Supplementary Information (SI) for Analyst.

Supplemental Information: Thermodynamic and Modeling Insights of DNA Molecular Beacons with Dual Target Binding to Design for Tunable Fluorescent Outputs

Esther E Stopps and Stephanie E McCalla

Ms. Esther E. Stopps: Department of Chemical and Biological Engineering, Montana State University, Bozeman, Montana, 59717, USA.

Prof. Dr. Stephanie E. McCalla: Department of Chemical and Biological Engineering, Montana State University, Bozeman, Montana, 59717, USA.

Contents

1 Molecular Beacon and Target Sequences

Table S1: Oligonucleotide sequences for all experiments. The molecular beacons (MB) are either *heterotropic* (Het) with two different target sites or *homotropic* (Hom) with two identical target sites. The heterotropic MBs are distinguished by having the *same toeholds* (STH) or *different toeholds* (DTH). The targets are denoted by the location of their complementary toehold on the MB, either at the toehold on the 3' fluorophore side (ToeF) or within the loop at the 5' quencher (LoopQ). Underlined sequences are the target binding sites. Bold, italicized sequences are the toeholds.

MBs	
HetB1-STH	/5'IABkFQ/ GCCCAC ***CTAT*** TAATGACTCT GTGGGC ***CTAT*** /3'6-FAM/
HetB2-DTH	/5'IABkFQ/ GTCCAC ***GCGA*** TAATGACTCT GTGGAC ***CTAT*** /3'6-FAM/
HomB	/5'IABkFQ/ GTGCAC ***CTAT*** TAATGACTCT GTGCAC ***CTAT*** /3'6-FAM/
FAM-Half-HomB	/5'/ ACTCT GTGCAC CTAT /3'6-FAM/
Targets	
TarB1-ToeF	/5'/ ***ATAG*** GCCCAC /3'/
TarB1-LoopQ	/5'/ ***ATAG*** GTGGGC /3'/
TarB2-ToeF	/5'/ ***ATAG*** GTCCAC /3'/
TarB2-LoopQ	/5'/ ***TCGC*** GTGGAC /3'/
TarHomB	/5'/ ***ATAG*** GTGCAC /3'/

2 NUPACK Figures

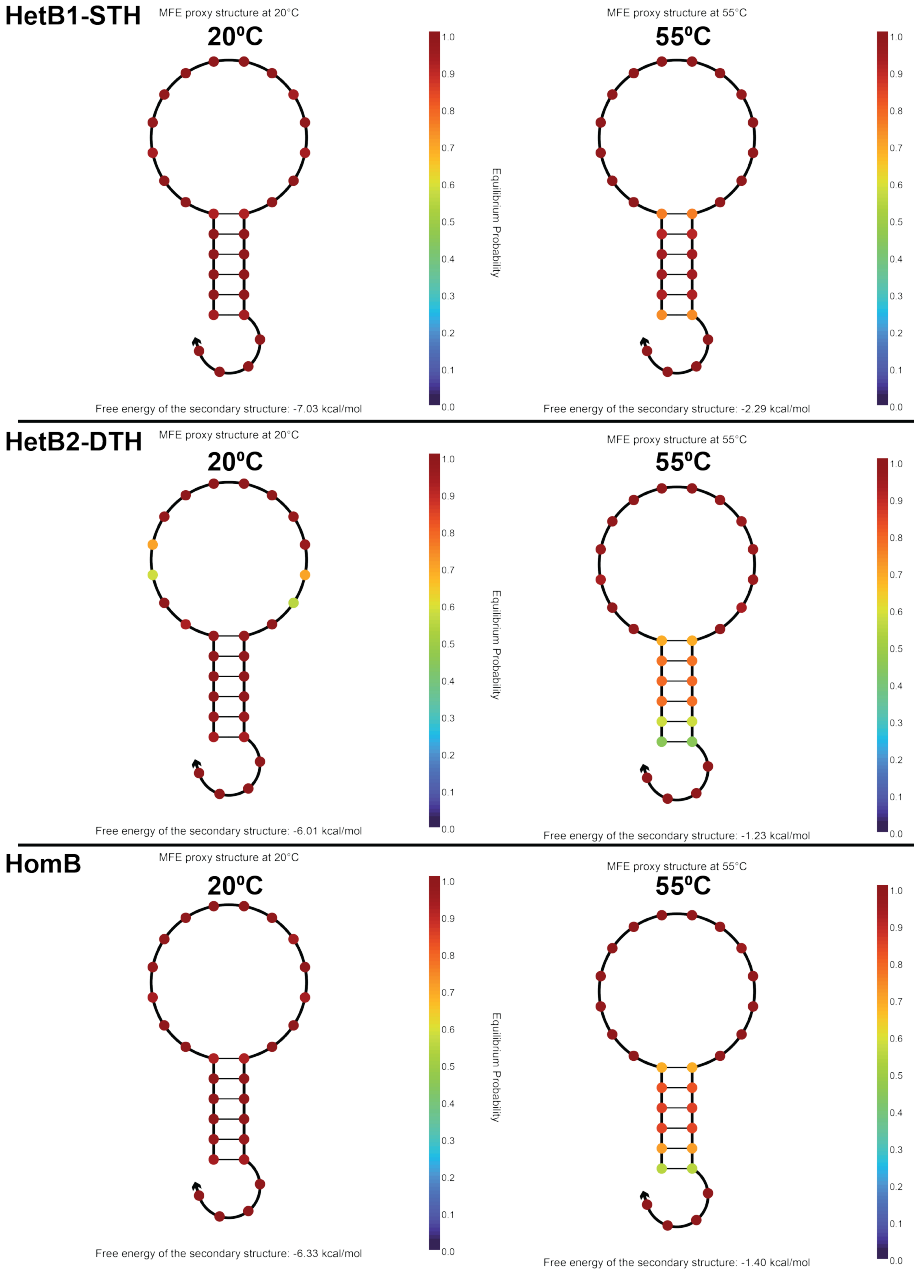

Figure S1: Predicted structures for each molecular beacon at 20 ^oC and 55 ^oC.

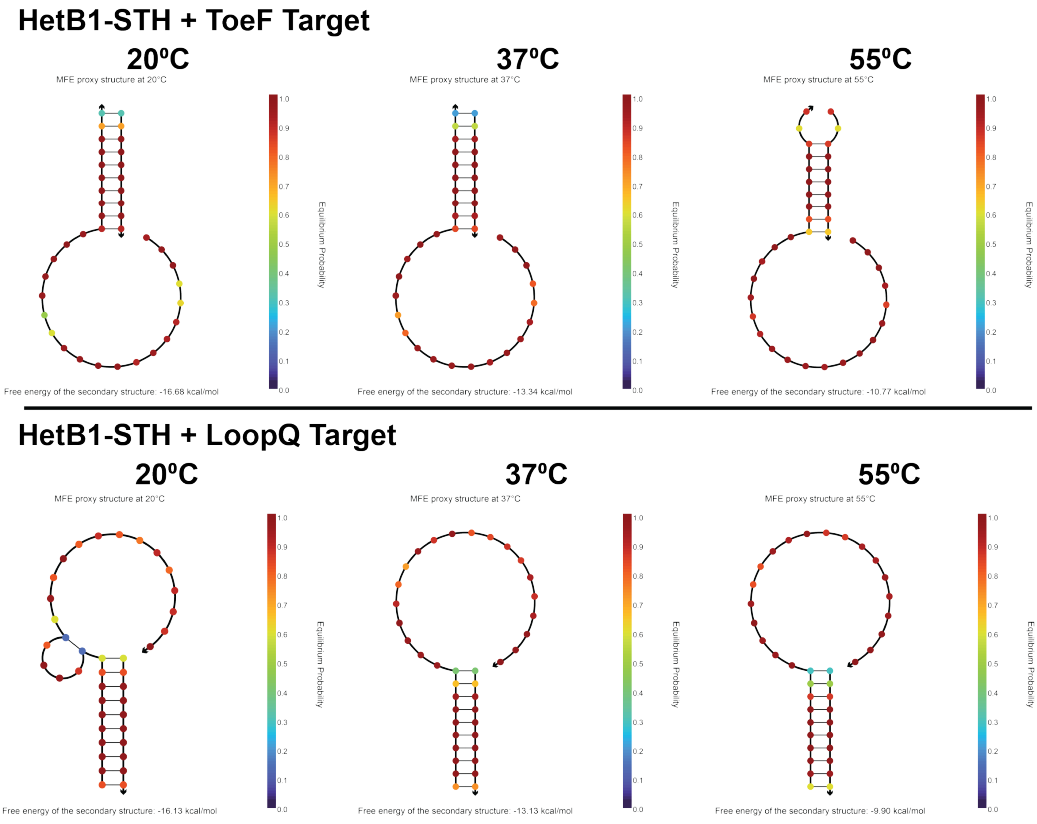

Figure S2: Predicted structures for the first heterotropic molecular beacon with identical binding site toeholds (HetB1-STH) and each target at 20 ^oC, 37 ^oC, and 55 ^oC.

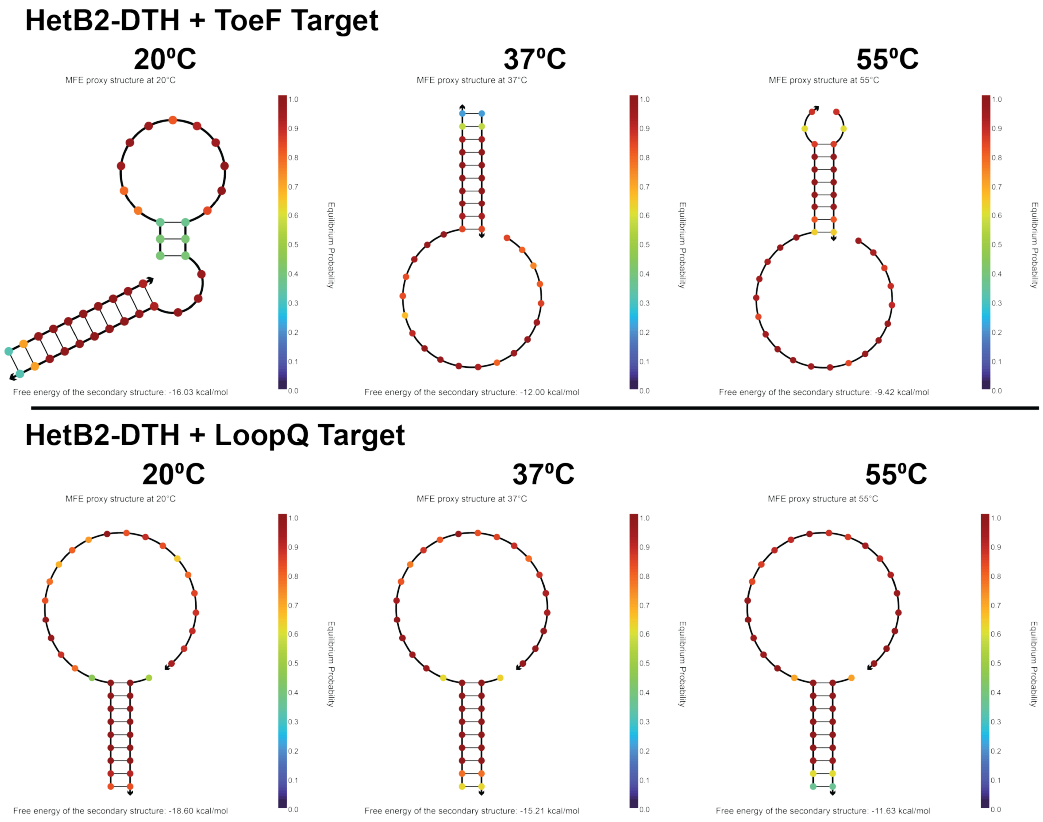

Figure S3: Predicted structures for the second heterotropic molecular beacon with different binding site toeholds (HetB2-DTH) and each target at 20 ^oC, 37 ^oC, and 55 ^oC.

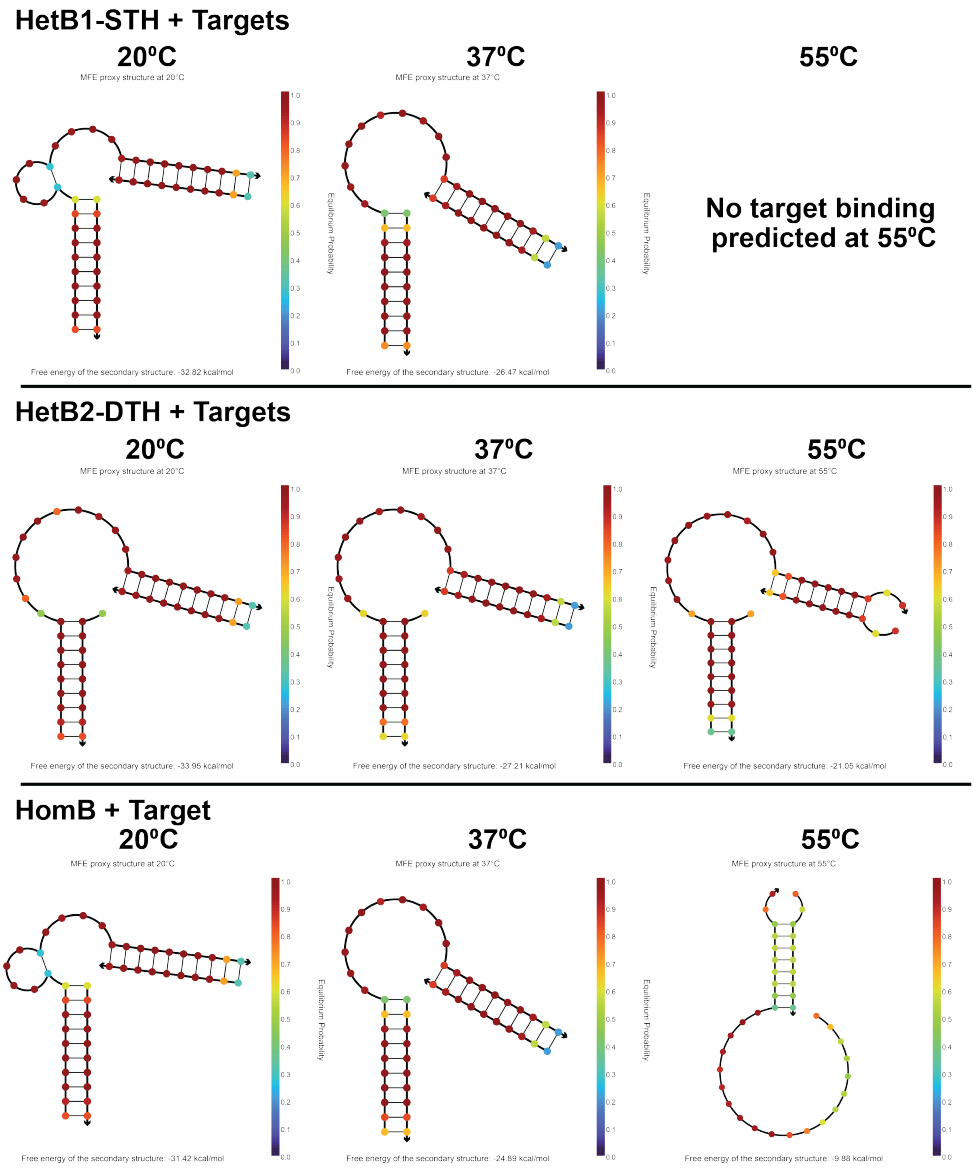

Figure S4: Predicted structures for each molecular beacon with both targets at 20 ^oC, 37 ^oC, and 55 ^oC.

3 Predicted thermodynamic parameters at 20 ^oC

Table S2: Predicted thermodynamic parameters for oligonucleotide secondary structures at 20 ^oC, using NUPACK and DINAMelt software. NUPACK model options were set at dna04.1, "All stacking", $[Na^+] = 0.06\ M$, $[Mg^{++}] = 0.006\ M$, max complex size = 3. DINAMelt parameters were analyzed using the Two-State Folding application with $[Na^+] = 0.06\ M$, $[Mg^{++}] = 0.006\ M$. The Two-State Folding application cannot make predictions for 3-strand complexes (ie Two-Site MBs + Target). *Incomplete binding occurs at the toehold regions.

	NUPACK	**DINAMelt**		
Molecular Beacon (MB)	**ΔG^o** (kcal/mol)	**ΔG^o** (kcal/mol)	**ΔH^o** (kcal/mol)	**ΔS^o** (cal/mol/K)
HetB1-STH	-7.03	-6.9	-47.7	-139.3
HetB2-DTH	-6.01	-5.8	-47.0	-140.4
HomB	-6.33	-6.2	-48.6	-144.8
One-Site MBs + Target	**ΔG^o** (kcal/mol)	**ΔG^o** (kcal/mol)	**ΔH^o** (kcal/mol)	**ΔS^o** (cal/mol/K)
HetB1-STH + TarB1-ToeF	-16.68	-14.3	-75.4	-208.5
HetB1-STH + TarB1-LoopQ	-16.13	-13.6	-67.6	-184.4
HetB2-DTH + TarB2-ToeF	-16.03	-13.0	-74.2	-208.9
HetB2-DTH + TarB2-LoopQ	-18.60	-16.0	-78.4	-212.8
Two-Site MBs + Target	**ΔG^o** (kcal/mol)	**ΔG^o** (kcal/mol)	**ΔH^o** (kcal/mol)	**ΔS^o** (cal/mol/K)
HetB1-STH + TarB1-ToeF/LoopQ	-32.82			
HetB1-STH + TarB1-ToeF/ToeF	-24.35*			
HetB1-STH + TarB1-LoopQ/LoopQ	-23.35*			
HetB2-DTH + TarB2-ToeF/LoopQ	-33.95			
HetB2-DTH + TarB2-ToeF/LoopQ	–			
HetB2-DTH + TarB2-ToeF/LoopQ	–			
HomB + TarHomB	-31.42			
Target Dimerization	**ΔG^o** (kcal/mol)	**ΔG^o** (kcal/mol)	**ΔH^o** (kcal/mol)	**ΔS^o** (cal/mol/K)
TarB1-ToeF	-10.10	-6.5	-38.2	-108.2
TarB1-LoopQ	–	-1.6	-17.4	-53.8
TarB1-ToeF + TarB1-LoopQ	-12.17	-9.5	-50.3	-139.0
TarB2-ToeF	-7.87	-4.4	-39.3	-119.0
TarB2-LoopQ	-11.22	-7.7	-47.6	-136.1
TarB2-ToeF + TarB2-LoopQ	-10.69	-8.0	-49.2	-140.5
TarHomB	-11.47	-8.4	-51.2	-145.9

4 Melting temperatures for each hybridized structure

NUPACK provides melting data as a fraction of unpaired bases at equilibrium over temperature. For each hybridized structure, the melting temperature is taken at the point where 50% of the complementary bases are unbound. For the molecular beacons (MBs) with a 6 nucleotide (nt) complementary region (12 nt involved in hybridizing), this is at NUPACK's 80% unpaired bases. This is calculated by dividing the number of unbound nt in one MB at 50% hybridized by the total nt:

$$\frac{24 \text{ unbound nt}}{30 \text{ total nt}} = 0.8 \tag{1}$$

For the one-site binding, the total number of nt from the MB and one target is 40 nt. Only 20 nt are involved in hybridization, so at 50% hybridized there are there are 10 nt bound and 30 nt unbound. Therefore, 30 unbound nt/40 total nt = 0.75. Similarly, for two-site binding, the total number of nt from the MB and two targets is now 50 nt with 40 nt involved in hybridization. At 50% hybridized there are there are 20 nt bound and 30 nt unbound, and 30 unbound nt/50 total nt = 0.6. Supplemental Figure S5 shows the fraction of unpaired bases at equilibrium for the unbound MBs (left), one-target bound MBs (middle), and two-target bound MBs (right) from NUPACK. Inset graphs detail how the melting temperatures were determined.

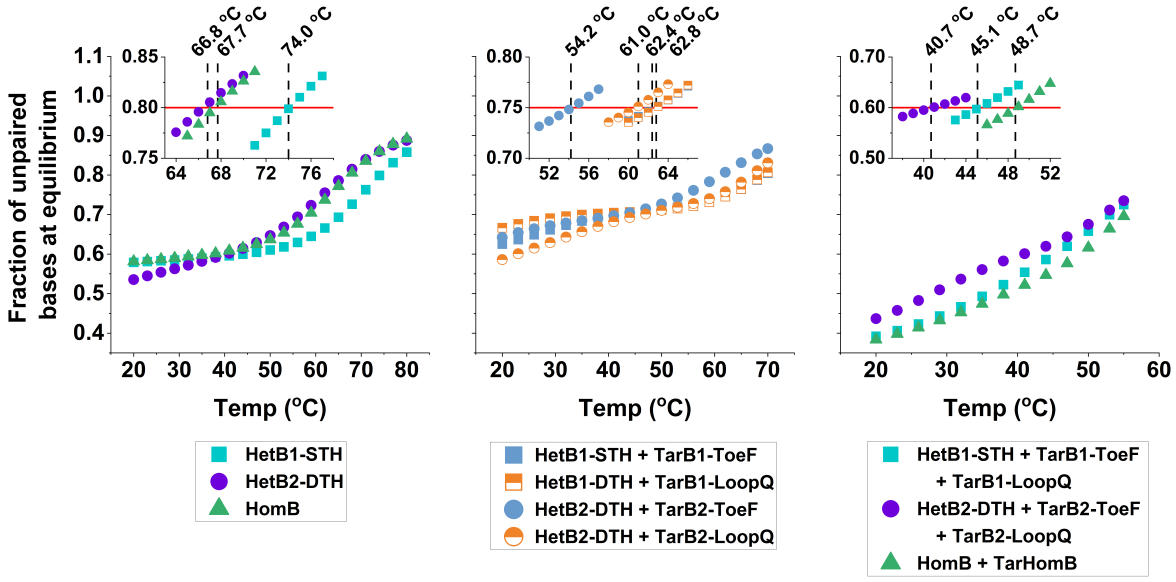

Figure S5: NUPACK predicted fractions of unpaired bases at equilibrium for the unbound MBs (left), one-target bound MBs (middle), and two-target bound MBs (right), with melting temperatures taken at 80% unpaired, 75% unpaired, and 60% unpaired, respectively (inset graphs).

For the target dimers, the number of complementary bases varies for each sequence. Table S3 details the total nt present for each structure (Total nt) and how many are involved in binding (100% bound and 50% bound) to calculate the fraction of unpaired bases from NUPACK predictions that should be used to calculate the melting temperature.

Supplemental Table S4 compares melting temperatures from NUPACK predictions, DINAMelt software, and emperical measurements.

Table S3: Calculation details for the fraction of unpaired bases from NUPACK predictions that should be used to calculate the melting temperature. MB = molecular beacon.

MB Structures	**Total nt**	**100% nt**	**50% nt**	**NUPACK fraction**
MB	30	12	6	$(30-6)/30 = 0.8$
MB + One Target	40	20	10	$(40-10)/40 = 0.75$
MB + Two Targets	50	40	20	$(50-20)/50 = 0.6$
Target Dimers				
TarB1-ToeF	20	8	4	$(20-4)/20 = 0.8$
TarB1-LoopQ	20	0	–	–
TarB1-ToeF + TarB1-LoopQ	20	12	6	$(20-6)/20 = 0.7$
TarB2-ToeF	20	8	4	$(20-4)/20 = 0.8$
TarB2-LoopQ	20	8	4	$(20-4)/20 = 0.8$
TarB2-ToeF + TarB2-LoopQ	20	12	6	$(20-6)/20 = 0.7$
TarHomB	20	12	6	$(20-6)/20 = 0.7$

Table S4: Predicted and experimental melting temperatures for oligonucleotide secondary structures comparing NUPACK, DINAMelt, and experimental results. NUPACK model options were set at "All stacking", $[Na^+] = 0.06\ M$, $[Mg^{++}] = 0.006\ M$, max complex size = 2. DINAMelt parameters were analyzed using the Two-State Folding application with $[Na^+] = 0.06\ M$, $[Mg^{++}] = 0.006\ M$. The Two-State Folding application cannot make predictions for 3-strand complexes (ie Two-Site MBs + Target). For all experimental data n = 4 except for *n = 16. A TarHomB oligonucleotide modified with a FAM molecule on the 5' end was used to experimentally measure the dimerization melting temperature; however, we expect that this is a higher T_m than for the unmodified TarHomB molecule due to the stabilizing effects of the fluorophore.

	T_m (^{o}**C**)		
Molecular Beacon (MB)	**NUPACK**	**DINAMelt**	**Experimental**
HetB1-STH	74.0	69.3	74.5 ±0.247*
HetB2-DTH	66.8	61.6	69.7 ±0.274*
HomB	67.7	62.8	68.7 ±0.271
One-Site MBs + Target			
HetB1-STH + TarB1-ToeF	62.4	50.8	34.1 ±0.537
HetB1-STH + TarB1-LoopQ	62.8	50.9	34.9 ±0.430
HetB2-DTH + TarB2-ToeF	54.2	45.0	33.9 ±0.465
HetB2-DTH + TarB2-LoopQ	61.0	57.6	45.4 ±0.437
Two-Site MBs + Target			
HetB1-STH + TarB1-ToeF/LoopQ	45.1	–	48.0 ±0.407
HetB2-DTH + TarB2-ToeF/LoopQ	40.7	–	50.1 ±0.421
HomB + TarHomB	48.7	–	48.0 ±0.0904
Target Dimerization			
TarB1-ToeF	36.5	21.5	no data
TarB1-LoopQ	–	-42.1	no data
TarB1-ToeF + TarB1-LoopQ	38.9	34.9	no data
TarB2-ToeF	17.3	6.6	no data
TarB2-LoopQ	44.6	28.9	no data
TarB2-ToeF + TarB2-LoopQ	27.7	25.4	no data
TarHomB	38.0	32.8	40.8** ±0.852

5 Hysteresis

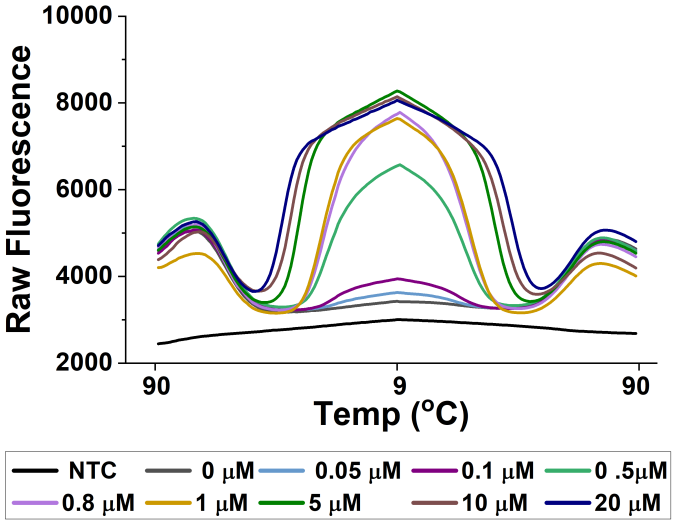

Figure S6: Raw fluorescent trace for one full experiment with the homotropic molecular beacon (HomB) and target (TarHomB), starting at 90 ^oC, cooling to 9 ^oC, and heating again to 90 ^oC at a rate of 1 ^oC per 10 minutes. The cooling and heating halves of the experiment result in nearly identical fluorescent curves, demonstrating a low level of hysteresis due to temperature changes.

6 Fluorescence of FAM depends on temperature

FAM fluorophores are known to have a temperature-dependent fluorescence, espcially in Tris-based buffers. To account for this, we measured the fluorescence of the FAM-Half-HomB oligonucleotide using the same experimental protocol as for the MB melting studies. FAM-Half-HomB is the 15 nucleotide sequence of the HomB molecular beacon (MB) from the 3' end, including the 3' FAM modification. This removed any interfering secondary structure effects of the MB while maintaining the same adjacent nucleotide sequence. The observed fluorescence decreased with temperature (Figure S7A). Two thermocyclers were used in this study, and each exhibited a slightly different response, highlighting the importance of performing these control experiments for each study. From the fluorescence, a correction ratio (Figure S7B was calculated:

$$CR_i = \frac{F_i}{F_{10^oC}} \tag{2}$$

The raw data was then multiplied by the CR values in the data normalization process (Supplemental Section 7).

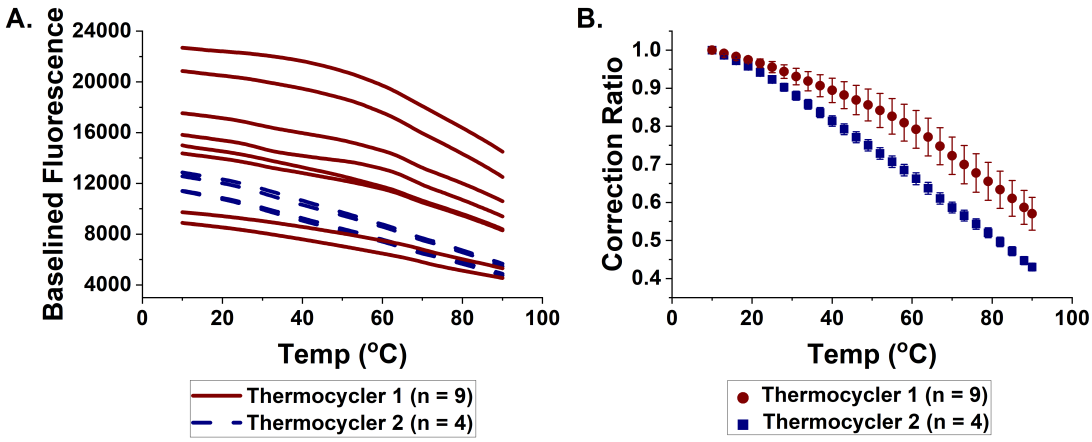

Figure S7: (A) Fluorescence as a function of temperature for each of the two thermocyclers used in this study. As temperature increases, the fluorescence decreases.**(B)** Average correction ratios for each of the two thermocyclers.

7 Data normalization

The experimental data were processed prior to model fitting using the following procedure: (1) average intra-experiment sample replicates (n = 3, SI Figure S8A), (2) baseline the data to the no-template controls (NTC) by subtraction (SI Figure S8B), (3) correct for FAM temperature dependence by dividing the data by the correction ratios (CR) shown in the SI Section 6 (SI Figure S8C), and (4) normalize the data to 1 at 90 ^oC (SI Figure S8D), where the molecular beacon is assumed fully open and unbound. After processing, the data for each experiment was fit to the corresponding model individually.

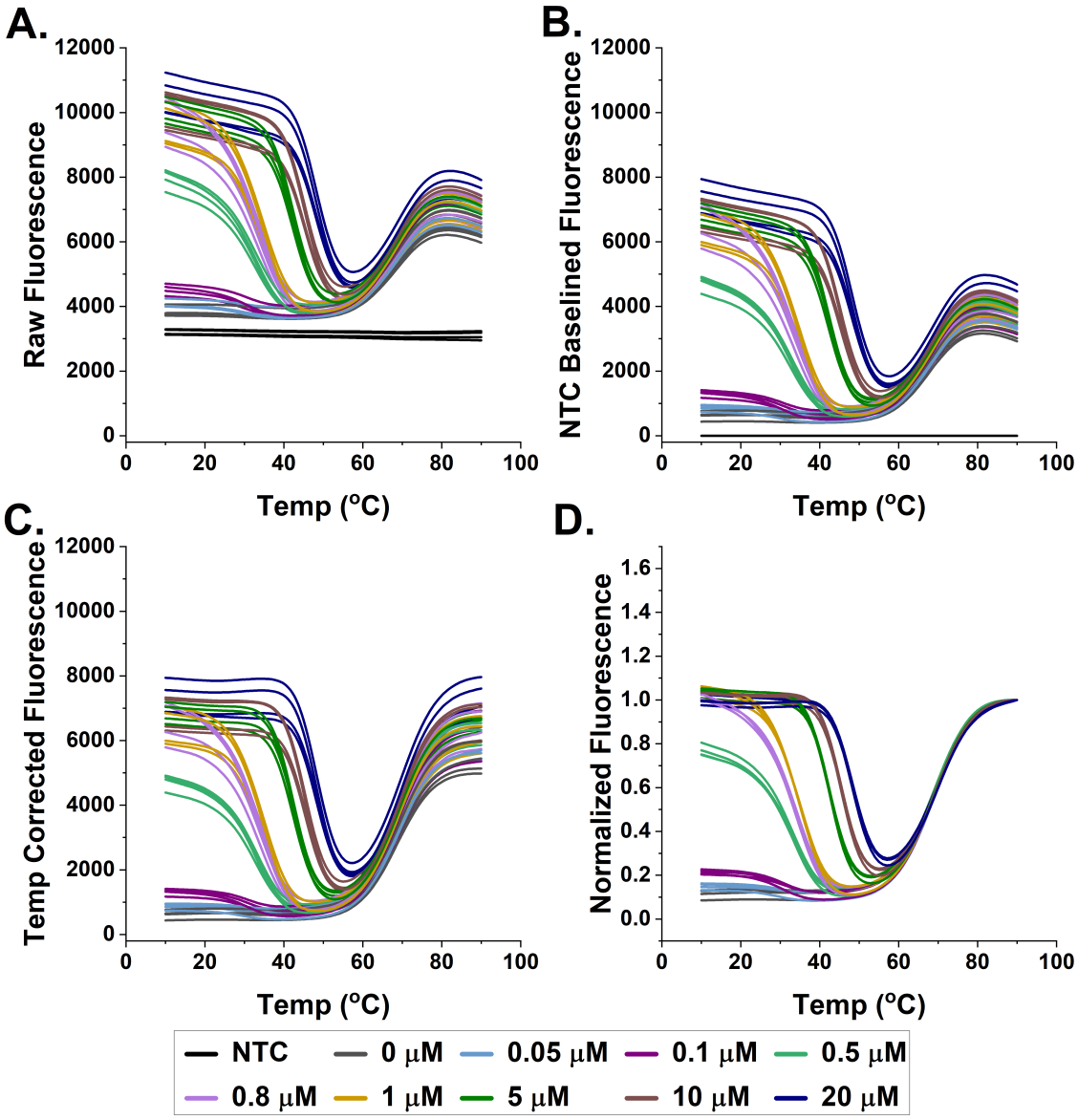

Figure S8: **(A)** Raw fluorescence data for HomB incubated with target for all replicate experiments (n = 4). Each experiment has triplicate sample replicates that are averaged. **(B)** The first normalization step was to baseline the data to the NTC fluorescence. **(C)** The second step was to correct for the temperature dependence of FAM by dividing the fluorescence at each temperature by the correction ratio (CR). **(D)** The final step was to normalize the data to one at 90 ^oC.

8 Detailed algebraic derivations for Two-Site Model Equations 8-11

8.1 Equation 8

$$\frac{[BYY]}{B_0} = \frac{[BYY]}{[BYY] + [BY] + [B_{closed}] + [B_{open}]}$$

Pull out $[BYY]$ *in denominator on the right-hand side (RHS).*

$$\frac{[BYY]}{B_0} = \frac{[BYY]}{[BYY]\left(1 + \frac{[BY]}{[BYY]} + \frac{[B_{closed}]}{[BYY]} + \frac{[B_{open}]}{[BYY]}\right)}$$

Cancel $[BYY]$ *terms and multiply RHS by* $\frac{K_{D,BYY}^{-1}}{K_{D,BYY}^{-1}}$.$K_{D,BYY}$ *is equal to* $\frac{[BY][Y]}{[BYY]}$ *(Equation 4).*

$$\frac{[BYY]}{B_0} = \frac{K_{D,BYY}^{-1}}{K_{D,BYY}^{-1} + \frac{[BY][BYY]}{[BYY][BY][Y]} + \frac{[B_{closed}][BYY]}{[BYY][BY][Y]} + \frac{[B_{open}][BYY]}{[BYY][BY][Y]}}$$

Cancel terms and multiply RHS by $\frac{Y}{Y}$.

$$\frac{[BYY]}{B_0} = \frac{K_{D,BYY}^{-1}}{K_{D,BYY}^{-1} + \frac{1}{[Y]} + \frac{[B_{closed}]}{[BY][Y]} + \frac{[B_{open}]}{[BY][Y]}} * \frac{[Y]}{[Y]}$$

Cancel terms and multiply RHS by $\frac{K_{D,BY}^{-1}}{K_{D,BY}^{-1}}$. $K_{D,BY}$ *is equal to* $\frac{[B_{closed}][Y]}{[BY]}$ *(Equation 5).*

$$\frac{[BYY]}{B_0} = \frac{K_{D,BYY}^{-1}[Y]}{K_{D,BYY}^{-1}[Y] + 1 + \frac{[B_{closed}]}{[BY]} + \frac{[B_{open}]}{[BY]}} * \frac{K_{D,BY}^{-1}}{K_{D,BY}^{-1}}$$

Cancel terms.

$$\frac{[BYY]}{B_0} = \frac{K_{D,BYY}^{-1}K_{D,BY}^{-1}[Y]}{K_{D,BYY}^{-1}K_{D,BY}^{-1}[Y] + K_{D,BY}^{-1} + \frac{[B_{closed}][BY]}{[B_{closed}][BY][Y]} + \frac{[B_{open}][BY]}{[BY][B_{closed}][Y]}}$$

Cancel terms and note that $K_{D,B} = \frac{[B_{open}]}{[B_{closed}]}$.

$$\frac{[BYY]}{B_0} = \frac{K_{D,BYY}^{-1}K_{D,BY}^{-1}[Y]}{K_{D,BYY}^{-1}K_{D,BY}^{-1}[Y] + K_{D,BY}^{-1} + \frac{1}{[Y]} + \frac{K_{D,B}}{[Y]}}$$

Pull out $K_{D,BYY}^{-1}K_{D,BY}^{-1}$ *from the nominator and denominator of the RHS.*

$$\frac{[BYY]}{B_0} = \frac{\left(K_{D,BYY}^{-1}K_{D,BY}^{-1}\right)[Y]}{\left(K_{D,BYY}^{-1}K_{D,BY}^{-1}\right)\left([Y] + K_{D,BYY} + (K_{D,BYY}K_{D,BY} + K_{D,BYY}K_{D,BY}K_{D,B})\frac{1}{[Y]}\right)}$$

Cancel terms and replace $[Y]$ *with* Y_f *for the final equation.*

$$\frac{[BYY]}{B_0} = \frac{Y_f}{Y_f + K_{D,BYY} + (K_{D,BYY}K_{D,BY} + K_{D,BYY}K_{D,BY}K_{D,B})\frac{1}{Y_f}}$$

8.2 Equation 9

$$\frac{[BY]}{B_0} = \frac{[BY]}{[BYY] + [BY] + [B_{closed}] + [B_{open}]}$$

Pull out $[BY]$ in denominator on RHS.

$$\frac{[BY]}{B_0} = \frac{[BY]}{[BY]\left(1 + \frac{[BYY]}{[BY]} + \frac{[B_{closed}]}{[BY]} + \frac{[B_{open}]}{[BY]}\right)}$$

Cancel $[BY]$ terms and multiply RHS by $\frac{K_{D,BYY}}{K_{D,BYY}}$. $K_{D,BYY}$ is equal to $\frac{[BY][Y]}{[BYY]}$ (Equation 4).

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}}{K_{D,BYY} + \frac{[BYY][BY][Y]}{[BYY][BY]} + \frac{[B_{closed}]K_{D,BYY}}{[BY]} + \frac{[B_{open}]K_{D,BYY}}{[BY]}}$$

Cancel terms and multiply RHS by $\frac{K_{D,BY}^{-1}}{K_{D,BY}^{-1}}$. $K_{D,BY}$ is equal to $\frac{[B_{closed}][Y]}{[BY]}$ (Equation 5).

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}}{K_{D,BYY} + [Y] + \frac{[B_{closed}]K_{D,BYY}}{[BY]} + \frac{[B_{open}]K_{D,BYY}}{[BY]}} * \frac{K_{D,BY}^{-1}}{K_{D,BY}^{-1}}$$

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}K_{D,BY}^{-1}}{K_{D,BYY}K_{D,BY}^{-1} + [Y]K_{D,BY}^{-1} + \frac{[B_{closed}]K_{D,BYY}[BY]}{[B_{closed}][Y][BY]} + \frac{[B_{open}][BY]K_{D,BYY}}{[B_{closed}][Y][BY]}}$$

Cancel terms.

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}K_{D,BY}^{-1}}{K_{D,BYY}K_{D,BY}^{-1} + [T]K_{D,BY}^{-1} + \frac{K_{D,BYY}}{[Y]} + \frac{[B_{open}]K_{D,BYY}}{[B_{closed}][Y]}}$$

Note that $K_{D,B} = \frac{[B_{open}]}{[B_{closed}]}$.

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}K_{D,BY}^{-1}}{K_{D,BYY}K_{D,BY}^{-1} + [Y]K_{D,BY}^{-1} + \frac{K_{D,BYY}}{[Y]} + \frac{K_{D,BYY}K_{D,B}}{[Y]}}$$

Pull out $K_{D,BY}^{-1}$ from the nominator and denominator of the RHS.

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}K_{D,BY}^{-1}}{K_{D,BY}^{-1}\left(K_{D,BYY} + [Y] + \frac{K_{D,BYY}K_{D,BY}}{[Y]} + \frac{K_{D,BYY}K_{D,BY}K_{D,B}}{[Y]}\right)}$$

Cancel terms and replace $[Y]$ with Y_f for the final equation.

$$\frac{[BY]}{B_0} = \frac{K_{D,BYY}}{Y_f + K_{D,BYY} + (K_{D,BYY}K_{D,BY} + K_{D,BYY}K_{D,BY}K_{D,B})\frac{1}{Y_f}}$$

8.3 Equation 10

$$\frac{[B_{closed}]}{B_0} = \frac{[B_{closed}]}{[BYY] + [BY] + [B_{closed}] + [B_{open}]}$$

Pull out $[B_{closed}]$ in denominator on RHS.

$$\frac{[B_{closed}]}{B_0} = \frac{[B_{closed}]}{[B_{closed}](\frac{[BYY]}{[B_{closed}]} + \frac{[BY]}{[B_{closed}]} + 1 + \frac{[B_{open}]}{[B_{closed}]}}$$

Cancel $[B_{closed}]$ terms. From Equation 5 we can solve for $\frac{[BY]}{[B_{closed}]} = \frac{[Y]}{K_{D,BY}}$ and substitute $K_{D,B} = \frac{[B_{open}]}{[B_{closed}]}$ from Equation 6.

$$\frac{[B_{closed}]}{B_0} = \frac{1}{\frac{[BYY]}{[B_{closed}]} + \frac{[Y]}{K_{D,BY}} + 1 + K_{D,B}}$$

Multiply RHS by $\frac{K_{D,BYY}}{K_{D,BYY}}$. $K_{D,BYY}$ *is equal to* $\frac{[BY][Y]}{[BYY]}$ *(Equation 4).*

$$\frac{[B_{closed}]}{B_0} = \frac{1}{\frac{[BYY]}{[B_{closed}]} + \frac{[Y]}{K_{D,BY}} + 1 + K_{D,B}} * \frac{K_{D,BYY}}{K_{D,BYY}}$$

$$\frac{[B_{closed}]}{B_0} = \frac{K_{D,BYY}}{\frac{[BYY][BY][Y]}{[B_{closed}][BYY]} + \frac{[Y]K_{D,BYY}}{K_{D,BY}} + K_{D,BYY} + K_{D,BYY}K_{D,B}}$$

Cancel terms and note again that $\frac{[BY]}{[B_{closed}]} = \frac{[Y]}{K_{D,BY}}$.

$$\frac{[B_{closed}]}{B_0} = \frac{K_{D,BYY}}{\frac{[Y][Y]}{K_{D,BY}} + \frac{[Y]K_{D,BYY}}{K_{D,BY}} + K_{D,BYY} + K_{D,BYY}K_{D,B}}$$

Pull out $K_{D,BYY}$ *in the denominator.*

$$\frac{[B_{closed}]}{B_0} = \frac{K_{D,BYY}}{K_{D,BYY}\left(\frac{[Y][Y]}{K_{D,BYY}K_{D,BY}} + \frac{[Y]}{K_{D,BY}} + 1 + K_{D,B}\right)}$$

Cancel terms and rearrange for the final equation, substituting Y_f *for* $[Y]$.

$$\frac{[B_{closed}]}{B_0} = \frac{1}{1 + K_{D,B} + \frac{Y_f}{K_{D,BY}}\left(\frac{Y_f}{K_{D,BYY}} + 1\right)}$$

8.4 Equation 11

$$\frac{[B_{open}]}{B_0} = \frac{[B_{open}]}{[BYY] + [BY] + [B_{closed}] + [B_{open}]}$$

Pull out $[B_{open}]$ *in denominator on RHS.*

$$\frac{[B_{open}]}{B_0} = \frac{[B_{open}]}{[B_{open}]\left(\frac{[BYY]}{[B_{open}]} + \frac{[BY]}{[B_{open}]} + \frac{[B_{closed}]}{[B_{open}]} + 1\right)}$$

Cancel $[B_{open}]$ *terms and multiply RHS by* $\frac{K_{D,B}}{K_{D,B}}$. *From equation 6,* $K_{D,B} = \frac{[B_{open}]}{[B_{closed}]}$.

$$\frac{[B_{open}]}{B_0} = \frac{1}{\frac{[BYY]}{[B_{open}]} + \frac{[BY]}{[B_{open}]} + \frac{[B_{closed}]}{[B_{open}]} + 1} * \frac{K_{D,B}}{K_{D,B}}$$

$$\frac{[B_{open}]}{B_0} = \frac{K_{D,B}}{\frac{[BYY][B_{open}]}{[B_{open}][B_{closed}]} + \frac{[BY][B_{open}]}{[B_{open}][B_{closed}]} + \frac{[B_{closed}][B_{open}]}{[B_{open}][B_{closed}]} + K_{D,B}}$$

Cancel terms and substitute $\frac{[BY]}{[B_{closed}]} = \frac{[Y]}{K_{D,BY}}$ *(Equation 4). Multiply RHS by* $\frac{K_{D,BYY}}{K_{D,BYY}}$. $K_{D,BYY}$ *is equal to* $\frac{[BY][Y]}{[BYY]}$ *(Equation 4).*

$$\frac{[B_{open}]}{B_0} = \frac{K_{D,B}}{\frac{[BYY]}{[B_{closed}]} + \frac{[Y]}{K_{D,BY}} + 1 + K_{D,B}} * \frac{K_{D,BYY}}{K_{D,BYY}}$$

$$\frac{[B_{open}]}{B_0} = \frac{K_{D,BYY}K_{D,B}}{\frac{[BYY][BY][Y]}{[B_{closed}][BYY]} + \frac{[Y]K_{D,BYY}}{K_{D,BY}} + K_{D,BYY} + K_{D,BYY}K_{D,B}}$$

Cancel terms and note again that $\frac{[BY]}{[B_{closed}]} = \frac{[Y]}{K_{D,BY}}$.

$$\frac{[B_{open}]}{B_0} = \frac{K_{D,BYY}K_{D,B}}{\frac{[Y][Y]}{K_{D,BY}} + \frac{[Y]K_{D,BYY}}{K_{D,BY}} + K_{D,BYY} + K_{D,BYY}K_{D,B}}$$

Pull out $K_{D,BYY}$ *in the denominator.*

$$\frac{[B_{open}]}{B_0} = \frac{K_{D,BYY}K_{D,B}}{K_{D,BYY}\left(\frac{[Y][Y]}{K_{D,BYY}KD,BY} + \frac{[Y]}{K_{D,BY}} + 1 + K_{D,B}\right)}$$

Cancel terms and rearrange for the final equation, substituting Y_f for $[Y]$.

$$\frac{[B_{open}]}{B_0} = \frac{K_{D,B}}{1 + K_{D,B} + \frac{Y_f}{K_{D,BY}}\left(1 + \frac{Y_f}{K_{D,BYY}}\right)}$$

9 Derivation of Y_f and B_f equations

9.1 Two-Site Model

Start with the reactions for target binding to the molecular beacon (MB) and target dimerization:

$$\mathrm{BYY} \overset{K_{D,BYY}}{\rightleftharpoons} \mathrm{BY} + \mathrm{Y}_f \overset{K_{D,BY}}{\rightleftharpoons} \mathrm{B}_f + 2\,\mathrm{Y}_f \tag{3}$$

$$\mathrm{YY} \overset{K_{D,YY}}{\rightleftharpoons} 2\,\mathrm{Y}_f \tag{4}$$

where Y_f is the free target and B_f is the free MB, which is the sum of the open and closed MBs ($B_f = B_{open} + B_{closed}$). We can write mass balances for the target and the MB:

$$\mathrm{B}_0 = \mathrm{B}_f + [\mathrm{BY}] + [\mathrm{BYY}] \tag{5}$$

$$\mathrm{Y}_0 = \mathrm{Y}_f + 2\,[\mathrm{YY}] + [\mathrm{BY}] + 2\,[\mathrm{BYY}] \tag{6}$$

The dissociation constant equations are as follows:

$$\mathrm{K}_{\mathrm{D,YY}} = \frac{Y_f^2}{[YY]} \tag{7}$$

$$\mathrm{K}_{\mathrm{D,BY}} = \frac{B_f Y_f}{[BY]} \tag{8}$$

$$\mathrm{K}_{\mathrm{D,BYY}} = \frac{[BY]Y_f}{[BYY]} \tag{9}$$

Solve equations 7-8 for $[YY]$, $[BY]$, and $[BYY]$ in terms of Y_f and B_f:

$$[\mathrm{YY}] = \frac{Y_f^2}{K_{D,YY}} \tag{10}$$

$$[\mathrm{BY}] = \frac{B_f Y_f}{K_{D,BY}} \tag{11}$$

$$[\mathrm{BYY}] = \frac{[BY]Y_f}{K_{D,BYY}} = \frac{B_f Y_f^2}{K_{D,BYY}K_{D,BY}} \tag{12}$$

Use equations 10-12 to solve the mass balance equations 5 and 6.
For the MB (B):

$$\begin{aligned} B_0 &= B_f + \frac{B_f Y_f}{K_{D,BY}} + \frac{B_f Y_f^2}{K_{D,BYY}K_{D,BY}} \\ &= B_f\left(\frac{Y_f}{K_{D,BY}} + \frac{Y_f^2}{K_{D,BYY}K_{D,BY}} + 1\right) \end{aligned} \tag{13}$$

$$0 = B_f \left(\frac{Y_f}{K_{D,BY}} + \frac{Y_f^2}{K_{D,BYY} K_{D,BY}} + 1 \right) - B_0 \tag{14}$$

For the target (Y):

$$\begin{aligned} Y_0 &= Y_f + \frac{2Y_f^2}{K_{D,YY}} + \frac{B_f Y_f}{K_{D,BY}} + \frac{2B_f Y_f^2}{K_{D,BYY} K_{D,BY}} \\ &= \frac{2B_f Y_f^2}{K_{D,BYY} K_{D,BY}} + \frac{2Y_f^2}{K_{D,YY}} + \frac{B_f Y_f}{K_{D,BY}} + Y_f \end{aligned} \tag{15}$$

$$0 = 2Y_f^2 \left(\frac{B_f}{K_{D,BYY} K_{D,BY}} + \frac{1}{K_{D,YY}} \right) + Y_f \left(\frac{B_f}{K_{D,BY}} + 1 \right) - Y_0 \tag{16}$$

9.2 One-Site Model

Start with the reactions for target binding to the MB and target dimerization:

$$\mathrm{BY} \rightleftharpoons \mathrm{B}_f + \mathrm{Y}_f \tag{17}$$

$$\mathrm{YY} \rightleftharpoons 2\,\mathrm{Y}_f \tag{18}$$

where Y_f is the free target and B_f is the free MB, which is the sum of the open and closed MBs ($B_f = B_{open} + B_{closed}$). We can write mass balances for the target and the MB:

$$\mathrm{B}_0 = \mathrm{B}_f + [\mathrm{BY}] \tag{19}$$

$$\mathrm{Y}_0 = \mathrm{Y}_f + 2\,[\mathrm{YY}] + [\mathrm{BY}] \tag{20}$$

The dissociation constant equations are as follows:

$$\mathrm{K}_{\mathrm{D,YY}} = \frac{Y_f^2}{[YY]} \tag{21}$$

$$\mathrm{K}_{\mathrm{D,BY}} = \frac{B_f Y_f}{[BY]} \tag{22}$$

Solve equations 21-22 for $[YY]$ and $[BY]$ in terms of Y_f and B_f:

$$[\mathrm{YY}] = \frac{Y_f^2}{K_{D,YY}} \tag{23}$$

$$[\mathrm{BY}] = \frac{B_f Y_f}{K_{D,BY}} \tag{24}$$

Use equations 23-24 to solve the mass balance equations 19 and 20.
For the MB (B):

$$B_0 = B_f + \frac{B_f Y_f}{K_{D,BY}} \tag{25}$$

$$0 = B_f \left(\frac{Y_f}{K_{D,BY}} + 1 \right) - B_0 \tag{26}$$

For the target (Y):

$$Y_0 = Y_f + \frac{2Y_f^2}{K_{D,YY}} + \frac{B_f Y_f}{K_{D,BY}} \tag{27}$$

$$0 = \left(\frac{2Y_f^2}{K_{D,YY}} \right) + Y_f \left(\frac{B_f}{K_{D,BY}} + 1 \right) - Y_0 \tag{28}$$

10 Weighted $K_{D,BY}$ calculations

To estimate the dissociation constant for the interaction of a molecular beacon (MB) with one target in the presence of both targets, we used the $K_{D,BY}$ values from the one-site fits to estimate a weighted $K_{D,BY}$. Because either target could in theory initiate the first binding event, $K_{D,BY}$ in the two-site model needed to account for the relative concentrations of the MB bound to each individual target ($[BY_i]$). We used the one-site model predictions to calculate BY_i at each temperature:

$$BY_i = Y_{f,i} K_{D,BY_i} \tag{29}$$

Figure S9 shows the calculated free target ($Y_{f,i}$) and K_{D,BY_i} values from the one-site model (LoopQ = orange squares; ToeF = blue triangles). Then the ratio of BY_{LoopQ} to BY_{ToeF} was used to calculate a weighted $K_{D,BY}$ average:

$$C(T) = \frac{[BY_{ToeF}]}{[BY_{LoopQ}]} \tag{30}$$

$$K_{D,BY,Weighted}(T) = \frac{C(T) * K_{D,BY,LoopQ} + K_{D,BY,ToeF}}{1 + C(T)} \tag{31}$$

This is in essence a weighted average, acknowledging the varying affinities of the different targets with temperature. Figure S9 shows the weighted $K_{D,BY}$ values (black circes) for each heterotropic MB in comparison to the individual K_{D,BY_i} values for each target.

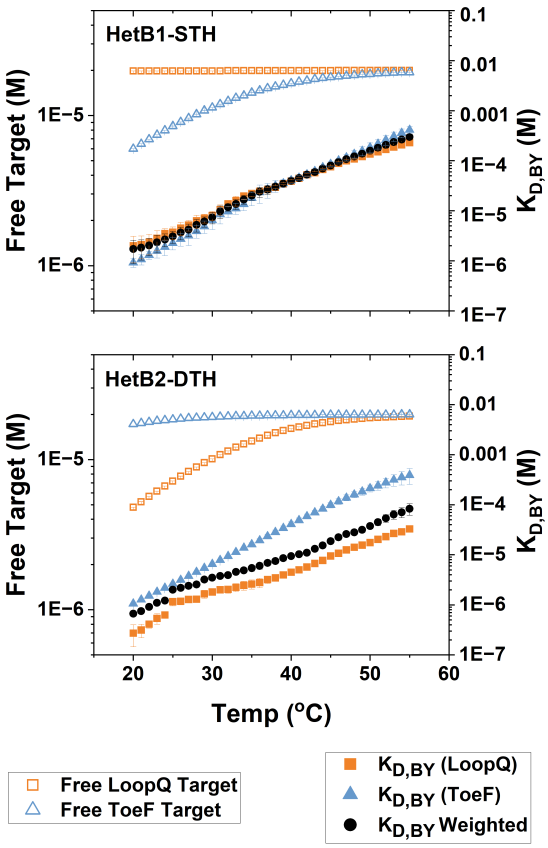

Figure S9: Predicted free target concentrations (open points, left axis) and dissociation constants (K_{D,BY_i}, closed squares and triangles, right axis) from the one-site model fits (n = 4) were used to calculate weighted $K_{D,BY}$ values (black circles) for the two-site model.

11 Fitting Parameters

The model used two Matlab algorithms to optimize the dissociation constant. *fmincon* was used minimize the sum of squares between the experimental fluorescence data and the predicted fluorescence, specifically employing the default 'interior-point' algorithm. *fsolve* was used to solve for the Y_f and B_f parameters with the 'levenberg-marquardt' algorithm. Specific *fsolve* tolerances are included in Tables S5-S8 below. Dissociation constants were constrained in different temperature regions to avoid local minima and maintain high goodness-of-fit measures (R^2).

Table S5: One-site fitting constraints for $K_{D,BY}$ for HetB1-STH.

	Temperature Range (^oC)	**Lower Bound**	**Upper Bound**	***fsolve* Tolerances:**		
				Optimality	**Function**	**Step**
HetB1-STH + TarB1-ToeF						
Exp 1	20	$2 * K^{o,i}_{D,BY}$	$35 * K^{o,i}_{D,BY}$	1e-20	1e-20	1e-30
	$21-39$	$K^{i-1}_{D,BY}$	$1.75 * K^{i-1}_{D,BY}$			
	$40-55$	$K^{i-1}_{D,BY}$	$1.2 * K^{i-1}_{D,BY}$			
Exp 2	20	$2 * K^{o,i}_{D,BY}$	$35 * K^{o,i}_{D,BY}$	1e-20	1e-20	1e-30
	$21-39$	$K^{i-1}_{D,BY}$	$2 * K^{i-1}_{D,BY}$			
	$40-55$	$K^{i-1}_{D,BY}$	$1.2 * K^{i-1}_{D,BY}$			
Exp 3-4	20	$2 * K^{o,i}_{D,BY}$	$25 * K^{o,i}_{D,BY}$	1e-20	1e-20	1e-30
	$21-39$	$K^{i-1}_{D,BY}$	$1.75 * K^{i-1}_{D,BY}$			
	$40-55$	$K^{i-1}_{D,BY}$	$1.2 * K^{i-1}_{D,BY}$			
HetB1-STH + TarB1-LoopQ						
Exp 1-2	20	$1.5 * K^{o,i}_{D,BY}$	100	1e-10	1e-60	1e-50
	$21-34$	$K^{i-1}_{D,BY}$	$2.5 * K^{i-1}_{D,BY}$			
	$35-55$	$K^{i-1}_{D,BY}$	$1.5 * K^{i-1}_{D,BY}$			
Exp 3-4	20	$5 * K^{o,i}_{D,BY}$	100	1e-10	1e-60	1e-50
	$21-34$	$K^{i-1}_{D,BY}$	$2 * K^{i-1}_{D,BY}$			
	$35-55$	$K^{i-1}_{D,BY}$	$1.5 * K^{i-1}_{D,BY}$			

*$K^{o,i}_{D,BY}$ is the initial guess for the dissociation constant at $Temperature = i$.

**$K^{i-1}_{D,BY}$ is the optimized dissociation constant at $Temperature = i - 1$.

Table S6: One-site fitting constraints for $K_{D,BY}$ for HetB2-DTH.

	Temperature Range (^oC)	**Lower Bound**	**Upper Bound**	***fsolve* Tolerances:**		
				Optimality	**Function**	**Step**
HetB2-DTH + TarB2-ToeF						
Exp 1-4	20	$2 * K_{D,BY}^{o,i}$	$25 * K_{D,BY}^{o,i}$	1e-20	1e-6	1e-20
	$21 - 34$	$1.2 * K_{D,BY}^{i-1}$	$2 * K_{D,BY}^{i-1}$			
	$35 - 55$	$K_{D,BY}^{i-1}$	$1.2 * K_{D,BY}^{i-1}$			
HetB2-DTH + TarB2-LoopQ						
Exp 1	20	$50 * K_{D,BY}^{o,i}$	0.01	1e-10	1e-60	1e-20
	$21 - 34$	$K_{D,BY}^{i-1}$	$5 * K_{D,BY}^{i-1}$			
	$35 - 55$	$K_{D,BY}^{i-1}$	$1.5 * K_{D,BY}^{i-1}$			
Exp 2	20	0	100	1e-100	1e-100	1e-100
	$21 - 34$	$K_{D,BY}^{i-1}$	$2 * K_{D,BY}^{i-1}$			
	$35 - 55$	$K_{D,BY}^{i-1}$	$1.5 * K_{D,BY}^{i-1}$			
Exp 3	20	$50 * K_{D,BY}^{o,i}$	1	1e-10	1e-60	1e-20
	$21 - 39$	$K_{D,BY}^{i-1}$	$5 * K_{D,BY}^{i-1}$			
	$40 - 55$	$K_{D,BY}^{i-1}$	$1.5 * K_{D,BY}^{i-1}$			
Exp 4	20	$10 * K_{D,BY}^{o,i}$	0.01	1e-20	1e-6	1e-20
	$21 - 29$	$K_{D,BY}^{i-1}$	$5 * K_{D,BY}^{i-1}$			
	$30 - 55$	$K_{D,BY}^{i-1}$	$1.5 * K_{D,BY}^{i-1}$			

*$K_{D,BY}^{o,i}$ is the initial guess for the dissociation constant at $Temperature = i$.

**$K_{D,BY}^{i-1}$ is the optimized dissociation constant at $Temperature = i - 1$.

Table S7: Two-site fitting constraints for $K_{D,BYY}$ for the heterotropic molecular beacons ($K_{D,BY}$ comes from the one-site fits).

	Temperature Range (^oC)	**Lower Bound**	**Upper Bound**	***fsolve* Tolerances:**		
				Optimality	**Function**	**Step**
HetB1-STH + TarB1-ToeF + TarB1-ToeF						
Exp 1-3	20	0	$K^{o,i}_{D,BYY}$	1e-6	1e-60	1e-20
	$21-34$	$K^{i-1}_{D,BYY}$	$1.3*K^{i-1}_{D,BYY}$			
	$35-55$	$K^{i-1}_{D,BYY}$	$1.7*K^{i-1}_{D,BYY}$			
Exp 4	20	0	$K^{o,i}_{D,BYY}$	1e-6	1e-60	1e-20
	$21-29$	$K^{i-1}_{D,BYY}$	$1.3*K^{i-1}_{D,BYY}$			
	$30-55$	$K^{i-1}_{D,BYY}$	$1.7*K^{i-1}_{D,BYY}$			
HetB2-DTH + TarB2-ToeF + TarB2-ToeF						
Exp 1	20	0	$0.5*K^{o,i}_{D,BYY}$	1e-6	1e-60	1e-20
	$21-29$	$K^{i-1}_{D,BYY}$	$1.5*K^{i-1}_{D,BYY}$			
	$30-39$	$1.1*K^{i-1}_{D,BYY}$	$1.7*K^{i-1}_{D,BYY}$			
	$40-55$	$K^{i-1}_{D,BYY}$	$1.2*K^{i-1}_{D,BYY}$			
Exp 2	20	0	$K^{o,i}_{D,BYY}$	1e-6	1e-60	1e-20
	$21-29$	$K^{i-1}_{D,BYY}$	$1.2*K^{i-1}_{D,BYY}$			
	$30-39$	$1.1*K^{i-1}_{D,BYY}$	$1.5*K^{i-1}_{D,BYY}$			
	$40-55$	$K^{i-1}_{D,BYY}$	$1.7*K^{i-1}_{D,BYY}$			
Exp 3	20	0	$0.5*K^{o,i}_{D,BYY}$	1e-6	1e-60	1e-20
	$21-29$	$K^{i-1}_{D,BYY}$	$1.5*K^{i-1}_{D,BYY}$			
	$30-39$	$1.1*K^{i-1}_{D,BYY}$	$1.8*K^{i-1}_{D,BYY}$			
	$40-55$	$K^{i-1}_{D,BYY}$	$2*K^{i-1}_{D,BYY}$			
Exp 4	20	0	$0.5*K^{o,i}_{D,BYY}$	1e-6	1e-60	1e-20
	$21-29$	$K^{i-1}_{D,BYY}$	$1.2*K^{i-1}_{D,BYY}$			
	$30-39$	$1.1*K^{i-1}_{D,BYY}$	$1.5*K^{i-1}_{D,BYY}$			
	$40-55$	$K^{i-1}_{D,BYY}$	$1.7*K^{i-1}_{D,BYY}$			

*$K^{o,i}_{D,BYY}$ is the initial guess for the dissociation constant at $Temperature = i$.

**$K^{i-1}_{D,BYY}$ is the optimized dissociation constant at $Temperature = i-1$.

Table S8: Two-site fitting constraints for $K_{D,BY}$ and $K_{D,BYY}$ for the homotropic molecular beacon (fit simultaneously).

	Temperature Range (^oC)	**Lower Bound**	**Upper Bound**	***fsolve* Tolerances:**		
				Optimality	**Function**	**Step**
HomB + TarHomB: $K_{D,BY}$						
Exp 1	20	0	10e-3	1e-6	1e-60	1e-60
	$21-34$	$K_{D,BY}^{i-1}$	$1.2 * K_{D,BY}^{i-1}$			
	$35-55$	$K_{D,BY}^{i-1}$	$1.5 * K_{D,BY}^{i-1}$			
Exp 2-4	20	0	10e-3	1e-6	1e-60	1e-60
	$21-34$	$K_{D,BY}^{i-1}$	$1.3 * K_{D,BY}^{i-1}$			
	$35-55$	$K_{D,BY}^{i-1}$	$1.5 * K_{D,BY}^{i-1}$			
HomB + TarHomB: $K_{D,BYY}$						
Exp 1	20	0	10e-3	1e-6	1e-60	1e-60
	$21-34$	$K_{D,BYY}^{i-1}$	$1.2 * K_{D,BYY}^{i-1}$			
	$35-55$	$K_{D,BYY}^{i-1}$	$1.5 * K_{D,BYY}^{i-1}$			
Exp 2-4	20	0	10e-3	1e-6	1e-60	1e-60
	$21-34$	$K_{D,BYY}^{i-1}$	$1.3 * K_{D,BYY}^{i-1}$			
	$35-55$	$K_{D,BYY}^{i-1}$	$1.5 * K_{D,BYY}^{i-1}$			

*$K_D^{o,i}$ is the initial guess for the dissociation constant at $Temperature = i$.

**K_D^{i-1} is the optimized dissociation constant at $Temperature = i - 1$.

12 Model fits for all experiments

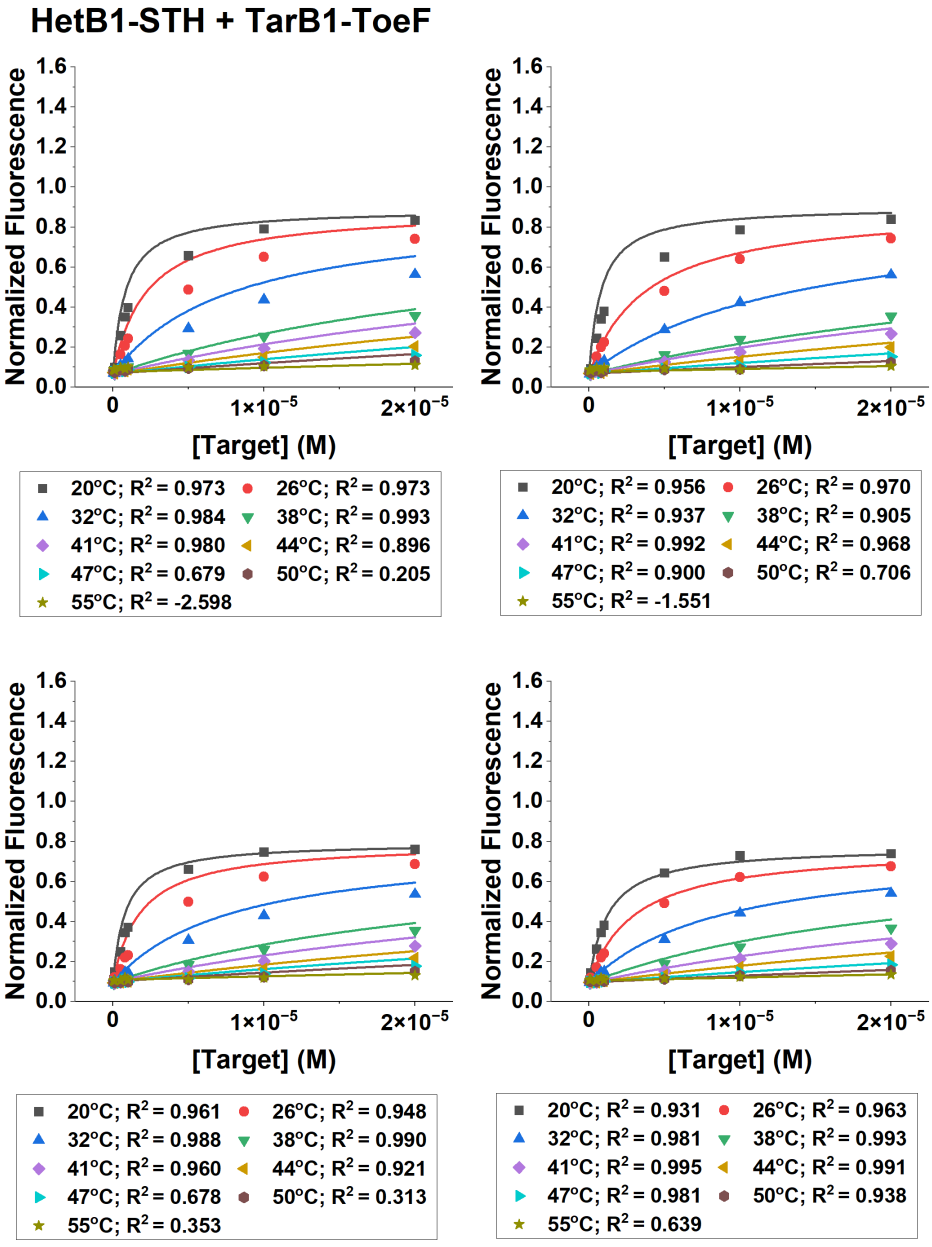

Figure S10: One-site model fits (lines) to the experimental melting data (points) for HetB1-STH with TarB1-ToeF target at selected temperatures. Each plot is a separate experiment. R^2 values for each fit are displayed in the legend.

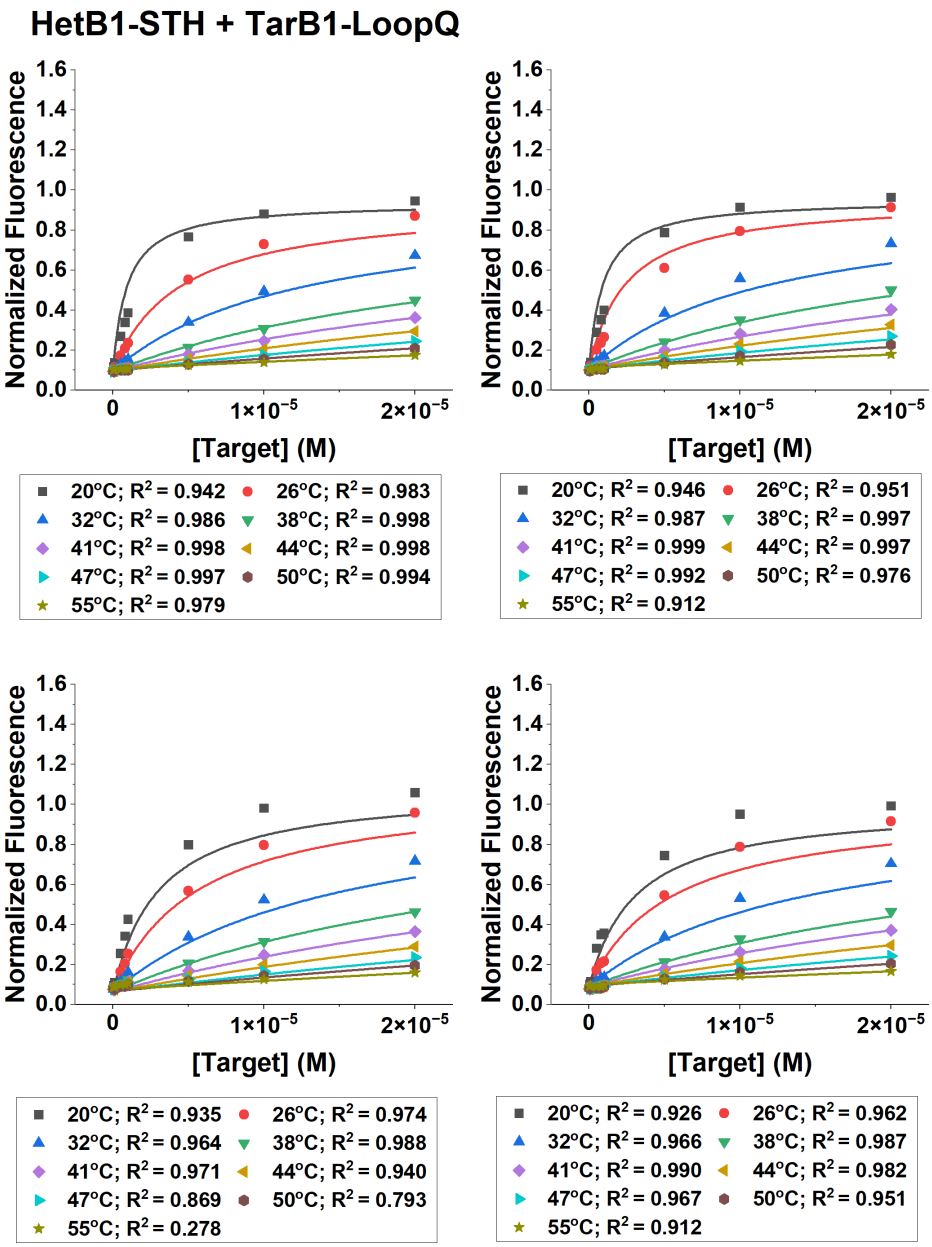

Figure S11: One-site model fits (lines) to the experimental melting data (points) for HetB1-STH with TarB1-LoopQ target at selected temperatures. Each plot is a separate experiment. R^2 values for each fit are displayed in the legend.

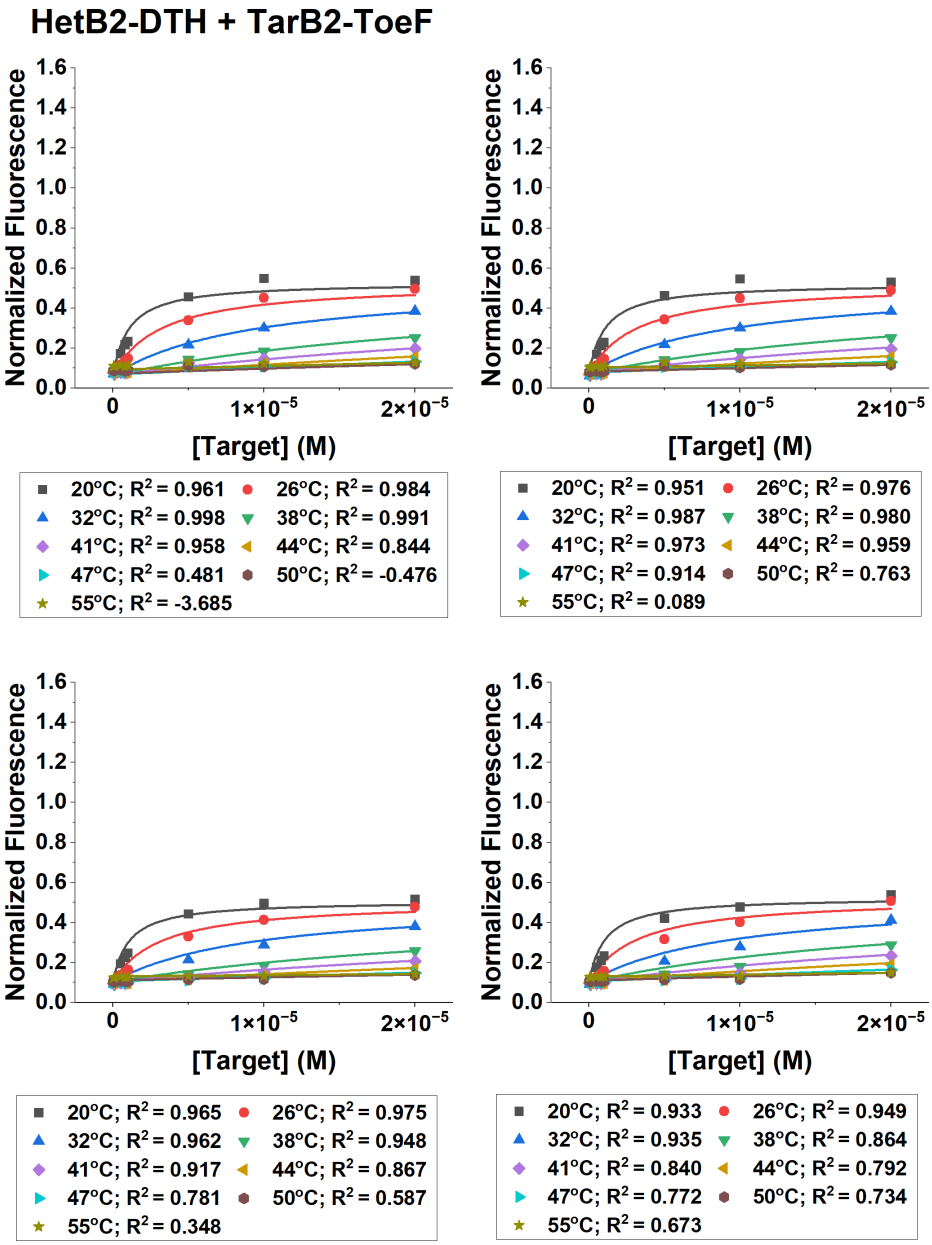

Figure S12: One-site model fits (lines) to the experimental melting data (points) for HetB2-DTH with TarB2-ToeF target at selected temperatures. Each plot is a separate experiment. R^2 values for each fit are displayed in the legend.

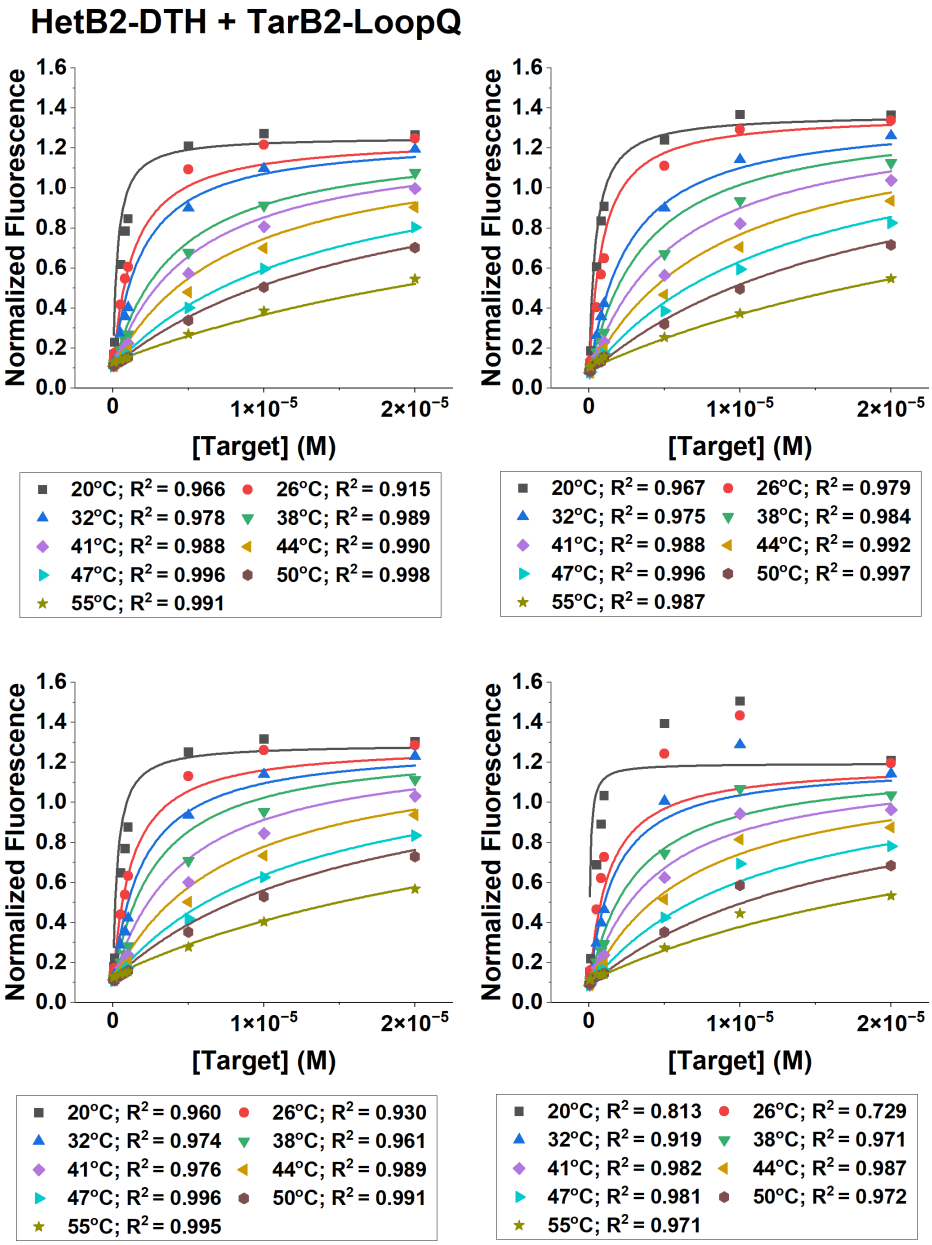

Figure S13: One-site model fits (lines) to the experimental melting data (points) for HetB2-DTH with TarB2-LoopQ target at selected temperatures. Each plot is a separate experiment. R^2 values for each fit are displayed in the legend.

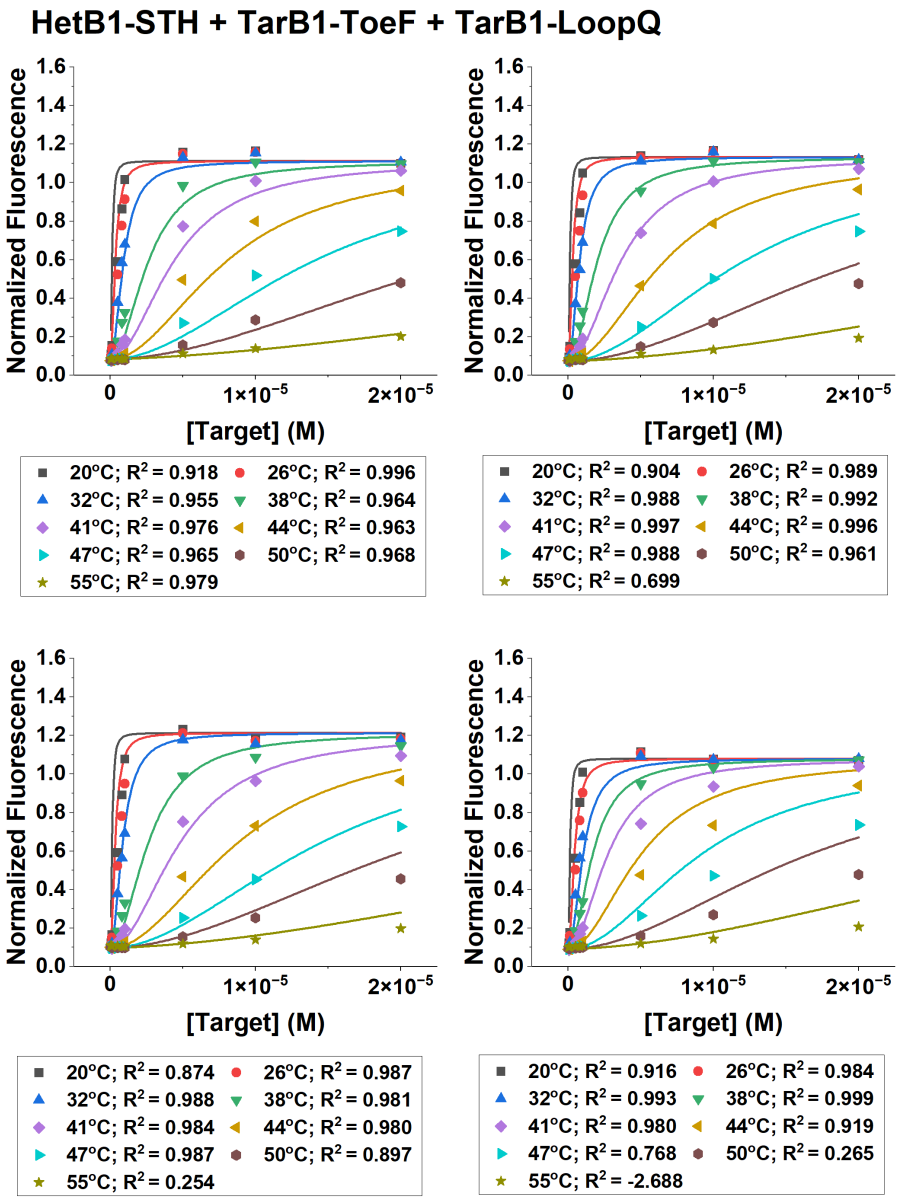

Figure S14: Two-site model fits (lines) to the experimental melting data (points) for HetB1-STH with both TarB1-ToeF and TarB1-LoopQ targets at selected temperatures. Each plot is a separate experiment. R^2 values for each fit are displayed in the legend. Weighted $K_{D,BY}$ from the one-site results were used in these fits (Supplemental Figure S9).

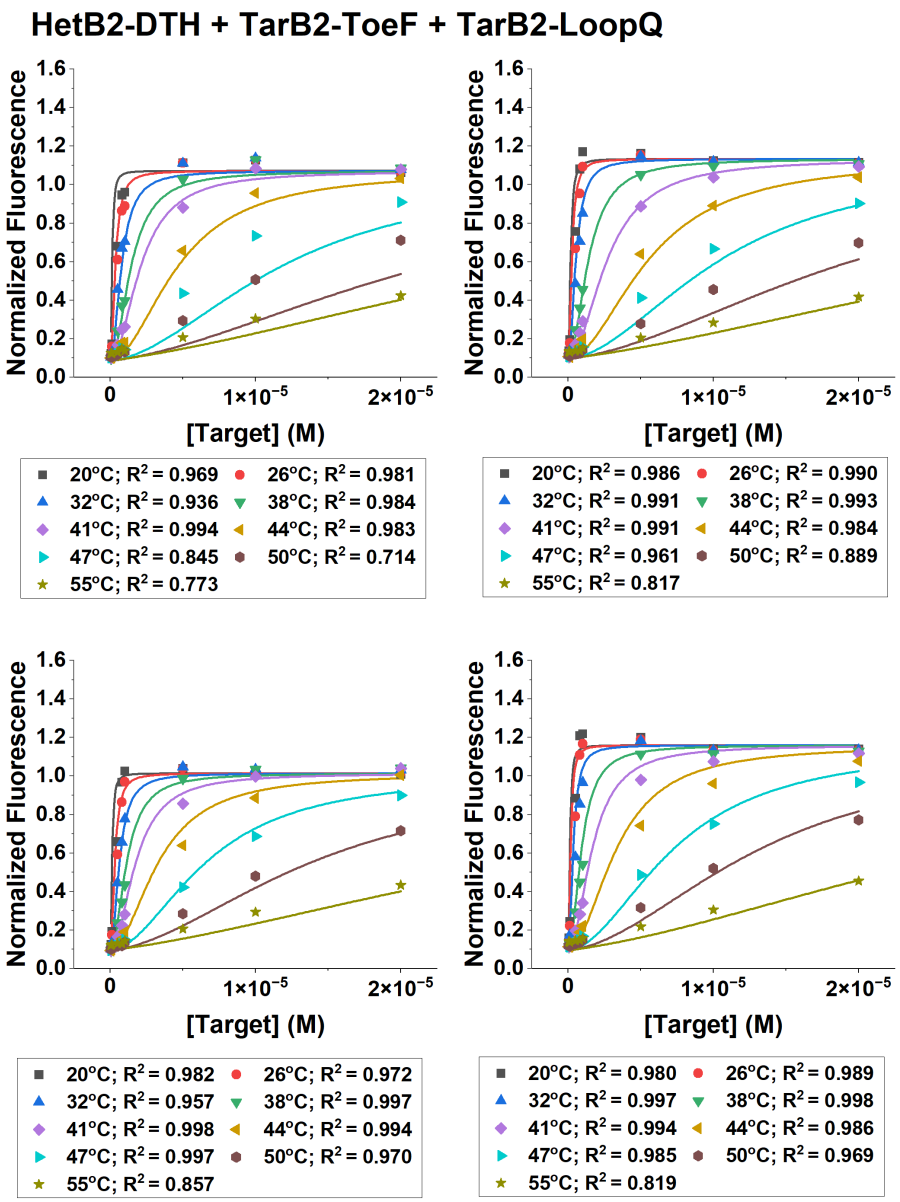

Figure S15: Two-site model fits (lines) to the experimental melting data (points) for HetB2-DTH with both TarB2-ToeF and TarB2-LoopQ targets at selected temperatures. R^2 values for each fit are displayed in the legend. Each plot is a separate experiment. Weighted $K_{D,BY}$ from the one-site results were used in these fits (Supplemental Figure S9).

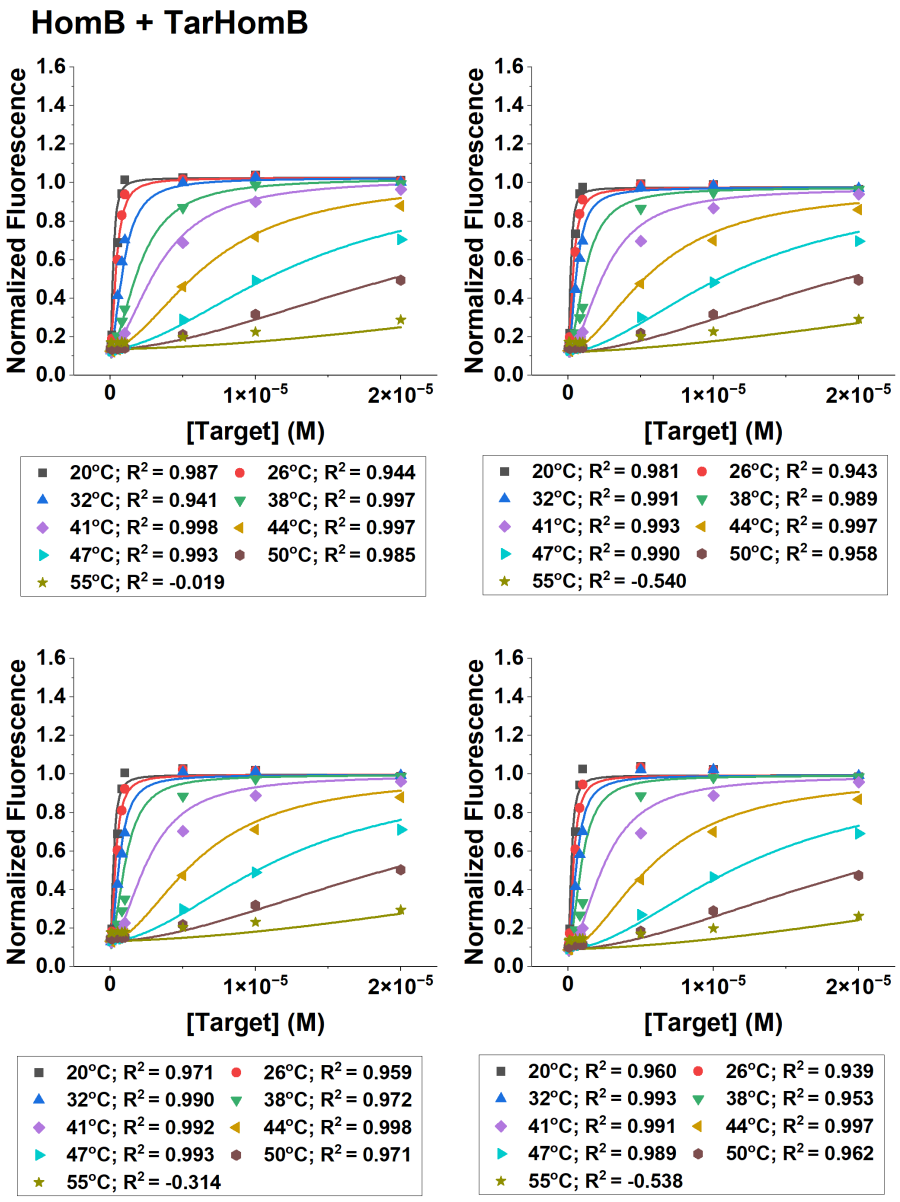

Figure S16: Two-site model fits (lines) to the experimental melting data (points) for HomB with TarHomB target at selected temperatures. Each plot is a separate experiment. R^2 values for each fit are displayed in the legend.

13 Sensitivity analysis on β estimates for the homotropic molecular beacon fits

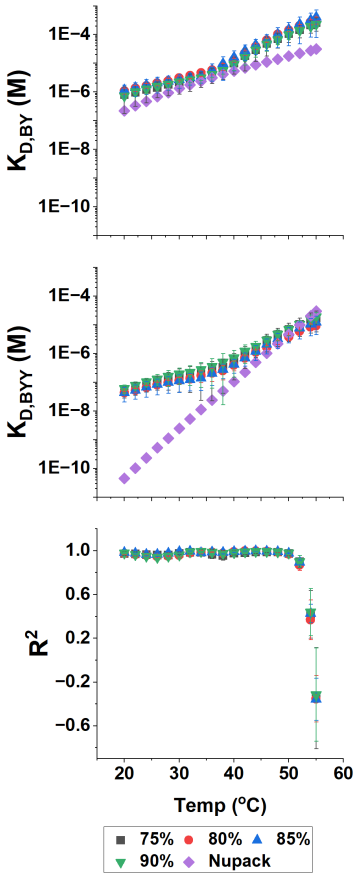

Figure S17: Sensitivity analysis on the β parameter for the homotropic molecular beacon (HomB). The β value was varied between $75 - 90\%$ of α for each experiment, and the resulting dissociation constants were compared. (Top) $K_{D,BY}$ and (middle) $K_{D,BYY}$ remain within less than an order of magnitude across all four β values, deviating by $1.3 - 2.3x$ within the tested temperature range. For comparison, NUPACK predictions are also shown, which align closely with the model predictions for $K_{D,BY}$ but deviate at lower temperatures for $K_{D,BYY}$. (Bottom) The corresponding R^2 values indicate no significant differences in the overall quality of fit across the tested β values.

14 Hill Equation Fits

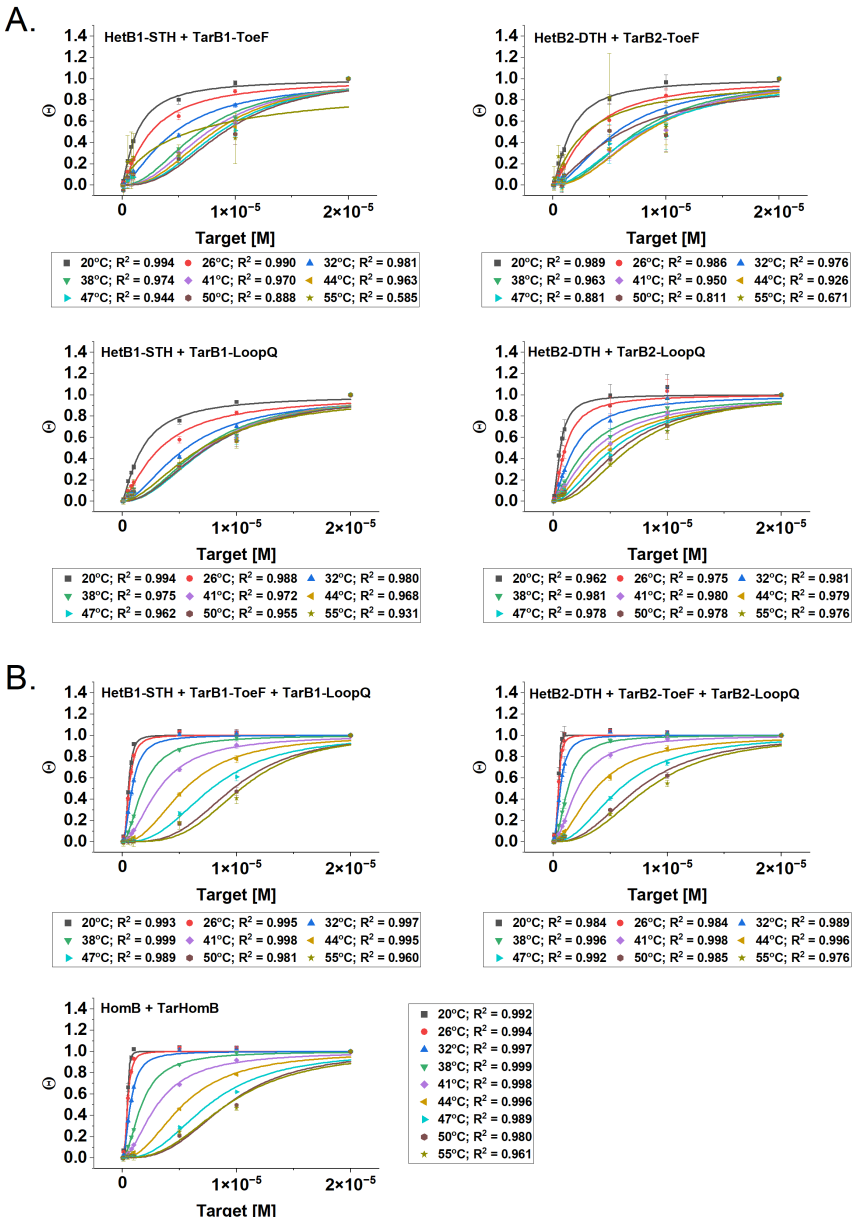

Figure S18: Hill equation fits for all data sets (n = 4), with R^2 values included in the legends. **(A)** Fits for the heterotropic molecular beacons (HetB1-STH and HetB2-DTH) in one-target experiments, with either the ToeF or LoopQ target. **(B)** Fits for the hetertropic molecular beacons in two-target experiments and the homotropic molecular beacon (HomB) with its target (TarHomB).

15 Comparison of fluorescence output for all molecular beacons

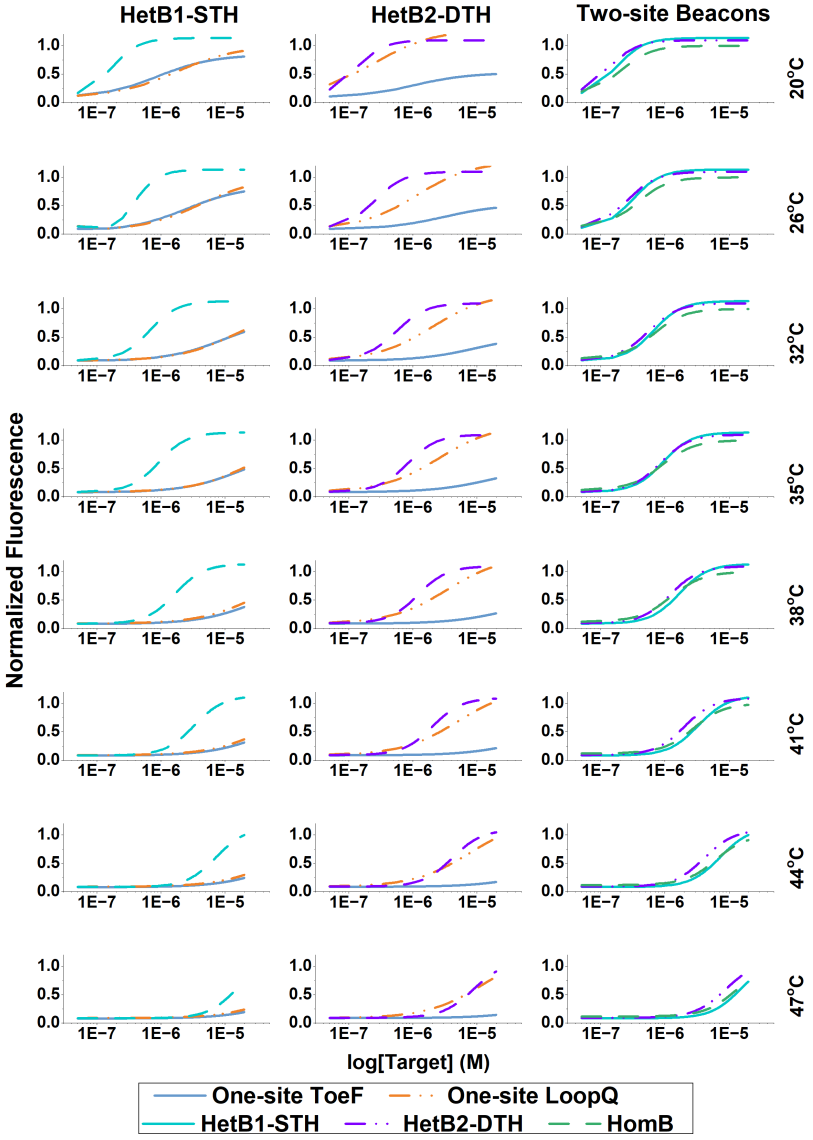

Figure S19: Comparison of fluorescence responses for all molecular beacons (MB). **(Left column)** HetB1-STH in the presence of TarB1-ToeF (solid blue), TarB1-LoopQ (dash-dot orange), and both targets (dashed teal). **(Middle column)** HetB2-DTH in the presence of TarB2-ToeF (solid blue), TarB2-LoopQ (dash-dot orange), and both targets (dashed purple). **(Right column)** HetB1-STH (solid teal) and HetB2-DTH (dash-dot purple) each with two targets and HomB (dashed green) with TarHomB.