

The method of single-nucleotide variations detection using capillary electrophoresis and molecular beacons

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Abstract We demonstrate that single-nucleotide variations in a DNA sequence can be detected using capillary electrophoresis (CE) and molecular beacons (MBs). In this method, the region surrounding the site of a nucleotide variation was amplified in a polymerase chain reaction, then hybridize PCR products with each of MBs. The sequences of the PCR products are different at the site of 2,044 in exon of interleukin (IL)-13 which to be identified. Through denaturation, the PCR product became single strand and hybridized with the completely complementary MB. The MB-target duplexes were separated using CE and solution-based fluorescence techniques. The results show that in each reaction a fluorescent response was elicited from the molecular beacon which was perfectly complementary to the amplified DNA, but not from the other MB whose probe sequence mismatched the target sequence. The method of CE based on MBs is able to identify single-nucleotide variations in a DNA sequence and can discriminate the genotyping of the SNP between the homo- and heteroduplexes of DNA fragments.

Keywords Capillary electrophoresis · Hybridization · Molecular beacon · Single-nucleotide variation

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Introduction

Single-nucleotide substitutions represent the largest source of diversity in the human genome. Some of these variations have been directly linked to human diseases [1, 2]. We introduced a method, electrophoresis (CE) based on molecular beacons (MBs) assay, which can detect the single-nucleotide variations and discriminate the genotyping of the SNPs.

MBs are oligonucleotide probes. A fluorescence moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other one. When the probe encounters a target molecule, the MB undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence which to be detect [3–5]. These probe-target duplexes were then separated by capillary electrophoresis, where the genotype of the sample could be determined by the shift in size of the target—products with bound MB probes which had a longer migration time than products without MB probes bound. From assessing the binding state of two MB (one for each allelic state) the genotype of the individual could be determined.

In this method, a polymerase chain reaction was carried out to amplify the DNA region, and then denatured and hybridized the PCR product with an MB in a sealed reaction tube. Because hybridization of these probes to their complementary targets is highly specific, we designed molecular beacons to detect an A2044G SNP in the exon 4 of IL-13 gene. IL-13 plays an important role in the induction of immunoglobulin E and in the pathogenesis of asthma [6, 7]. Recently mutations in IL-13 have been identified are associated with asthma mechanism, especially A2044G SNP in exon 4 which results in an amino

acid change in residue 110 from Arg to Gln [8–10]. The A allele is reportedly associated with increased serum IgE levels in allergic patients [11].

We hybridized the 178 bp PCR fragment including 2,044 site with the specific MB in a liquid phase environment to determine the presence of the A2044G mutation. The specific molecular recognition of MBs are based on their hybridization to complementary target oligonucleotide sequences, which include conformational change through denaturation, causing a separation of the fluorophore from the quencher allowing it to fluoresce [12]. While increased solution fluorescence implies an opening up of the hairpin structure upon hybridization [13]. The properties of CE can separate the hybridized and unhybridized MB depending on the alteration in the shape of the molecules.

Materials and methods

PCR products

The samples were put three groups: (1) 2044 G/G; (2) 2044 A/A; (3) 2044A/G. There are 15 cases in each group. All the sequences were verified by sequencing. DNA was extracted and concentrations were measured spectrophotometrically and diluted concentration of 20 ng/μl was used for PCR amplification. A 178 bp PCR fragment including the A2044G SNP was generated with use of the primes 5'-CCCTGGTTTGTGCGAGTC-3' and 5'-CGCCTACCCAAGACATTTT-3'. PCR reactions were carried out in a total volume of 50 μl containing approximately 100 ng of genomic DNA, 30 mmol/l Tris-HCl (pH 8.3), 150 mmol/l potassium chloride, 5 mmol/l MgCl₂, 600 μmol/l of each deoxynucleotide triphosphate, 100 ng of each primer and 0.5 units of TaqDNA polymerase (invitrogen). Samples were denatured at 94°C for 2 min followed by 30 cycle of 94°C for 30 s, 51°C for 30 s and 72°C for 30 s and a final extension for 3 min at 72°C, then ice bathing. Sequencing of IL-13 gene in all samples were performed by cycle sequencing of overlapping polymerase chain reaction (PCR) amplified DNA fragments covering the region containing the polymorphism.

Synthesis of molecular beacons

MB probes were designed following the guide-line described by Tyagi and Kramer [14]. Two MBs were synthesized; each containing 5-nucleotide-long arms and a 12-nucleotide-long probe sequence (Fig. 1). The only different nucleotide between the two sequences is the 11th nucleotide.

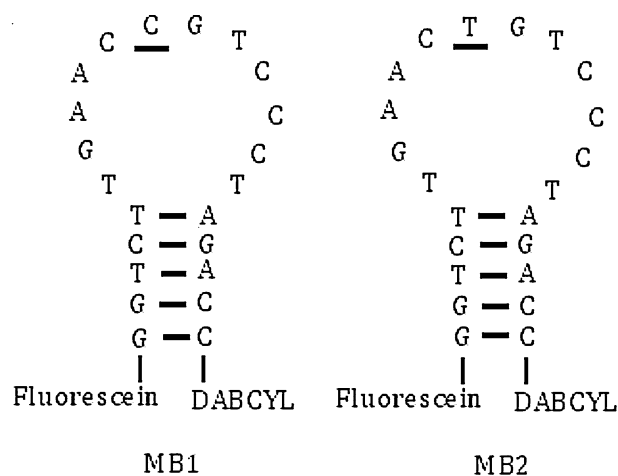


Fig. 1 Structure of molecular beacons. MBs consist of 12-nucleotide-long-probe sequence embedded within two complementary 5-nucleotide-long arm sequences. The fluorophore is joined to the 5'-terminal phosphate, and the quencher is joined to the 3'-terminal. The underlined nucleotides identify the site of variation

MB1: fluorescein-5'-GGTCT TGAACCGTCCCTAGACC-3'-DABCYL.

MB2: fluorescein-5'-GGTCT TGAAC7GTCCCTAGACC-3'-DABCYL.

The italicized nucleotides indicate the site of variation. The loop region was complementary to the true target. A fluorescent group attached to the 5' end, and a black hold quencher (BHQ) molecule attached to the 3'-end. Each MB was purified by high-pressure liquid chromatography [15].

Thermal denaturation profiles and hybridization condition

To determine thermal denaturation profiles of the hybrid formation by the two molecular beacons and their perfect and mismatch oligonucleotide targets. We monitored the fluorescence of a 50 μl solution containing 25 μM MB probe, 20 mmol/l Tris-HCl, and 1 mmol/l MgCl₂(pH8) at a function of temperature. Fluorescence was monitored during each period by using a fluorescence reader with a programmed temperature control (Applied Biosystems Prism 7700). The temperature was increased from 10 to 80°C in 1°C steps of 60 s each. Fluorescence was monitored during each step.

One sample of PCR product was put into two sealed tubes containing MB1 and MB2, respectively. Hybridization reactions were carried out in a total volume of 100 μl containing approximately 20 mmol/l Tris-HCl (pH 8.0), 1 mmol/l MgCl₂, 25 μM MB, 25 μl PCR product. Samples were denatured at 95°C for 5 min, hybridized at 30°C for 30 min then ice bathing for detecting. MB1 and MB2 have been done by this process, respectively.

Capillary electrophoresis

A P/ACE™ System 5500 instrument equipped with laser-induced fluorescence (LIF) detection (Beckman instruments) was used for the CE runs. Post-run data analysis was performed with Gold (version 7.0) software. Capillary columns, 37 cm length (30 cm to detection window) \times 50 μ m ID, were assembled in a P/ACE LIF cartridge. This cartridge contains an ellipsoidal mirror to collect fluorescence.

Specifications for the run were as follows: capillary fused-silica tube, 30 cm (inlet-to-detector distance), 37 cm (total length) \times 50 μ m ID; running buffer: 50 mM phosphate (pH 7.0); running temperature: 20°C; running voltage: +20 kV; 488 nm emitted laser, 20 s by hydrodynamic injection. CE was performed with each of the target alone (target control), the MB alone (MB control) and the mixture containing probe-target duplexes, respectively.

Results

Thermal denaturation profiles of MB

Real-time measurements were performed in a fluorometric thermal cycle containing molecular beacons in sealed tubes. When the temperature of solution containing molecular beacons increased, fluorescence became remarkable in a manner that is characteristic of the melting of a nucleic acid double helix. Figure 2 showed the fluorescence pattern of the MBs observed at temperatures ranging from 10 to 80°C.

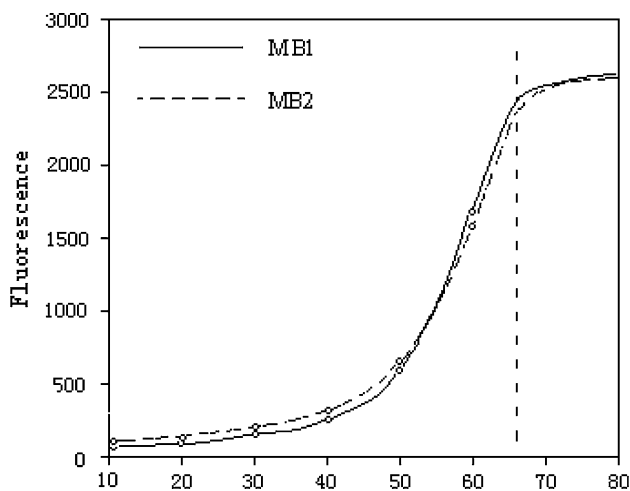


Fig. 2 Thermal transition profile of molecular beacons. The fluorescence intensity in solution containing molecular beacon A and B were plotted as a function of temperature on a linear scale from 10 to 80°C. Fluorescence became remarkable between 50 and 66°C and the fluorescence line converge the same fluorescence level above 66°C.

The thermal denaturation profile confirms that at low temperatures the arms from a hairpin stem that brings the fluorophore and quencher together, thus inhibiting fluorescence. However, at higher temperatures, the fluorophore and quencher lead to be apart from each other, thus enabling the fluorophore to emit a fluorescent signal. Fluorescence produced and became remarkable between 50 and 66°C and the fluorescence line converge the same fluorescence level above 66°C.

Hybridization mixture studies by CE

Based on the fluorescence data, three type targets were analyzed using CE. Figure 3 showed the electropherograms of the MBs (Fig. 3a, b) and the respective MB-target interactions (see Fig. 3c–h) obtained by LIF detection. As expected, MB1 and MB2 controls have been denatured evidenced by the single peak in Fig. 3a and b, respectively. The sample of group 1(2044G/G) hybridized almost completely with MB1 while no hybridization was taken place with MB2. This can be seen in Fig. 3c and d. Conversely, the sample of group 2(2044A/A) hybridized completely with MB2 and little with MB1, as showed in Fig. 3e and f, respectively. While there were two peaks obtained from the hybridization reactions (Fig. 3g, h) of the group 3 sample (2044A/G). The former peak is the supernumerary MB, the latter is the hybridization of probe-target duplexes.

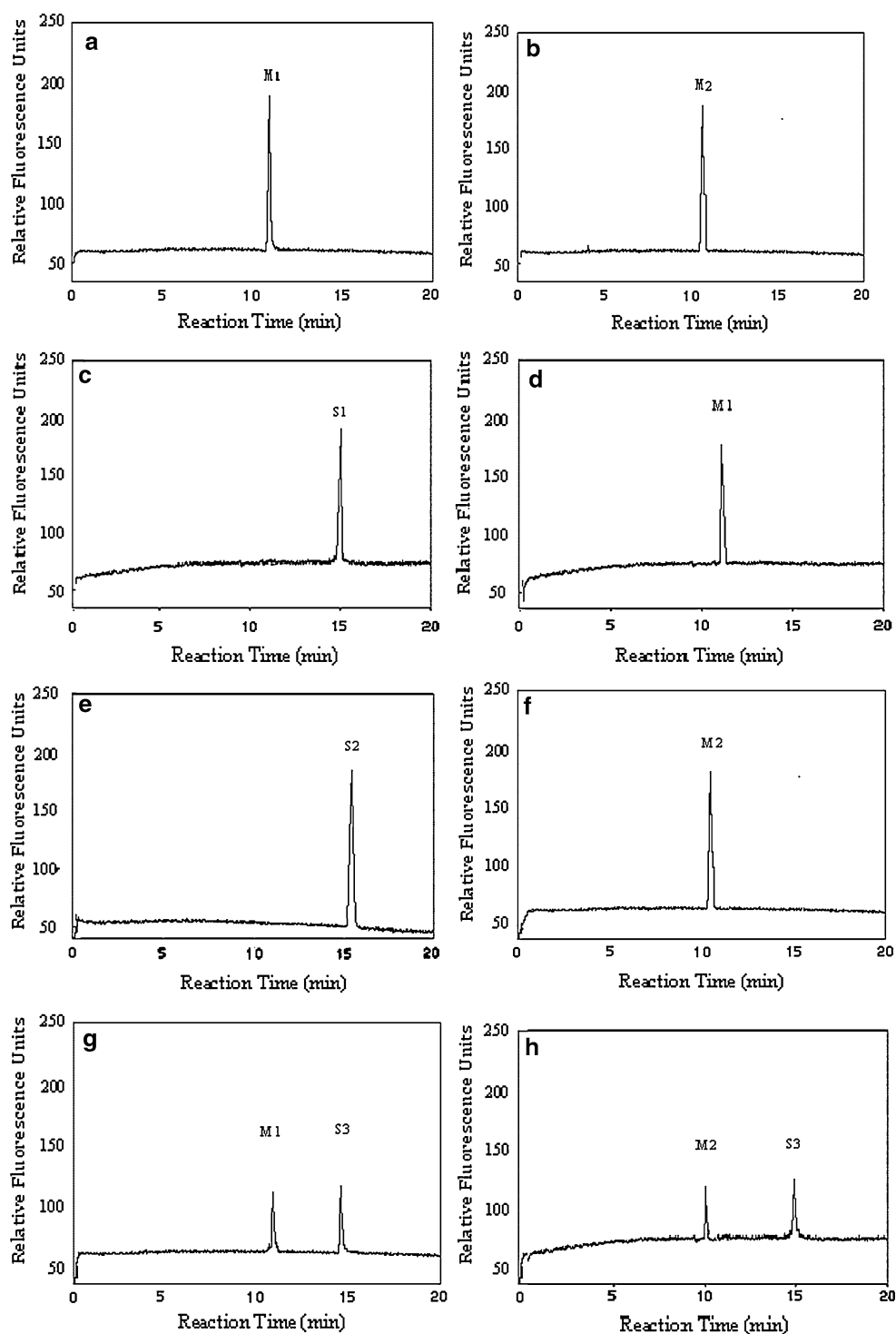
The sequencing results of the samples have been showed as Fig. 4.

Discussions

Capillary electrophoresis is a sensitive and versatile technique which has grown steadily in popularity. Its applicability is enhanced by short separation time, facile and rapid methods of development and a requirement for very small amount of analyte [16–18]. Molecular beacons are oligonucleotide probes that can report the presence of specific nucleic acids.

In this study, we used CE and MB to detect the SNP in exon 4 of IL-13 A2044G. The PCR products hybridized with each of MBs in liquid phase environment independent of temperature change. The MB-target duplexes denatured at 95°C for 5 min, and then hybridized at 30°C for 20 min. It is noteworthy that above 66°C, the fluorescence lines converge more or less to the same fluorescence level, so we denatured MB at 95°C. The MBs became one single strand; fluorophore and quencher were separated due to a conformational change. Each MB and targets were mixed in a total volume of 100 μ l per tube in a soluble environment that is beneficial to make the molecular hybridized completely. Each target should hybridize with the complete

Fig. 3 Electropherograms of the MBs hybridized with three group samples. Condition: fused-silica capillary, 30 cm to the detector, 37 cm (total length) \times 50 μ m ID; running buffer: 50 mM phosphate, pH 7.0; running temperature: 20°C; running voltage: +20 kV; pressure injection at 20 psi for 20 s; detection wavelength 488 nm. MB controls separated as Fig. 3a (MB1) and b (MB2); MB-sample type1(2044G/G) duplex showed as c and d (c MB2-sample type1 duplex; d unhybridization in MB1 and sample type1 mixture); MB-sample type2(2044A/A) duplex showed as e and f (e MB1-sample type2 duplex; f unhybridization in MB2 and sample type2 mixture); MB-sample type3(2044A/G) duplex showed as g and h (g MB1-sample type3 duplex; h MB2-sample type3 duplex); *S* sample; *M* molecular beacon



complementary sequence of MB. Fluorescence produced as a result of the true target hybridization was the highest. It was hypothesized that ΔG is the free energy of MB1 hybridized complementary target, while $\Delta G'$ is the MB2'. Since only single-nucleotide variations to be detected, distance between the ΔG and $\Delta G'$ should be large as far as possible [19, 20]. The targets that mismatch the given MB

sequence were ineffective in producing a stable structure. We suggest that the loop of the MB should be less than 15 bp in order to hybridize accurately and specificity. At the same time, the hybridization target molecules should not only bind to a significant portion of the loop, but complementary to the stem to produce more stable structure also.

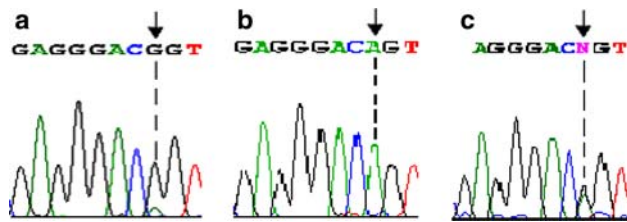


Fig. 4 Sequence results of the PCR products. **a** group 1 sample (2044G/G), **b** group 2 sample (2044A/A), **c** group 3 sample (2044A/G). The arrows indicate the location of the A2044G mutation. (partial sequence of exon 4 in IL-13 in asthma patients and controls)

The mixture of MB and targets were separated by CE. Figure 3 showed the electropherograms for the MBs and the MB-target interactions. In an electropherogram, the area under the peak for a particular DNA fragment can be correlated to the quantity of that fragment, at the same time the peak length of a DNA fragment is related to its residence time in the detector [21–23]. Slower migrating (large) fragments will remain in the detector window longer than a faster migrating (small) fragment [24, 25]. The migration time mostly depend on the variation length of fragments. So we can presume that the peaks around 10 min represent MBs, while the peak around 15 min represent MB-target helices. These probe-target duplexes were then separated by capillary electrophoresis, where the genotype of the sample could be determined by the shift in size of the target—products with bound MB probes which had a longer migration time than products without MB probes bound. From assessing the binding state of two MB (one for each allelic state) the genotype of the individual could be determined.

The present study describes the hybridization interaction of an MB with complementary target sequence in a soluble environment. Then the hybridization mixtures were electrophoresis to read fluorescence in a stable temperature at 20°C. As temperature regulation is not required during electrophoresis, the method can be performed easily. Here, we designed two MBs because the mutation has been described or confirmed. If we do not know which kind of the mutation nucleotide turned except the position, we should design three MBs at least to detect which kind nucleotide it turned.

The largest advantage of this method should be the ability to use MB probes without fluorescent moieties to genotype samples—this would greatly reduce the cost for typing large numbers of samples. Additionally, by carefully choosing the size of the PCR amplicon, it would be possible to multiplex this reaction (provided the MB probes were also carefully designed, to avoid complementarity). The results of the fluorescence and CE studies indicate that this method can effectively discriminate single-nucleotide variations and can be used for genotyping. And it is no problem to distinguish

between homozygous and heterozygous samples. The method of CE and MB can be performed independence on temperature changes. This study has demonstrated the potential of CE in the field of molecular biology for assessing hybridization reaction and structural changes occurring in DNA molecules as well as performing simultaneous separation assays.

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