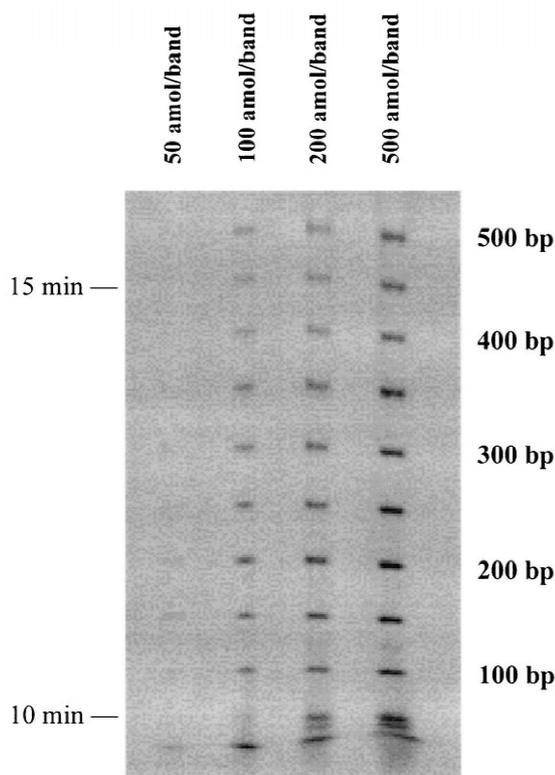
Fig. 5. Detection sensitivity of the ultra-thin-layer agarose gel electrophoresis-laser-induced fluorescence detection system. Lanes correspond to the injected amount of 50 amol per band to 500 amol per band Cy5-labeled 50-bp sizing ladder. Separation conditions: gel: 2% agarose; buffer: 0.5× TBE (pH 8.3); separation voltage: 750 V; effective separation length: 6 cm; gel thickness: 190 μm; temperature: ambient; sample loading: membrane mediated, 0.5 μl/lane.

agarose gel electrophoresis–laser-induced fluorescent detection system was evaluated by loading various concentrations of the 50-bp sizing ladder sample (50, 100, 250 and 500 amol per band) onto the separation platform. Fig. 5 exhibits the resulting picture of this evaluation, showing 100 amol per band as the practical detection limit (LOD) of this system for the Cy5-labeled 50-bp ladder.

As a practical example, PCR products were amplified out of the polymorphic region of 48 basepair repeats of human dopamine D_4 receptor (D_4DR) exon III region of ten individuals. Genotyping of the D_4DR exon III region might have an important application in the investigation of genetic influences on the novelty seeking personality traits, and in determining the risk factors regarding smoking and depression, obsessive–compulsive disorder, opioid-dependency and others [31,32]. The example in Fig. 6 clearly shows that the amplified fragments of the individual samples (lanes 1–10) correspond either to $2\times$ or 4×48 bp repeats ($171+2\times48=267$ and $171+4\times48=363$ bp, respectively) of the examined polymorphic region. Please note that multilane format separation and visualization of these PCR products was accomplished in less than 15 min in an automated fashion, enabling rapid genotyping of multiple samples. For fragment length assessment, Cy5-labeled 50-bp sizing ladder was also separated along with the samples (lane S in Fig. 6).
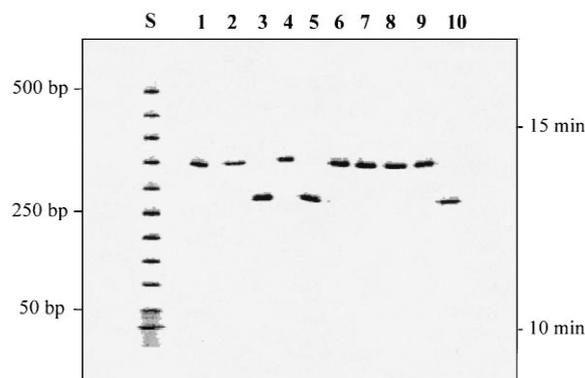
Fig. 6. Genotyping of ten individuals for the 48-bp repeat polymorphism of the D_4DR exon III region by ultra-thin-layer agarose gel electrophoresis. Conditions are the same as in Fig. 5. Lanes: S: separation of the 50-bp Cy5-labeled DNA sizing ladder. Lanes 1–10 correspond to samples from ten individuals showing $2\times(267$ bp, lanes: 3, 5 and 10) and $4\times(363$ bp, lanes: 1,2,4,6–9) 48-mer repeats.

4. Conclusion

Ultra-thin-layer agarose gel electrophoresis, a combination of the established methodologies of slab gel electrophoresis and capillary electrophoresis, provided a novel multilane separation format with rapid analysis time and excellent detection sensitivity. In this paper, we have studied the effects of gel concentration and separation temperature during ultra-thin-layer agarose gel electrophoresis analysis of Cy5-labeled dsDNA fragments. We observed convex Ferguson plots similar to that observed earlier in conventional agarose gel electrophoresis of smaller (<500 bp) DNA fragments. The activation energy (E_a) values derived from the slopes of the Arrhenius plots of ln μ vs. $1/T$ exhibited increasing characteristics with increasing fragment size, suggesting the higher activation energy requirement for the separation of the longer fragments.

The limit of detection (LOD) of the system for Cy5-labeled dsDNA fragments was 100 amol per band. A practical genotyping example of ten individuals checking the number of their 48 base pair repeats in the dopamine D_4 receptor (D_4DR) exon III region, proved the high-throughput separation capabilities of the system. The separation speed in conjunction with real time laser-induced fluorescence detection provided an easy to use and very sensitive dsDNA analysis technique. Multiplexing the PCR reactions would further increase the throughput of the system.

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