Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information

	Description	Sequence (5'—3')
Hybridization experiments	Primer sequence	5' ACATGGCTATGACGGGTAACG 3'
	Target sequence	5' CGTTACCCGTCATAGCCATGT 3'
	Primer-analog fluorescent	5' /fluorophore*/ ACATGGCTATGACGGGTAACG 3'
	Target-analog BHQ2 0 offset	5' CGTTACCCGTCATAGCCATGT /BHQ2/ 3'
	Target-analog BHQ2 5 offset	5' CGTTACCCGTCATAGCCATGTTAGGT /BHQ2/ 3'
	Target-analog BHQ2 10 offset	5' CGTTACCCGTCATAGCCATGTTAGGTCAATA /BHQ2/ 3'
	Target-analog guanine 0 offset	5' CGTTACCCGTCATAGCCATGTG 3'
PCR Experiments	Forward primer	5' TGGTAAATGTGCTCATGTGTTT 3'
	Reverse primer	5' AGTTTCGGATGTTACAAAACTATAGT 3'
	Target DNA	5' TGGTAAATGTGCTCATGTGTTTAAACTTATTTTTAAAGAGATTAA GGATAATATTTTTATTTATATTTTAAGTATTATTTATTTAAGTGTATG TGTAATGAATACAATTTTTGCTAAAAGAACTTTAAACAAAATTGGTA ACTATAGTTTTGTAACATCCGAAACT 3'
	Hydrolysis probe	5' /Texas Red/ GTAATGAA(T)(A)(C)(A)A(T)T(T)TTGCT /IBRQ/ 3'
	L-DNA primer-analog	5' /FAM/ TGGTAAATGTGCTCATGTGTTT 3'
	L-DNA target-analog BHQ2 0 offset	5' AAACACATGAGCACATTTACCA /BHQ2/ 3'
	L-DNA target-analog BHQ2 10 offset	5' AAACACATGAGCACATTTACCAAGACAAGAAC /BHQ2/ 3'
	L-DNA target-analog guanine 0 offset	5' AAACACATGAGCACATTTACCAG 3'

Table S1. List of DNA sequences used in this study. Hybridization experiments were performed using a 21-base primer sequence for malaria detection (see main text reference 26) and the complementary portion of the target sequence (all D-DNA). Unlabeled primers were tested using SYTO 82 intercalating dye. *Fluorescently-labeled primer-analogs were labeled with FAM, HEX, Texas Red, ROX, ATTO Rho101, or Cy5. PCR experiments used D-DNA primers, targets, and hydrolysis probe, along with L-DNA primer-analogs and target-analogs (see main text reference 27).

BHQ2 = Black Hole Quencher® 2. IBRQ = Iowa Black® RQ quencher. Nucleotides in parentheses are locked nucleic acid bases incorporated as a part of the hydrolysis probe.

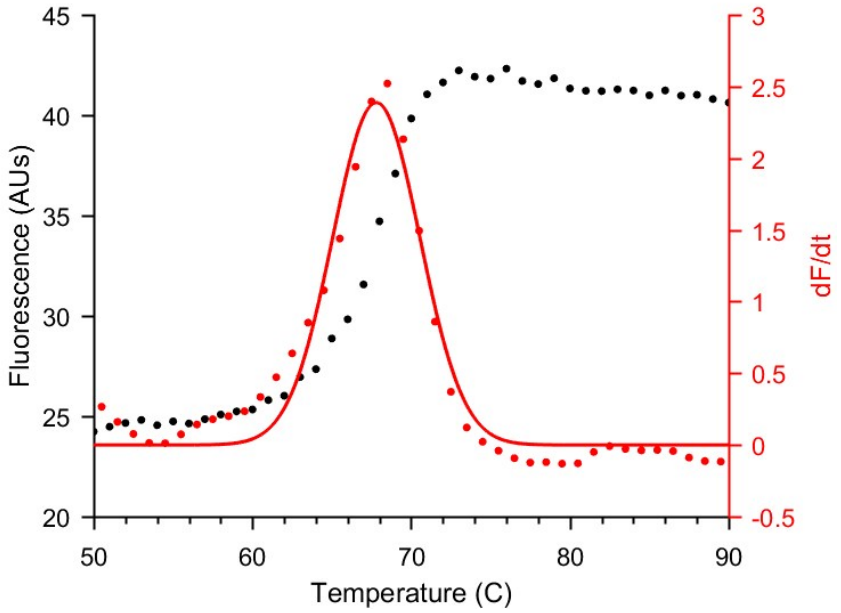

Figure S1. Example annealing curve. The raw fluorescence (black dots) and derivative with respect to time (red dots) were obtained on a Rotor-Gene Q instrument, and plotted as a function of temperature in Matlab. A Gaussian curve (red line) was then fit to the derivative data, and the temperature at which the peak occurred was taken as the annealing temperature.

	0-offset	5-offset	10-offset	guanine
FAM	94.1	91.2	89.9	49.0
HEX	96.5	92.6	90.7	57.9
Texas Red	99.3	98.4	98.5	12.2
ROX	98.9	98.5	98.1	12.3
RHO	98.3	97.8	97.7	14.1
Cy5	99.6	98.8	97.1	10.4

Table S2. Table of calculated quenching efficiencies for each fluorophore and quenching method. 0, 5, and 10-base offset refers to quenching with a Black Hole Quencher 2 which is offset from the fluorophore on the complementary strand by 0, 5, or 10 bases. Efficiencies were calculated by comparing the quenched fluorescence to the fluorescence of a sample containing no quencher. Fluorescence of a buffer sample was used as a baseline.

	BHQ 0-base offset	BHQ 5-base offset	BHQ 10-base offset	Guanine quenching
FAM	67.3 ± 0.0	63.3 ± 0.1	62.8 ± 0.1	62.7 ± 0.1
HEX	68.5 ± 0.1	64.3 ± 0.1	63.5 ± 0.1	63.3 ± 0.2
Texas Red	70.1 ± 0.1	67.3 ± 0.0	66.1 ± 0.2	64.4 ± 0.5
ROX	70.8 ± 0.1	68.3 ± 0.0	67.1 ± 0.3	NA
RHO	69.8 ± 0.1	68.1 ± 0.1	67.1 ± 0.0	NA
Cy5	69.3 ± 0.1	67.5 ± 0.1	66.8 ± 0.0	NA
SYTO 82	62.3 ± 0.1			

Table S3. Complete list of measured annealing temperatures for each fluorophore and quenching scheme, measured using D-DNA. Mean and 95% confidence intervals are listed. Guanine quenching was ineffective for ROX, RHO, and Cy5 fluorophores. The hybridization of unmodified DNA was monitored using SYTO 82 intercalating dye.

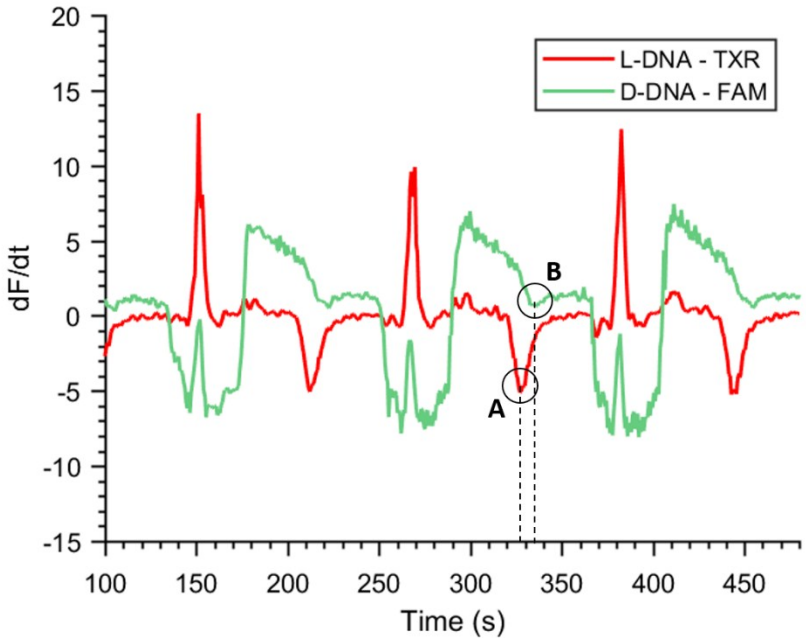

Figure S2. Example dual waveform of the hybridization of Texas Red-labelled L-DNA (red) and FAM-labelled D-DNA (green). The derivative with respect to time of the fluorescence is plotted, and the local minimum peak corresponds to the annealing point. Three cycles are shown here, with the annealing point of the second cycle circled for each fluorescent marker (A and B for Texas Red and FAM-labeled, respectively). The difference in annealing points is the time between the two vertical lines. FAM is a temperature-sensitive fluorophore; its fluorescence decreases as temperature increases.[1] Texas Red does not exhibit this temperature sensitivity, which is why the two waveforms have different baselines.

Labels	Difference in annealing point (seconds)									Mean
	Trial 1			Trial 2			Trial 3			
L-DNA-FAM D-DNA-TXR	6.8	7.5	6.8	7.5	7.6	7.5	7.6	6.9	7.0	7.2 ± 0.23
L-DNA-TXR D-DNA-FAM	5.5	6.2	7.5	10.2	5.5	6.9	8.2	6.9	8.2	7.2 ± 0.98
L-DNA-FAM D-DNA-HEX	1.1	0.9	1.0	0.6	1.5	2.4	0.5	1.3	1.5	1.2 ± 0.37

Table S4. Complete list of the differences in observed annealing point during rapid thermal cycling. Mean and 95% confidence interval are listed. FAM-labeled oligomers annealed approximately 7.2 seconds later than those labeled with Texas Red. Replacing Texas Red with HEX reduced this effect to approximately 1.2 seconds. Fluorophores are quenched with BHQ2 with no offset.

References

1. Liu, W.-T.; Wu, J.-H.; Li, E. S.-Y.; Selamat, E. S., Emission characteristics of fluorescent labels with respect to temperature changes and subsequent effects on DNA microchip studies. *Appl. Environ. Microbiol.* **2005,** *71* (10), 6453-6457.