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A molecular beacon DNA microarray system for rapid detection of *E. coli* O157:H7 that eliminates the risk of a false negative signal

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Abstract

A DNA hybridization based optical detection platform for the detection of foodborne pathogens has been developed with virtually zero probability of the false negative signal. This portable, low-cost and real-time assaying detection platform utilizes the color changing molecular beacon as a probe for the optical detection of the target sequence. The computer-controlled detection platform exploits the target hybridization induced change of fluorescence color due to the Förster (fluorescence) resonance energy transfer (FRET) between a pair of spectrally shifted fluorophores conjugated to the opposite ends of a beacon (oligonucleotide probe). Unlike the traditional fluorophore-quencher beacon design, the presence of two fluorescence molecules allows to actively visualize both hybridized and unhybridized states of the beacon. This eliminates false negative signal detection characteristic for the fluorophore-quencher beacon where bleaching of the fluorophore or washout of a beacon is indistinguishable from the absence of the target DNA sequence. In perspective, the two-color design allows also to quantify the concentration of the target DNA in a sample down to $\leq$1 ng/μl. The new design is suitable for simultaneous reliable detection of hundreds of DNA target sequences in one test run using a series of beacons immobilized on a single substrate in a spatial format.

Keywords: *Escherichia coli* O157:H7; DNA hybridization; Fluorescence detection platform; FRET (Förster or fluorescence resonance energy transfer); Microarray; Molecular beacon

1. Introduction

Food contamination is an emerging concern due to an increased emphasis of a healthy diet and the recognition of new foodborne pathogens such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* (Hedberg et al., 1994; Tauxe, 1992). While the recommended practices from the publicly published sources (see, for example, FDA, 1998) provide a framework for minimizing risk, availability of rapid and accurate methods for detection of pathogens is needed in order to provide real time assessment of risks. The current culture-based methods are time-consuming and may require long incubation times to obtain results. Antibody methods rely on the detection of surface antigens but have numerous drawbacks including cross-reactivity and possible antigen expression affection by environmental conditions (Harry et al., 1995).

DNA hybridization techniques are also used in the detection and identification of foodborne pathogens (Hill et al., 1998) mainly with the extensive use of polymerase chain reaction (PCR). The assays involve both amplification of single DNA targets and multiple targets (multiplex PCR) for specific detection of pathogens. A single-target amplification of a portion of *flaA* of *C. jejuni* and *C. coli* was utilized to detect their presence in human stool samples (Oyofo et al., 1992). Linton et al. (1997) also used PCR to identify and differentiate between *C. jejuni* and *C. coli* in diarrheic samples. Fratamico et al. (2000) used multiplex PCR to detect *E. coli* O157:H7 in foods and in bovine feces using targets specific for the H serogroup and spe-

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cific genes associated with O157 specific virulence factors. A method for the detection of viable *L. monocytogenes* was developed using reverse transcriptase PCR (RT-PCR) for the detection of the mRNA transcripts from the genes associated with virulence (Klein and Juneja, 1997). The assay was able to detect 10–15 CFU/g in pure culture and 3 CFU/g in a cooked meat sample after two hours of enrichment. Similar approaches have also allowed the simultaneous detection of *Salmonella* strains and *E. coli* O157:H7 in a single enrichment (Sharma and Carlson, 2000).

Nucleic acid hybridization for the detection of target DNA or mRNA is not limited to foodborne pathogen detection, it has also been used extensively in a variety of applications such as the development of the optical, electrochemical, gravimetric, surface plasmon resonance-based and electric biosensors (Piunno et al., 1994; Millan et al., 1994; Minunni et al., 2003; Sawata et al., 1999; Kelley et al., 1999; Drummond et al., 2003). Unlike protein-based detection, nucleic acid recognition layers can be readily synthesized and regenerated for multiple uses. The technique utilizes a nucleic acid probe, a labeled or label-free single-stranded DNA or RNA, which can hybridize to its complimentary DNA or RNA sequence. Probe and target base sequences have to share a significant amount of similarity but depending on hybridization conditions, do not necessarily have to be exactly identical (Kane et al., 2000; Ramachandran et al., 2004). Once hybridization occurs, the probe-target duplex is detected using an analytical signal by the transducer as described above. Detection of specific bacteria in the environment using various DNA hybridization techniques can also provide quantitative analysis of the microbial ecosystem (Applegate et al., 1995).

The aim of this work is to develop a portable, low-cost and real-time assaying detection platform for the detection of specific foodborne pathogens, but most importantly to minimize and ultimately eliminate the risk of false negative signal to effectively tackle the mounting concern of recent microbial contamination of food. The prototype detection platform developed in this work is a fluorescence-based assay, utilizing FRET change to detect hybridization of a set of target DNA sequences associated with each pathogen. The detection platform exploits the molecular beacon method pioneered by Tyagi and Kramer (Tyagi et al., 1998, 2000; Tyagi and Kramer, 1996). The molecular beacon approach uses an internal probe that forms a secondary structure (hairpin loop), placing the donor and quencher in proximity to each other, allowing the quenching of donor fluorescence. When the internal probe hybridizes to its target sequence the fluorophores are spatially separated, resulting in unquenched fluorescence of donor. The molecular beacon approach has been utilized in a solid-state format by attaching the probe to a solid surface (Ramachandran et al., 2004; Fang et al., 1999). Fang et al. (1999) have used a biotinylated molecular beacon to demonstrate the hybridization dynamics of immobilized beacons with complimentary DNA molecules with a subnanomolar concentration detection limit. The approach utilized a glass surface coated with avidin followed by attachment of the biotinylated molecular beacon while we used amino-linker for immobilization. However, the use of fluorescing donor and quenching acceptor had the inherent limit of significantly high probability of false negative signal generation caused by the photo-bleaching or wash-off of donor fluorophore because the damaged donor could also produce the negative signal (no fluorescence). This possibility of a false negative signal may have dramatic consequences as a product with pathogen could be erroneously labeled as pathogen-free. To eliminate this kind of false negative signal, we employed the color changing molecular beacon whose acceptor is not a dark quencher but a second fluorophore which quenches the donor fluorescence (green) and emits color changed fluorescence (red). Once it is hybridized with the target sequence, the spatial separation of the two fluorophores makes the donor's green fluoresce dominant as it is illustrated in Fig. 1A. In this arrangement, the hybridization state of molecular beacons can be established by measuring the ratio of the fluorescence of the excitation donor to that of the acceptor. Because this technique always requires the

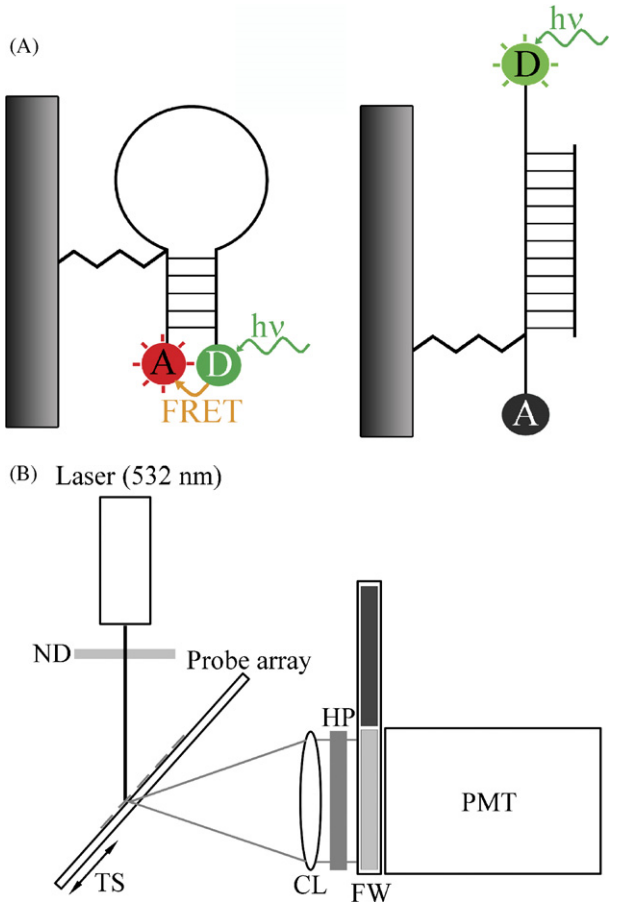

Fig. 1. (A) Operation of a color changing molecular beacon that is immobilized through amine-binding linkage on a glass surface. In the absence of target DNA sequence (left), the 'red' acceptor fluorophore A is strongly fluorescent due to the efficient FRET from the nearby donor molecule D that is excited by a green laser. Upon hybridization with the target sequence (right), the rigid loop structure keeps the donor and acceptor fluorophores apart making the donor fluorophore strongly fluorescent. (B) Simplified design of the prototype detection platform. ND: neutral density filter, TS: translation stage, CL: collimating lens, HP: high-pass orange glass filter, FW: interference filter wheel, PMT: photomultiplier. Two band-pass interference filters centered at 570 and 670 nm are automatically swapped during each measurement. TS, FW and PMT are controlled by a computer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

measurement of fluorescence of both fluorophores, damage to any of the two fluorophores will be immediately detected. Unlike the case of a "dark" quencher, the fluorescence of the acceptor visualizes the presence of the coiled single-stranded beacon and ensures virtually zero false negative signal occurrences.

In this paper, we report the development of a negative-signal-free DNA de/hybridization based optical detection platform with the immobilized two-color molecular beacon probes to detect food pathogens. The prototype of the proposed detection platform was built and its ability of rapid and accurate detection of food pathogens is demonstrated.

2. Materials and methods

2.1. Hairpin probe design and microarray production

Each "hairpin" probe was designed to harbor complementary nucleotide to each target gene to facilitate Watson–Crick base-pairing with the target gene sequence (Watson and Crick, 1953). Six additional bases were added at the 3′ end, which were complementary to the first six bases in the probe sequence, representing a palindromic sequence that formed a "stem" to closely position the two different fluorophores, which were chemically added to the extreme ends of the DNA sequence, and facilitate Förster (or fluorescence) resonance energy transfer (FRET). FRET signaling was achieved by adding a fluorescent group at the 3′ ends (Cyanine-3 or Cy-3, FRET donor) and a different fluorescent group at the 5′ end (Cyanine-5 or Cy-5, FRET acceptor). To facilitate binding of the fluorophore-labeled probe to the microarray surface, a modified thymidine nucleotide was incorporated at the base of the "stem" motif that contained a 6-carbon (C6) amino-linker, which was covalently bound to the chemically activated microarray surface during microarray construction. Each probe was synthesized (IDT Technologies, Coralville, IA) and HPLC purified (Table 1). Hairpin probes were diluted to 20 μM in 150 mM phosphate buffer and spotted on an amino-reactive microarray substrate (CodeLink, Amersham) using modified titanium spotting pins, resulting in 750 μm diameter spots. Microarrays were spotted using a Genomic Solutions Flexys Microarray Spotter (Ann Arbor, MI).

2.2. Sample preparation and microarray hybridization

Double-stranded DNA sequences of the target genes were derived through PCR amplification from *E. coli* O157:H7 genomic DNA. PCR primers specific for *eaeA*, *hlyC*, *rfbE*, and *stxI* resulted in double-stranded PCR products of 126, 150, 119, and 140 bp, respectively. No other sample preparation steps were taken. PCR products were diluted 1:10 and 1:50 into 1× or 2× SSC and heated to 94 °C for 3 min, then 5 μl was placed on the microarray surface (under a 1 cm^2 cover slip). The microarray hybridization involved incubation at 94 °C for 2 min, 50 °C for 2 min, and then 30 °C for 1 min (total of 5 min hybridization time). Once complete, microarrays were immediately scanned and no post-hybridization washing was required. Microarrays were imaged using a Genomic Solutions LSIV microarray scanner (Ann Arbor, MI). FRET signaling was determined by scanning the microarray with the green laser to excite the FRET donor (Cy-3) and detecting fluorescence emission from both fluorophores.

2.3. Fluorescence detection system of prototype detection platform

The molecular beacon probes were immobilized on the glass slide and excited by a 532 nm green laser (GMP-532, Lasermate). The slide was mounted on the computer-controlled translation stage (KT-LS28, Zaber Technologies). Neutral density filters were inserted permanently at the output of the laser to mute the intensity of the laser in order to minimize the photobleaching of the fluorophores while keeping the signal to noise ratio of the fluorescence detector at acceptable level. The fluorescence of the molecular beacon was collected by a lens and filtered by either 570 or 670 nm band-pass optical interference filters (10 nm full width at half maximum, F10-570.0-4-1.00 and F10-670.0-4-1.00, CVI). The filters were mounted into the computer controlled motorized filter wheel (FW102, Thorlabs). The resulting fluorescence signal was quantified by a photomultiplier (P25232-05, Electrontubes) in photon counting mode and recorded by a computer. A high-pass glass filter (OC12, Lomo) was inserted between the beacon and the filter wheel to minimize the yield of the scattered excitation laser. The simplified design of the prototype detection platform is shown in Fig. 1B. Translation stage, filter wheel and photomultiplier were all equipped with RS232 serial interfaces and connected to a single standard computer USB port using the external serial port hub (USA-49WLC, Keyspan). This approach made the system compatible for connection with the wide variety of modern computers. The prototype controlling software was written using Microsoft Visual basic.net. The program enabled individual spot measurements as well as automated multi-spot measurements of multiple probes in the spatial array format. The program was also capable to automatically analyze the presence of multiple pathogens according to the set of bi-fluorescent beacons on the test slide. Since both states of the beacon were visualized and detected, the possibility of a false negative signal occur-

Table 1
Color changing molecular beacon probe sequences

Target gene	Hairpin probe sequence (5′–3′)
eaeA	(Cy-3)CATTTCCCGTGGTTGCTTGCGTTTGAGACT(C6-amino)GGAAATG(Cy-5)
hlyC	(Cy-3)TTCAGAGCAATAAGGTTAGATGAGAGCAGT(C6-amino)CTCTGAA(Cy-5)
rfbE	(Cy-3)TTACTACAGGTGAAGGTGGAATGGTTGTCA(C6-amino)GTAGTAA(Cy-5)
stxI	(Cy-3)AATGGCGGCGGATTGTGCTAAAGGTAAA(C6-amino)CGCCATT(Cy-5)

rence due to damage to beacon, laser or PMT was essentially eliminated.

2.4. Fluorescence spectrum acquisition

To acquire wavelength-dependent fluorescence spectra, the interference filters in the prototype detection platform were replaced by a computer controlled grating monochromator (MS257, Oriel), while the samples were excited by the same green laser and dependent fluorescence signal detected by the same PMT. The spectra were not corrected for the spectral response of the detection system. The temperature of the molecular beacon solution was controlled by water circulating bath (2602, Thermo Forma) with the water-thermostated cell holder (B0080819, Perkin-Elmer).

3. Results

3.1. Thermal response of molecular beacons

To characterize the thermal response of the custom designed molecular beacons, the beacons were dissolved in 2× SSC buffer and fluorescence spectrum was measured in the temperature range from 15 to 85 °C. The melting temperature of a molecular beacon was computed to be around 56 °C based on the stem sequence of two pairs of G–C and four pairs of A–T. In the first sequence of spectral measurements, the temperature was lowered from 85 to 15 °C and series of fluorescence spectra were recorded at fixed temperatures with 15 °C intervals. The procedure was then repeated while raising the temperature back from 15 to 85 °C, producing essentially the same spectra at all temperatures, indicating that the melting-hybridization process is fully reversible. The normalized fluorescence spectra of molecular beacon whose hairpin probe sequence was *hlyC* gene from *E. coli* O157:H7 strain are shown in Fig. 2A. Spectra measured at 40 and 15 °C were essentially identical to that measured at 25 °C and are not shown. As expected, the temperature drop from 85 to 25 °C causes sharp decrease of the green pigment fluorescence band centered at ∼570 nm and simultaneous rise of the red pigment fluorescence band centered at ∼670 nm. This confirmed that the stem base-pairing of beacons was disrupted at high temperatures and restored at low temperatures. The thermal conformational change of molecular beacons showed high reproducibility during multiple thermal cycles (not shown).

The fluorescence ratio of the green (570 nm) to red (670 nm) signals (green/red fluorescence ratio) reflects FRET efficiency from donor to acceptor, and was measured to be 2.59 at 85 °C and 0.67 at 25 °C. Three additional molecular beacons with hairpin probe sequences containing the three other marker genes of *E. coli* O157:H7 were characterized in a similar manner. The 2.5–4-fold change of green/red fluorescence ratio at different temperatures (85 and 25 °C) was observed throughout all the molecular beacons independent of the probe sequences. Thus, the thermal response of all four molecular beacons was highly reproducible and their conformation reliably reflected in fluorescence spectra, confirming the potential applicability of our beacons for pathogen detection. No significant photobleaching of fluorophores was detected during several thermal cycles.

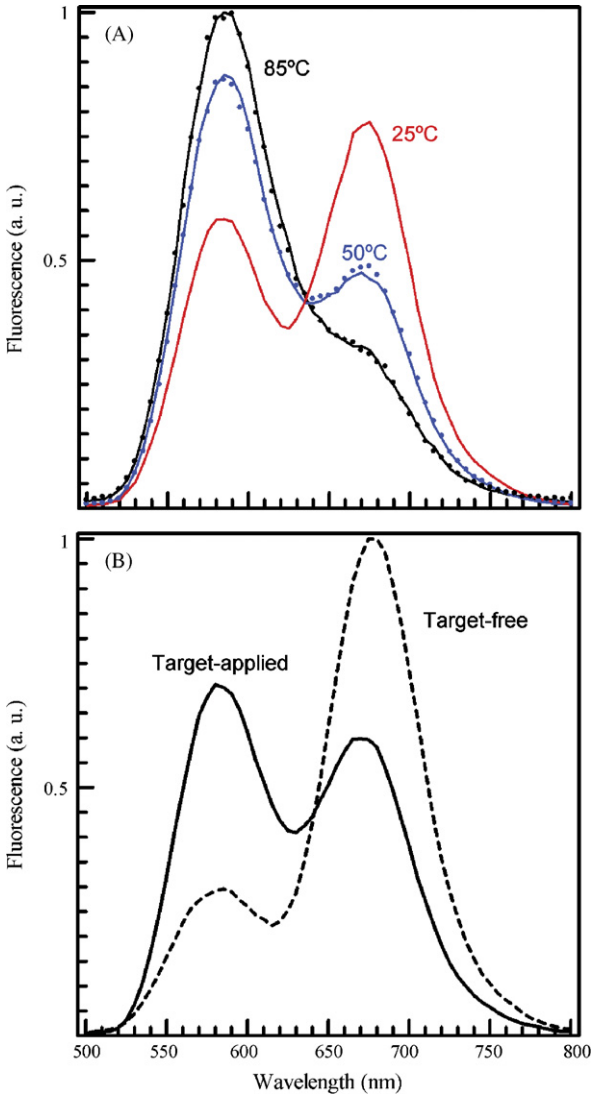

Fig. 2. Normalized fluorescence spectra of the color changing molecular beacons (A) in 2× SSC buffer at several temperatures and (B) immobilized on the glass slide. (A) The hairpin probe sequence of beacon is *hlyC* gene from *E. coli* O157:H7. Temperature was first lowered from 85 to 25 °C (lines), and then raised back to 85 °C (circles). (B) Control spots (no hybridization, dashed line) and 50 ng/μl concentrated target applied spots (solid line) on the glass surface. The hairpin probe sequence of the beacon is *eaeA* from *E. coli* O157:H7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Immobilization, scanner imaging and hybridization

Circular spots (∼1 mm in diameter) of amine modified molecular beacons were immobilized on the amine reactive glass slide using microarray techniques according to the conventional amine binding protocol (Kane et al., 2000; Schena, 2003). The beacon binding efficiency was quantified each time by scanning the green and red fluorescence images of the immobilized spots under green laser illumination through respective optical band-pass filters. The conformational state of beacons was

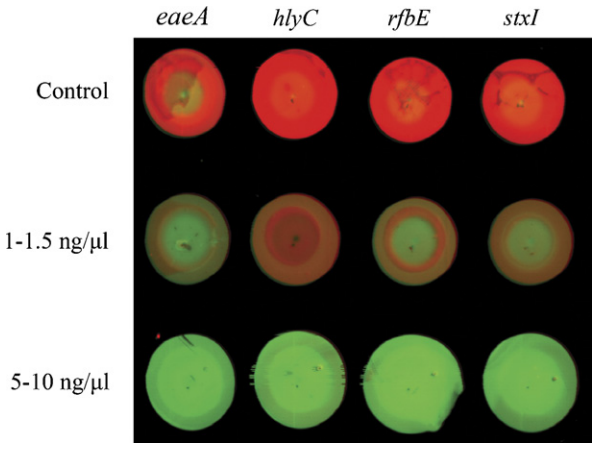

Fig. 3. Color mapped images of the molecular beacon probe spots visualized by microarray scanner. Amount of green and red colors reflects fluorescence intensities of the respective fluorophores. Each column represents the probe spots of the different molecular beacons whose hairpin probe sequences are respectively *eaeA*, *hlyC*, *rfbE* and *stxI*, four marker genes of *E. coli* O157:H7. The probes in the first row were free of complementary hybridization sequences and in the next two rows the specific complementary sequences were applied with concentrations of 1.0–1.5 and 5–10 ng/μl, respectively.

visualized by comparing the intensities of red and green fluorescence (Fig. 3). In the absence of the complimentary hybridization sequences, the green/red fluorescence ratio was around 0.2–0.3 for all types of beacons and did not depend on the absolute intensity variations from spot to spot.

To test the hybridization operation of immobilized molecular beacons, the PCR amplified complementary sequences specific to each probe sequence of the spotted beacons were applied. Two sets of spots were treated with complimentary sequences at concentrations of 1.0–1.5 and 5–10 ng/μl, respectively, and no complimentary sequence was applied to the control set of beacon spots. All four types of molecular beacons displayed similar dramatic change upon hybridization with the complementary sequences, as documented in Table 2 and shown in Fig. 3. In the absence of the target gene(s), the microarray features (spots) appear red due to nearly complete FRET signaling (i.e. excitation of the green fluorophore results in red fluorophore emission due to FRET). In the presence of the target gene, the complementary strand hybridizes to the probe in a sequence-specific manner, thereby disrupting the stem base-pairing in the probe and increasing the distance between the two fluorophores, resulting in diminished FRET. The decrease in FRET leads to a decrease in red fluorescence complimented with the increase in green fluorescence. It is important to note that the hybridization process involves increasing the temperature to denature the PCR product (double-stranded DNA), which far exceeds the melting temperature of the stem portion of the probe attached to the microarray. Therefore, any reduction in initial hybridization temperature would detrimentally affect the detection process by failing to denature the PCR products derived from the sample, even if the stem portion of the beacon probe is adequately denatured ("opened"). At high concentration of complementary sequence (5–10 ng/μl, Fig. 3), the green/red fluorescence ratio increased at least 30-fold from that of the control sample as shown at the bottom row of Table 2. The green/red fluorescence ratio increased only about 5 times at the lower concentration (1.0–1.5 ng/μl). Thus, the 1.0–1.5 ng/μl represents a concentration that is close to the lower limit at which DNA sequence could be detected using the proposed method. This concentration is about 50 times lower than that obtained directly from the PCR amplified complementary DNA sample.

The effect of the hybridization on fluorescence spectra was detailed by measuring emission of the microarray spots using grating monochromator. Fig. 2B shows two normalized fluorescence spectra of *eaeA* probe spots, one for control with no complimentary sequence, and one for the spot hybridized with target strands at concentration of 50–100 ng/μl. The target hybridization induced clear transition of fluorescence spectrum through the increase of the green fluorescence band and the decrease of red band. The green/red fluorescence ratio increased about four times upon target hybridization and was very similar to that caused by thermal response. These data unambiguously demonstrate reliable operation of the proposed beacons.

Table 3
Green/red fluorescence ratios of *eaeA* sequenced beacon probes upon target DNA hybridization measured with prototype detection platform

Target DNA concentration	Green/red fluorescence ratio
Control (none)	0.24 ± 0.03
0.5–1.0 ng/μl	0.23 ± 0.03 (0.96 ± 0.17)[a]
5–10 ng/μl	0.53 ± 0.06 (2.21 ± 0.40)
50–100 ng/μl	0.86 ± 0.09 (3.58 ± 0.64)

[a] In parentheses: degree of increase of the measured green/red fluorescence ratio compared to that of the control probe.

3.3. Characterization of the prototype detection platform

The automated prototype detection platform described in the Materials and methods section was tested for the applicability for DNA detection in the environments similar to the detection with the microarray scanner. In each measurement, the green/red fluorescence ratio was measured automatically. Table 3 shows the test data with *eaeA* sequenced molecular beacon probes upon hybridization with the specific complementary sequences. As the concentration of complementary strands is

Table 2
Green/red fluorescence ratios of beacon probes upon target DNA hybridization measured with microarray scanner

Target DNA concentration	*eaeA*	*hlyC*	*rfbE*	*stxI*
Control (none)	0.30 ± 0.03	0.10 ± 0.01	0.20 ± 0.02	0.20 ± 0.02
1.0–1.5 ng/μl	1.9 ± 0.02	0.30 ± 0.03	1.0 ± 0.1	1.0 ± 0.1
5–10 ng/μl	12.8 ± 1.4	5.2 ± 0.6	7.3 ± 0.8	6.2 ± 0.7

increased, the green/red fluorescence ratio shows about four-fold increase, which is in line with the optical spectra measurements, as expected. The data indicates that the detection platform is capable of detecting the presence of complimentary sequences at concentrations of about 1–5 ng/μl, which is similar to the lower detection limit of the microarray scanner described in the previous section.

4. Discussion

In the conventional molecular beacon technique with fluorescent marker and a quencher, only the state where the beacon is hybridized with the target sequence is actively visualized through emission. Thus, the presence of fluorescence (positive signal) unambiguously indicates contamination of the sample with the target gene and the occurrence of the false positive signal is essentially impossible. The absence of the target gene, on the other hand, is visualized passively through the absence of fluorescence (negative signal). This leads to the finite probability of a false negative signal, where fluorescence is muted due to the degradation of the fluorophore or due to the beacon washout. To avoid false negative signal, the conventional beacon probe must be tested each time with the artificial complimentary DNA sequence. This opens a window of possibility for the probe contamination and further degrades the reliability of the target gene detection. Second drawback of such a probe is its inability to assess the fraction of the hybridized beacons as the total amount of beacons may vary between the measurements due to the possible washout.

The proposed design employs a beacon with a pair of spectrally shifted fluorescent molecules that, unlike the conventional design, enables active visualization of both hybridized and unhybridized states of the beacon. The ratio of the hybridized and unhybridized beacons can be assessed in a single run, virtually eliminating the possibility for a false negative signal without the necessity to use the artificial complimentary DNA sequences and extra runs. Moreover, as both signals from the hybridized and unhybridized beacons are measured simultaneously, the new design opens an opportunity to quantify the amount of the target DNA in the sample, provided that the green/red fluorescence ratio is pre-calibrated in respect to the target gene concentration in the manner illustrated in Table 3. Since the green/red fluorescence ratio is automatically compensated for possible probe loss between consequent runs, the reuse of the hybridization slide does not impair the reliability of the detection.

In the particular realization of the dual fluorophore beacon, we utilized the pair of fluorescent molecules—Cy-3 as FRET donor, and Cy-5 as FRET acceptor. Both fluorophores are characterized with sufficient fluorescence quantum yield (∼0.2, Mujumdar et al., 1993; Yu et al., 1994; Ha, 2001). The large spectral separation of their emission spectra (∼100 nm) eases the signal separation by just a pair of relatively cheap interference filters. At the same time, the good energetic overlap between the fluorescence of the donor and the absorbance of the acceptor ensures effective FRET. The Cy-3 molecule can be easily excited by the relatively cheap and compact 532 nm laser commonly used in green laser pointers.

Vet et al. (1999) reported real time multiplexed PCR detection of target genes that relied on the use of different pairs of fluorophores for each molecular beacon and consequent spectral separation of the signals from different probes. This format, however, limits simultaneous detection to few target genes due to the limited number of distinguishable fluorescence markers. We propose to use a set of different beacons that all share the same pair of fluorophores and are, instead, separated in a spatial format. The four different beacons characterized in this study displayed similar dependence of the green/red fluorescence ratio on the conformation states of molecular beacons as well as on concentration of the target DNA sequence used for hybridization (Fig. 3). The observed uniform response would facilitate simultaneous detection of virtually thousands of genes using multiplexed PCR technique combined with a single platform spotted with large number of different spatially isolated beacons as no beacon-dependent calibration is required. The detection in such a multiplexed format could be completed more rapidly than analysis that utilizes conventional assays such as electrophoresis. The proposed format will also allow direct hybridization of isolated DNA, rRNA, and mRNA (viability assays), avoiding the need for amplification reactions and yielding quantitative results.

The target gene detection limit of 1 ng/μl determined in this work is an upper limit of the sensitivity as the hybridization procedure was not optimized. More efficient hybridization protocol may benefit from longer incubation times, optimize temperature regime and buffer composition (Tyagi and Kramer, 1996; Schena, 2003).

One of the drawbacks of using the Cy-3/Cy-5 pair of fluorophores is irreversible photochemical bleaching of the Cy-5 molecule (Ha, 2001). However, in our experiments excitation light intensity was kept at minimum and the fluorophores could be kept under excitation laser beam for tens of measurements, making the proposed beacons suitable for multiple reuses. Further improvement of the beacon photo-stability may involve the choice of more stable fluorophore pairs, or stabilizing the Cy-5 fluorophore. One of the major factors leading to photodegradation is the presence of the molecular oxygen (Foote, 1976). The photo-stability of the fluorophore may be increased through using low oxygen conditions (nitrogen flow) in the sample compartment. Alternatively, active molecular protection mechanisms may be utilized, such as incorporation of carotenoid molecules that are known to provide very effective photoprotection in natural pigment–protein photosynthetic complexes by diffusing harmful singlet oxygen (Frank and Cogdell, 1996; Krinsky, 1979).

In conclusion, we demonstrated that an unconventional color-changing molecular beacon can be successfully used as a biosensor that virtually eliminates the false negative signals, and ultimately can serve as an on-site genomic testing instrument in food safety and other applications involving biodetection such as water quality, soil flora and bioterrorism risk monitoring, as well as the detection of single nucleotide polymorphisms (SNPs) in the clinical and agricultural arena. The utilization in SNP detection will involve positioning the variant allele (nucleotide) in the stem portion of the FRET-probe construct, and the presence or

absence of a given allele would result in FRET-acceptor (open) or FRET-donor (closed) fluorescence, respectively.

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