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## Capillary electrophoresis and fluorescence studies on molecular beacon-based variable length oligonucleotide target discrimination

Molecular beacons (MBs) are oligonucleotide probes having a compact hairpin structure, with a fluorophore attached to one end and a quencher molecule attached to the other end. In its native state, the fluorophore is quenched by virtue of its proximity to the quencher molecule. Upon hybridization with its complementary oligonucleotide target, fluorescence is elicited due to a conformational change that results in separation of the fluorophore and quencher molecule. The present study describes the hybridization interaction of an MB to various complementary target sequences. The effects of temperature and length of complementary target sequences on hybridization were investigated using capillary electrophoresis and solution-based fluorescence techniques. Hybridization efficiency was dependent on the ability of the target sequences to destabilize the stem region by binding directly to the stem region. Optimal hybridization occurred between 40 and 50°C for all targets tested, with the true target forming a more stable hybrid complex.

**Keywords:** Capillary electrophoresis / Fluorescence / Hybridization / Molecular beacon

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### 1 Introduction

Molecular beacons (MBs), first described by Tyagi and Kramer in 1996 [1], are important probes for quantitative and qualitative genomic studies. MBs are single-stranded DNA (ssDNA) molecules, composed of a hairpin-shaped oligonucleotide (*i.e.*, stem-and-loop structure) that is tagged with a fluorophore and a quencher molecule at either end (see Fig. 1) held close to each other by a stem portion. MBs act as molecular switches that turn fluorescence “off” when the fluorophore and quencher are in proximity, and turn fluorescence “on” when the fluorophore and quencher are separated due to a conformational change [1]. This on/off fluorescing properties have made MBs useful in many applications including biosensors [2, 3], protein studies [4, 5], monitoring of RNA/DNA amplification during PCR [1, 6], gene typing and mutation detection [7, 8], enzymatic cleavage assays [9], real-time

detection of DNA/RNA hybridization in living cells [10], the detection of pathogenic retroviruses [11], *etc.*; for recent reviews see [12, 13].

The most specific molecular recognition of a given MB is based on its hybridization to a complementary target oligonucleotide sequence, which induces a conformational change, causing a separation of the fluorophore from the quencher allowing it to fluoresce [1, 14]. The resulting fluorescence will be of varying intensities depending on the spatial arrangement of the quencher with respect to the fluorophore. Molecular beacons are highly sensitive probes and have been reported to detect subnanomolar concentrations of target in real time [3], which makes them useful in the detection of highly infectious bacterial organisms, zoonotic agents or biological warfare agents. As a prelude to using MBs for the actual detection of organisms *in situ*, and keeping in mind the eventual application of this research, we studied the conformational behavior of a MB designed to target a unique sequence in the 16S rRNA of *Francisella tularensis*, a highly infectious bacterial organism and a bioweapons agent (15, 16; see references therein). The 16S rRNA forms the structural and functional unit of ribosomes, which are present on the order of approximately ten thousand per cell. In an average, *Escherichia coli* cell ribosomes could vary in number from 6700 to 71 000 depending on the growth conditions [17], making rRNA a favorable target for bacterial detection. Specificity and temperature-stability of

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**Abbreviation:** MB, molecular beacon

**Table 1.** Alignment of various target sequences across the length of the beacon (HP1)

Description	Target sequence alignment with probe	Bases ( <i>n</i> )
HP1 probe <sup>a)</sup>	3'-BHQ- <b>CGCGG</b> AACCCCCTCCTGCAAT <b>CGCGG</b> -Cy3-5'	26
CS11 (true target)	5' GCCTTGGGGGAGGACGTTAC 3'	20
CS12	CTTGGGGGAGGACGTTAC	18
CS13	TTGGGGGAGGACGTTA	16
CS14	CGCCTTGGGGGAGGAC	16
CS15	CCTTGGGGGAGGACGT	16
CS16	GGGGGAGGACG	11
CS17	TTACGCG	7
CS21 (noncomplementary)	AGCTGTTGGATTGGGTAAAGG	23

a) Bold underlined bases denote the stem of the molecular beacon. Italicized bases at the 3'-end represents true-target binding sites within the stem

the beacon were assessed using solution fluorescence and capillary electrophoresis (CE). While increased solution fluorescence implies an opening up of the hairpin structure upon hybridization, capillary electrophoresis provides additional evidence of such a structural event occurring.

## 2 Materials and methods

### 2.1 Reagents and materials

#### 2.1.1 Targets

All genebank entries for the 16s rRNA sequence of *Francisella* spp. were pooled and a consensus sequence was made using the software Vector NTI (Informax, Bethesda, MD, USA). Using the BLAST program [18], four regions were identified in the consensus sequence that contained oligonucleotide sequences unique to *Francisella* organisms. A 20 base segment (CS11, see Table 1) derived from one of these sequences (gccttgggggaggacgttac-Gene bank accession No. Z21931, bases 454–473), was used as the true target in this study. Affinity of other sequences (CS12 to CS17, see Table 1) that were only partially complementary to the MB probe was also investigated. Target sequences were obtained from the core facility at Oklahoma State University, (Stillwater, OK, USA). For fluorescence assays, 800 nM solutions of the target were made in 10 mM Tris/3.5 mM MgCl<sub>2</sub> buffer, pH 8, and for CE studies, 160 μM solutions were used.

#### 2.1.2 Molecular beacon

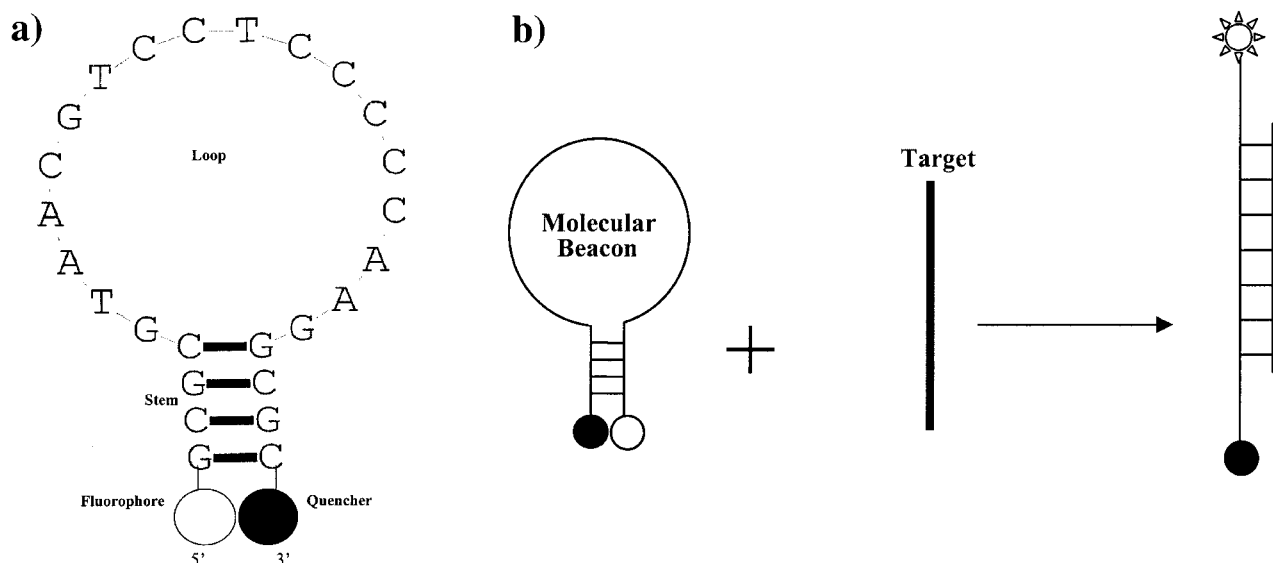
An MB (HP1, Table 1) was designed to probe the true target sequence (CS11) using the mfold program (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>

[19] and the DNA fold option in the RNA structure software [19, 20] (<http://128.151.176.70/RNAstructure.html>). The MB was designed to have a 20 base loop region that was complementary to the true target, a 4 bp stem of which 2 bases were complementary to the true target, a Cy3 fluorescent group attached to the 5'-end, and a black hole quencher (BHQ) molecule attached to the 3'-end (see Fig. 1). The MB was obtained from IDT (Coralville, IA, USA). For fluorescence studies, 400 nM solutions in 10 mM Tris/3.5 mM MgCl<sub>2</sub> were used, and roughly 80 μM solutions for CE. Modified beacons that had a fluorescent group (Cy3) but no quencher (IDT) were used to determine correction factors for temperature-dependent effects on fluorescence that are independent of hybridization.

### 2.2 Instruments

#### 2.2.1 Fluorescence studies

Hybridization was performed in triplicates in a 96-well microtiter plate and fluorescence was read using an ABI 7700 instrument (Applied Biosystems, Foster City, CA, USA). Volumes of 25 μL each of the molecular beacon and individual targets were mixed in a 96-well microtiter plate to give a final concentration of 200 nM and 400 nM, respectively, in a total volume of 50 μL per well. Three wells were also dedicated to the modified beacon (beacon without a quencher), which was dispensed in the same concentration as the beacon (200 nM) at a final volume of 50 μL per well. Measurements were taken at 5°C decrements ranging from 90°C to 5°C at an exposure time of 25 ms. To account for the temperature-effect, fluorescence at different temperatures from all wells involving the MB was divided by the average fluorescence from the modified beacon at the corresponding temperature.



**Figure 1.** (a) Structure of the MB (HP1) used in this study; (b) mechanism of MB-DNA probe operation. Binding of MB to a target oligonucleotide sequence results in a conformational shift that moves the quencher (solid circle) away from the fluorophore (open circle) thereby allowing it to fluoresce.

## 2.2.2 Capillary electrophoresis

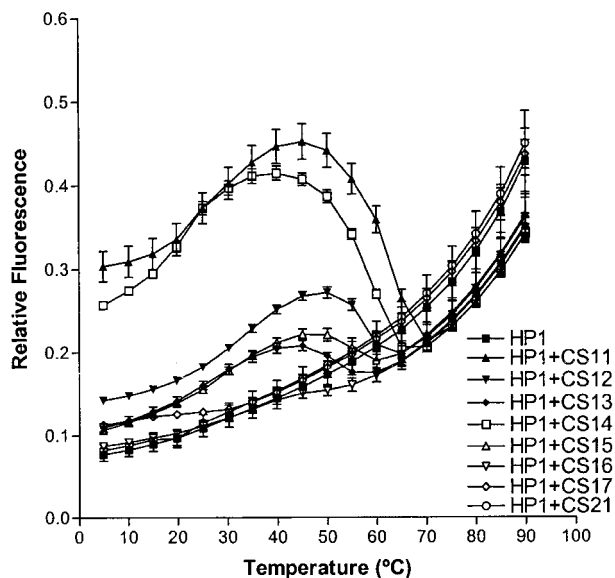
Two different instruments were used in the CE studies. One of the instruments was assembled in-house from commercially available components. It consisted of two 30 kV dc power supplies of positive and negative polarity, Models MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, USA) and a UV-Vis variable wavelength detector Model 200 from Linear Instrument (Reno, NV, USA) equipped with a cell for on-column detection. The electropherograms were recorded with a Shimadzu data processor Model CR6A (Kyoto, Japan). Fused-silica capillaries of 50  $\mu\text{m}$  ID and 360  $\mu\text{m}$  OD were from Polymicro Technologies (Phoenix, AZ, USA). The second CE instrument was a Model 5510 Beckman P/ACE instrument (Fullerton, CA, USA). The instrument was equipped with a diode array detector, a personal computer and P/ACE station software for data handling purposes. CE studies were performed in capillaries maintained either at room temperature or at fixed temperatures ranging from 15 to 50°C in order to investigate conformational changes occurring in MBs in the presence and absence of target sequences. In all cases, the final concentration of HP1 was  $\sim 40 \mu\text{M}$  and that of targets were  $\sim 80 \mu\text{M}$  in 10 mM Tris/3.5 mM  $\text{MgCl}_2$  solution. Specifications for the room-temperature run were as follows: capillary, fused-silica tube, 50 cm (inlet-to-detector distance), 65 cm (total length)  $\times$  50  $\mu\text{m}$  ID; running buffer, 50 mM phosphate, pH 7.0; running voltage, +25 kV; injection, 30 s by hydrodynamic injection from a height of 20 cm above the outlet reservoir. Four targets were used

in this study, namely CS11, CS14, CS17 and CS21. CE was performed with each of the targets alone (target control), the MB alone (beacon control) and the reaction mix containing target and MB. Specifications for the multiple-temperature runs were as follows: capillary, fused-silica tube, 50 cm (inlet-to-detector distance), 57 cm (total length)  $\times$  50  $\mu\text{m}$  ID; running buffer, 50 mM phosphate, pH 7.0; running voltage, +20 kV; pressure injection at 20 psi for 10 s. The temperatures considered were 15, 20, 30, 40 and 50°C. Only two targets, namely CS11 and CS14, were studied. Control runs were performed as for the CE experiments at room temperature.

## 3 Results and discussion

### 3.1 Fluorescence studies

Fluorescence readings from MB in the presence or absence of various targets at different temperatures were divided by the fluorescence readings from the modified beacon without a quencher at the corresponding temperatures to correct for temperature effect on fluorescence independent of the hybridization event. Figure 2 shows the fluorescence pattern of the MB observed at temperatures ranging from 5°C to 90°C following hybridization with the different targets. Fluorescence produced as a result of the true target (*i.e.*, CS11) hybridization was the highest. It is noteworthy that the sequence CS12 that binds to the loop sequence alone was ineffective in producing a high fluorescence, but the shorter sequence



**Figure 2.** Fluorescence signals of HP1 and its hybridization product with various oligonucleotide targets as a function of temperature.

CS14, which binds to three bases in the stem region, produces a fluorescence that is comparable to the fluorescence resulting from binding of the true target CS11. CS15 which was of the same length as CS14 but complementary to only one base in the stem, and CS17 which was only 7 bases long but complementary to all bases but one in the stem, did not produce significantly high fluorescence. It is believed that the chance of ssDNA to fold on itself forming a secondary structure (*i.e.*, stem-and-loop structure), which is an intramolecular event, is much higher than binding to other smaller DNA molecules. Moreover, the rigidity of the probe-target helix thus formed by the hybridization of a small DNA molecule to the stem region of the probe will not be sufficient to introduce a conformational shift of the quencher away from the fluorophore resulting only in a minimal emission of fluorescence [14]. The cases with the other target sequences, CS13 and CS16, were the same, each giving a small signal comparable to the background signal. CS21 was a noncomplementary sequence and was not expected to produce any fluorescence. Thus, the specificity of an MB towards target oligonucleotides comes from its loop-and-stem structure [12]. These results show that for a significant fluorescence to be produced, the hybridizing target molecules must not only bind to a significant portion of the loop, but must also destabilize the stem.

It is interesting to note that above 70°C, all the fluorescence lines converge more or less to the same fluorescence level including the molecular beacon HP1 by itself. This suggests that the steady increase in fluorescence is

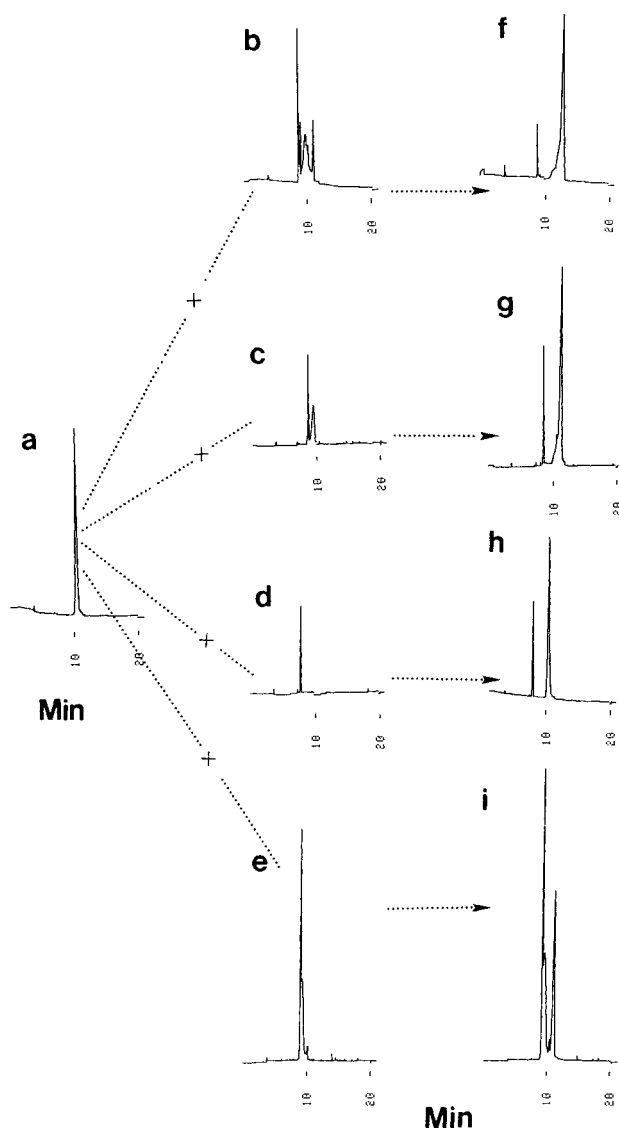
due to the melting of the stem with increasing temperature and the resultant displacement of the quencher away from the fluorophore. On the other hand, partial- and complete-complementary targets hybridize to the MB resulting in the beacon “opening up” to various degrees at lower temperatures. As the temperature increases the fluorescence produced increases and reaches a maximum around 45°C for CS11 (the true target). For CS14, the fluorescence dips earlier, around 40°C, because the hybrid is less stable. All other sequences fail to produce as high a rise in fluorescence at any temperature. The optimum temperature for hybridization seems to be between 40°C and 50°C for this particular molecular beacon.

### 3.2 Capillary electrophoresis

Based on the fluorescence data, different target sequences were selected to be analyzed using CE and further look into the hybridization behavior. The migration rate of a particle through the capillary column is dependent on the charge-to-mass ratio and on the shape of the molecule. Since the charge-to-mass ratio is almost the same in all DNA, the main factor here will be the alteration in the shape of the molecule. Therefore, in principle, CE should be a simple and useful technique that complements fluorescence studies for investigating changes upon probe binding to targets requiring only minute amounts of materials. Moreover, the CE approach does not require labeling the MB with fluorophore and quencher, instead it relies directly on physical properties of the MB and hybridization products; thus, potentially greatly decreasing the expense for MB synthesis.

#### 3.2.1 Hybridization studies by CE

Based on the fluorescence data, four targets were selected for CE. Figure 3 shows the electropherograms for the MB HP1 (Fig. 3a), the targets CS11, CS14, CS17 and CS21 (see Figs. 3b–e, respectively) and the respective MB-target interactions (see Figs. 3f–i) obtained by UV detection (see Section 2.2.2). Although the targets CS11, CS14, CS17 and CS21 are composed of 20, 16, 7 and 23 bases, respectively, they migrated within a narrow time window extending from 6.9 min to 8.8 min (see Figs. 3b–e). This is because the target oligonucleotides have approximately the same charge-to-mass ratios in the electrophoretic medium used in this study. As expected, and in accordance with the fluorescence data, HP1 hybridizes almost completely with CS11 and CS14 as evidenced by the single peak obtained for the hybridization reactions in Figs. 3f and g, respectively. For the CS17 and CS21 targets, little or no hybridization has



**Figure 3.** Electropherograms of the MB under investigation, the oligonucleotide targets, and their hybridization products. Conditions: fused-silica capillary, 50 cm (to the detector), 65 cm (total length)  $\times$  50  $\mu$ m ID; running buffer, 50 mM phosphate, pH 7.0; running voltage, + 25 kV; 30 s hydrodynamic injection from a height of 20 cm above the outlet reservoir; detection wavelength, 260 nm. (a) HP1; (b) CS11; (c) CS14; (d) CS17; (e) CS21; (f) HP1+CS11; (g) HP1+CS14; (h) HP1+CS17; (i) HP1+CS21.

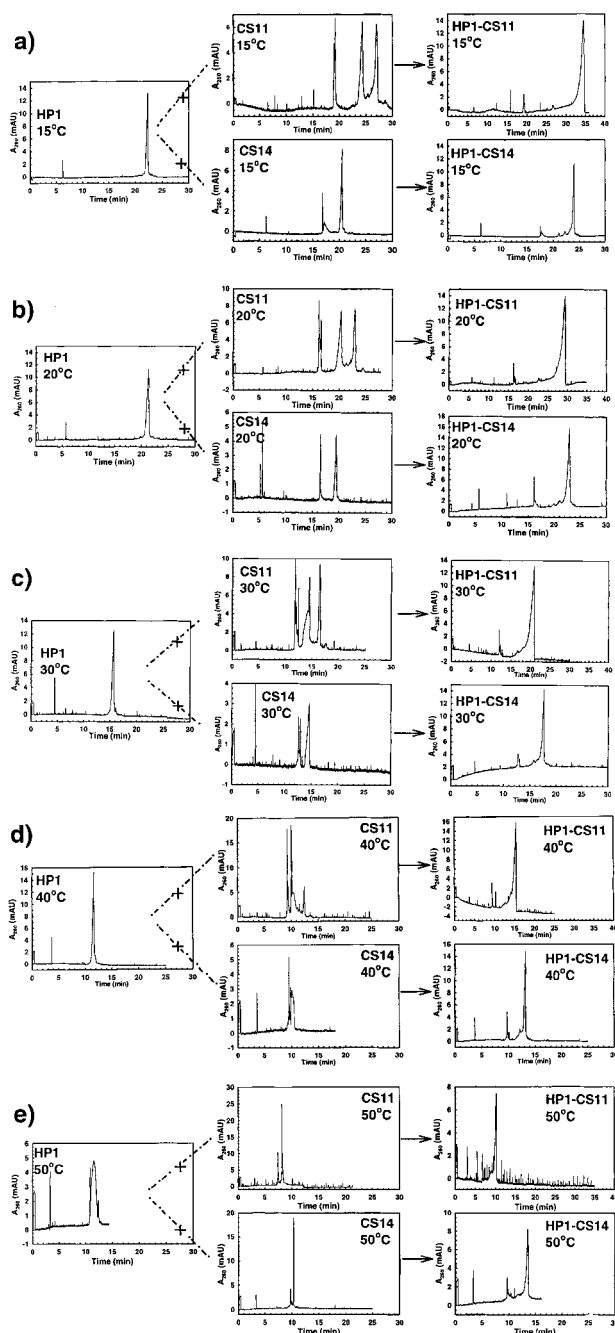
taken place with HP1. This can be seen in Figs. 3h and i, where no new peaks have appeared on the electropherograms. Under these conditions, the peak heights of HP1 and the targets in Figs. 3a, d and e should in principle stay the same as their corresponding peaks in Figs. 3h and i. The observed slight deviation in the peak heights can be attributed to the imperfection in the sample introduction on the in-house built manual instrument used.

### 3.2.2 Effects of temperature

To gain further insight into the extent of hybridization, the effects of temperature on the hybridization of CS11 or CS14 with HP1 were studied by CE in the temperature range of 15 to 50°C. The theoretical melting temperature of the two probe-target hybrids (*i.e.*, HP1-CS11 and HP1-CS14) as determined by the “Hybridization Server” (<http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form6.cgi>) developed by Michael Zuker was found to be around 72°C in both cases. The CE instrument used in this study had a temperature limit of 50°C. Hence, we expected the probe and target to remain in the hybridized state at all temperatures tested. The migration patterns of the MB (*i.e.*, HP1), targets and the hybridizations as well as the conformational changes occurring in each case with changes in temperature are shown in Fig. 4.

In order to correlate probable conformations and CE elution peaks, we first generated the possible secondary structure predictions for each oligonucleotide under investigation, namely, the target sequences, CS11 and CS14, as well as the probe HP1, using the DNA mfold server by Michael Zuker (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>) at 15 and 50°C and a Na<sup>+</sup> concentration of 0.05 M using the default settings on the server. The results of the structural conformation predictions are shown in Fig. 5. While HP1 is most likely to exist in a single conformation at 15°C ( $\Delta G = -3.8$  kcal/mol), a second but less stable conformation seems to be possible at 50°C ( $\Delta G = 0.7$  kcal/mol). In fact, a single and relatively sharp CE peak is obtained for HP1 at all temperatures studied except at 50°C (see Fig. 4) where the peak becomes broader indicating the possibility of two conformations: the stem-and-loop structure ( $\Delta G = -0.2$  kcal/mol) and a more linear conformation due to the melting of the secondary structure at 50°C. The conservation of the stem-and-loop structure even at 50°C may be viewed as an important feature of the molecular beacon HP1 in the sense that it conserves its molecular recognition conformational energetics towards complementary targets over a wide range of temperature.

The target CS11 seems to assume four stable conformations at 15°C and five less stable conformations at 50°C. This is well observed upon CE analysis at various temperatures as shown in Fig. 4. The few peaks observed at low temperatures converge into one major peak at 50°C indicating that CS11 may become linear at 50°C due to the melting of the secondary structure at this relatively high temperature. This fact may explain why in fluorescence studies, the HP1-CS11 hybridization is most effective at about 40°C, temperature at which maximum fluorescence is observed. Also, the target CS14 can assume four conformations at 15°C and five less stable conforma-



**Figure 4.** Electropherograms of the MB under investigation, the oligonucleotide targets and their hybridization products. Conditions: fused-silica capillary, 50 cm to the detector, 57 cm (total length)  $\times$  50  $\mu$ m ID; running buffer, 50 mM phosphate, pH 7.0; running voltage, +20 kV; pressure injection at 20 psi for 10 s; detection wavelength, 260 nm. (a) 15°C; (b) 20°C; (c) 30°C; (d) 40°C; (e) 50°C.

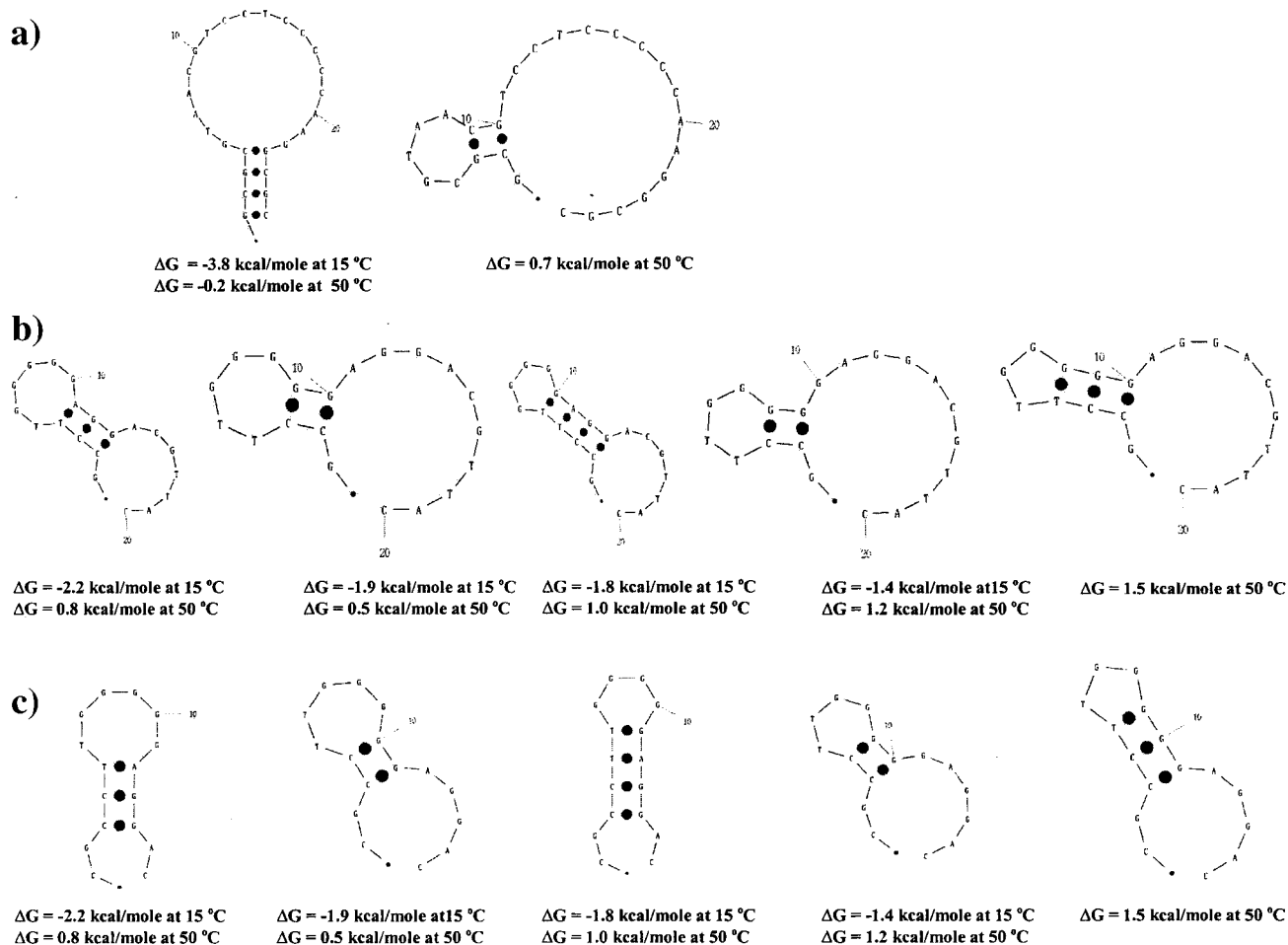
tions at 50°C. Being a shorter molecule, the various conformations of CS14 appear similar to one another (see Fig. 5c). This may explain why only two distinct

peaks are seen for CS14 at low temperature, which converge into one single major peak at 50°C. Again, as the temperature approaches 50°C, CS14 exists most probably as a linear molecule thus explaining the higher fluorescence of the HP1-CS14 at 40°C. Using the above-mentioned “Hybridization Server” by Michael Zuker, the formation of stable target-target dimers in the case of both CS11 and CS14 was ruled out at the temperatures studied. As expected, in all cases and in the temperature range studied, the hybridization reactions yield one major peak as can be seen in Fig. 4. The linear configuration of the hybridization product makes the counter-directional mobility of HP1-CS11 or HP1-CS14 with respect to the electroosmotic flow (EOF) higher than the individual oligonucleotides HP1, CS11 or CS14, and consequently the apparent mobility of the hybridization product (*i.e.*, HP1-CS11 and HP1-CS14) is slower. It should be noted that in all these hybridization reactions the molar ratio of target to HP1 was 2:1. However, very little residual of the target molecules (*i.e.*, CS11 and CS14) remained after hybridization, which does not amount to half the initial amount offered. This may reflect two discrepancies: the original concentration as stated by the supplier of the oligonucleotides may not have been very accurate and/or sample evaporation during the experiment may have introduced some alteration to the actual concentration especially that only a few microliters ( $\sim$  25  $\mu$ L) were available for the CE experiments.

In all cases, increasing the temperature of the electrolyte decreases the viscosity of the medium, thus increasing both the electrophoretic mobility of the analyte and the electroosmotic mobility of the medium and the net result is a decrease in the migration time of the individual oligonucleotides and the hybridization products. From Fig. 4 it can be seen that the probe-target hybrid is stable at all temperatures and is represented by a single peak, which denotes the presence of only a single entity in accordance with the fluorescence data.

#### 4 Concluding remarks

The results of the fluorescence and CE studies indicate that the MB can effectively discriminate between true and false targets. CE elution profiles correlated well with the different structural forms that the oligonucleotides may assume at various temperatures and they also validated the results obtained by fluorescence studies. This study has demonstrated the potential of CE in the field of molecular biology for assessing hybridization reactions and structural changes occurring in DNA molecules as well as performing simultaneous separation assays. CE



**Figure 5.** Secondary structure prediction of the target sequences CS11 and CS14 as well as that of the probe HP1 at 15 and 50°C and their associated free energy changes.

also has the advantage that unlabelled MBs can be used for hybridization studies thereby reducing the expenses involved and the amount of material used.

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