Supplementary Information for Stemless molecular beacon probes for single-molecule detection of supercoil-induced DNA denaturation

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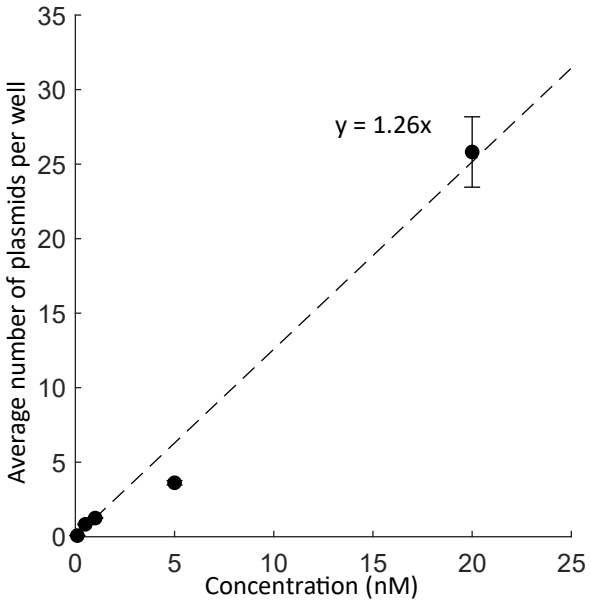
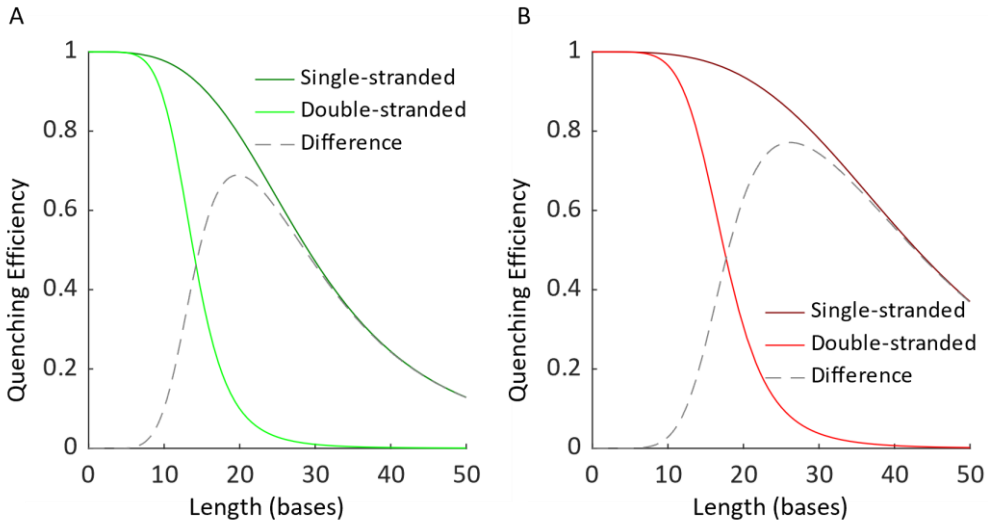
Supplementary Table 1: Summary of the various probe designs used throughout this study. Cy3 and Cy5 refer to the cyanine based fluorophores, while BHQ2 and BHQ3 denote Black Hole Quencher 2 and 3 fluorescence quenchers respectively. All probes were synthesized by Integrated DNA Technologies (IDT) and purified by high-performance liquid chromatography (HPLC).

Probe name	Sequence
Targeting the FUSE region	
Singly-labelled probe	5' - Cy3 - CCC GAG GGA ATA TAC ATT ATA - 3'
Singly-labelled target	5' - ATA ATG TAT ATT CCC TCG GG - Cy5 - 3'
Hairpinning beacon	5' - Cy3 - CCC GAG GGA ATA TAC ATT ATT CGG G - BHQ2 - 3'
FUSE probe (stemless beacon)	5' - Cy3 - CCC GAG GGA ATA TAC ATT ATA - BHQ2 - 3'
Unlabelled target	5' - ATA ATG TAT ATT CCC TCG GG - 3'
Targeting the AT-insert	
AT-insert probe	5' - Cy5 - AAA AAA AAA AAA AAA AAA AAA AAA AA - BHQ3 - 3'
Singly-labelled probe	5' - Cy5 - AAA AAA AAA AAA AAA AAA AAA AAA AA - 3'
Unlabelled target	5' – TTT TTT TTT TTT TTT TTT TTT TTT TT – 5'

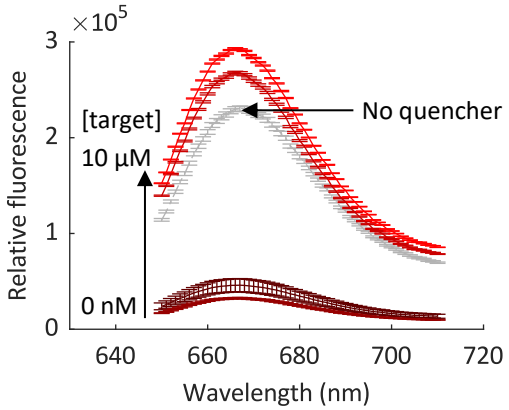


Supplementary Figure 1 Calibration of the relationship between bulk concentration and number of plasmids per well. Calibration performed by measuring the mean number of YOYO-1 labelled plasmids per well as a function of concentration, and using the slope of the line of best fit, when the fit is forced through the origin. For concentrations where there were less than 5 plasmids per well, the number of plasmids per well was directly counted. For concentrations with more than 5 plasmids per well, the relationship between well intensity (I) and the number of

plasmids per well was found to be $\boldsymbol{N = 0.103I - 56.115}$. It was found that a concentration of 1 nM corresponded to 1.26 ± 0.07 plasmids per well. Error bars are the standard error of the mean over 10 videos for each data point.

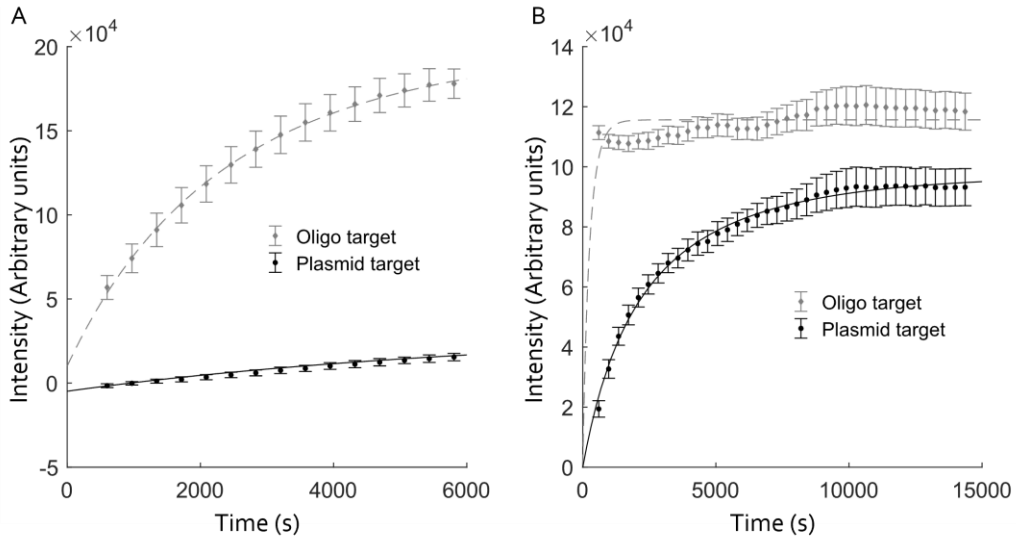


Supplementary Figure 2 Calculated quenching efficiencies as a function of length of single-stranded vs double-stranded DNA end-labelled with **A** Cy3 and BHQ2 or **B** Cy5 and BHQ3. Quenching efficiency was estimated assuming a length per base pair of 0.34 nm for dsDNA and 0.65 nm for ssDNA[1]; an end-to-end distance of aN^{ν} (where a is the length per base, N is the number of bases and ν~0.588 is the Flory constant)[1]; and Förster radii of 4.712 nm for Cy3-BHQ2 and 5.935 nm for Cy5-BHQ3[2]. dsDNA was treated as a rigid rod with an end-to-end distance of aN. Dashed lines indicate the difference in quenching efficiency between the single-stranded and double-stranded DNA. The optimal length was estimated to be 21 bases for the Cy3-BHQ2 probe and 26 bases for the Cy5-BHQ3 probe.

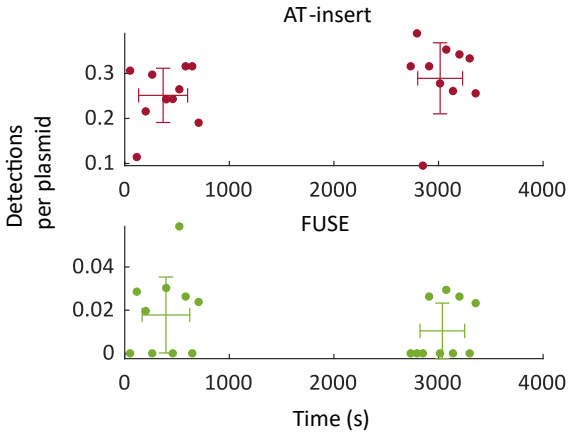


Supplementary Figure 3 Relative fluorescence emission of 50 nM of Cy5 and BHQ3 labelled stemless beacons as a function of (unlabelled) target concentration (0 nM, 1 nM, 100 nM and 10 µM). Probes were annealed to the target by heating to 95 °C and slowly cooling to room temperature before measuring. All measurements were taken at room temperature. The grey curve is the relative fluorescence emission from 50 nM of a Cy5 labelled probe with no target.

Error bars are the standard error of the mean from 3 measurements. All buffers contained 50 mM NaCl, 15.84 mM tris HCl (pH 8), 1 mM KCl, 20 mM EDTA, and 1% glycerol.

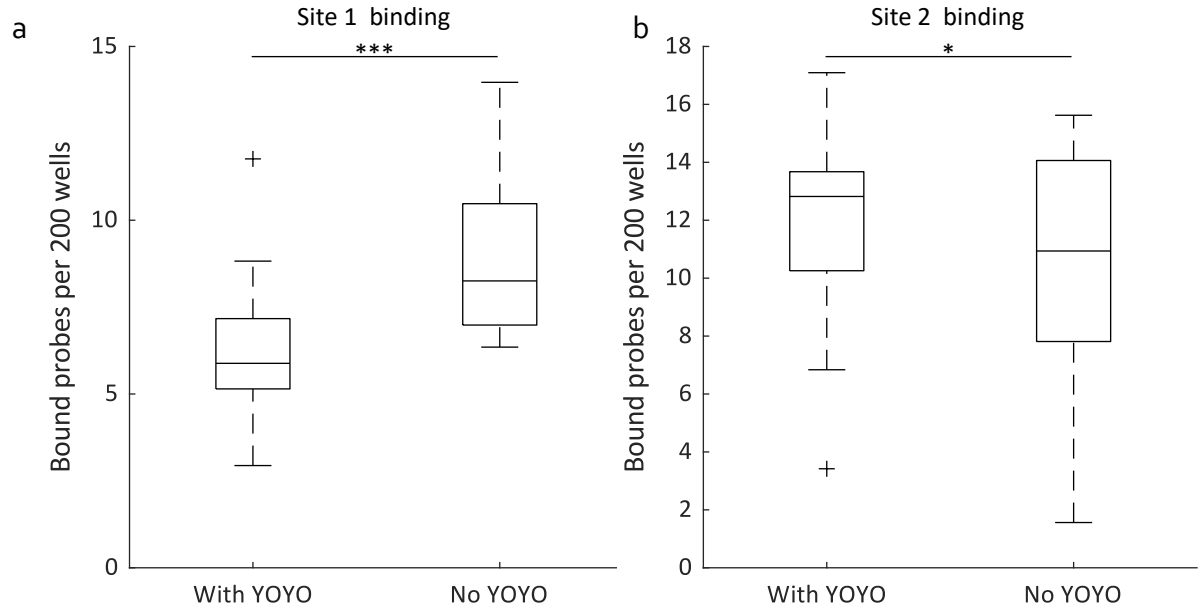


Supplementary Figure 4 Bulk change in intensity of **A** the Cy3 labelled stemless beacon and **B** the Cy5 labelled stemless beacon binding to both a complementary unlabelled oligonucleotide and the complementary denaturation site within pFLIP-FUSE-40AT. Samples contained 20 nM of probe and 200 nM of target, and the reaction was conducted at 37 °C. Measurements were taken following the same protocol as Figure 1E in the main text. Error bars are standard error of the mean from averaging three independent intensity measurements. Fits are from fitting Equation 1 to the intensity data. These data are consistent with the hypothesis that the denaturation rates of these sites is slow compared to the timescale of an experimental measurement at these conditions, and that only a small percentage of plasmids have a melted FUSE region at these conditions.



Supplementary Figure 5 Fraction of plasmids with a red probe bound to the AT-insert or green probe bound to the FUSE region of a plasmid at two different times after dilution. There is no significant difference in the proportion of bound molecules before and after incubation (chi-square, 1 df, $p = 0.235$ for red channel data and $p = 0.385$ for green channel data). 20 nM of plasmids were incubated for 1h with 400 nM of each probe, then diluted to a concentration

of 0.5 nM plasmid into a solution containing 50 nM of YOYO-1. All buffers contained 10 mM tris, 20 mM NaCl and the imaging buffer contained PCA and PCD. Error bars are standard deviation for time and weighted standard deviation for detections per plasmid. Each datapoint represents the detections per plasmid in one video (N = 10 videos per condition). Overall, 394 plasmids were analyzed at early times and 384 plasmids were analyzed at long times.



Supplementary Figure 6 Difference in probe binding to plasmid samples after dilution and staining with YOYO-1. **A** shows the average number of Cy 3 probes bound to site 1 observed per 200 wells from samples mixed at a concentration of 1000 nM Cy 3 probe: 20 nM plasmid, incubated for 1h. Sample was diluted to a final concentration of 0.0625 nM plasmid and 50 nM YOYO-1 (in the YOYO-1 sample). N = 19 videos for the 'with YOYO-1' sample and N = 20 videos for the 'no YOYO-1' sample. The amount of binding per 200 wells (~ the number of wells in a field of view) is displayed. *** Indicates statistically significant difference between results (two-tail t-test, 37 df, p = 0.0008). **B** shows average number of Cy 5 labelled probes bound to site 2 from samples mixed at a concentration of 1000 nM Cy 5 probe: 60 nM plasmid and incubated for one hour. Sample was diluted to a final concentration of 0.187 nM plasmid and 50 nM YOYO-1 (in the YOYO-1 sample). N = 30 videos for each sample. * Indicates statistically significant difference between results (two-tail t-test, 58 df, p = 0.02).

Supplementary Table 2 Binding of the FUSE and AT-insert probes to relaxed (σ = 0) pFLIP-FUSE-40AT plasmids under the same imaging conditions as for Figure 4 in the main text.

Total plasmids observed	AT-probe binding	FUSE probe binding	Both probes binding	Neither probe binding
226	2	2	0	222

(1) Tree, D. R.; Muralidhar, A.; Doyle, P. S.; Dorfman, K. D. Is DNA a Good Model Polymer? *Macromolecules* **2013**, *46* (20), 8369–8382. https://doi.org/10.1021/ma401507f.
(2) Lambert, T. J. FPbase: A Community-Editable Fluorescent Protein Database. *Nat Methods* **2019**, *16* (4), 277–278. https://doi.org/10.1038/s41592-019-0352-8.
(3) Zhabinskaya, D.; Madden, S.; Benham, C. J. SIST: Stress-Induced Structural Transitions in Superhelical DNA. *Bioinformatics* **2015**, *31* (3), 421–422.