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# Supplementary Information

# Multiplex Fluorophore Molecular Beacon; Detection of Target sequence Using Large Stokes Shift and Multiple Emission Signal Properties

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## Solid-phase oligonucleotide synthesis

The phosphoramidite of $\mathbf{A^{py}}$, fluorescein-dT ($\mathbf{dT^{Fam}}$) and TAMRA-dT($\mathbf{dT^{TAMRA}}$) was introduced at the 5'end position to produce fluorescent oligodeoxynucleotides (ODNs) on a controlled-pore glass (CPG) solid support (1-O-Dimethoxytrityl-propyl-disulfide, 1'-succinyl-lcaa-CPG), using a standard phosphoramidite approach and an automated DNA synthesizer (MERMADE DNA-Synthesizer) . The synthesized oligonucleotides were cleaved from the solid support with 30% aqueous $NH_4OH$ (1.0 mL) for 10 h at 55 °C. The crude products obtained from the automated ODN synthesis were lyophilized and diluted with distilled water (1 mL). The ODNs were purified using high-performance liquid chromatography (HPLC; Merck LichoCART C18 column; 10 × 250 mm; 10 µm; pore size: 100 Å). The HPLC mobile phase was isocratic for 10 min (5% MeCN/0.1 M triethylammonium acetate (TEAA) (pH 7.0)) at 2.5 mL/min. The gradient was linearly increased over 10 min from 5 to 50% MeCN/0.1 M TEAA at the same flow rate. The fractions containing the purified ODN were cooled and lyophilized. Subsequently, 80% aqueous AcOH was added to the ODNs. After 1 h at ambient temperature, the AcOH was lyophilized under reduced pressure. The residue was diluted with water (1 mL) and this solution was purified via HPLC using the same conditions as described above.

## Exonuclease III treating method

All DNA samples were diluted with distilled water(40$\mu l$) and Exonuclease III (E. coli) enzyme 2 $\mu l$ were added with 10x NEB Buffer 5 $\mu l$(10x NEB Buffer : 100mM Bis Tris Propane-HCl, 100mM $MgCl_2$, 10mM DTT, pH7.0) and stored at 37 °C for 1 hour.

## Fluorescence and UV/visible spectroscopy experiments

The fluorescence emission spectra for the ODNs were collected at different excitations 386nm, 485nm, 555nm depends on experiment at 25 °C using a quartz cuvette (path length: 1 cm) on a JASCO FP-6500 Cary spectrofluorometer. The UV/visible spectra were recorded using a Cary 100 UV-Vis spectrophotometer (Agilent Technologies) and a quartz cell (path length: 1 cm)

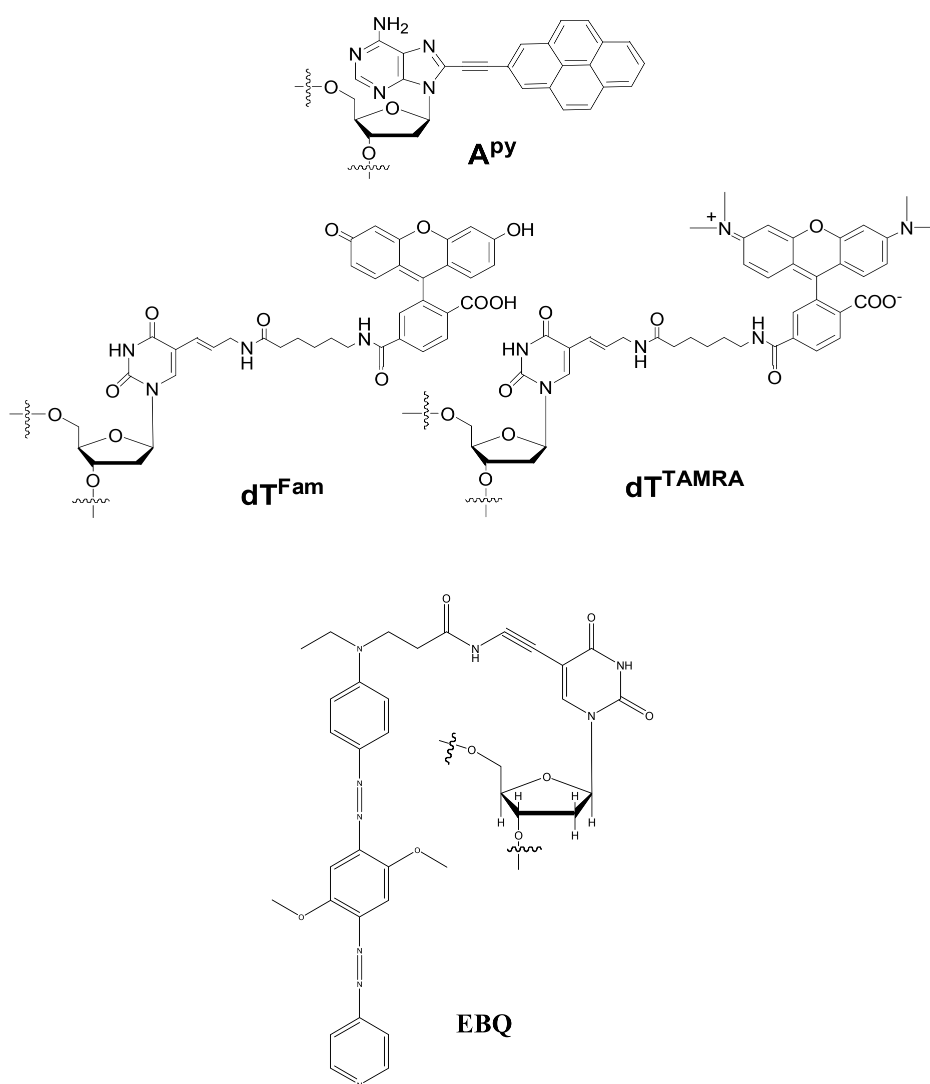



**Figure S1**. Structures of each fluoropores and universal quencher **EBQ**. **EBQ** was provided from Bioneer Company.

| | **absorbance($\lambda_{max}$)** | **emission($\lambda_{max}$)** |
|---|---|---|
| **$A^{py}$** | 386nm | 450nm |
| **Fluorescein-dT ($dT^{Fam)}$** | 485nm | 520nm |
| **TAMRA-dT ($dT^{TAMRA)}$** | 550nm | 580nm |

**Figure S2.** Absorbance($\lambda_{max}$) and emission($\lambda_{max}$) data of used fluorophores **$A^{PY}$**, **$dT^{Fam}$** and **$dT^{TAMRA}$**.

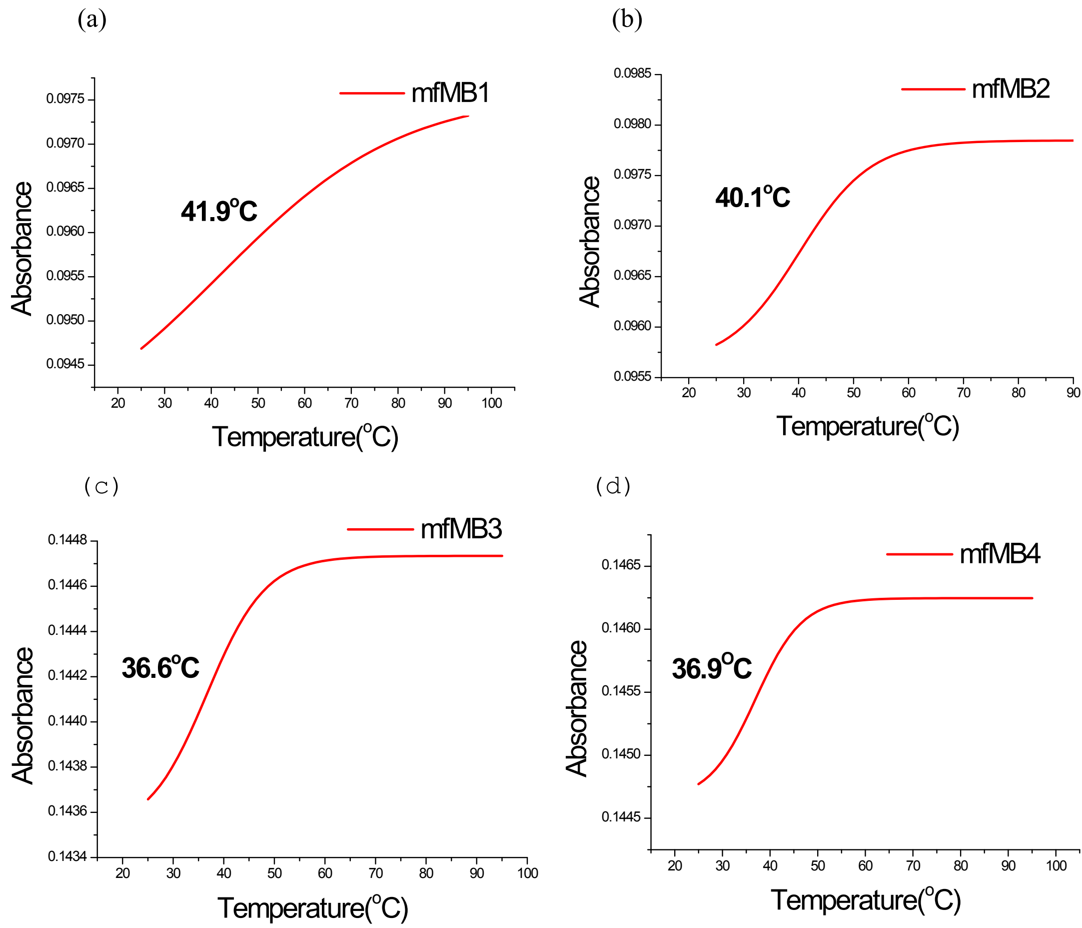



**Figure S3.** Melting temperature of (a)**mfMB1**, (b)**mfMB2**, (c)**mfMB3**,(d)**mfMB4**. All DNA samples were prepared at a concentration of 1$\mu$M in 100mM Tris-HCl buffer at 25°C at PH 7.2(1mM $Mgcl_2$)

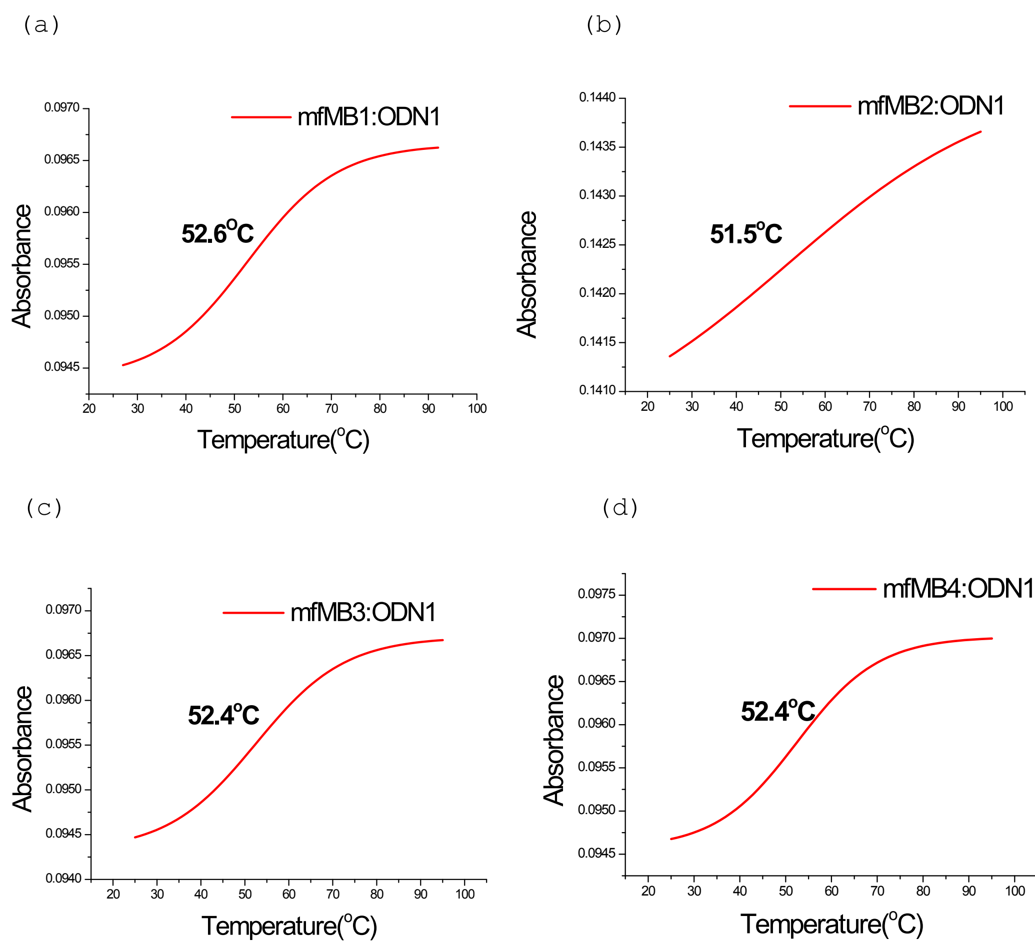



**Figure S4.** Melting temperature of (a) **mfMB1:ODN1,** (b) **mfMB2:ODN1,** (c) **mfMB3:ODN1,** (d) **mfMB4:ODN1.** All DNA samples were prepared at a concentration of 1μM in 100mM Tris-HCl buffer at 25°C at PH 7.2(1mM $Mgcl_2$)

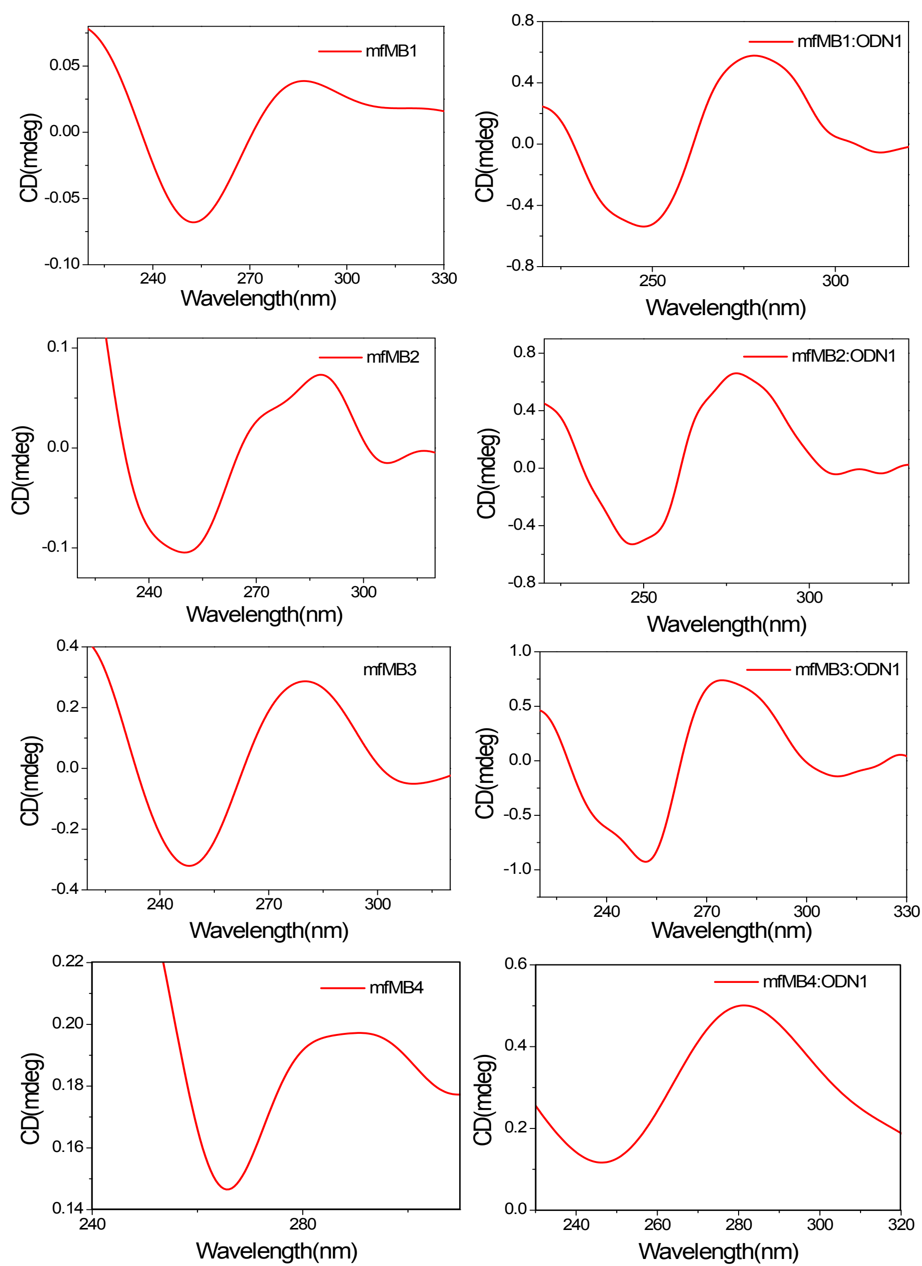
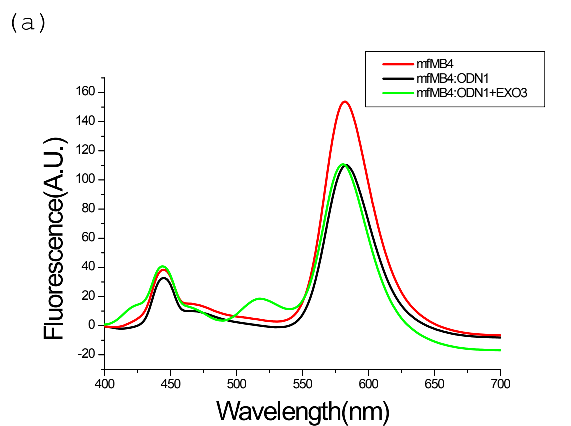



**Figure S5.** Circular dichroism data of **mfMB1, mfMB2, mfMB3, mfMB4** and with **ODN 1.** All DNA samples were prepared at a concentration of 1μM in 100mM Tris-HCl buffer at 25°C at pH 7.2(1mM $_{MgCl2}$).

(a)




(b)

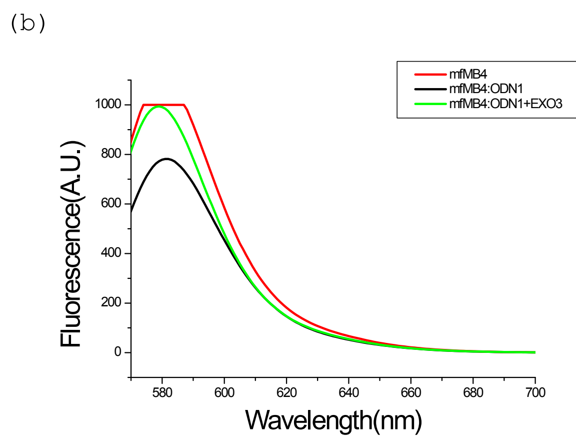



**Figure** S6. Fluorescence data of (a) at 386nm excitation and (b) at 555nm excitation of **mfMB4**, **mfMB4:ODN1** and **mfMB4:ODN1** with exonuclease III. All DNA samples were prepared at a concentration of 1$\mu$M in 100mM Tris-HCl buffer at 25°C at PH 7.2(1mM $MgCl_2$)

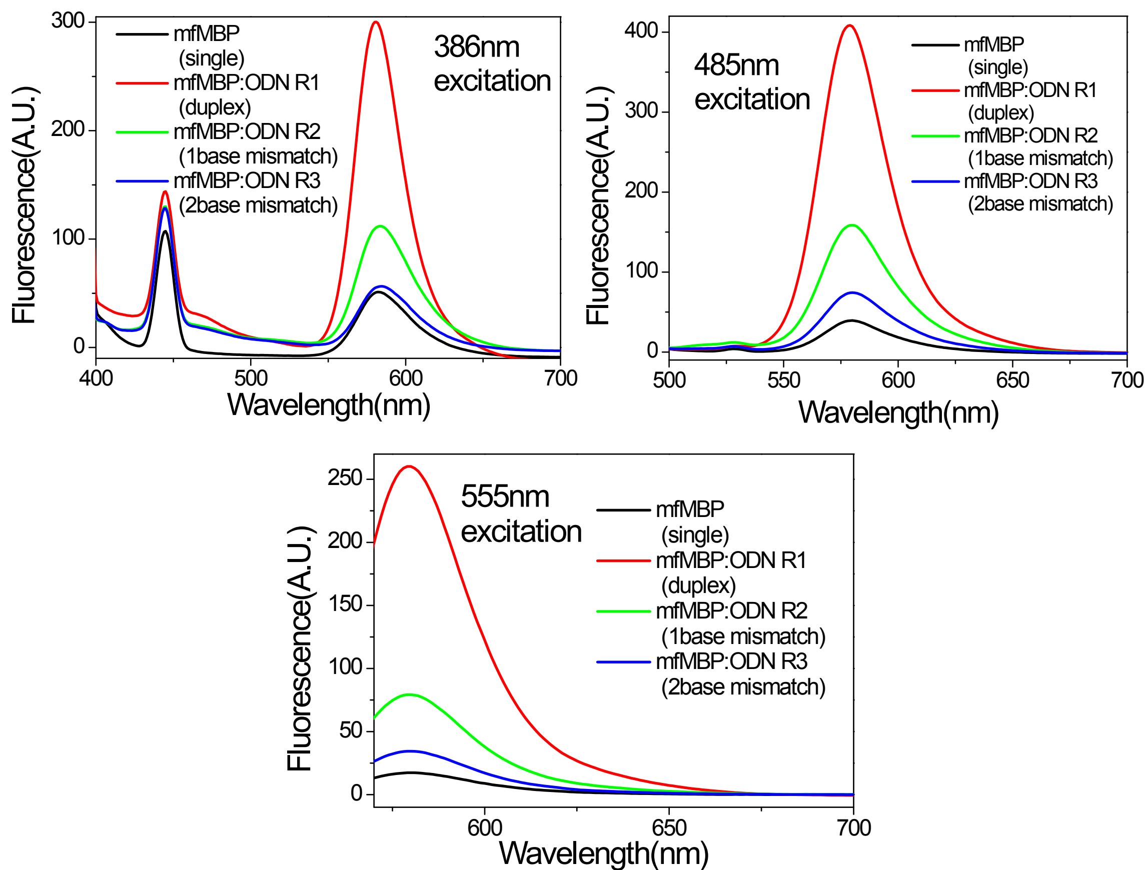



**Figure S7.** Fluorescence data of (a) at 386nm excitation and (b) at 485nm excitation and (c) at 555nm excitation of **mfMBP**, mfMBP:ODN R1(Perfect match), **mfMBP:ODN R2**(1base mismatch), **mfMBP:ODN R3**(2base mismatch) . All DNA samples were prepared at a concentration of 1$\mu$M in 100mM Tris-HCl buffer at 25°C at PH 7.2(1mM $MgCl_2$)

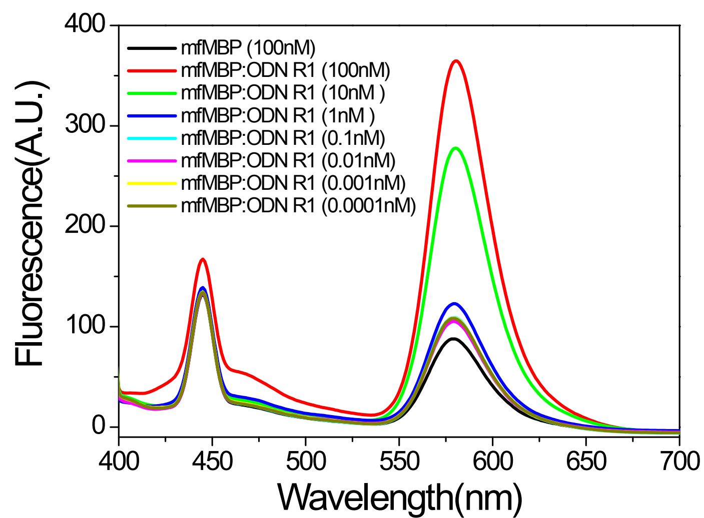



**Figure S8**. Fluorescence data of (a) at 386nm excitation of **mfMB1**(100nM) with different concentration of target sequences **ODN R1**. All DNA samples were prepared at different concentration in 100mM Tris-HCl buffer at 25°C at PH 7.2(1mM $MgCl_2$).

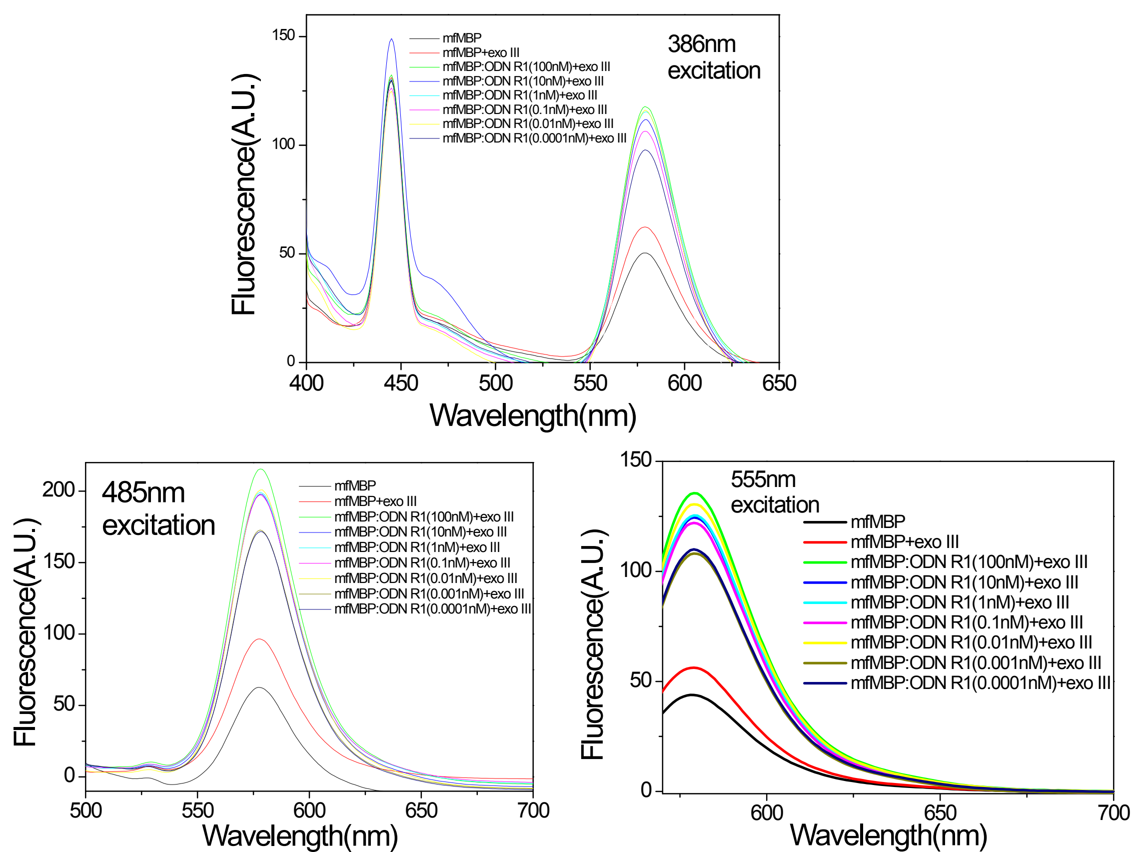



**Figure S9.** Fluorescence data of (a) at 386nm excitation and (b) at 485nm excitation and (c) at 555nm excitation of **mfMBP** with different concentration of target sequences **ODN R1** in exonuclease III. All DNA samples were prepared at different concentration in 100mM Tris-HCl buffer at 25ºC at PH 7.2(1mM $MgCl_2$).

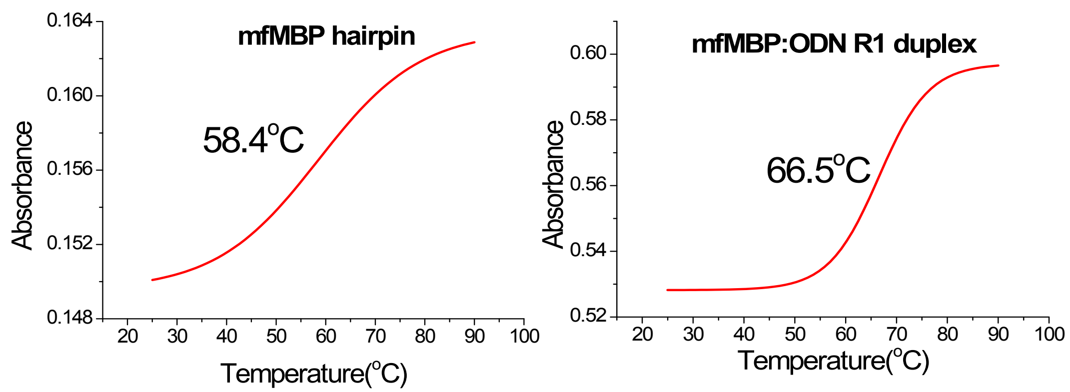



**Figure S10.** Melting temperature of **mfMBP** and **mfMBP:ODN R1** duplex. All DNA samples were prepared at a concentration of 1$\mu$M in 100mM Tris-HCl buffer at PH 7.2(1mM $MgCl_2$)