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Novel DNA probes with low background and high hybridization-triggered fluorescence

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ABSTRACT

Novel fluorogenic DNA probes are described. The probes (called Pleiades) have a minor groove binder (MGB) and a fluorophore at the 5′-end and a non-fluorescent quencher at the 3′-end of the DNA sequence. This configuration provides surprisingly low background and high hybridization-triggered fluorescence. Here, we comparatively study the performance of such probes, MGB-Eclipse probes, and molecular beacons. Unlike the other two probe formats, the Pleiades probes have low, temperature-independent background fluorescence and excellent signal-to-background ratios. The probes possess good mismatch discrimination ability and high rates of hybridization. Based on the analysis of fluorescence and absorption spectra we propose a mechanism of action for the Pleiades probes. First, hydrophobic interactions between the quencher and the MGB bring the ends of the probe and, therefore, the fluorophore and the quencher in close proximity. Second, the MGB interacts with the fluorophore and independent of the quencher is able to provide a modest (2–4-fold) quenching effect. Joint action of the MGB and the quencher is the basis for the unique quenching mechanism. The fluorescence is efficiently restored upon binding of the probe to target sequence due to a disruption in the MGB–quencher interaction and concealment of the MGB moiety inside the minor groove.

INTRODUCTION

Fluorogenic hybridization probes are probes whose fluorescence signal changes upon hybridization with a target sequence. Several types of such probes have been described. They can generally be divided into two groups: dual-labeled and single-labeled probes.

The first group of probes utilizes an alteration of dye–dye interactions resulting from hybridization to target sequence to generate a change in fluorescence intensity. Molecular beacons (1) belong to this group. These probes are stem-loop structures with the sequence recognition region located in the loop and the stem serving to bring fluorescent dye and non-fluorescent quencher in close proximity. It has been shown that so-called contact quenching is responsible for the efficient quenching effect (2). Upon hybridization with a target sequence, the dyes are spatially separated which results in a significant fluorescence increase. A similar design is used in the so-called scorpion PCR primers (3). Peptide nucleic acid (PNA) versions of molecular beacons have been developed (4,5). Several stemless dual-labeled probe formats (also called linear probes) have been described. The simplest linear probe contains two dyes at the opposite ends of an oligonucleotide sequence. One of the dyes is fluorescent, while the second one can be fluorescent (6) or non-fluorescent (7,8). The transition from single-stranded, randomly coiled probe to double-stranded DNA duplex is accompanied by a measurable change in fluorescence due to a median increase in distance between the dyes. A decrease in efficiency of fluorescence resonance energy transfer (FRET) from the donor to the acceptor dye is the major mechanism of signal generation for this type of probe (6). PNA versions (9,10) of linear probes demonstrate improved signal-to-background ratios compared to their DNA analogs (8). Since the quenching effect does not significantly depend on the distance between the dyes and their spectral overlap, it has been suggested that direct contact is the primary mode of energy transfer for this type of probe (9). Another way to improve quenching in DNA-based dual-labeled probes without using stem structures is described by Johansson *et al.* (11). It relies on intramolecular

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heterodimer formation between Cy3.5 or fluorescein and a non-fluorescent quencher called Black Hole Quencher. Dual-labeled hybridization probes with fluorophores and various intercalators have also been used (12–15). The fluorescence in these probes is quenched by the intercalator in the absence of a target sequence and becomes unquenched on hybridization with a target sequence due to the hindrance in quenching by intercalation.

The second group of hybridization probes, single-labeled probes, relies on alteration of fluorescence efficiency of a single dye that takes place due to a hybridization event. Oxazole yellow-oligonucleotide (16) or thiazole orange-PNA (17) conjugates (light-up probes) have been described. These probes utilize the ability of the dyes to enhance fluorescence upon intercalation into double-stranded DNA. An improved version of the light-up probes exploits a thiazole orange-base in place of a regular PNA base to increase probe performance (18). The hybeacon probes (19) are also single-labeled oligonucleotides that increase their fluorescence upon hybridization. The increase is due to a disruption of quenching interactions in the single-stranded probe between fluorophore and nucleobases. The inherent quenching ability by deoxiguanosine nucleotides has been used to develop several single-labeled probe formats (20–22).

Our focus in the DNA probe development has been on the exploration of minor groove binder (1,2-dihydro-3*H*-pyrrolo[3, 2-*e*]indole-7-carboxylate tripeptide) oligonucleotides and their application in genetic analysis. DNA probes with conjugated MGB groups form extremely stable duplexes with complementary single-stranded DNA targets, allowing shorter probes to be used for hybridization assay. This property provides improved mismatch discrimination (23) and allows more efficient probe synthesis. Two types of fluorogenic MGB probes have been previously reported. The first type, MGB-TaqMan (23,24) has the MGB ligand and a quencher located at the 3′-end of the probe while the fluorophore is attached at the 5′-end. This configuration allows the fluorophore to be cleaved and unquenched during PCR using the 5′->3′ nucleolytic activity of *Taq* DNA polymerase (25,26). The second type, the MGB-Eclipse™ (27,28), has the MGB and quencher located at the 5′-end and the fluorophore at the 3′-end. The 5′-positioning of the MGB protects the MGB-Eclipse probe from being cleaved during PCR and makes the probe available for post-PCR melting curve analysis (27,28). The fluorescence increase is generated by a transition of the probe from a randomly coiled to linearized state upon hybridization to target DNA. It has been observed that the MGB-probes have reduced fluorescence background compared to non-MGB analogs (23). This was attributed to a reduced length of the MGB probes and, therefore, relative proximity of the fluorophore and the quencher. Both MGB-TaqMan and MGB-Eclipse probe formats have fluorophore and the MGB attached at opposite ends of the DNA probe.

We have recently discovered that when a fluorescent dye is positioned in close proximity to the MGB moiety, its fluorescence is substantially reduced. This surprising finding prompted us to investigate a novel design of hybridization probes, which we have called Pleiades probes (after one of the brightest star constellations in the night sky), wherein the fluorophore is located directly adjacent to the MGB at one of the ends of the probe, whereas the quencher is attached at the opposite end. We have found that this design provides extremely low background fluorescence. Moreover, when such probes are hybridized to complementary targets the fluorescence is efficiently released.

The aim of the current study was to investigate fluorescence and hybridization properties of the novel probes and determine structural elements underlying the efficient fluorescence quenching for free probes and strong hybridization-triggered fluorescence.

MATERIALS AND METHODS

Synthesis of oligonucleotide probes

5′-DNA phosphoramidites (Pierce) were used for the preparation of Pleiades and Eclipse probes. Pleiades probes were synthesized on a MGB-modified DNA synthesis support (29) by first incorporating a fluorophore (LL-FAM, SL-FAM or YY, see Figure 1A and D) using either LL-FAM phosphoramidite (29) or SL-FAM phosphoramidite (Glen Research, Corp., 6-fluorescein phosphoramidite, cat# 10-1964) or Yakima Yellow™ (YY) phosphoramidite followed by incorporation of oligonucleotide sequence. At the last step, an Eclipse Dark Quencher moiety was introduced at the 3′-end using an Eclipse Quencher phosphoramidite (Glen Research Corp., Epoch Eclipse Quencher Phosphoramidite, cat# 10-5925). For the synthesis of Eclipse probes, MGB-Quencher-modified DNA synthesis support (30) was utilized. After the incorporation of nucleotide sequence, spacer C-18 was introduced using the corresponding phosphoramidite (Glen Research Corp., Spacer Phosphoramidite 18, cat# 10-1990) followed by a fluorophore phosphoramidite 6-FAM or yakima yellow (Glen Research, Corp., Epoch Yakima Yellow phosphoramidite, cat# 10-5921). Molecular beacons were synthesized on Eclipse Quencher solid support (Glen Research Corp., epoch eclipse quencher CPG, cat# 205925) using standard 3′-DNA phosphoramidites. 6-FAM or Yakima Yellow phosphoramidites were incorporated at the last step of the synthesis. After deprotection, all probes were carefully purified by reverse-phase HPLC to ensure complete removal of free dyes or quencherless probe fragments. After HPLC purification, the probes were dried and re-dissolved in water. Structures of MGB, dye and linkers for all three types of probes are shown in Figure 1. A nearest-neighbor model was applied to calculate extinction coefficients (ε_{260}) of probes (31). A_{260} measurements were made in 0.1 M Tris-HCl pH 8.5 at ambient temperature and probes were assumed to have a random coil DNA structure in solution. For each MGB–Quencher, Eclipse Quencher, LL-FAM, SL-FAM and YY substitution a ε_{260} correction of +53 500, +6600, +33 300, +10 000 and +28 600 $M^{-1}cm^{-1}$ was used, respectively.

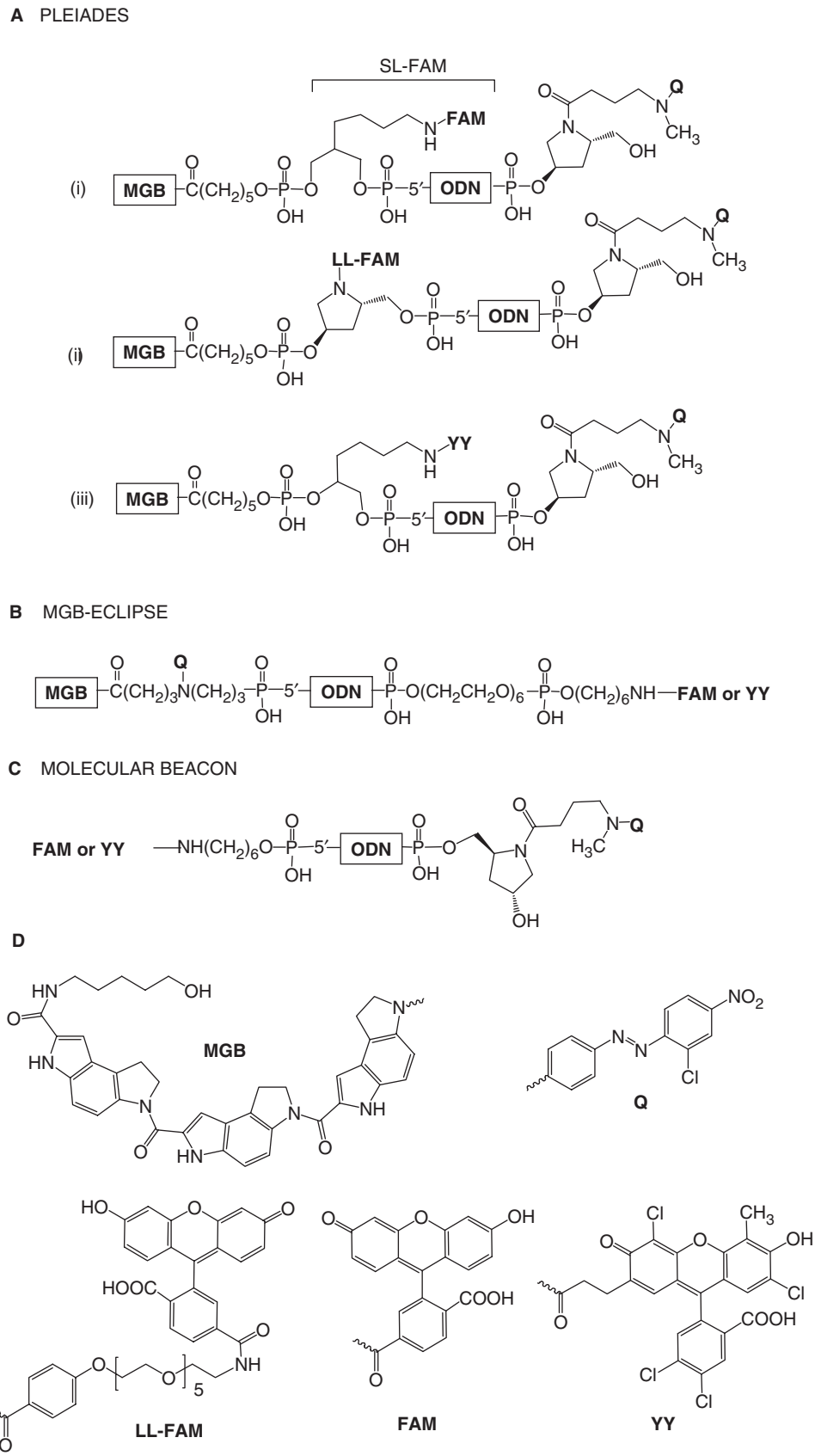

Figure 1. Structures of (**A**) Pleiades probes (i) with 'short-linker' fluorescein (SL-FAM), (ii) with 'long-linker' fluorescein (LL-FAM) or (iii) with Yakima Yellow; (**B**) MGB-Eclipse probes; (**C**) Molecular beacons and (**D**) MGB, Quencher and fluorophores used in this study. ODN stands for oligonucleotide sequence.

UV-vis and fluorescence studies

UV-vis experiments were done on Perkin Elmer PE lambda2S or Varian Cary 400Bio UV-visible spectrophotometers. All fluorescence studies were done on a Varian Cary Eclipse fluorescence spectrometer equipped with a temperature-controlled cell holder. The buffer contained 40 mM NaCl, 10 mM Tris-HCl pH 8.9 and 5 mM $MgCl_2$. Fluorescence was measured at 518 nm for FAM-labeled probes and 550 nm for YY-labeled probes with excitation at 496 and 530 nm, respectively.

Fluorescence signals for single-stranded (0.1 or 0.2 μM) or hybridized probes (in the presence of 0.2 or 0.4 μM complement) were measured at 20°C. To assure equilibrium conditions were reached, duplex mixtures were heated at 60°C for 3 min and cooled to 20°C for measurement.

Fluorescence versus temperature profiles for single-stranded probes and duplexes were obtained by increasing the temperature from 20 to 90°C at the ramp rate of 0.5°C/min. Probe concentration was 200 nM. For the sensitivity study (Figure 4), complement concentration varied from 0.16 to 4 nM.

Fast hybridization kinetics of Pleiades probes and molecular beacons were studied with a rapid kinetics spectrophotometer accessory (Applied Photophysics, Surrey, UK) over a temperature range of 30–55°C. Probe and complement concentrations were 100 and 200 nM, respectively.

RESULTS

Background and hybridization fluorescence of Pleiades, MGB-Eclipse and molecular beacon probes

Four sets of Pleiades (PL), MGB-Eclipse (EC) and molecular beacon (MB) probes were prepared (Table 1) to compare fluorescence properties of the three probe formats. Each set had a common core sequence and approximately equal duplex stabilities (Table 2). PL1 and PL2 sequences were shifted two bases downstream relatively to EC1 and EC2 sequences to avoid quenching by guanosine in the complementary strand (quenching data not shown). The molecular beacons had additional 5′-CGGC and GCCG-3′ stem-forming sequences (underlined in Table 1).

Fluorescence signals for probes before and after hybridization with complementary targets are summarized in Table 2. FAM-labeled Pleiades probes (PL1, PL1R) and molecular beacons (MB1, MB1R) show very low background fluorescence and high hybridization signal with signal-to-background (S/B) ratios of approximately 80–100 for Pleiades and 78–130 for molecular beacons. Hybridization signals for corresponding MGB-Eclipse (EC1, EC1R) are approximately equal to those

Table 1. Sequences, background signal, hybridization signals and signal-to-background ratios for Pleiades (PL), MGB-Eclipse (EC) and molecular beacon (MB) probes. Fluorescence signals for single-stranded (0.1 μM) and hybridized probes (in the presence of 0.2 μM complement) were measured at 20°C. C1, C2, C1R and C2R are hybridization targets

Name	Sequence	Dye	Background	Hybridization Signal	Ratio
PL1	5′-GTCAGAGACATACACC	LL-FAM	3.1	249.2	80.4
EC1	5′-CAGAGACATACACCA	FAM	32.5	267.3	8.2
MB1	5′-CGGCGAGTCAGAGACATACACCAGCCG	FAM	2.9	226.0	77.9
C1	3′-GTGCCTCAGTCTCTGTATGTGGTGGGACG				
PL2	5′-GTCAGACACATACACC	YY	0.5	96.6	193.2
EC2	5′-CAGACACATACACCA	YY	6.4	123.6	19.3
MB2	5′-CGGCGAGTCAGACACATACACCAGCCG	YY	1.8	137.0	76.1
C2	3′-GTGCCTCAGTCTGTGTATGTGGTGGGACG				
PL1R	5′-GTATGTCTCTGACTCC	LL-FAM	3.7	356	96.2
EC1R	5′-GTATGTCTCTGACTCC	FAM	21.6	336	15.6
MB1R	5′-CGGCGTGTATGTCTCTGACTCCGGCCG	FAM	2.8	368.0	131.4
C1R	3′-CCACCACATACAGAGACTGAGGCACTG				
PL2R	5′-GTATGTGTCTGACTCC	YY	0.5	173.6	347.2
EC2R	5′-GTATGTGTCTGACTCC	YY	8.3	165	19.9
MB2R	5′-CGGCGTGTATGTGTCTGACTCCGGCCG	YY	1.8	203.0	112.8
C2R	3′-CCACCACATACACAGACTGAGGCACTG				

Table 2. Melting temperatures for fully matched and single nucleotide mismatched duplexes

Probe	Dye type	T_m (match)	T_m (mismatch)	ΔT_m
PL1	LL-FAM	64°C (G/C)	55.5°C (G/G)	8.5°C
EC1	LL-FAM	64°C (G/C)	57.5°C (G/G)	6.5°C
MB1	LL-FAM	66°C (G/C)	60°C (G/G)	6°C
PL2	YY	64°C (C/G)	47°C (C/C)	17°C
EC2	YY	64°C (C/G)	53°C (C/C)	11°C
MB2	YY	66°C (C/G)	53°C (C/C)	13°C

Probe and complement concentrations were 200 and 100 nM, respectively.

of Pleiades. However, their high background fluorescences (6–10 times higher than that of Pleiades or molecular beacons) result in relatively low S/B ratios of only about 8–16.

YY-labeled Pleiades (PL2 and PL2R) have extremely low background fluorescence and high S/B ratios (193 and 347 respectively). The ratio is 2–3 times higher than for the corresponding FAM analogs (PL1 and PL1R). Interestingly, the molecular beacon analogs (MB2, MB2R) are quenched about 3 times less efficiently even though they have the fluorophore and quencher conveniently brought together by the stem structure. YY-labeled MGB-Eclipse probes (EC2, EC2R) demonstrate even higher background fluorescence and relatively low S/B ratios (19 and 15, respectively).

Temperature dependence of background fluorescence

Stable temperature-independent fluorescence background is important for accuracy and sensitivity of post-PCR melting curve analysis. Therefore, we studied the temperature dependence of background fluorescence for molecular beacon, Pleiades and MGB-Eclipse probes (Figure 3A and B). Background signals increased approximately 1.5-fold between 20 and 60°C (PCR relevant temperature) for the Pleiades probes PL1, PL1R, PL2 and PL2R. This slight change may indicate a presence of weak secondary structures at low temperature which become unstable at high temperature. Still, the absolute value of the background fluorescence remained very low throughout the temperature range. Profiles of background fluorescence versus temperature for the MGB-Eclipse probes were less stable and substantially varied from one probe to another. For example, the EC1 and EC2R probes showed some sort of melting transition. The EC1R probe demonstrated a drop in fluorescence, while fluorescence of the EC2 probe remained mostly unchanged at any temperature. The nature of these changes for the Eclipse probes is not clear, and likely reflects either presence of secondary structures (especially for EC1, EC2R) or statistical/conformational reorganizations within the probes. On the other hand, the fluorescence increase between 50 and 80°C for molecular beacons is expected and indicates opening of the stem structure, which is accompanied by loss of quenching. The drop in quenching efficiency (~5 times at 60°C) significantly reduces the S/B ratios for molecular beacons at temperatures approaching T_m of the stem and, consequently, diminishes usefulness of molecular beacons at real-time PCR temperatures.

Hybridization specificity

In order to compare the specificity of the probes, melting temperatures were determined for duplexes with fully matched and a single nucleotide mismatch sequences. Probes PL1, EC1 and MB1 were hybridized with C1 template (Table 1) to form fully matched duplex or with C2 template to form a G/G mismatch. Correspondingly, probes PL2, EC2 and MB2 were hybridized with C2 template to form fully matched duplex or with C1 template to form a C/C mismatch. First derivative melting curve analysis of these duplexes (Figure 2) showed that the discrimination, which could be estimated as a first derivative area overlap between matched and mismatched complexes, was approximately equivalent for the molecular beacons (Figure 2E and F) and the Pleiades probes (Figure 2A and B). The molecular beacons demonstrated the highest cooperativity of the melting transitions whereas the Pleiades probes had the largest ΔT_m values (Table 2). The MGB-Eclipse probes (EC1, EC2) showed both relatively low ΔT_m and reduced cooperativities of the melting transitions thus resulting in a slightly reduced discrimination for both mismatches (Figure 2C and D) compared to the other two types of probes.

Sensitivity

Melting curve assay can be used to compare sensitivity limits for different probes. An example of such an assay is shown in Figure 4 wherein a Pleiades and a MGB-Eclipse probe are compared. Both probes (see Figure 4 legend for sequence) at 200 nM concentrations were annealed with reducing amounts of complementary target followed by fluorescence melting curve analysis. The Pleiades probe showed clear specific melting transition down to 0.16 nM concentration; whereas the corresponding MGB-Eclipse analog started to show anomalous melting at 1 nM concentration. Therefore, under the conditions of the experiment the sensitivity limits for Pleiades and MGB-Eclipse were 0.16 and 4 nM correspondingly. Similar, unpredictable melting curves were observed at subnanomolar concentrations of the targets for the EC1 and EC2R MGB-Eclipse probes.

Hybridization kinetics

Figure 5 shows representative kinetics of hybridization for a FAM-labeled Pleiades probe (PL1) and analogous molecular beacon (MB1). One can see that depending on temperature the molecular beacon hybridizes about 4–2 times slower to the complementary DNA than the corresponding Pleiades probe. It has been previously shown that Beacon's stem structure, while beneficial for improved quenching and mismatch discrimination (32,33), substantially reduces hybridization rate with its target (8,33). At higher temperatures the stem structure starts to open, leading to hybridization rates closer to those of Pleiades probes.

Investigation of quenching mechanism

The absence of melting transitions (Figure 3) and fast kinetics of hybridization (Figure 4A) indicate that the efficient quenching for Pleiades probes is not generated by a unique secondary structure. The quenching effect may be attributed to potential interactions of fluorophore with quencher, MGB and nucleotide sequence. It is also possible that some or all of these factors act cooperatively. To investigate the contribution of MGB and quencher to the quenching effect, four sets of four probes were prepared (Table 4A). The first set had all components (MGB, fluorophore and quencher) present. The second set had the quencher absent. The third set had the quencher

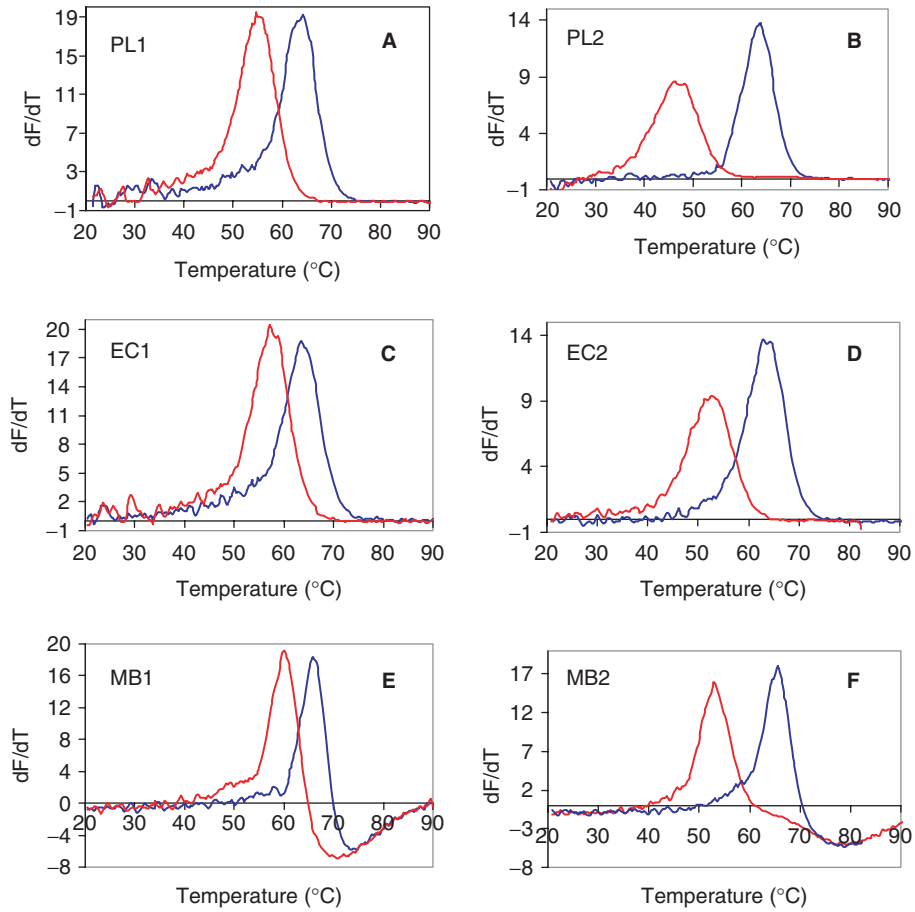

Figure 2. Differential melting curves for the complexes PL1 + C1 (or C2) (**A**), PL2 + C2 (C1) (**B**); EC1 + C1 (or C2) (**C**), EC2 + C2 (C1) (**D**); MB1 + C1 (or C2) (**E**), MB2 + C2 (C1) (**F**).

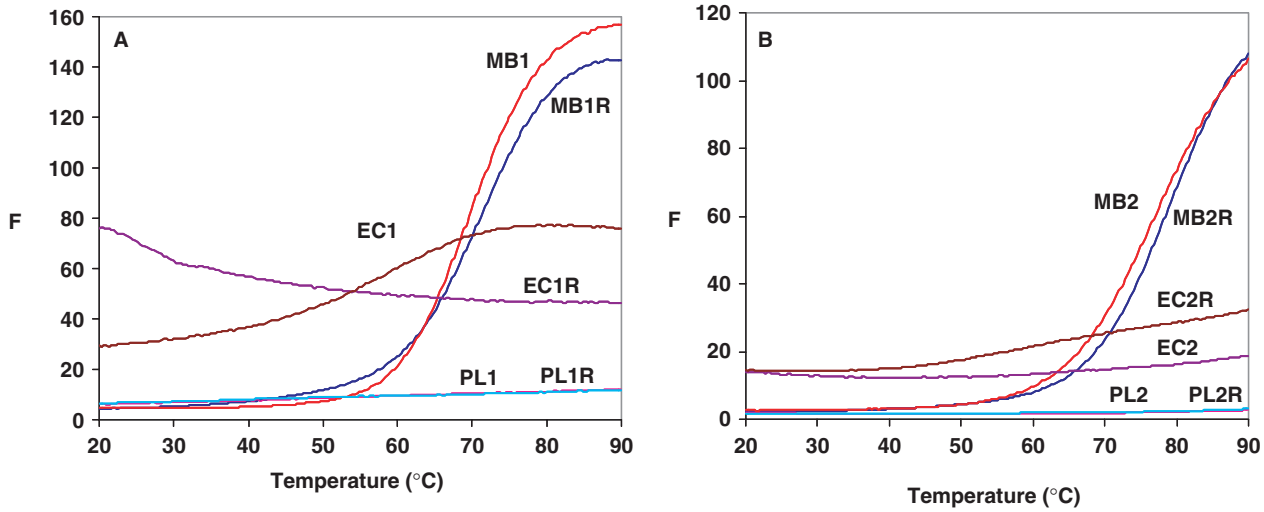

Figure 3. Temperature dependence of background fluorescence of Pleiades (PL) probes, MGB-Eclipse (EC) probes and molecular beacons (MB) labeled with **(A)** FAM or **(B)** YY fluorophores. Structures of the probes are shown in Table 1. Probe concentration was 200 nM. Excitation and emission slits were 5 nm.

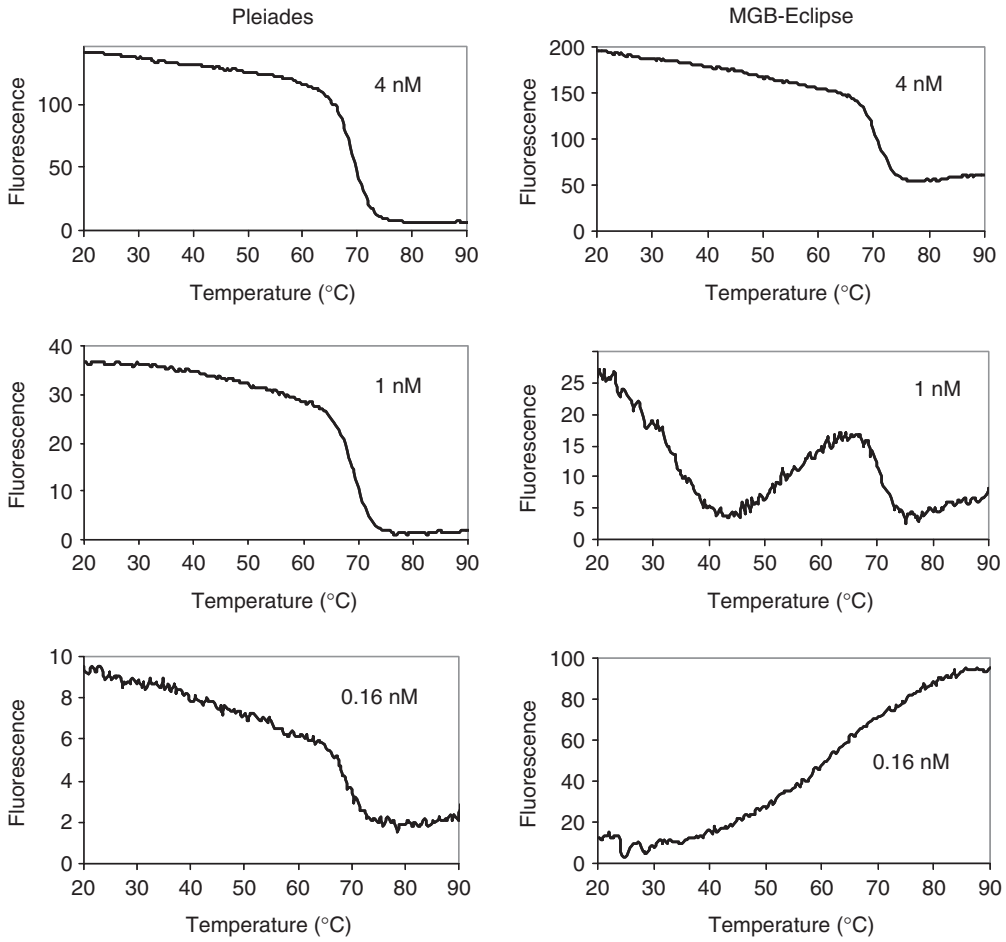

Figure 4. Determination of sensitivity limits for Pleiades and MGB-Eclipse probes by a fluorescence melting curve analysis. Probe sequence was 5′-GATGTGTCCGTGTCTC for both Pleiades and MGB-Eclipse. Complement sequence was 5′-AAAGAGACACGGACACATCAATCCAT. The probes (200 nM) were hybridized at 4.0, 1.0 or 0.16 nM complement concentrations. Excitation and emission slits were 10 nm. Fluorescence background (probe without the target) was first recorded for each probe in a separate run. The resultant background curves were subtracted from all of the subsequent melting curves of the same probes in the presence of target. It has to be noticed that MGB-Eclipse probes generally have high fluorescence background (~600 RFU under the experimental conditions), complicated temperature dependence of the background and a tendency to aggregate and adhere to the cuvette at high temperature, especially in the presence of magnesium cations. Therefore the background curves can substantially fluctuate from run to run. Due to these problems significant fluctuations in the final duplex melting curve shape and signal are observed for the MGB-Eclipse probe.

and the fluorophore but no MGB, and the fourth set had both MGB and quencher absent. The fluorescence of the probes was measured before and after hybridization with a complement to determine S/B ratios. The minor groove binder alone clearly quenches FAM fluorescence (probes PL7–PL10), although the effect is fairly modest (S/B 1.6–2.3). As expected, the quencher alone produces the major quenching effect (S/B ~ 6–10). However, the cooperative effect of the MGB and the quencher (S/B ~ 45–75) is much greater than expected from the individual contributions.

All data presented in Table 4A were obtained with probes containing a relatively long hexaethylene glycol linker (Figure 1A (ii)) between MGB and FAM. To further investigate interactions between the components of the Pleiades probes, a shorter, six-atom linker (Figure 1A (i)) was tested (Table 4B). The background fluorescence of such probes is about two times lower than those with the long, hexaethylene glycol linker. However, a general drop (20–30%) in hybridization fluorescence is also observed. Overall, S/B ratios are improved by 1.4–1.9-fold. Presumably, the improvement is due to an enhancement in an MGB-FAM interaction. Similar enhancement in quenching (1.2–1.6-fold) and reduction in hybridization signal are observed for the MGB-FAM probes (PL23–PL26) in which quencher is absent. The drop in hybridization signal could be a result of quenching by nucleotide sequence. This may also explain greater variations in hybridization signal among different sequences. For example, quenching by 5′-terminal guanosine may be responsible for the reduced signal for PL19, PL23, PL27 and PL31 probes. The G-quenching is even more evident for other sequence contexts (data not shown). We have found that the use of the hexaethylene glycol linker substantially reduces these undesired effects.

As a next step we investigated absorption spectra of the Pleiades probes before and after addition

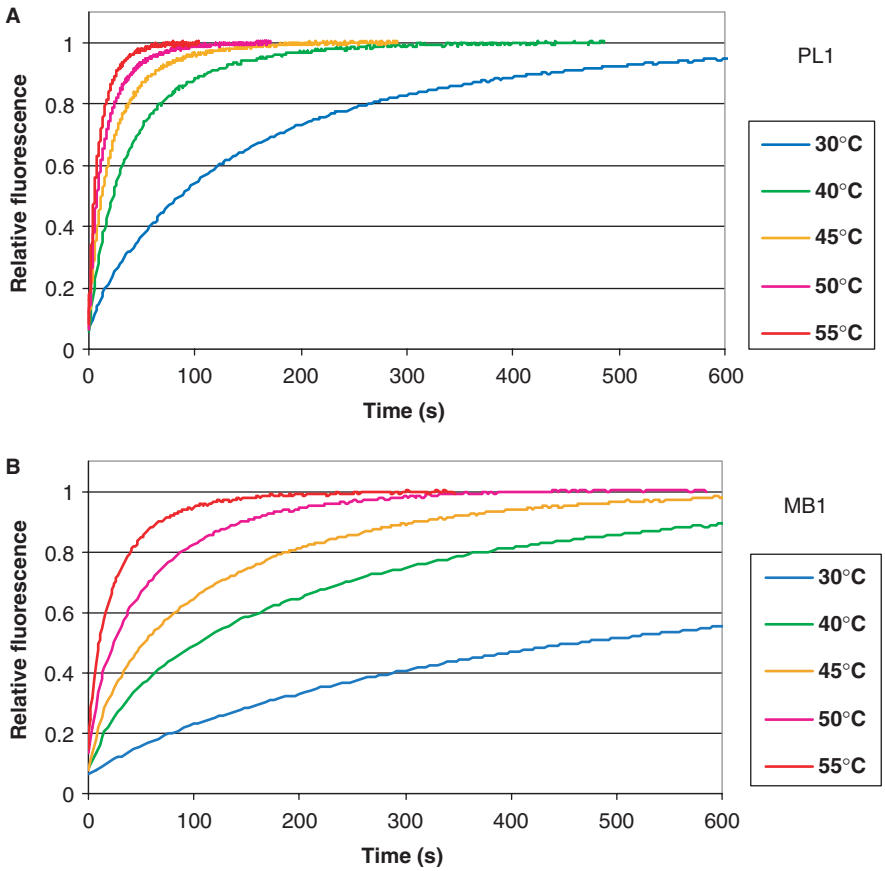

Figure 5. Kinetics of hybridization of (**A**) FAM-labeled Pleiades (PL1) and (**B**) FAM-labeled molecular beacon (MB1) probes with complementary sequence at various temperatures. Second-order rate constants for molecular beacons and Pleiades probes labeled with two different dyes are summarized in Table 3.

Table 3. Second-order rate constants (k_h, ($M^{-1}s^{-1}$)) for hybridization of Pleiades probes (PL1 and PL2) and their molecular beacon analogs (ML1 and ML2) to complementary target at different temperatures

	k_h (30°C)	k_h (40°C)	k_h (45°C)	k_h (50°C)	k_h (55°C)
PL1	4.1×10^4	1.6×10^5	2.8×10^5	4.3×10^5	6.5×10^5
MB1	1.0×10^4	2.7×10^4	5.4×10^4	1.0×10^5	2.4×10^5
PL2	6.8×10^4	2.7×10^5	6.2×10^5	1.0×10^6	1.4×10^6
MB2	2.0×10^4	5.0×10^4	1.2×10^5	1.9×10^5	2.3×10^5

of a complement. We used the PL3 probe as a typical example to illustrate the results of this study. The absorption spectrum of unhybridized probe clearly differs from the spectrum of a duplex with a complement (Figure 6A). Shifts in absorbance maxima to the shorter wavelength region are observed for all components of the spectrum above 300 nm: MGB (which absorbs around 340 nm) by 2 nm; fluorescein (which absorbs around 495 nm) by 6 nm and quencher (which absorbs around 550 nm) by 23 nm. In addition to the shift, the absorbance intensity is increased for fluorescein and decreased slightly for the MGB. These changes in absorption spectra are indicative of possible interaction between the components of the probe. However, some of the changes could be due to differences in interactions of the dyes and MGB with DNA in single- and double-stranded states. To distinguish between these two situations we investigated spectra of simplified analogs of the PL3 probe in which one or two components were absent.

The results for single-labeled analogs are shown in Figure 6B–D. No appreciable change in absorption spectrum is observed for the FAM-only labeled analog when complementary sequence is added (Figure 6B). A minor decrease (~2%) in absorbance is due to dilution of the sample on addition of the complement. The MGB-labeled probe shows (Figure 6C) a 6 nm red shift upon duplex formation and a slight drop in absorbance at 320 nm. This change can be attributed to the MGB binding to the minor groove. An 8 nm blue shift is observed for the quencher-only-labeled analog (Figure 6D); an interaction with the minor groove could also be responsible for the change.

Figure 6E–G illustrates the results for dual-labeled analogs. The most dramatic spectral change is seen for the MGB-Quencher probe (Figure 6E). The absorbance maximum of the quencher is at 550 nm for free probe (compared to 534 nm for the Quencher-only analog (Figure 6D)) and is blue shifted by 25 nm to 525 nm when complementary sequence is added. The MGB absorbance maximum in the free probe (346 nm) also differs from the one observed in the MGB-only

Table 4. Effects of MGB and quencher on background and hybridization fluorescence. (**A**) Fluorescent label (FAM) is attached via a hexaethylene glycol spacer (LL); (**B**) fluorescent label attached via C6-spacer (SL). Seq1 is 5′-GTGTCCGTGTCTCTTT; Seq2 is 5′-GATGT GTCCGTGTCTC; Seq3 is 5′-TTGATGTGTCCGTGTC; Seq4 is 5′-ATTGATGTGTCCGTGT. Complement is 3′-CTACCTAACTACAC AGGCACAGAGAAA. Fluorescence signals for single-stranded (0.2 μM) and hybridized probes (in the presence of 0.4 μM complement) were measured at 20°C using excitation at 496 nm and emission at 518 nm

Name	Sequence	Background	Hybridization signal	Ratio
(A)				
PL3	**MGB-(LL-FAM)**-Seq1-**Q**	11.4	517	45.1
PL4	**MGB-(LL-FAM)**-Seq2-**Q**	7.6	571	75.0
PL5	**MGB-(LL-FAM)**-Seq3-**Q**	14.1	630	44.7
PL6	**MGB-(LL-FAM)**-Seq4-**Q**	14.9	524	35.1
PL7	**MGB-(LL-FAM)**-Seq1	354	732	2.1
PL8	**MGB-(LL-FAM)**-Seq2	361	825	2.3
PL9	**MGB-(LL-FAM)**-Seq3	454	741	1.6
PL10	**MGB-(LL-FAM)**-Seq4	441	796	1.8
PL11	**(LL-FAM)**-Seq1-**Q**	100	577	5.7
PL12	**(LL-FAM)**-Seq2-**Q**	65	614	9.5
PL13	**(LL-FAM)**-Seq3-**Q**	92	681	7.4
PL14	**(LL-FAM)**-Seq4-**Q**	104	617	5.9
PL15	**(LL-FAM)**-Seq1	790	861	1.1
PL16	**(LL-FAM)**-Seq2	856	891	1.0
PL17	**(LL-FAM)**-Seq3	902	874	1.0
PL18	**(LL-FAM)**-Seq4	905	863	1.0
(B)				
PL19	**MGB-(SL-FAM)**-Seq1-**Q**	5.25	336	64
PL20	**MGB-(SL-FAM)**-Seq2-**Q**	4.28	459	107
PL21	**MGB-(SL-FAM)**-Seq3-**Q**	7.21	504	70
PL22	**MGB-(SL-FAM)**-Seq4-**Q**	6.11	401	66
PL23	**MGB-(SL-FAM)**-Seq1	170	483	2.8
PL24	**MGB-(SL-FAM)**-Seq2	161	618	3.8
PL25	**MGB-(SL-FAM)**-Seq3	279	708	2.5
PL26	**MGB-(SL-FAM)**-Seq4	257	532	2.1
PL27	**(SL-FAM)**-Seq1-**Q**	42	277	6.6
PL28	**(SL-FAM)**-Seq2-**Q**	30	414	13.8
PL29	**(SL-FAM)**-Seq3-**Q**	52	487	9.4
PL30	**(SL-FAM)**-Seq4-**Q**	64	452	7.1
PL31	**(SL-FAM)**-Seq1	327	424	1.3
PL32	**(SL-FAM)**-Seq2	459	577	1.3
PL33	**(SL-FAM)**-Seq3	682	728	1.1
PL34	**(SL-FAM)**-Seq4	506	459	0.9

analog (341 nm). This result clearly points to a prominent interaction between the MGB and the quencher in the unhybridized probe. Duplex formation disrupts this interaction and restores the absorption spectra characteristic for MGB and quencher in single-labeled analogs.

Absorption spectra analysis of the MGB-FAM dual-labeled probe (Figure 6F) supported an interaction between MGB and FAM. The existence of such interaction is consistent with the fluorescence data for the MGB-dependent quenching described above. The FAM absorbance maximum is at 500 nm for the free probe and moves to 494 nm with a slight increase in intensity after the duplex formation. The latter number is characteristic for FAM in the single-labeled FAM probe. The same change in the FAM absorbance is found for the fully equipped PL3 probe (Figure 6A). An existence of an aggregate between MGB and FAM is, therefore, a possibility.

The last of the dual-labeled probes, FAM-Quencher analog (Figure 6G) did not reveal any contact interactions. The small change in the quencher absorbance maximum is similar to that observed in the single-labeled Quencher analog (Figure 6D). This result suggests that the fluorescence quenching for dual-labeled FAM-Quencher probes PL11–14 (Table 4A) is predominantly FRET based.

It appears from the data presented that all the differences in absorption spectra observed between free and hybridized probes may be explained by three types of major interactions: MGB–quencher, MGB–minor groove and MGB–FAM. In addition, the quencher seems to interact with the nucleotide sequence, via the minor groove or otherwise.

Additional evidence for the strong MGB–quencher intramolecular interaction may be obtained from a comparison of absorption spectra of unhybridized probes of variable length. To eliminate the effect of nucleotide sequence on fluorescence signal, simplified oligoT probes were designed (Table 5). Two guanosine bases were introduced in the middle of the sequence to avoid frame shifting upon hybridization with complementary target. The strongest red shift in both MGB and quencher portions of spectrum is observed for the 10-mer probe (Figure 7). The shift is progressively reduced as the length of the probe is increased to 18 bases.

Background fluorescence and hybridization signals increased proportionally with probe length, with almost constant signal-to-background ratios (Table 5). However, the 10-mer probe has a hybridization signal less than half versus the 18-mer. Therefore, to maximize hybridization signal it is preferable to use probes of 16 bases and longer. If real-time PCR is the assay format, the probes require T_m relevant to PCR conditions. Fortunately this requirement goes well with MGB probes which have T_m of 60–70°C for 16–18-mers almost independent of sequence content.

Mechanism of action of Pleiades probes

Based on the data generated in this study we suggest a hypothetical mechanism of action of Pleiades probes (Figure 8). The bulk of the quenching effect for unhybridized probe results from an interaction between the MGB and the quencher. Hydrophobic interaction between these two groups, possibly an intramolecular heterodimer structure (11), brings the ends of the probe and, therefore, the dyes in close proximity. It should be noted that such mechanism is absent in MGB-Eclipse probes in which the MGB and the quencher are located next to each other. The second level of quenching is generated by an effect of the MGB. Since the MGB lacks appropriate spectral overlap with the fluorophores used in this study, its quenching effect cannot be explained by traditional FRET. Contact-mediated quenching (2) driven by hydrophobic forces may be one mechanism. It is also possible that the electron-rich dihydropyrroloindole ring structure of the minor groove binder is involved in the

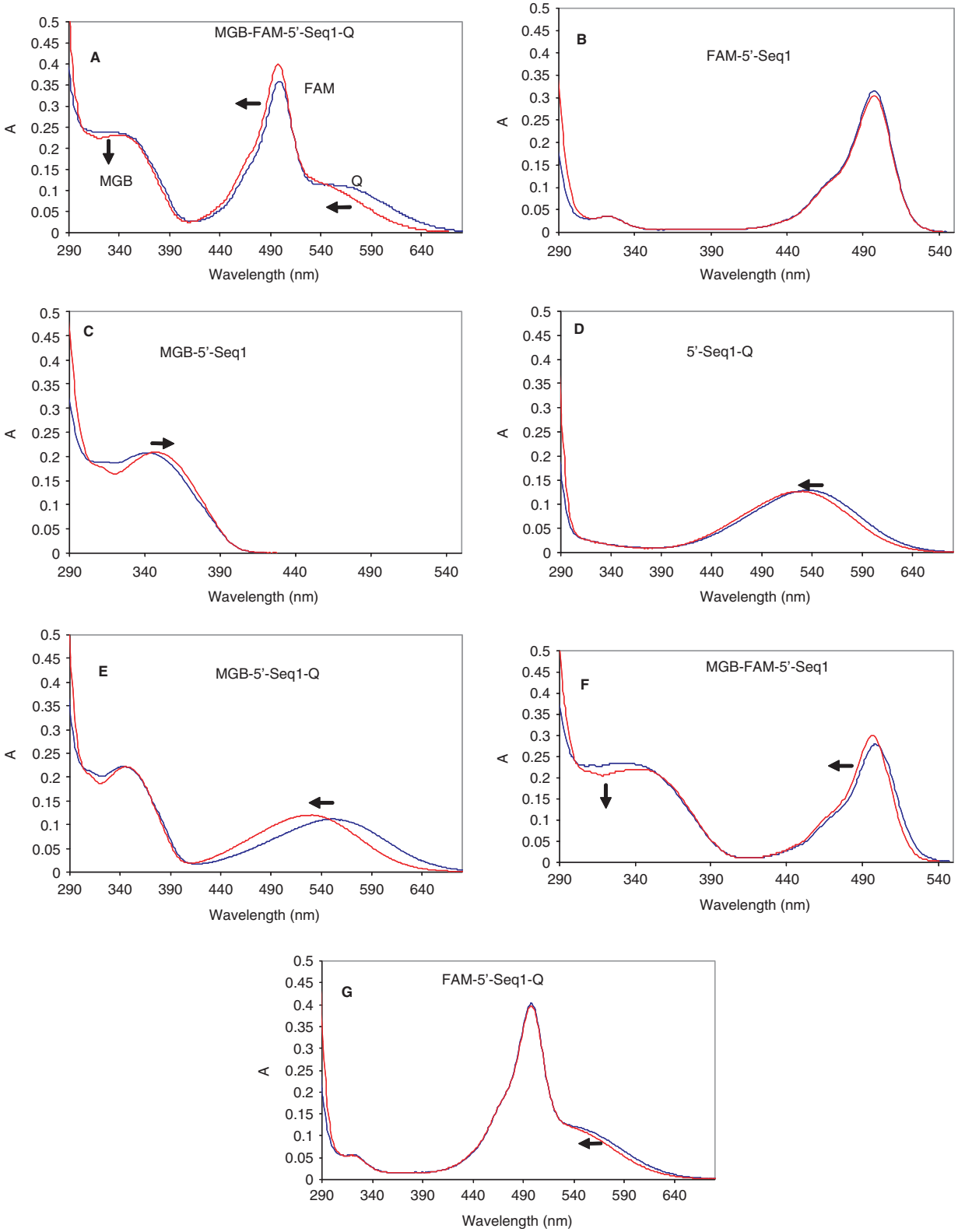

Figure 6. Effects of duplex formation on absorption spectra of the PL3 probe (**A**) and its simplified analogs without the MGB and the quencher (**B**); without the fluorophore and the quencher (**C**); without the MGB and the fluorophore (**D**); without the fluorophore (**E**); without the quencher (**F**); and without the MGB (**G**). Blue line is unhybridized probe. Red line is duplex. Arrows indicate the directions of spectral changes. Probe and complement concentrations were 4 and 5 μM, respectively.

Table 5. Effect of probe length on background and signal fluorescence

Sequence	Length	Background	Signal	Ratio
MGB-(LL-FAM)-5′-TTTTGGTTTT-Q	10-mer	7.3	324	44
MGB-(LL-FAM)-5′- TTTTTGGTTTTT-Q	12-mer	9.5	500	53
MGB-(LL-FAM)-5′-TTTTTTGGTTTTTT-Q	14-mer	12.5	616	49
MGB-(LL-FAM)-5′-TTTTTTTGGTTTTTTT-Q	16-mer	14.4	734	51
MGB-(LL-FAM)-5′-TTTTTTTTGGTTTTTTTT-Q	18-mer	19.3	862	45

Sequence of the complement –CCCTCTAAAAAAAACCAAAAAAAATCTCCC

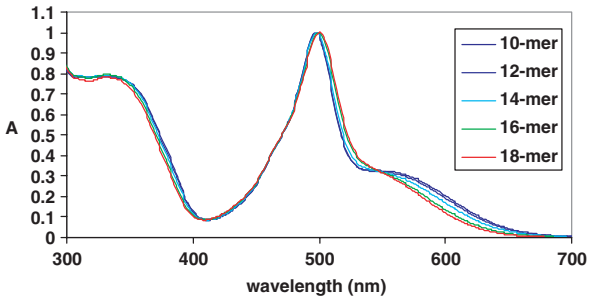

Figure 7. Effect of probe length on absorption spectra of unhybridized probes. Structures of the probes are shown in Table 5. The spectra were normalized at the FAM maximum with the assumption that its intensity remained constant for all probes.

photoinduced electron transfer (34) to a fluorescent dye to generate the quenching effect. This process is observed between some dyes and nucleotide bases, especially guanines (35). The MGB alone is only a modest quencher. However, the combination of the two quenching effects is the basis for the extraordinary low fluorescence background. In addition to these two major factors, other effects may also contribute to the efficiency of quenching. These effects are sequence-dependent and include the possible presence of secondary structures or quenching by interaction with DNA bases. These additional factors may explain the approximately 2-fold variations in fluorescence background between Pleiades probes presented in Tables 1 and 4A.

The second critical step in the mechanism of action is the efficient hybridization-triggered fluorescence release. It is achieved through a disruption of the MGB–quencher interaction upon probe binding to target sequence and concealment of the MGB inside the minor groove as illustrated in Figure 8. The FRET-based quenching of the quencher group is greatly reduced as the median distance between quencher and fluorophore is increased in the hybridized state.

DISCUSSION

Performance of a fluorogenic DNA hybridization probe can be characterized by several important parameters. The first is the degree of quenching in unhybridized probe (background fluorescence). The second factor is the fluorescence intensity after hybridization with a target (signal fluorescence). Together they can be characterized by the value of signal-to-background (S/B) ratio—the ultimate measure of assay sensitivity. Furthermore, it is crucial for some applications to keep the background fluorescence stable at any temperature of the assay. For example, post-PCR melting curve analysis is an important tool in genetic analysis (36–38); it assures specificity of target detection and allows discrimination between fully and partially matched duplexes. However, a probe that has an unstable fluorescence background due, for instance, to an intrinsic secondary structure, may generate a complicated melting profile and lead to erroneous conclusions. The third factor is the efficiency of hybridization, which depends on the probe's T_m, assay conditions and presence of secondary structures in probe or target. Secondary structure in a probe can reduce hybridization rates and result in inefficient hybridization and, consequently, low fluorescent signal. This is crucial for applications such as fast cycling PCR assays. The fourth factor is the sequence specificity of hybridization, such as the ability to discriminate single nucleotide polymorphisms. The fifth factor is the resistance to enzymatic degradation during PCR reaction. This property allows a post-PCR thermal melt analysis to be performed.

The molecular beacons tested in this study demonstrated outstanding S/B ratios at low temperatures, similar to that reported in literature (1). However, at PCR relevant conditions the value of this parameter was substantially compromised. A relatively stable stem is needed to keep molecular beacon's background fluorescence low at elevated temperatures. The stem structure is also required for the improved mismatch discrimination by molecular beacons (32). However, the benefits of having this structure come at the price of reduced hybridization kinetics (8,33), which may render molecular beacons inefficient for some applications. Melting profile analysis for molecular beacons is also complicated by the presence of the stem-loop to random coil transition. In addition, substantial probe degradation is observed for molecular beacons during PCR reaction (28).

MGB-Eclipse, the second type of probes compared, does not possess very low fluorescence background. The S/B ratios for these probes are only slightly better than those for corresponding non-MGB linear probes. Unstable, temperature-dependent background fluorescence is another weakness of MGB-Eclipse probes. On the upside, due to the stabilizing effect of the MGB, MGB-Eclipse probes are relatively short and, therefore, have a good mismatch discriminating ability. Another benefit is that their rates of hybridization are not

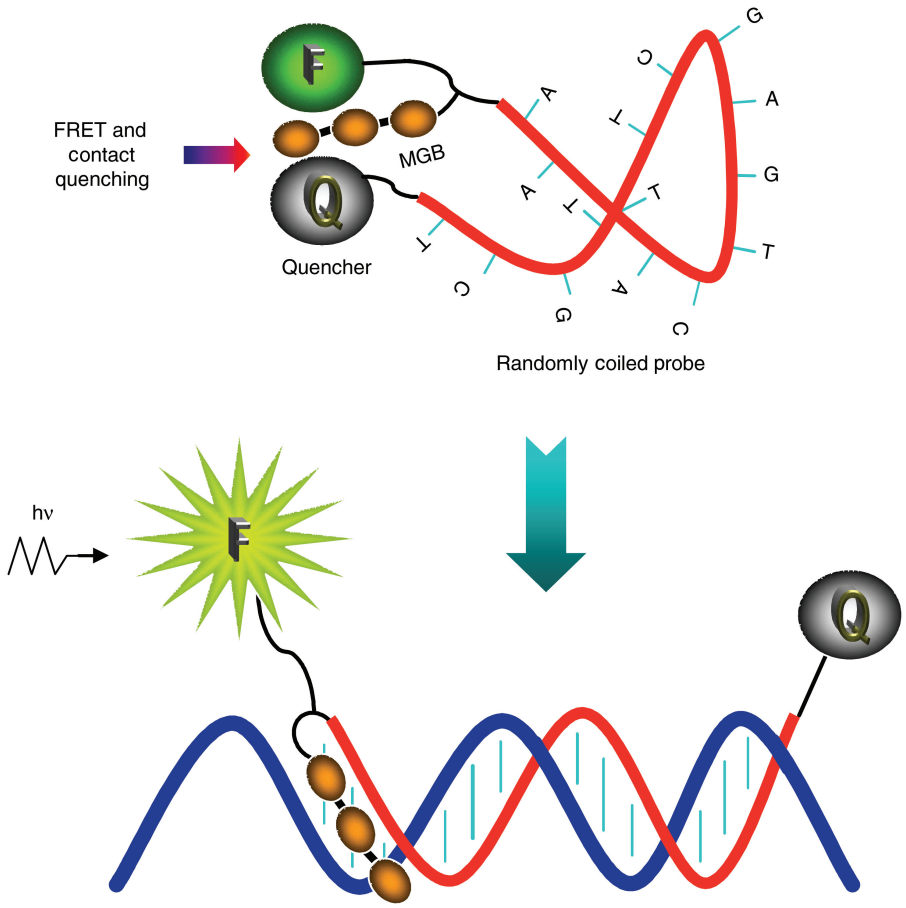

Figure 8. Proposed mechanism of action of Pleiades probes.

inhibited by purposefully introduced secondary structures. In this and other aspects MGB-Eclipse probes are similar to no-stem PNA beacons (8). PNA beacons also demonstrate signal-to-background ratios of approximately 10–15 at room temperature and of ~7 at 43°C (39), which are similar to those of MGB-Eclipse. Also like MGB-Eclipse, short no-stem PNA beacons are more sensitive to sequence mismatches compared to longer linear DNA probes. For many applications both no-stem PNA and MGB-Eclipse probes will provide good performance.

Pleiades probes combine the advantages and address some of the drawbacks of the other technologies. They possess low background fluorescence and high S/B ratios both at ambient temperature and PCR-relevant temperatures. Unlike MGB-Eclipse, Pleiades probes do not demonstrate peculiar, duplex-unrelated melting transitions. Combination of high hybridization signal with low and stable background is the basis for increased sensitivity of the Pleiades probes. In addition to providing a higher sensitivity, low background fluorescence allows the use of increased concentrations of the probes without overwhelming detection devices. Another useful feature of the Pleiades probes is that the background fluorescence, at least in part, depends on the distance (spacer length) between the MGB and the dye. This offers a way to fine tune properties of the probes, which may be desirable for certain applications. For example, the long, hexaethylene glycol spacer between branching point and fluorophore slightly raises the background fluorescence. On the other hand, this allows higher hybridization fluorescence and reduced quenching by the nucleotide sequence. This may be important for high-throughput probe design for PCR application. Alternatively, for applications, such as solid phase-immobilized light-up hybridization (40–42) or *in vivo* studies, a lower background and higher S/B ratios may be more beneficial. In this case shorter spacer, such as C6-spacer, should be used. Similar to MGB-Eclipse, they demonstrate fast hybridization kinetics and excellent stability against 5′->3′-exonuclease cleavage. Like all MGB-containing oligonucleotides, Pleiades probes possess excellent specificity due to their short length and the mismatch discriminating effects of the MGB (43).

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