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## Fast and sensitive diagnosis of thalassemia by capillary electrophoresis

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**Abstract** This paper demonstrates the diagnosis of  $\beta$ -thalassemia by capillary electrophoresis in conjunction with laser-induced fluorescence using poly(ethylene oxide) (PEO) solutions in the presence of electroosmotic flow (EOF). During the electrophoretic separation, PEO solution entered a capillary from the anodic vial by EOF. The separation of a mixture of the polymerase chain reaction (PCR) products (330 and 334 base pairs) from a healthy person and a  $\beta$ -thalassemia patient was accomplished within 15 min at 15 kV using 1.5% PEO containing 2 M urea at 30 °C. The electropherogram patterns instead of migration times were used to diagnose  $\beta$ -thalassemia, with an accuracy of 100% for the analyses of 11 blood samples from suspected patients. After injecting a large volume of the mixture to the capillary filled with 800 mM Tris-borate buffer (pH 10.0), the DNA fragments stacked due to increases in viscosity and sieving when migrating into 1.5% PEO solution. As a result of improved sensitivity, only 15 PCR cycles were required when using 500 ng of DNA templates. The results shown in this study indicate the potential of this simple, rapid, and cost-effective method for the diagnosis of  $\beta$ -thalassemia.

**Keywords** Capillary electrophoresis · DNA · Laser-induced fluorescence · Polymer solutions · Thalassemia

**Abbreviations** CE: Capillary electrophoresis · EOF: Electroosmotic flow · EtBr: Ethidium bromide · LIF: Laser-induced fluorescence · PCR: Polymerase chain reaction · PEO: Poly(ethylene oxide) · TRIS: Tris(hydroxymethyl)aminomethane · TB: TRIS-borate

### Introduction

Thalassemia results from quantitative reductions in globin chain synthesis [1]. Those with decreased  $\alpha$ -globin chain production are called  $\alpha$ -thalassemias, whereas those with diminished  $\beta$ -globin chains are termed  $\beta$ -thalassemias. The manifestations of thalassemias range from mild anemia with microcytosis to fatal severe anemia (Hb Barts hydrops fetalis or  $\beta$ -thalassemia major).  $\beta$ -thalassemia major presents in infancy and requires life-long transfusion therapy and/or bone marrow transplantation [2], and thus its diagnosis is of considerable importance.

Slab gel electrophoresis has become one of the most popular tools for diagnosis of  $\beta$ -thalassemia [3, 4]. Compared to slab gel electrophoresis for DNA analysis, capillary electrophoresis (CE) provides the advantages of rapidity, use of minute amounts of samples, and easy automation [5, 6]. With such advantages, numerous CE techniques have been tested and validated for DNA sequencing, genotyping, mutation analysis, and forensic human identification [7–9]. The success of CE in DNA separation stems in part from the use of replaceable polymer solutions that provide the capability of self coating (dynamic coating) and single-base resolution [10–12]. Dynamic coating of polymer molecules on the capillary wall is a simple approach to minimizing the variation of electroosmotic flow (EOF) and DNA adsorption, leading to reproducibility and better resolution.

Temperature and electric field strength induce differential changes in the conformation (mobility) of various sizes and sequences of DNA, and thus temperature gradient and voltage programming in CE have shown powerful for the analyses of single-point mutations [13–15]. The applications of temperature gradient-CE and CE-using strongly acidic, isoelectric buffers to the analysis of point mutants [16] and screening  $\beta$ -39 mutations in thalassemia [17] have been demonstrated. Adding denaturant reagents such as urea and glycerol to the background electrolytes to improve resolving power

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of CE for DNA has also been recognized [18–20]. Recently, we have demonstrated DNA separation in the presence of EOF under discontinuous conditions [21–23]. Polymer solutions enter the capillaries filled with Tris-borate (TB) buffer by EOF after injecting DNA samples. In the course of separation, DNA fragments possessing negatively charges enter PEO (neutral molecules) and they slow down as a result of sieving and an increase in viscosity. Since DNA migrates against EOF, large DNA fragments with small electrophoretic mobilities are detected earlier at the cathode end. In the presence of EOF, we have also developed on-line concentration and separation techniques for the analysis of DNA [24–26]. Unlike other conventional on-line concentration methods [27–32], stacking in our approaches is due to sieving and increases in viscosity when DNA migrating into PEO solution.

With the capability of single-base resolution and improved sensitivity (up to 450-fold), these recently developed on-line concentration techniques have established high potentials for clinical applications. In this study, one of the on-line concentration CE techniques was applied to the analyses of a mixture containing the polymerase chain reaction (PCR) products from a healthy person and a suspected thalassemia patient [24]. Our experimental goal also aimed at evaluating the effects of temperature and urea on resolution and speed for the diagnosis of thalassemia.

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## Materials and methods

### Equipment

The basic design of the separation system has been previously described [21]. Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL, USA) was used to drive electrophoresis. The entire CE system was enclosed in a black box with a high-voltage interlock. The high-voltage end of the separation system was put in a laboratory-made plexiglass box for safety. A 4.0-mW He-Ne laser with 543.6-nm output from Uniphase (Mantence, CA, USA) was used for excitation. The light was collected with a 20X objective (numerical aperture = 0.25). One RG 610 cutoff filter was used to block scattered light before the emitted light reached the phototube (Hamamatsu R928). The amplified currents were transferred directly through a 10-k $\Omega$  resistor to a 24-bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a PC. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75  $\mu$ m i.d. and 365  $\mu$ m o.d. were used for DNA separations without any further coating process.

### Chemicals

All chemicals for preparing buffer and PEO (M. W. 8,000,000) solutions were from Aldrich (Milwaukee,

WI, USA). Ethidium bromide (EtBr) that is cheap and weakly fluorescent was obtained from Molecular Probes (Eugene, OR, USA). TB buffers were prepared from Tris adjusted with boric acid to pH 9.0 and 10.0, respectively. In this manuscript, the molarity of TB buffer refers to that of Tris. Buffers of 800 mM TB (pH 10.0) and 200 mM TB (pH 9.0) were used to fill capillaries and prepare 1.5% PEO (with/without urea), respectively [21]. QIAamp DNA blood mini kit was purchased from QIAGEN (Hilden, Germany).

### DNA extraction and PCR products

The blood samples were collected from patients that possibly suffer from thalassemia (lifelong transfusion) in Mackey Memorial Hospital. Human genomic DNA from buffy coat was extracted using QIAamp DNA blood mini kit according to the manufacturer's instructions. Amplification of the DNA samples was conducted as suggested by Chang's group [33]. Briefly, a certain amount of human genomic DNA was mixed with 100 ng of the primer, 200  $\mu$ M dNTP, and 2.5 U *Taq* polymerase in 100- $\mu$ l PCR reaction buffer consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.01% gelatin. The settings for conducting PCR were: initial incubation at 94 °C for 10 min; cycling at 94 °C for 1.1 min, at 55 °C for 1.1 min, and at 72 °C for 2.1 min; and stored at 4 °C after the PCR cyclings (15 and 30) were finished. The times for 15 and 30-cycles PCR reactions were 90.0 and 170.0 min, respectively.

### Stacking and separation

Prior to analyses, capillaries were treated with 0.5 M NaOH overnight. At least three times capillary-volume 800 mM TB (pH 10.0) was used to flush NaOH out of the capillary. DNA samples were prepared by mixing equal volumes of the PCR products either from a healthy person and a  $\beta$ -thalassemia patient, from two healthy people, or from two  $\beta$ -thalassemia patients. Hydrodynamic injection (30-cm height; 12–360 s) was applied to inject DNA samples into the capillary filled with 800 mM TB (pH 10.0). During separation (and stacking), PEO entered the capillary by EOF and acted as sieving (and stacking if large-volume injection was applied) matrices. Owing to self-adsorption capability of PEO, EOF gradually decreased in the course of electrophoretic separation. For reproducibility, the capillary was equilibrated with 0.5 M NaOH at 1 kV for 10 min after each run.

To investigate the urea dependence, buffers consisting of 200 mM TB (pH 9.0) and urea (1–7 M) were used to prepare 1.5% PEO. After sample injection, the DNA fragments migrated into PEO solution, wherein they encountered urea that entered the capillary by EOF from the anodic end. For investigating the temperature effect, a portion of the capillary, from the point of 5 cm

from the inlet to the point of 15 cm from the outlet, was surrounded by a teflon tube, wherein water flowed through at a rate of 5 mL/min. Using a temperature controller (YIH DER Instruments Co. Ltd., R.O.C.), the system temperature was stable ( $\pm 1$  °C) in the range of 20–80 °C.

## Results and discussion

### Analysis of PCR products

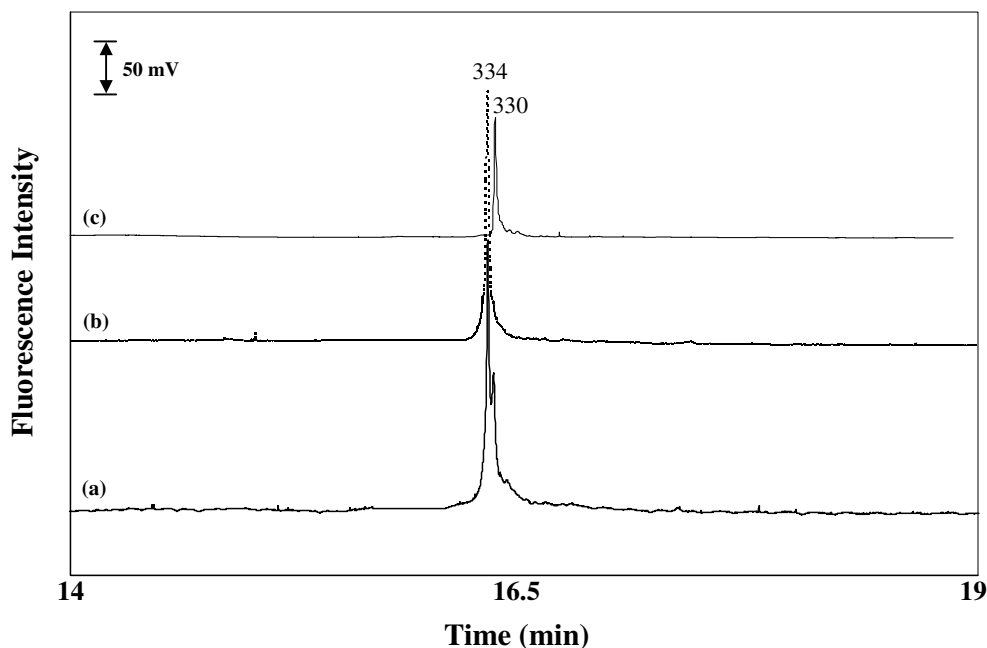
Owing to a 4-bp difference between the PCR products from a healthy person (334 bp) and a  $\beta$ -thalassemia patient (330 bp), a technique providing high-resolving power is essential for diagnosis of  $\beta$ -thalassemia. In a traditional method for  $\beta$ -thalassemia, enzymatic digestion of the PCR products is conducted prior to the separation by slab gel electrophoresis, in which only the PCR products from  $\beta$ -thalassemia patients can be digested [33]. However this method is slow and is difficult for automation, and thus it is not suitable for high-throughput diagnosis of  $\beta$ -thalassemia. With the advantages of simplicity, sensitivity, high-resolving power, and ease of automation [21–26], CE-LIF using PEO solutions should be suitable for fast diagnosis of  $\beta$ -thalassemia. To test our hypothesis, the CE-LIF technique was applied to the separations of three different mixtures of the PCR products, which were from one healthy person and a suspected  $\beta$ -thalassemia patient, from two different healthy persons, and two suspected  $\beta$ -thalassemia patients. Electropherogram (a) in Fig. 1 presents two peaks corresponding to the PCR products from a healthy person and a  $\beta$ -thalassemia patient, respectively. We note that the first peak corresponds to the 334-bp fragment since DNA migrated

against EOF. Electropherograms (b) and (c) in Fig. 1 show only one peak when separating the mixtures of the PCR products from two healthy persons and two  $\beta$ -thalassemia patients, respectively. The relative standard deviation (RSD) values ( $n=3$ ) of the migration times for the two peaks are 2.4 and 2.5%, respectively. We note that the time ( $t_0$  in Table 1) at which the baseline shifted can be used as an internal standard if the correction of the variation of the migration times for the 334- and 330-bp fragments is needed [21]. When PEO migrated through the detection window, the shift occurred as a result of the change in refractive index and a greater quantum yield of EtBr in PEO. Based on the results shown in electropherogram (b) and (c), the migration times for the PCR products could not be reliably used to tell the samples obtained from two healthy people or suspected  $\beta$ -thalassemia patients. It is also impossible to tell samples whether from healthy person or  $\beta$ -thalassemia patients if we only separate the PCR product from a person's blood, based on the migration time data. Fortunately, two partially resolved peaks were always detected for the mixtures containing the PCR products from a healthy person and a suspected patient although their differential migration time is so small. In other words, one should easily detect  $\beta$ -thalassemia from the electropherogram patterns regardless a small difference and variation of migration times for the two peaks around 16.0 min (more details see Sect. 3.3).

### Effect of urea and temperature on DNA analysis

To further speed up the separation and optimize resolution, we conducted the separations at various temperatures and urea concentrations. DNA conformation and the viscosity of the background electrolyte are

**Fig. 1a–c** Electropherograms of separating three mixtures each containing two PCR products. Blood samples from a healthy person and a  $\beta$ -thalassemia patient in (a), two healthy persons in (b), and two  $\beta$ -thalassemia patients in (c). The PCR products of 5.0 ng/ $\mu$ L DNA templates were amplified by 30 cycles. Capillary: 50 cm in total length and 40 cm in effective length, filled with 800 mM TB, pH 10.0. PEO containing 5  $\mu$ g/ml EtBr was prepared in 200 mM TB, pH 9.0. Hydrodynamic injections were conducted at 30-cm height for 60 s and separations were conducted at 25 °C and 15 kV in the presence of EOF using 1.5% PEO



**Table 1** Effects of urea and temperature on migration time and resolution of the two PCR products from a healthy person and a  $\beta$ -thalassemia patient

Temperature (°C)	$t_0$ (min)			Migration time (min)			Resolution <sup>a</sup>		
	0 M	1 M	2 M	0 M	1 M	2 M	0 M	1 M	2 M
20	12.06 (0.75) <sup>b</sup>	11.82 (0.62)	11.50 (0.56)	16.37 <sup>c</sup> / 16.40 <sup>d</sup>	15.62/15.65	15.25/15.28	0.9	0.9	0.7
30	11.16 (0.58)	11.18 (0.55)	10.42 (0.50)	15.27/15.31	14.90/14.93	14.02/14.04	0.9	1.0	0.8
40	10.31 (0.59)	9.80 (0.49)	9.80 (0.46)	14.15/14.19	13.15/13.17	13.10/13.13	1.0	1.1	0.8
50	9.05 (0.49)	9.02 (0.48)	8.82 (0.45)	12.56/12.59	12.15/12.18	11.83/11.85	1.0	1.1	0.8
60	8.23 (0.52)	8.60 (0.49)	8.23 (0.46)	11.49/11.51	11.61/11.63	11.11/11.13	1.0	1.1	0.8
70	7.89 (0.67)	7.88 (0.57)	7.47 (0.54)	11.14/11.17	10.74/10.77	10.21/10.23	1.1	1.1	0.9
80	7.67 (0.71)	7.33 (0.58)	7.28 (0.50)	11.10/11.13	10.08/10.10	10.10/10.12	1.1	1.0	0.9

<sup>a</sup> $R_s = 0.589 (t_2 - t_1) / w_{1/2av}$ ;  $t_1$  and  $t_2$  are the migration times for the 334- and 330-bp fragments, respectively;  $w_{1/2av}$  is the average width at the half heights for the two peaks corresponding to the 334- and 330-bp fragments, respectively

<sup>b</sup>RSD ( $n = 3$ ; %)

<sup>c</sup>334-bp fragment

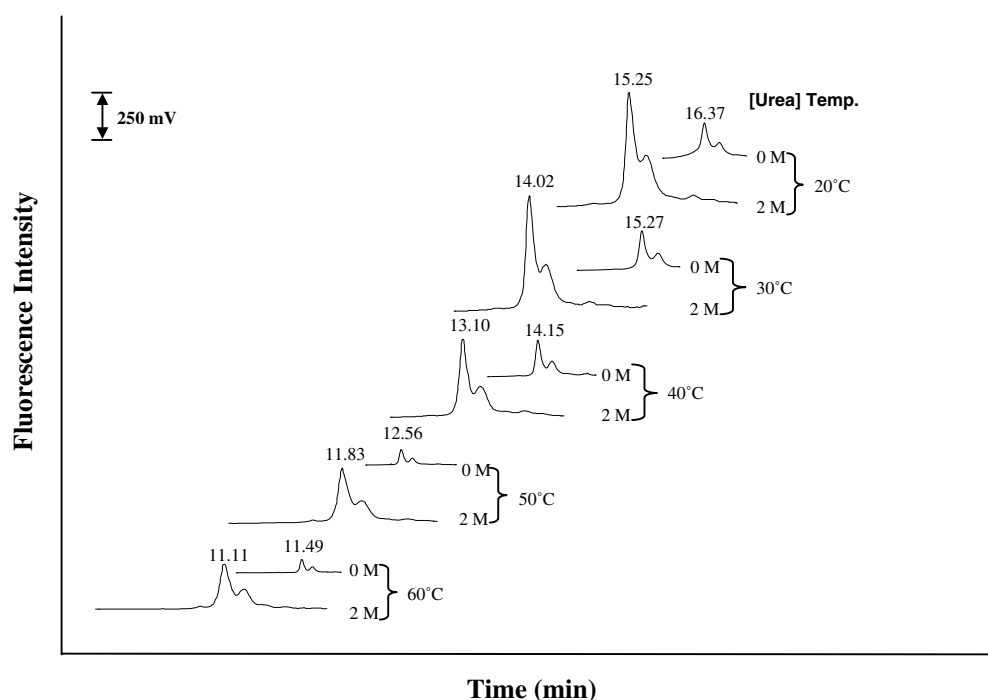
<sup>d</sup>330-bp fragment

temperature dependent, and thus the electrophoretic mobility of DNA should be different at various temperatures. Figure 2 presents the analysis was faster at higher temperature as a result of decreases in viscosity (increases in EOF). For example, in the absence of urea, the separation time and the bulk EOF mobility were 16.40 min and  $1.84 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$  at 20 °C, while they were 11.51 min and  $2.70 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$  at 60 °C, respectively. Next, we turned our attention to exploring the effect of urea on DNA separation at constant temperature. Although our measurements shows that the effect of urea on the viscosity of 1.5% PEO solutions is negligible (less than 3.0% in the presence of 0 and 7 M urea), which is in good agreement with a reported result [18], Fig. 2 does show the analysis time was slightly shorter in the presence of urea at a

constant temperature. This is mainly due to a suppression of PEO adsorption on the capillary wall, presumably because hydrogen bonding between the hydroxyl groups in PEO and the Si-OH on the capillary surface reduced in the presence of urea [34–39]. Our reasoning is supported by the fact that the bulk EOF mobility values were  $1.84 \times 10^{-4}$  and  $1.93 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$  in the absence and presence of 2 M urea at 20 °C. It is important to note that the reproducibility is better in the presence of urea, partially because of reduced adsorption of PEO (more constant EOF) on the capillary wall. For example, the RSD values of the migration time for the 330-bp fragment were 2.5 and 1.5% in the absence and presence of 2 M urea, respectively.

Temperature and urea also play some roles in determining the fluorescence intensity and resolution for the

**Fig. 2** Effects of temperature and urea on the separation of a mixture of the PCR products from a healthy person and a  $\beta$ -thalassemia patient. The migration times for the 334-bp fragment were shown in the electropherograms. Other conditions were the same as in Fig. 1



two DNA fragments. At a constant urea concentration, the fluorescence intensities (peak heights) of the EtBr-DNA complexes slightly varied (less than 10%) in the range of 20–35 °C, while decreased with increasing temperature in the range of 35–80 °C. For example, the fluorescence intensities of the EtBr-DNA complexes at 80 °C were about threefold lower than those at 30 °C. Unlikely, the fluorescence monotonically decreased with increasing temperature from 20–80 °C under static conditions (measured with a fluorometer), resulting from dynamic quenching. At high temperature, the changes in DNA conformation (denaturation) also contribute to the loss of fluorescence intensity because EtBr binds more strongly to ds DNA than to ss DNA

**Table 2** Diagnosis of  $\beta$ -thalassemia by CE in the presence of EOF using 1.5% PEO containing 2 M urea at 30 °C

Sample	Total peaks	Codon 41-42 (-4 nt) frame shift	
		This work	Traditional method <sup>b</sup>
H1 + P1 <sup>a</sup>	2	Positive	Positive
H1 + P2	2	Positive	Positive
H1 + P3	2	Positive	Positive
H1 + P4	1	Negative	Negative
H1 + P5	2	Positive	Positive
H1 + P6	2	Positive	Positive
H1 + P7	2	Positive	Positive
H1 + P8	2	Positive	Positive
H1 + P9	1	Negative	Negative
H1 + P10	2	Positive	Positive
H1 + P11	1	Negative	Negative

<sup>a</sup> H1 means the PCR product was from a healthy person and P1–P11 mean the PCR products were from patients 1 to 11, respectively

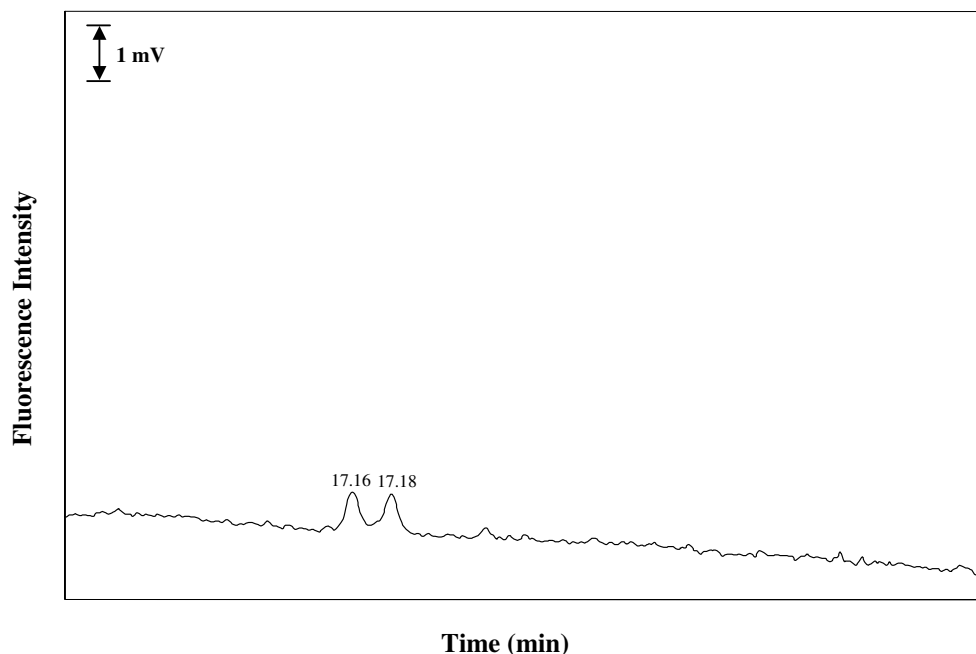
<sup>b</sup> Ref. [33]

[40, 41]. A differential temperature dependence of fluorescence in CE from that in the static state suggested another mechanism involved in CE. In the absence of urea, narrower and more symmetric peak profiles were observed at high temperature, which is a result of weaker DNA adsorption and small diffusion (shorter migration times). Thus we suggest that quenching, stability of intercalated DNA, and peak profiles affect the sensitivity of the two DNA fragments in CE. We note that the resolving power for the two peaks increases with increasing temperature in the range of 30–70 °C (Table 1) as a result of narrower peak profiles. The RSD values ( $n=3$ ) for the resolution were less than 6.0%. Overall, we concluded that the separation conducted at 30–40 °C using solutions consisting of 1.5% PEO and 1–2 M urea is proper. Hereafter, the separations were conducted at 30 °C in the presence of 2 M urea. Using this condition, the resolution for the two DNA fragments was 0.8, with a RSD of 3.6% in five consecutive runs.

### Diagnosis of $\beta$ -thalassemia

Figures 1 and 2 suggests that the electropherogram pattern is useful for diagnosing  $\beta$ -thalassemia. One peak and two partially resolved peaks around 14.0 min shown in the electropherograms (30 °C; 2 M urea) indicate that the patients do not and do suffer from  $\beta$ -thalassemia, respectively. To further validate this method for the diagnosis of  $\beta$ -thalassemia, we collected bloods from one healthy person and eleven patients who may suffer from  $\beta$ -thalassemia. The electropherogram patterns of separating 11 mixtures each consisting of the PCR products from the healthy person and one of the suspected

**Fig. 3** Separations of a large-volume mixture of the 15-cycle PCR products from a healthy person and a  $\beta$ -thalassemia patient using 1.5% PEO containing 2 M urea at 30 °C. The concentrations of the DNA templates were 5.0 ng/ $\mu$ l. Hydrodynamic injection was conducted at 30-cm height for 180 s. Other conditions were the same as in Fig. 1



**Table 3** Summary of the analyses of PCR products amplified after 30 and 15 cycles

Method <sup>a</sup>	Template (ng)	Injection volume (nl)	Time (min) <sup>b</sup>				Advantages
			PCR	Injection	Separation	Total	
A	500	7.5	170.0	0.2	14.1	184.3	Simple
B	500	132	90.0	3.0	17.2	110.2	Fast

<sup>a</sup>Conditions for methods A and B were as in Fig. 2 (30 °C; 2 M urea) and Fig. 3, respectively

<sup>b</sup>Total analysis time was about 320 min using a conventional method (Ref. [33])

patients were used to read the diagnostic results. The sensitivity of this method tabulated in Table 2 is 100% when compared to those diagnosed by a traditional method [33], indicating the potential of this method for fast diagnosis of  $\beta$ -thalassemia. Unlike most traditional methods, the variation of migration time does not affect our diagnostic results because the reading relies on the electropherogram patterns in our method. Without conducting enzyme digestion prior to separation at an electric field of 375 V cm, this new method is simple, low costly, and fast.

#### On-line concentration

The total analysis time (PCR, injection, and separation time) increases with increasing PCR cycles, and thus low PCR cycles are favorable for fast diagnosis of  $\beta$ -thalassemia. However, a highly sensitive detection method is required for small amounts of PCR products. For the past years, our efforts have made to develop on-line CE concentration and separation techniques for the analyses of trace amounts of DNA [24–26]. These techniques allow the analyses of DNA prepared in high- and low-conductivity media without tedious sample pretreatment. Together with the result shown in Table 2, we believed that our on-line CE concentration and separation techniques should be highly suitable for fast diagnosis of  $\beta$ -thalassemia. To demonstrate the beauty of our method, a large volume (ca. 132 nl) of the mixture of the PCR products from a healthy person and  $\beta$ -thalassemia patient after 15-cycle amplification was injected. Figure 3 presents the separation was accomplished within 17.2 min, with two well separated peaks. The RSD values ( $n=3$ ) of the migration times for the two peaks were 3.8%. Due to relatively stronger PEO adsorption at low ionic strengths, the separation time was slightly longer when compared to that shown in Fig. 2 (30 °C; 2 M urea). It is important to point out that the total analysis time was shortened from 184.3 min (30 cycles) to 110.2 min (15 cycles) as shown in Table 3, revealing the potential of this method for diagnosing  $\beta$ -thalassemia from a great number of blood samples, especially when using a capillary array electrophoresis (CAE) system [42, 43]. When conducting DNA separation in a CAE system, the migration times for the DNA fragments obtained in different capillaries may be so different,

mainly due to the differential capillary properties, the variation of the electric field strength in different capillaries, and so on. To correct the variation, an internal standard is usually added to the sample. By applying our method, an internal standard is not required since the diagnosis is according to the electropherogram patterns, not migration times, and the shift in the baseline is useful to correct the migration times. Thus our suggested method should hold great potential for high-throughput diagnosis of  $\beta$ -thalassemia using a CAE system.

#### Concluding remarks

We have progressed in the investigation of diagnosis of  $\beta$ -thalassemia by CE in the presence of EOF using 1.5% PEO. From the electropherogram pattern of separating a mixture of the PCR products from a healthy person and a suspected  $\beta$ -thalassemia patient, one can easily and accurately diagnose whether the patient suffers from  $\beta$ -thalassemia. Together with applying the on-line CE concentration technique, we have demonstrated the analysis of a mixtures of 15-cycle PCR products from a healthy person and a  $\beta$ -thalassemia patient. Because electropherogram patterns instead of migration times are used to diagnose  $\beta$ -thalassemia, the variation of migration times if obtained in different runs is not problematic. With the advantages of sensitivity, rapidity, simplicity, and low costs, the proposed method has great potential for screening  $\beta$ -thalassemia from a large number of samples when using a CAE system.

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