Thermal melt of the 2bp CPB. We performed a thermal melt of the 2bp construct using a Varian Cary Eclipse Fluorimeter with a temperature gradient of 1° C/min (Fig. SI1). The CPB was at a concentration of 20 nM, and was excited at 480 nm and monitored as fluorescence intensity at 515 nm. An apparently cooperative transition was observed with a midpoint of approximately 290K. After the completion of this apparently cooperative transition (above ~295K) the fluorescence intensity increases monotonically.

The fluorescence intensities (F) were fitted to a standard two state thermal melt with a sloped upper baseline:

$$F = F_f + \frac{F_u + \alpha_u T - F_f}{1 + e^{-(\Delta H - T\Delta S)/RT}} \qquad \text{SI1}$$

Where F_f and F_u are the fluorescence of the fully "folded" and "unfolded" stem loop respectively, ΔH and ΔS are the enthalpy and entropy of stem-loop formation and R and a_u are the gas constant and the slope of the upper baseline respectively. The best fit curve produces estimates of ΔH and ΔS of 412 kJ/mol and 1.43 kJ/mol.K respectively. These values, which are approximately half those of a typical 4bp stem-loop DNA molecular beacon[1], indicate that the free energy of folding is approximately -7.1 kJ/mol (corresponding to an equilibrium constant of ~20) at the temperature employed in our studies.

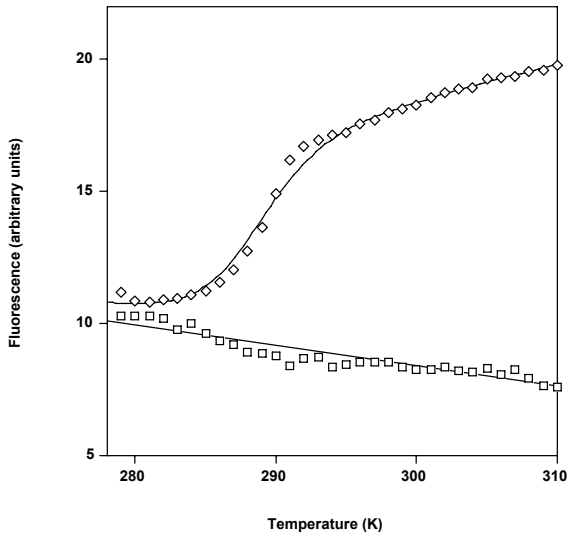

Figure SI1: A thermal melt of target-free 2bp CPB (diamonds) exhibits a cooperative transition with a melting temperature of 288K, above which a linear increase in fluorescence is observed. This differs significantly from the behavior of tryptophan-quenched Bodipy (squares), the fluorescence of which decreases monotonically with increasing temperature. The solid curve represents a fit of the thermal melt to standard two state thermal transition with a sloped upper baseline, as given by equation SI1.

The gain observed upon thermal melting is poorer than the nearly 3-fold gain observed upon target binding. Comparison of the absolute fluorescence of the "folded," unbound state at low

temperature and the thermally melted state above 295K with those of the bound states at low temperatures suggests that this lower gain stems from excessive quenching in the latter; the thermally unfolded state at, for example, 295K, is significantly less emissive than the target-bound CPB at 283K (Fig. 2). This suggests in turn that, after the completion of the cooperative melting of the PNA stem, the thermally unfolded state remains relatively compact, enhancing quenching (in contrast to the bound state, in which the two ends of the construct are rigidly separated), as might be expected to occur due to the relatively hydrophobic nature of PNA[2]. The strong, linear increase in fluorescence observed at higher temperatures thus likely represents the non-cooperative disruption of this state. In contrast, the emission of solutions of both free Bodipy (data not shown) and of Bodipy plus free tryptophan (Fig. SI1) decrease with increasing temperature, providing further evidence that the strong, positive slope observed after the cooperative thermal transition reflect the disruption of interactions specific to the thermally-unfolded CPB.

Saturable binding of the 3bp CPB. The fluorescence of the 3bp CPB does not increase as much as that of the 2bp CPB upon binding to the target antibody. Titration studies (Fig. SI2) suggest that this discrepancy does not reflect a significantly poorer binding affinity for the 3bp construct: while the sensor exhibits saturable binding the observed gain remains limited even under saturating conditions. Instead the poor gain of the three-base-pair CPB apparently arises because its longer stem supports Bodipy/tryptophan collisional quenching even when complexed with the target antibody, an argument that is supported by the observation that the quantum yield of both the bound and unbound 3bp construct are similar to that of the unbound 2bp construct (Fig. 2).

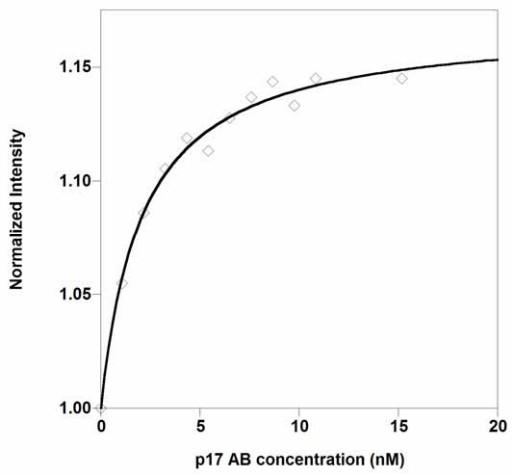

Figure SI2: A titration of the 3bp CPB illustrates its relatively poor gain. Even at saturating concentrating conditions, fluorescence only increases by 15%. Shown here is a titration of the CPB (at 5 nM) with the target antibody. The experiment was conducted at 10° C in 50 mM phosphate buffer, pH 7.4 containing 200 mM NaCl and 200 mM $MgCl_2$.

[1] A. Tsourkas, M. A. Behlke, S. D. Rose and G. Bao, *Nuc. Acid Res.,* 2003, **31**, 1319-1330.

[2] H. Kuhn, V. V. Demidov, J. M. Coull, M. J. Fiandaca, B. D. Gildea and M. D. Frank-Kamenetskii, *J. Am. Chem. Soc.*, 2002, **124**, 1097-1103.