Phthalocyanine dimerization-based molecular beacons using near-IR fluorescence

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SUPPORTING INFORMATION

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Experimental procedures

DBMB synthesis

The syntheses of the succinimidyl ester **Pc1** and asymmetric amino-modified **Pc2** (see Scheme 1) are reported elsewhere.[1, 2]

*Preparation of DBMB1-**Pc1** and DBMB2-**Pc1***. Stock solutions of **Pc1** (10 mM) was prepared in DMSO (dimethyl sulfoxide). Labeling buffer (100 mM sodium carbonate, pH 7.5) was prepared in-house by dissolving the appropriate amount of sodium carbonate in water and adjusting the pH using HCl and/or NaOH. The oligonucleotide modified with amino groups at the 5' and 3' ends was obtained either from IDT (Coralville, IA) or Trilink Biotechnology (San Diego, CA). The oligonucleotide was purified by ethanol precipitation before the conjugation reaction to remove any amino-containing impurities. The purified oligonucleotide was re-dissolved in water to yield a solution with a concentration of ~2.7 mM.

The reaction mixture included (in order of addition): Labeling buffer (66 μL), oligonucleotide (4 μL, 10.8 nmol), and succinimidyl ester **Pc1** (30 μL, 302 nmol). The reaction was incubated at 40^0C for 3 h. Labeled oligonucleotides were purified by ethanol precipitation (to partially

remove excess unreacted dye) using 250 μL of cold absolute ethanol and 10 μL of 3 M NaCl added to the reaction mixture. The solutions were mixed and kept at -20^{0}C for 30-60 min and centrifuged at 12,000 rpm with the supernatant discarded. The precipitate was dried in air and reconstituted in 1000 μL of 0.1 M TEAA (triethylammonium acetate) for chromatographic analysis. The isolated fractions were combined and concentrated in a rotary evaporator. The excess TEAA was removed by drying in high vacuum (<0.01 mm Hg) at room temperature.

*Preparation of DBMB1-**Pc2***. Stock solution of **Pc2** (20 mM) was prepared in methanol. Labeling buffer (100 mM sodium acetate, pH 5.5) was prepared by dissolving the appropriate amount of sodium acetate in water and adjusting the pH using acetic acid and/or NaOH. Sodium borocyanohydrate ($NaCNBH_3$) solution (10 mM) was prepared by dissolving the appropriate amount of the salt in methanol. The oligonucleotide modified with the aldehyde groups at their 5' and 3' ends was obtained from Trilink Biotechnology (San Diego, CA). The oligonucleotide was purified by ethanol precipitation prior to the conjugation reaction. The purified oligonucleotide was re-dissolved in water to yield a solution with a concentration of ~5 mM.

The reaction mixture included (in order of addition): Labeling buffer (50 μL), oligonucleotide (4 μL, 20 nmol), **Pc2** (50 μL, 10 μmol), and $NaCNBH_3$ solution (10 μL, 100 μmol). The reaction was incubated at room temperature overnight. Labeled oligonucleotides were purified by ethanol precipitation to remove excess unreacted dye as described above. The precipitate was dried on air and reconstituted in 100 μL of 0.1 M TEAA (triethylammonium acetate) for chromatographic analysis and further purification. The isolated fractions were combined and concentrated in a rotary evaporator. The excess TEAA was removed by drying in high vacuum (<0.01 mm Hg) at room temperature.

High Performance Liquid Chromatography (HPLC)

The HPLC separations were performed using a JASCO (Easton, MD) 2000-series HPLC equipped with a quaternary gradient pump, autosampler, and fluorescence and diode-array detectors. The analytical column (Zorbax C18, 5 μm 4.6 mm ×150 mm) was purchased from Agilent Technologies (Santa Clara, CA). The following gradient resulted in the best separation between unlabeled oligonucleotide, mono-labeled oligonucleotide, double-labeled oligonucleotide (dimerization-based molecular beacon, DBMB) and unreacted **Pc1**: Flow rate = 1.0 mL/min; Mobile phase – gradient from 0.05M TEAA to 80/20 MeOH/THF; Gradient: Initial hold at 95% TEAA/5% MeOH/THF for 5 min, ramp to 5% TEAA/95% MeOH/THF in 30 min, hold for 5 min and 15 min wash with 100% MeOH/THF at 1.5 mL/min. The column was allowed to equilibrate at the initial mobile phase conditions for 20 min before the next injection. The fluorescence detector was set to an excitation wavelength of 677 nm and the emission wavelength was set to 687 nm, the corresponding maxima for Pc's absorption and emission.[3] A representative chromatogram is presented in Figure S1.

Absorption and fluorescence measurements

All absorption spectra were acquired using an Ultrospec 4000 spectrophotometer (Pharmacia Amersham Biosciences, Piscataway, NJ) or Cary 50 UV-Vis (Varian, Palo Alto, CA) with 10 mm path length quartz cuvettes. Emission spectra were acquired using a FLUOROLOG-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) equipped with a 450 W xenon lamp and a cooled Hamamatsu R928 photomultiplier operated at 900 V in the photon-counting mode. All spectral measurements were performed under ambient conditions within 3 h of solution preparation.

DBMB - target hybridization

The hybridizations of DBMBs with target DNAs were performed in a buffer containing 10 mM Tris-HCl, 4 mM $MgCl_2$ and 15 mM KCl at pH 8.5. The solution containing both MB and buffer was heated to 80^0C to allow complete stem melting, held at 80^0C for 30 min and then slowly cooled to room temperature.

DNA sequences used as fully matched and single base mismatch templates

The sequences used as a random DNA target and as a target for SNP detection along with the loop recognition sequence are summarized in the Table below.

	5' – 3' sequence
Loop Sequence of DBMB1	GAGTCCTTCCACGATACCA
Random template	ACTGGCCGTCGTTTTAC-(T_{12})-AACGTCGTGACTGGGAA
SNP template*	(($T)_{20}$TGGTATCGTCGAAGGACTCGTCAG($T)_{20}$

* Underlined base indicates the SNP site where "G" is replaced with "C" in the SNP template.

The random DNA sequence was equivalent to bases 6291-6324 of the M13mp18 phage cloning vector with a T_{12} insert between bases 6307 and 6308. The sequence for the DBMB loop was a specific probe to exon 6 of the human GAPDH gene.[4] Also shown in this table is the sequence of the SNP template with the location of the mismatch noted as the underlined base.

DBMB identification

After DBMB synthesis, the reaction mixture was separated by HPLC. The separation products were detected by absorption (photodiode array) or fluorescence detection. The possible components of the mixture that would demonstrate absorption signals include: Unreacted

oligonucleotide, unreacted Pc, mono-labeled Pc-oligonucleotide conjugation product, and double-labeled Pc-oligonucleotide conjugation product. The corresponding controls (e.g. unreacted oligonucleotide and unreacted Pc) were analyzed to allow determination of the elution patterns of those compounds. Thus, the post-reaction mixture chromatogram contained (besides unreacted Pc and oligonucleotide) two reaction products – "product peak 1" and "product peak 2" (see Figure S1). It should be noted that "product peak 1" does not produce any fluorescence emission signal, but "product peak 2" did display a fluorescence emission signal. The absorption spectra of product peaks 1 and 2 were extracted from the photodiode array data and are presented in Figures S2 and S3, respectively. Major conclusions based on the appearance of these spectra include: (i) Both compounds possessed characteristics for oligonucleotide absorption at approximately 260 nm and characteristics of Pc absorption at approximately 680 nm; (ii) the spectrum for "product peak 1" indicates a higher degree of Pc aggregation (band at 680 nm is split and shifted to the blue) compared to "product peak 2". Evidence of Pc aggregation states in the absorption spectrum for "product peak 1" explains the absence of strong emission from this particular chromatographic band. Thus, the observations presented here result in the conclusion that "product peak 1" is likely the double-labeled Pc-oligonucleotide conjugate (DBMB) while "product peak 2" is the monolabeled product. Additional mass spectral conformation studies are currently underway to confirm this observation.

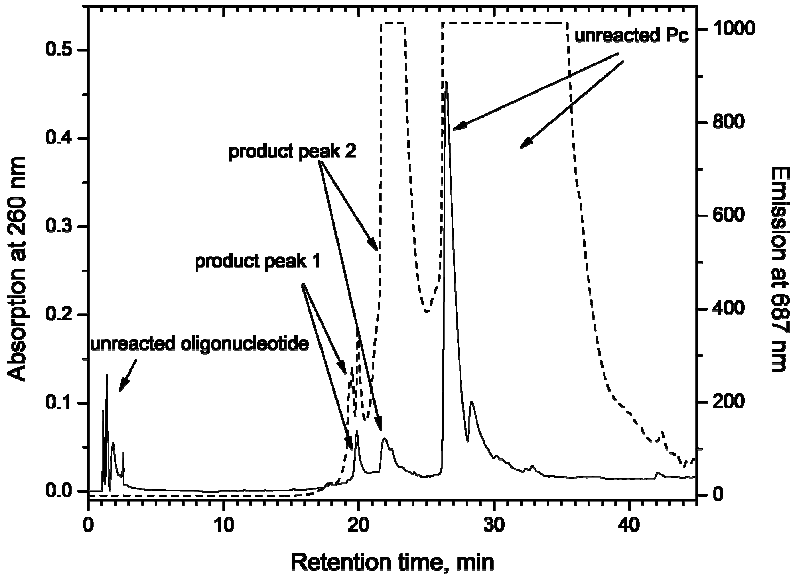


Figure S1. Chromatogram of the reaction mixture (solid: absorption at 638 nm, dash: emission).

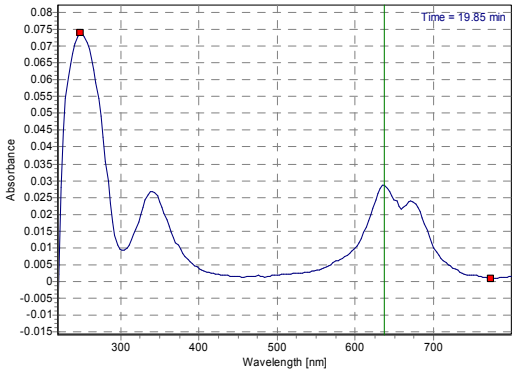


Figure S2. Absorption spectrum for "product peak 1."

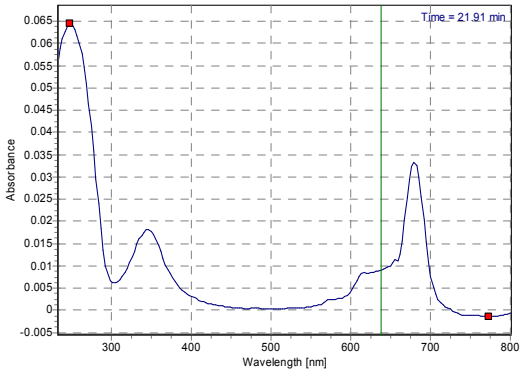
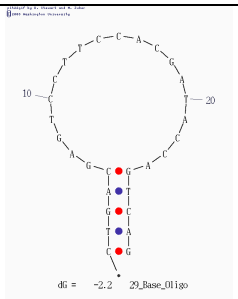
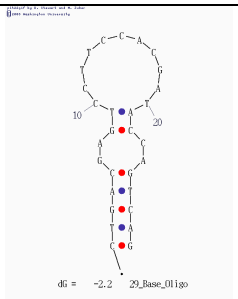
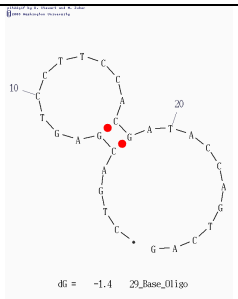


Figure S3. Absorption spectrum for "product peak 2."

DBMB structure in solution

Because the molecular beacon (MB) sequences we employed in these studies have already been successfully utilized in other MB experiments,[5] we suspected that secondary structure artifacts would be inconsequential. The performance of the sequence content of this MB-based structure reported in the literature indicates that the beacon does exist in a thermodynamically favorable "stem and loop" conformation.[5] To verify this, we also modeled potential secondary structure artifacts using *mfold* (www.idtdna.com) and found that the two "stem and loop" conformations (see Structures 1 and 2 in the Table below) possessed almost twice the ΔG's compared to a potential non-"stem-and-loop" structure, indicating that the stem-loop structures would be the favorable conformations.

Parameter	Structure 1	Structure 2	Structure 3
Conformation			
Δ G, kcal mole^{-1}	-2.23	-2.17	-1.35
Description	Stem-and-loop structure		

Sticky-end pairing evaluations

The sticky end formation mechanism with respect to MBs and their targets has been described recently by Li and Tan[6] and is schematically depicted in Figure S4. If a MB is hybridized to a complementary DNA target of approximately similar length as the beacon's loop, there is a possibility of the opposite stem fragments from different hybrids interacting between each other forming rather stable structures. In those structures, the Pc moieties would potentially be

brought into close proximity and could potentially dimerize, causing loss of fluorescence emission due to H-type dimer formation. We assumed that those sticky end structures in Pc-modified DBMB have increased stability due to additional interactions between Pc's. Therefore, to circumvent this artifact, the model studies employed DNA targets with lengths longer than the MB's loop length, which is typically performed in most MB applications.

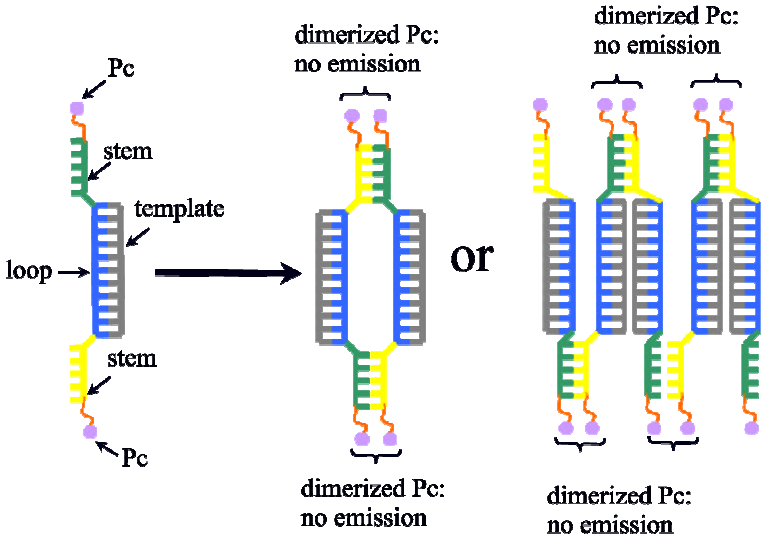


Figure S4. Sticky end formation mechanism of DBMB with short complementary DNA targets.

DBMB characterization

The absorption spectrum of DBMB was recorded (see Figure S5) and compared to a typical absorption spectrum of an oligonucleotide end-labeled with a single Pc molecule. The differences observed in the spectra of DBMB and the singly-labeled oligonucleotide indicated increased degree of dimerization (Q-band is shifted to the blue) compared to the mono-labeled oligonucleotide. The differences in absorption properties can also be observed with the naked eye (see Figure S6) – the colors of mono- and double-labeled oligonucleotides differ despite the use of the same dye (**Pc1**) for labeling in both cases.

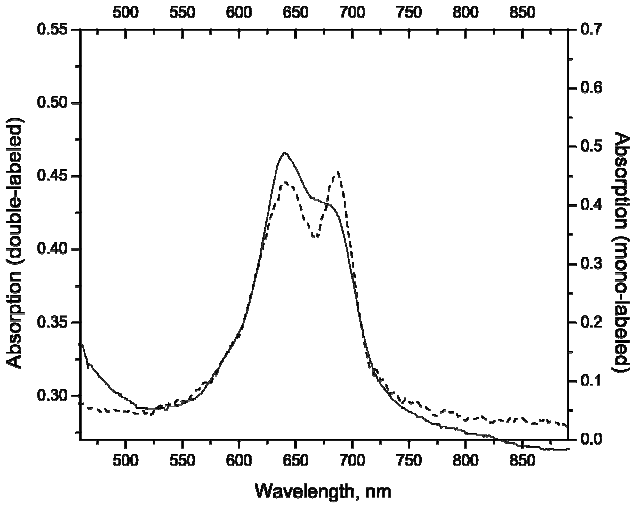


Figure S5. Absorption spectra of DBMB (solid) dual-labeled with **Pc1** and a 17-bp oligonucleotide end-labeled with a single molecule of **Pc1** (dashed line).

Figure S6. Solution of DBMB labeled with two molecules of **Pc1** (right) and a 17-bp oligonucleotide labeled with a single molecule of **Pc1** (left). Both dye/oligonucleotide conjugates are at a concentration of ~10 μM.

SNP detection

The ability of DBMB1-**Pc1** to detect a SNP has been demonstrated by comparing the response in the presence of complementary DNA (T3) to the response in the presence of a single-base mismatched DNA ($(T)_{20}$TGGTATCGT**C**GAAGGACTCGTCAG$(T)_{20}$, location of mismatch is in bold). The results (Figure S7) indicated 5-times higher signal-to-background ratio for complementary DNA in comparison with the 1 base mismatched DNA. While the perfectly matched template versus single base mismatch template hybridization to the DBMB resulted in only an approximate 5-fold difference in the signal-to-background ratio, replacing **Pc1** with **Pc2**

in the DBMB as well as designing the loop sequence for SNP detection near the stem will significantly improve the discriminating power of this DBMB for SNP detection.

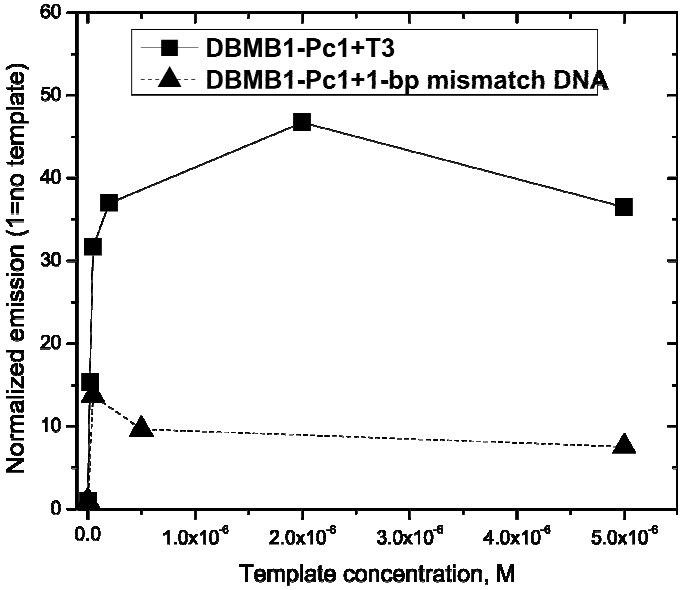


Figure S7. Changes in emission of DBMB1-**Pc1** (~200 nM) upon addition of various amounts of complementary target, T3 or 1-bp mismatched DNA.

DBMB1 and DBMB2 open vs. closed form fluorescence

The emission from the "open" (excess of the MB loop complementary target) and "closed" (no complementary template added) forms of DBMB1-**Pc1** and DBMB2-**Pc1** are shown in Figure S8. In addition, an expanded view of the closed forms is also shown for both of these constructs. For the longer linker, there is a higher level of fluorescence in the absence of the MB's complementary sequence indicating not as efficient quenching resulting from **Pc1** dimerization as seen for the case of DBMB2-**Pc1**, which possesses a shorter linker structure. This observation could be explained by entropic effects with the longer linkers allowing less ground state interactions between the **Pc** dyes due to a larger number of ground state conformations. However, inspection of Figures S8(a) and S8(b) seem to indicate that the fluorescence yield in the "open" form for DBMB1-**Pc1** is nearly 3-fold higher compared to

DBMB2-**Pc1**. This observation could be explained by quantum yield differences for **Pc1** mitigated by linker effects, in which the identity of the linker structure affects the fluorescence quantum yield of the dye or the shorter linker provides higher proximity of the dyes to the duplexed oligonucleotides that may affect **Pc1**'s quantum yield. Conversely, the higher degree of dimerization for the shorter linker structures afforded by DBMB2-**Pc1** may compete favorably with the complement's hybridization to the loop structure of the MB and as such, the number of "open" forms of the MB is less for DBMB2-**Pc1**, giving rise to reduced fluorescence yields.

If we use the nearest-neighbor thermodynamic model[7] adjusted for the sodium concentration used in the hybridization buffers to calculate ΔG for the duplex formed between the MB's loop structure and its complement, K_d ~1×10^{-11} M. While we did not determine the dimerization dissociation constant for **Pc1** directly, literature reports for tetracarboxylated Zn Pc's with a similar structure to **Pc1** indicates that K_d ~9.6×10^{-10} M.[8] While the relative difference in these two K_d values in nearly 100, we should note that DNA duplex stability is typically degraded by the presence of negatively charged dyes appended to the duplex.[9] Therefore, the presence of the two **Pc1** dyes would be expected to reduce the stability of the hybrid formed between the MB loop and its complement, especially in the case of the shorter linker structures used for DBMB2. In addition, the **Pc1** dyes used herein would be expected to possess a higher propensity to aggregate compared to the Zn Pc dyes referenced above based on structural considerations. Therefore, we believe that **Pc1** dimers may interfere with the efficiency of opening the MB causing some to remain in their "closed" form even in the presence of the complement generating less fluorescence. Because the dimerization is more pronounced for DBMB2, its apparent open form fluorescence is less than DBMB1 as observed in Figure S8.

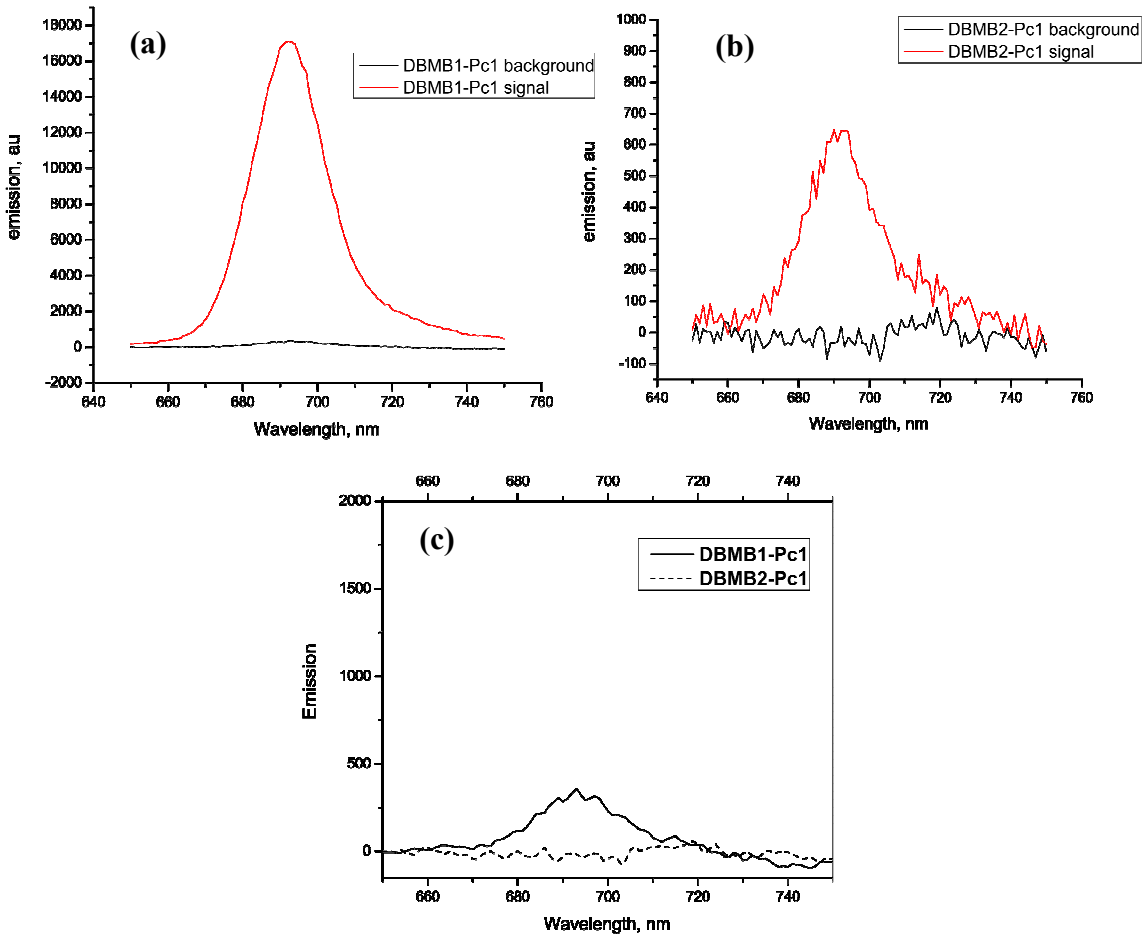


Figure S8. Emission of the "open" forms (complementary DNA to the MB loop added) for DBMB1-**Pc1** (a) and DBMB2-**Pc1** (b). Also shown is an expanded view of the closed forms of both DBMB1-**Pc1** and DBMB2-**Pc1** in the absence of the MB loop's complementary sequence (c). For these measurements, each MB was at approximately 200 nM concentration. In these spectra, the background as measured from a buffer blank was subtracted at each wavelength value for all of the spectra shown in this figure. In these figures, the background refers to the "closed" form of the MB and the "signal" refers to the "open" form of the MB.

References

1. Nesterova, I. V.; Verdree, V. T.; Pakhomov, S.; Strickler, K. L.; Allen, M. W.; Hammer, R. P.; Soper, S. A., *Bioconjugate Chem.* **2007,** 18, (6), 2159-2168.
2. S. Sibel Erdem, Irina V. Nesterova, Brooke Delhomme, Steven A. Soper and Robert P. Hammer, *manuscript in preparation* **2009**.
3. Verdree, V. T.; Pakhomov, S.; Su, G.; Allen, M. W.; Hammer, R. P.; Soper, S. A., *J. Fluoresc.* **2007,** 17, 547-563.
4. Fujimoto, K.; Shimizu, H.; Inouye, M., *J. Org. Chem.* **2004,** 69, (10), 3271-3275.
5. Tsourkas, A.; Behlke, M. A.; Bao, G., *Nucleic Acids Res.* **2002,** 30, (19), 4208-4215.
6. Li, J. W. J.; Tan, W. H., *Anal. Biochem.* **2003,** 312, (2), 251-254.
7. SantaLucia, J., *Proc. Natl. Acad. Sci. U.S.A.* **1998,** 95, (4), 1460-1465.
8. Lebedeva, N. S., *Russ. Chem. Bull.* **2004,** 53, (12), 2674-2683.
9. Randolph, J. B.; Waggoner, A. S., *Nucleic Acids Res.* **1997,** 25, (14), 2923-2929.