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Separation of DNA fragments for fast diagnosis by microchip electrophoresis using programmed field strength gradient

We evaluated a novel strategy for fast diagnosis by microchip electrophoresis (ME), using programmed field strength gradients (PFSG) in a conventional glass double-T microfluidic chip. The ME-PFSG allows for the ultrafast separation and enhanced resolving power for target DNA fragments. These results are based on electric field strength gradients (FSG) that use an ME separation step in a sieving gel matrix poly(ethylene oxide). The gradient can develop staircase or programmed shapes FSG over the time. The PFSG method could be easily used to increase separation efficiency and resolution in ME separation of specific size DNA fragments. Compared to ME that uses a conventional and constantly applied electric field (isoelectrostatic) method, the ME-PFSG achieved about 15-fold faster analysis time during the separation of 100 bp DNA ladder. The ME-PFSG was also applied to the fast analysis of the PCR products, 591 and 1191 bp DNA fragments from the 18S rRNA of *Babesia gibsoni* and *Babesia caballi.*

Keywords: DNA separation / Microchip electrophoresis / Miniaturization / Programmed field strength gradients
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1 Introduction

Since the first demonstrations by Manz *et al.* [1] and Harrison *et al.* [2], microchip electrophoresis (ME) is fastly becoming an important technique for diagnosing DNA fragments in analyses because of analytical throughput, speed, small reagent volume, automation, miniaturization, high resolution, and so on [3–6]. One of the significant advantages of ME in DNA fragment analysis is its high speed in comparison to the traditional slab gel electrophoresis and capillary electrophoresis (CE). Fast DNA fragment separations have been reported in glass [7] and plastic [8, 9] microfluidic devices. The DNA fragment separation in ME can be achieved by the mobility difference of DNA molecules, which are influenced by the sizes of the DNA fragments and the magnitude of the electric field. Small DNA molecules are able to pass through pores within the gels and elute first, whereas larger molecules are retarded by the gel and elute later under constant electric field strength. Although the ME provides a faster analysis than the conventional gel electrophoresis and CE, a major limitation of DNA fragment analysis is also the use of a sieving matrix for the gel electrophoretic separation of DNA fragments. Because DNA fragment separation depends on the DNA size in the ME separation, large DNA molecules always show long elution time compared to small DNA molecules, when under constant electric field (isoelectrostatic method). This fact increases the analysis time for long DNA fragments. Thus, a novel ME technique is needed for the fast analysis of specific-size DNA fragments, although the DNA samples contain a mixture of different-size DNA fragments.

Different field operation techniques have been described to achieve better and faster separation of differently sized DNA molecules resolution. The techniques mainly use slab gel electrophoresis [10] and CE [11]. Voltage-controlled programming techniques also decreased the analysis time in CE separation [12–14]. Under the influence of an electric field, an electrically negative charged DNA molecule will migrate through a buffer with an electrophoretic velocity [15]. Separation is achieved because DNA molecules migrate through the microchip at different velocities. Consequently, elution time can be reduced by using field strength gradients (FSG) in the microchip.

In this study, we explore how this new ME technique and programmed FSG (PFSG), can accelerate the elution of late-eluting DNA molecules, and enhance the

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Abbreviations: EtBr, ethidium bromide; **FSG**, field strength gradients; **ME**, microchip electrophoresis; **PFSG**, programmed field strength gradients; **TBE**, Tris-borate-EDTA

resolving power. The ME-PFSG is demonstrated using the separation of a mixture of DNA fragments, 100 bp DNA ladder in sieving matrix poly(ethylene oxide) (PEO). The ME-PFSG does not involve special requirements and/or devices, and it has a short analysis time. The ME-PFSG method is also analyzed to evaluate fast analysis of PCR DNA products in a clinical sample without any modification.

2 Materials and methods

2.1 Reagents

1 × Tris-borate-EDTA (TBE) buffer (pH 8.3) was prepared by dissolving a premixed powder (Amerosco, Solon, OH, USA) in deionized water. Poly(vinylpyrrolidone) (PVP) was obtained from Polysciences (Warrington, England). PEO and ethidium bromide (EtBr) were purchased from Sigma (St. Louis, MO, USA). A 100 bp DNA ladder was purchased from Invitrogen (Carlsbad, CA, USA), and it was diluted to 0.1 μg/μL with 1 × TBE buffer before use. For the PCR of *Babesia gibsoni* and *Babesia caballi*, a 10 × PCR buffer and 0.25 mM dNTP mix were purchased from Promega (Madison, WI, USA). The DNA polymerase (5 U/μL) was obtained from Super-Bio (Suwon, South Korea). A 1191 bp DNA fragment from the 18S rRNA of *Babesia* was amplified using forward primer (5'-GCC AGT AGT CAT ATG CTT GTC-3') and reverse primer (5'-CAA ATC ACT CCA CCA ACT AAG A-3'). A 519 bp DNA fragment from the 18S rRNA of *Babesia* was amplified with forward primer (bg18F, 5'-CTT GCC TTG TCT GGT TTC-3') and reverse primer (bg18R, 5'-AAC TTT GTC TGG ACC TGG TG-3').

2.2 PCR sample preparation

In the *B. gibsoni* PCR product and the *B. caballi* PCR product, the amplified fragments of 519 and 1191 bp DNA from the 18S rRNA of *B. gibsoni* and *B. caballi* were obtained from 2 μL of a purified DNA sample [16–19]. The reaction was performed in a thermal cycler (Perkin-Elmer model 2400, Norwalk, CT, USA) using the following temperature protocol: 3 min incubation at 95°C; 35 cycles of denaturing at 95°C for 60 s, annealing at 52°C for 40 s, and extension at 72°C for 60 s for the 519 bp DNA fragment, and 3 min incubation at 95°C; 40 cycles of denaturing at 95°C for 60 s, annealing at 58°C for 1 min 30 s, and extension at 72°C for 2 min; followed by a 7-min hold at 72°C for the 1191 bp DNA fragment. The 20-μL PCR reaction mixture was composed as follows: 1 μL of each 10 × PCR buffer 1 and 2, 0.25 mM dNTP, 2 μL each of forward and reverse primer, 0.3 μL *Taq* DNA polymerase, and 2 μL purified DNA. Each amplified PCR product sample was introduced into the MGE system.

2.3 ME

ME was performed on a DBCE-100 Microchip CE system (Digital Bio Technology, Suwon, South Korea), equipped with a diode-pumped solid-state laser (exciting at 532 nm and collecting fluorescence at 605 nm), and a high-voltage device (DBHV-100, Digital Bio Technology). The microfluidic chip, composed of Schott Borofloat glass, was purchased from Micralyne (Edmonton, Alberta, Canada). The injection design involved a double-T channel with a 100-μm offset. The chip channel was 50 μm wide and 20 μm deep. The reservoirs were 2.0 mm in diameter and 1 mm deep. The injection channel length (from reservoir 2 to reservoir 4 as shown in Fig. 1) was 8.0 mm. The separation channel (from reservoir 1 to reservoir 3 as shown in Fig. 1) was 85 mm long. Detection was performed at 32.5 mm from the injection-T. All the reservoir positions are shown in Fig. 1. The ME run buffer was 1 × TBE buffer (pH 8.3) with 0.5 μg/mL EtBr. The dynamic coating matrix was made by dissolving 0.5% w/v of M_r 1 000 000 PVP into the 1 × TBE buffer with 0.5 μg/mL EtBr, and shaking the mixture for 2 min. To remove bubbles, the mixture was allowed to stand for 2 h. The sieving matrix was made by dissolving 0.5% w/v of M_r 8 000 000 PEO into the 1 × TBE buffer with 0.5 μg/mL EtBr, slowly stirring overnight. The mixture was shaken for 2 min and left standing for 2 h to remove any bubbles. The sieving matrix was hydrodynamically filled by applying a vacuum (EYELA A-3S vacuum aspirator, Tokyo Rikakikai, Japan) to the ME reservoir 3 for 4 min. The sample was pipetted into the sample inlet reservoir 2 of the microchip. The DNA sample injection by a conventional electrokinetic injection was accomplished into the injection-T region by applying a potential of 480 V at the sample outlet reservoir 4 followed by grounding the sample inlet reservoir 2 for 60 s (Fig. 1). Subsequently, voltage gradient separation was achieved by applying voltage in the range of 0–5 kV to the buffer inlet (1) and sample outlet (4).

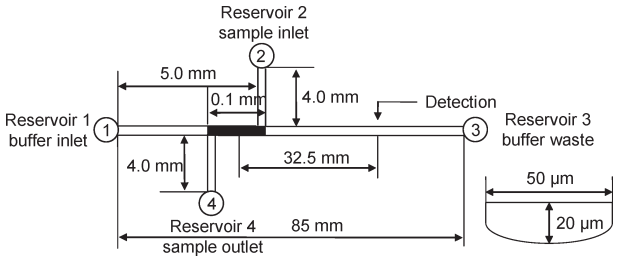

Figure 1. Schematic diagram of the microfluidic chip.

3 Results and discussion

3.1 ME-PFSG

Under the influence of an electric field (*E*), negatively charged DNA molecules will migrate through a buffer with an electrophoretic velocity (ν_{EP}) in cm/s, which can be expressed by the product of the electric field and the electrophoretic mobility (μ_{EP}) at a given field strength [11, 15]

$$\nu_{EP} = \mu_{EP} \cdot E$$

Since *E* is voltage/length, changing the voltage is an easy way to control the velocity of DNA molecules because it produces a variation in the electric field. An increase in electric field strength increases the velocity of DNA fragments and reduces migration time, which leads to shorter analysis times (Fig. 2). This suggests that one should use the highest voltage available on the instrument. However, higher voltages lead to higher currents and increased Joule heating. Therefore, at higher voltages, the resulting higher current can cause an increase in heat production. Increased heat in the microchannel may lead to broader peaks, nonreproducible migration times, sample decomposition, or even boiling of the buffer, which can cause electrical discontinuity through the channel. These effects can shut down the ME system and/or decrease resolving power and efficiency. Figure 3 illustrates the interrelation of electric field and efficiency. The efficiency fluctuates throughout the electric field strength.

Using a relatively high electric field, DNA molecules can be separated with high resolution in a relatively short time. However, at high field strengths, the electrophoretic mobility of DNA molecules becomes field-dependent [20]. The chain entanglement also plays a significant role in the

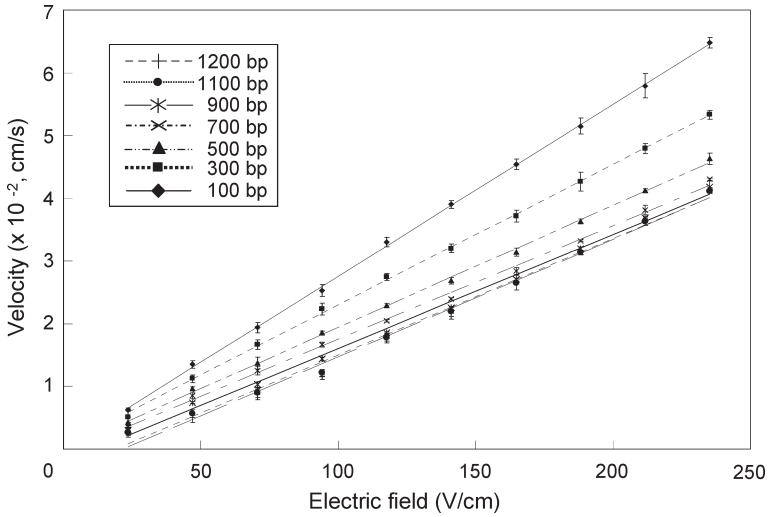

Figure 2. Velocity of DNA fragments as a function of the applied electric field in the ME system. ME conditions: applied electric field, from 23.5 to 235.3 V/cm; electrokinetic injection, 60 s at 480 V; running buffer, 1 × TBE buffer (pH 8.3) with 0.5 ppm EtBr; microchip injector design, double-T chip, total channel length, 85 mm; access hole diameter, 2 mm; width, 50 μm; effective length, 32.5 mm; coating matrix, 1 × TBE buffer with 0.5 ppm EtBr plus 0.5% PVP (M_r 1 000 000); sieving matrix, 1 × TBE buffer with 0.5 ppm EtBr plus 0.5% PEO (M_r 8 000 000). *RFU: relative fluorescence unit. Vertical bars represent the standard deviations (SDs) of the respective means (n = 3).

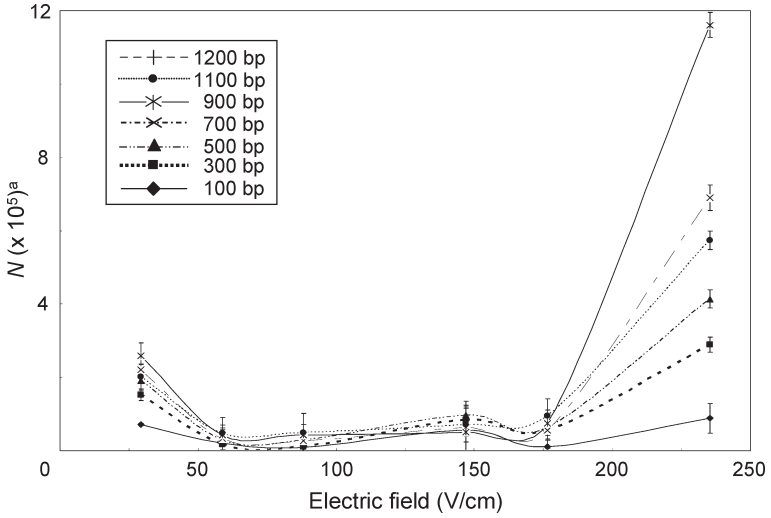

Figure 3. Relationship between the applied electric field and the efficiency (*N*) of some DNA fragments. ME conditions as shown in Fig. 2. Vertical bars represent the SDs of the respective means (n = 3). [a]N = 5.55 (migration time/peak width at half of the peak height)^2.

separation of DNA molecules with different chain lengths in a gel of a given pore size [21]. The entanglement is a function of the molecule size and the applied electric field [22]. Therefore, the main objective is to find the appropriate field strength for the optimal separation of a mixture of DNA molecules which have different chain lengths in a given gel matrix. Generally a higher electric field strength provides shorter analysis times and decreases the resolving power of DNA fragments above 800 bp in CE [11], which means it is best to find the optimum condition for the field strength gradient in ME. In order to show how the applied electric field affects resolution, the resolution of a critical pair of 100 bp DNA ladders was measured at different types of electric field strengths such as a constant electric field (isoelectrostatic), and a staircase gradient electric field (Fig. 4). The 100 bp DNA ladder showed a baseline separation within 630 s under the constant electric field strength of 47.1 V/cm (Fig. 4A). When the electric field strength of 23.5 V/cm was increased at regular intervals of 50 s like a staircase gradient (Fig. 4B), the separation time of DNA fragments decreased from 630 to 310 s. The resolving power of long DNA fragments (>1000 bp), however, was significantly reduced.

Another approach for decreasing the separation time of different sizes of DNA fragments is to program the field strength to change during analysis, that is, to use a PFSG. We can develop the PFSG separation as follows: First, find the constant electric field strength for the separation of all DNA fragments. From this separation, decide whether FSG or constant strength is best. If FSG is chosen, eliminate portions of the gradient prior to the first DNA peak and following the last DNA peak or decrease the portion between the two DNA base pair fragments in the regions of interest. Finally, if the separation in the second step is acceptable, try reducing the gradient time to reduce the run time. Different electric fields are optimum for different-sized DNA fragments; thus, the PFSG can be programmed to give the best separation of all DNA fragments with resolutions >1.5 (Fig. 5). The separation volt-

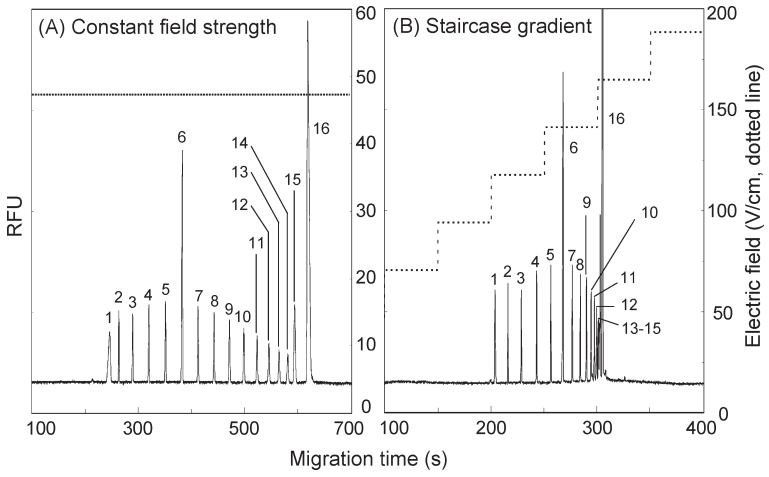

Figure 4. ME separation of 100 bp DNA ladder fragments under (A) the constant field strength and (B) the staircase FSG method. ME conditions: applied separation (A) constant electric field, 47.1 V/cm and (B) FSG at regular field strength, 23.5 V/cm from 0 to 50 s, 47.1 V/cm from 50 to 100 s, 70.6 V/cm from 100 to 150 s, 94.1 V/cm from 150 to 200 s, 117.6 V/cm from 200 to 250 s, 141.2 V/cm from 250 to 400 s. Dotted line represents the applied electric field. Peaks: 1 = 100, 2 = 200, 3 = 300, 4 = 400, 5 = 500, 6 = 600, 7 = 700, 8 = 800, 9 = 900, 10 = 1000, 11 = 1100, 12 = 1200, 13 = 1300, 14 = 1400, 15 = 1500, and 16 = 2070. Other ME conditions as in Fig. 2.

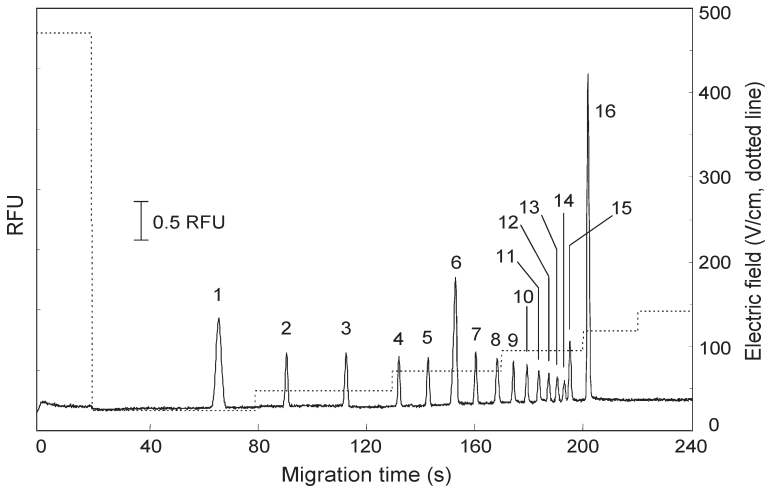

Figure 5. ME separation of 100 bp DNA ladder fragments at PFSG method. ME conditions: applied separation electric field, 470.6 V/cm from 0 to 20 s, 23.5 V/cm from 20 to 80 s, 47.1 V/cm from 80 to 130 s, 70.6 V/cm from 130 to 170 s, 94.1 V/cm from 170 to 200 s, 117.6 V/cm from 200 to 220 s, 141.2 V/cm from 220 to 240 s. Other ME conditions as in Fig. 4.

age of PFSG was applied as follows: initial applied separation electric field, 470.6 V/cm for 20 s; increase the electric field from 23.5 to 188.2 V/cm at variable intervals, 470.6 V/cm from 0 to 20 s, 23.5 V/cm from 20 to 80 s, 47.1 V/cm from 80 to 130 s, 70.6 V/cm from 130 to 170 s, 94.1 V/cm from 170 to 200 s, 117.6 V/cm from 200 to 220 s, 141.2 V/cm from 220 to 240 s; electrokinetic injection, 60 s at 480 V. Under these conditions, DNA fragments were analyzed about 1.5-times faster, and with better resolution, than when using the simple staircase FSG (Fig. 5 *vs.* 4B). Using a constant field strength (Fig. 4A), all the fragments were separated in about 650 s. With the PFSG (Fig. 5), however, the separation was achieved with the same efficiency, and with resolution $>$1.5, in only about 200 s.

Electroosomotic flow (EOF) is substantially reduced in a PEO matrix due to dynamic coating [23]. However, at a higher pH 7.0, the PEO coating is unstable [24]. The migration times in 0.1 and 1% PVP solutions were much longer than those in the buffer itself. For M_r 1 000 000 PVP added into the 1 $\times$ TBE buffer (pH 8.3) with 0.5 μg/mL EtBr, the results indicate no significant difference from the migration times of DNA fragments in consecutive runs, with the same ME condition. The addition of 0.5% PVP as a dynamic coating matrix effectively reduced the EOF and the adsorption of DNA fragments into the microchip. This result in ME-PFSG corresponds to the evidence that suggests polymer that suppresses EOF, and presents the adsorption of DNA molecules into the fused-silica surface in CE [25, 26].

3.2 Clinical PCR sample applications

The enhanced and faster separation of DNA fragments can be achieved by applying a nonuniform FSG, and PFSG at the same time. For quantitative analysis, a resolution $>$1.5 is highly desirable. In this way, all specific size DNA fragments are exposed to the PFSG which is optimal for their separation. The electric field strengths at the beginning and end of the gradient play an important role in determining the adequacy of the applied electric field, and also in the rapid separation of all-size DNA fragments and specific-size DNA fragments.

Figure 6 shows the separation of PCR products, 519 and 1191 bp DNA fragments from the 18S rRNA of *B. gibsoni* and *B. caballi*, in rapid diagnosis of Babasia by employing the PFSG in a microchip (effective microchannel length: 32.5 mm). The PFSG method for the diagnosis of *B. gibsoni* consisted of three consecutive steps: 517.6 V/cm from 0 to 26 s, 105.9 V/cm from 26 to 36 s, and 258.8 V/cm from 36 to 60 s for the separation of 519 bp DNA fragment (Fig. 6A). We then employed the following four consecutive steps to diagnose *B. caballi*: 70.6 V/cm from 0 to 5 s, 582.4 V/cm from 5 to 30 s, 82.4 V/cm from 30 to 38 s, and 388.2 V/cm from 38 to 60 s for the long separation of 1191 bp DNA (Fig. 6B). All target PCR products were analyzed separately within a minimum of 40 s. The perfectly baseline-separated peaks show that the PCR products can also be determined by simply calculating the peak areas of each of the separated DNA peaks. When we calculated the resolutions for the 500 and 600 bp DNA fragments at PFSG I (Fig. 6A) and 1100 and 1200 bp DNA fragments at PFSG II (Fig. 6B), they showed values of 2.46 and 1.61, respectively. These data show that migration time, resolution, and peak efficiency of DNA fragments in ME separations can largely be controlled by the PFSG. In PGFS, final electropherogram should contain no blank space near the beginning or end of the gradient, as these represent wasted time in gradient elution.

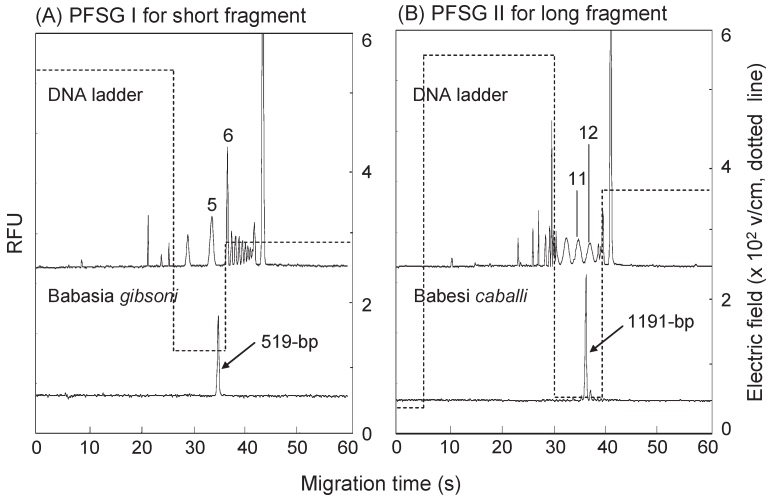

Figure 6. PFSG separation of PCR products, 519 and 1191 bp DNA fragments from the 18S rRNA of (A) *B. gibsoni* and (B) *B. caballi* for the fast diagnosis of *Babasia* in ME. Other conditions are the same as those shown in Fig. 4. *Arrows indicate the amplified DNA peaks.

4 Concluding remarks

A simple PFSG method was introduced in order to increase the resolving power and decrease the separation time of DNA fragments in ME. The ME with PFSG will provide identical migration curves for all DNA fragments, in regards to the beginning elution time and terminating eluting time of DNA fragments in the microchip. The electric field strengths at the beginning and end of the gradient played a major role in determining the adequacy of the applied electric field. The applied electric field shape of PFSG can be designed in linear or varying degrees of convex and concave. Most commercial ME systems used in microchips offer a software with gradient voltage shapes. Thus, the ME-PFSG method for fast separation of DNA fragments is applied easily, by simply adjusting the field strength without any additional devices and/or tools. The primary goal of PFSG is faster separation of DNA fragments, without the risk of losing more resolving power than the staircase FSG in ME. Gradient steepness is expressed in various ways, and depends on V/s changes in the applied voltage to the DNA molecules that enter the microchannel. All DNA fragments and/or specific DNA molecules of interest will be eluted during the gradient or soon after the completion of the gradient with continued elution of amplified DNA fragments by PCR.

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