SUPPLEMENTARY INFORMATION

Design Strategies for Countering the Effect of Fluorophore-Quencher Labelling on DNA Hairpin Thermodynamics

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Section S1. Experimental Methods

Materials

All DNA oligonucleotides used in this study were purchased from Integrated DNA Technologies (IDT) with HPLC purification. The DNA sequences are listed in Table S1, Supporting Information. The lyophilized oligonucleotide was reconstituted in 1× Tris–EDTA buffer (1× TE, pH 8.0) to give 100 μM stock and stored at 4°C and protected from light.

The following chemicals were used as received: sodium chloride (NaCl, ≥ 99.5%, # S3014), magnesium chloride ($MgCl_2$, ≥ 98%, M8266), sodium acetate (#S8625) and absolute ethanol (#E7023) were purchased from Sigma Aldrich. 1× TE (pH 8.0) was purchased from 1st BASE. Phosphate buffer was prepared using sodium phosphate monobasic dehydrate and sodium phosphate dibasic anhydrous purchased from Acros Organics and Fisher Scientific respectively. Milli-Q water with resistance >18.2 MP/cm was used throughout the experiment.

Conjugation of Fluorophore to HCR Hairpin (HP1)

The list of modified HP1 with different combinations of fluorophore/quencher placement, fluorophore and quencher identity, is summarized in Table S2. All quenchers were modified at the point of oligonucleotide synthesis by IDT. For fluorophores which were post-modified onto HP1, the succinimidyl ester form of the fluorophore was conjugated to HP1 at the location of the amine group modification. Briefly, 250 μM of HP1 was mixed with 2.5 mM of fluorophore succinimidyl esters in 0.1M sodium bicarbonate (pH 8.3) reaction buffer. The conjugation reaction took place at room temperature for 6 h under vigorous thermomixer shaking (700 rpm). The dye-HP1 conjugate was purified using ethanol precipitation. Briefly, 0.3 M sodium acetate (pH 5.2) was added to the conjugation product and mixed with 2.5 times volume of absolute ethanol (stored at -20 °C). After 30 min incubation at -20 °C, the slurry was centrifuged at 14 000 rpm for 15 min at 4 °C. The pellet was washed twice with ice-cold 70% ethanol and reconstituted in 1× TE buffer (pH 8.0).

Annealing of Oligo Duplexes and Hairpins

The reaction buffer used in this study was 10 mM phosphate buffer (pH 7.4), 140 mM NaCl, 10 mM $MgCl_2$ and 0.005% Tween-20. A low concentration of Tween-20 was added to disrupt the accumulation of labelled DNA at the air/liquid interface[24] and to improve the run-to-run consistency. Stock HP1 and HP2 were diluted to 10 μM working concentration in the reaction buffer. For oligo hairpins, they were heated to 95°C for 5 min and snap cooled on ice for 30 min in separate tubes. For oligo duplexes, they were heated to 95°C for 5 min and slowly cooled to 25 °C at a rate of – 0.1 °C/s. For longer term storage of up to 3 months, the annealed oligos were kept in DNA LoBind tube (Eppendorf). The exact concentration for each batch of annealed oligos was calculated from A260 measurement using Nanodrop.

Performing Oligo Hybridization Reaction

The same assay reaction buffer was used in this study, i.e. 10 mM phosphate buffer (pH 7.4), 140 mM NaCl, 10 mM $MgCl_2$ and 0.005% Tween-20. All reactions proceeded at room temperature (22 - 25 ºC, based on the temperature reading of the Tecan Spark 10M plate reader in which measurements were performed) for a reaction volume of 10 μL in the 384-well black plate (Grenier, #784900). For each reaction, 5 μL trigger strand and 5 μL oligo duplex or hairpins were mixed to obtain a final reaction condition of 20 nM probes (duplex or hairpins) and the required trigger strand concentration. The real-time fluorescence signal was measured every minute for a total analysis time of up to 1 h, unless stated otherwise. The excitation and emission wavelengths used for each fluorophore-quencher pair are summarized in Table S2.

For each set of experiment (i.e. each figure represents the results from one experimental set), different permutations of probes (duplex or hairpins) and trigger strands were prepared from the same working tube and measured in a single plate reader run. Each experiment was repeated in triplicates. Due to the slight time lag from the pipetting of one well to another, the signal evolution particularly nearer t = 0 was not accurate. However, such experimental design

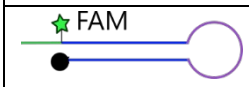
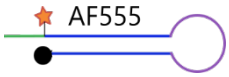
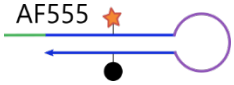
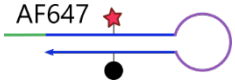
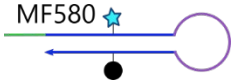
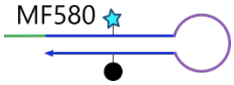
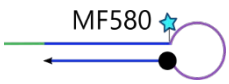
ensured that critical observations (e.g. the same DT9 generated high signal when used with FAM-quencher duplex probe but not much lower signal with FAM-modified hairpin probe) were not artefacts from batch-to-batch variations in the reagents or unintended effects, e.g. loss of reagent at working concentration due to non-specific adsorption to tube surface over time. To avoid cluttering the graph, error bars were omitted from the kinetics curve in the main text but can be found in Section S4.

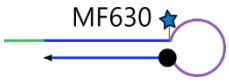
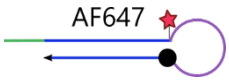
Section S2. List of Oligonucleotide Sequences and Fluorophore / Quencher Combinations

Table S1. List of oligonucleotide (oligo) sequences used in this study.

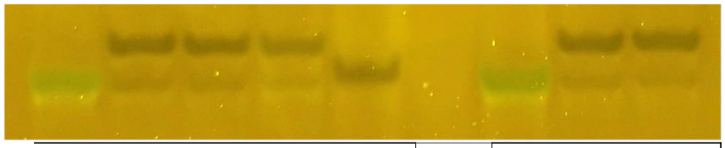
Oligo Strand	Sequences
F (end)	5'- GTT GGA ATT GGG AGT AAG GGC /36-FAM/ -3'
Q (end)	5'- /5IABkFQ/ GCC CTT ACT CCC -3'
F (HP equivalent)	5'- GTT GGA ATT /i6-FAMK/ GGG AGT AAG GGC -3'
Q (HP equivalent)	5'- GCC CTT ACT CCC /3IABkFQ/ -3'
F (int)	5'- GTT GGA ATT GGG AGT /i6-FAMK/ AAG GGC -3'
Q (int)	5'- GCC CT/iBHQ-1dT/ ACT CCC -3'
HP1 (3' FAM)	5'- GTT GGA AT/i6-FAMK/ GGG AGT AAG GGC TCT TAC TTT GCC CTT ACT CCC /3IABkFQ/ -3'
HP1 (3' BHQ_2)	5'- GTT GGA AT/iAmMC6T/ GGG AGT AAG GGC TCT TAC TTT GCC CTT ACT CCC /3BHQ_2/ -3'
HP1 (int BHQ_2)	5' - GTT GGA ATT GGG AG/iAmMC6T/ AAG GGC TCT TAC TTT GCC CT/iBHQ-2dT/ ACT CCC -3'
HP1 (int BHQ_2) (2)	5' - GTT GGA ATT GGG AGT AAG GGC /iAmMC6T/CT TAC TT/iBHQ-2dT/ GCC CTT ACT CCC -3'
HP2	5'- GCC CTT ACT CCC AAT TCC AAC GGG AGT AAG GGC AAA GTA AGA -3'
DT9	5'- GCC CTT ACT CCC AAT TCC AAC -3'
DT9-HP4	5'- GCC CTT ACT CCC AAT TCC AAC **GTC CTT TTG GAC** -3'
DT9-HP6	5'- GCC CTT ACT CCC AAT TCC AAC **GTT GGC TTT TGC CAA C** -3'
DT9-HP10	5'- GCC CTT ACT CCC AAT TCC AAC **GTT GAG CGC GTT TTC GCG CTC AAC** -3'

Table S2. List of different combinations of HP1 strand, fluorophore and quencher modification.

HP1 Construct	Oligo Strand	Fluorophore	Quencher	Excitation / Emission λ
	HP1 (end FAM)	FAM (as synthesized)	Iowa Black® FQ (as synthesized)	495 nm / 525 nm
	HP1 (3' BHQ_2)	AF555 (post modified) #1166-1, Fluoroprobes	Black Hole Quencher®-2 (as synthesized)	550 nm / 580 nm
	HP1 (3' BHQ_2)	AF555 (post modified)	Black Hole Quencher®-2 (as synthesized)	550 nm / 580 nm
	HP1 (3' BHQ_2)	AFDye 647 (post modified) #1121-1, Fluoroprobes	Black Hole Quencher®-2 (as synthesized)	595 nm / 670 nm
	HP1 (int BHQ_2)	AFDye 647 (post modified)	Black Hole Quencher®-2 (as synthesized)	595 nm / 670 nm
	HP1 (int BHQ_2)	mFluor™ Blue 580 SE (post modified) #1178, AAT Bioquest	Black Hole Quencher®-2 (as synthesized)	490 nm / 575 nm
	HP1 (int BHQ_2)	mFluor™ Blue 630 SE (post modified) #1164, AAT Bioquest	Black Hole Quencher®-2 (as synthesized)	490 nm / 630 nm
	HP1 (int BHQ_2) (2)	mFluor™ Blue 580 SE (post modified) #1178, AAT Bioquest	Black Hole Quencher®-2 (as synthesized)	490 nm / 575 nm

	HP1 (int BHQ_2) (2)	mFluor™ Blue 630 SE (post modified) #1164, AAT Bioquest	Black Hole Quencher®-2 (as synthesized)	490 nm / 630 nm
	HP1 (int BHQ_2) (2)	AFDye 647 (post modified) #1121-1, Fluoroprobes	Black Hole Quencher®-2 (as synthesized)	595 nm / 670 nm

(a)



	HP1 (int)					HP (3')		
Dye	Unconj.	AF555	AF647	MF580	MF630	Unconj.	AF555	AF647
MW	-	930	956	833	710	-	930	956
% Yield	-	92.2 (±7.18)	90.1 (±0.86)	88.6 (±2.60)	100	-	90.7 (±0.66)	90.1 (±0.13)

(b)

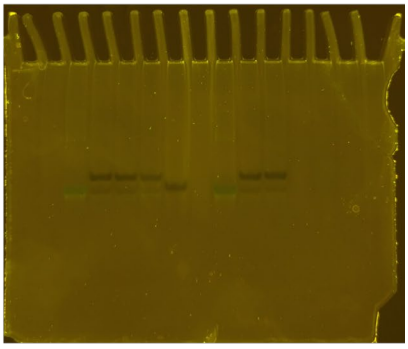


Figure S1. (a) The conjugation yield of the in-house conjugated hairpins was characterized using 20% native PAGE gel and stained with 1X SYBR Gold nucleic acids stain. An estimated 90% conjugation yield was achieved using Image J gel analysis tool over two gel runs. Note that a negative staining pattern was obtained as the SYBR Gold stain was quenched by the presence of the quencher, i.e. the band appeared darker than the background gel staining. The gel image has been cropped and adjusted for brightness and contrast for better visualization of the gel band. (b) Raw image of the gel as captured under blue light illumination and orange filter using Huawei P30 smartphone camera.

Section S3. Reaction Pathways for Different Trigger Combinations

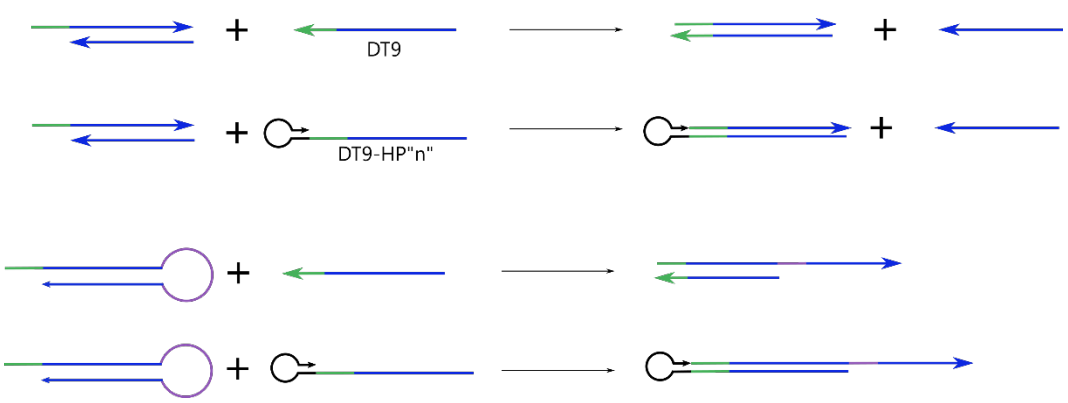


Figure S2. Products formed when different combinations of oligo reactants (fluorophore-quencher and trigger strands) were reacted.

Section S4. Further Characterisation of Different Probe and Trigger Configurations

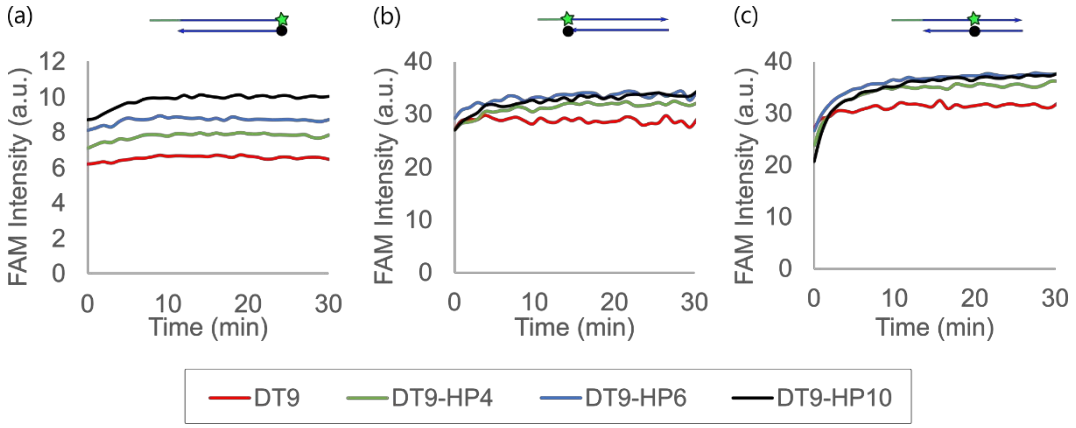


Figure S3. Evolution of fluorescence signal when various configurations of direct triggers were reacted with duplex probes labelled with FAM and quencher at different positions: (a) most common design at the distal ends, (b) modification at the start of the strand displacement region (similar to end-modified hairpin probe) and (c) modification in the middle of the strand displacement region (similar to internally-modified hairpin probe).

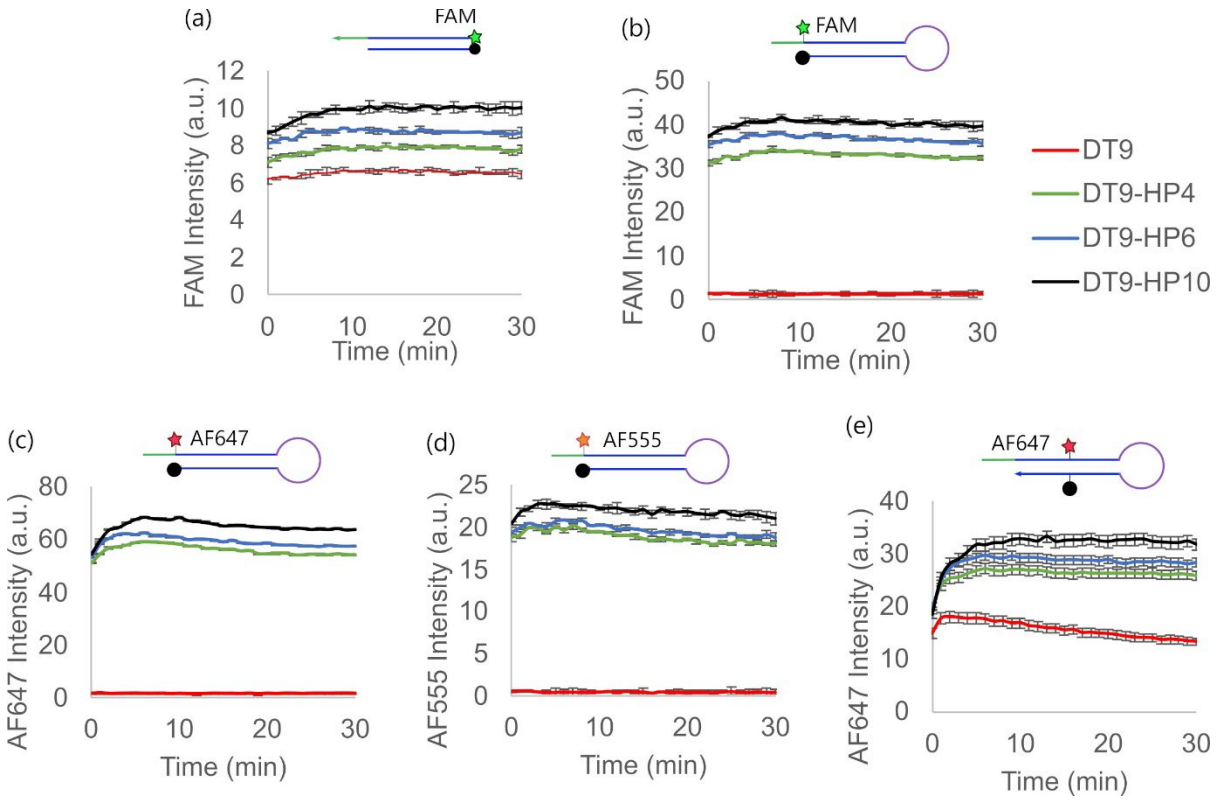


Figure S4. Representation of some of the fluorescence kinetics curve from Figure 2, with error bars (standard deviation) included to indicate the reproducibility of the fluorescence measurement across triplicate runs.

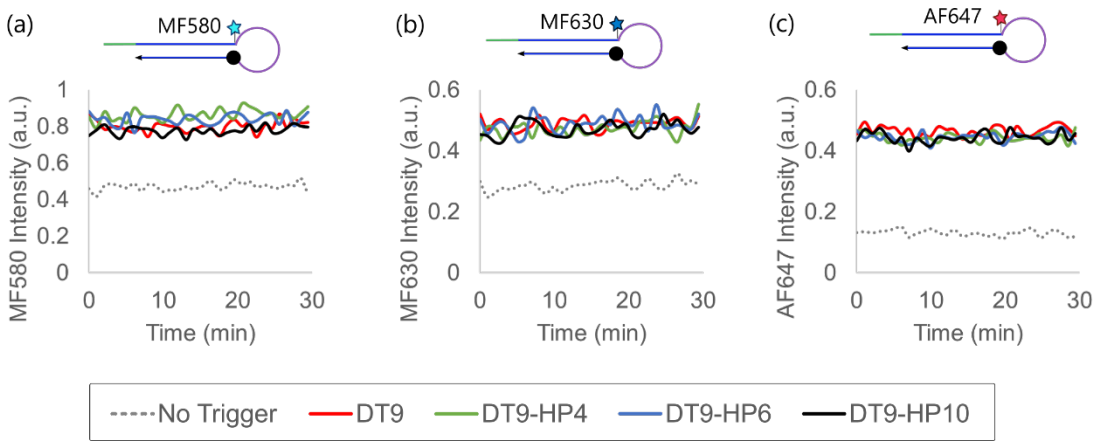


Figure S5. Evolution of fluorescence signal when various configurations of direct triggers were reacted with hairpin modified with the respective fluorophore (and Black Hole Quencher 2) (a) mFluor 580 (MF580), (b) mFluor 630 (MF630) and (c) Alexa Fluor 647 (AF647). Note that the absolute turn-on fluorescence signal (upon opening of the hairpin in the presence of the trigger strand, versus the closed hairpin state) was modest as the separation of the fluorophore and quencher after hairpin opening was only 9 nt (ca. 3 nm, where the quenching efficiency remained high) using the hairpin design consistent with the rest of the study. The baseline case of no trigger strand was included to confirm the successful hairpin opening despite the modest final fluorescence intensity measured.

Section S5. Characterisation of the Melting Temperature of Hairpin-Trigger Complexes Formed

Table S3. Melting temperature of the respective systems in presence of 20 nM of each stated reactant. All data are shown as mean ± standard deviation (n = 3).

System	Melting Temperature (°C)
HP1	68.8 (±1.2)
HP1 + DT9	69.2 (±1.1)
HP1 + DT9-HP4	74.3 (±0)
HP1 + DT9-HP6	75.8 (±0.07)
HP1 + DT9-HP10	79.0 (±0.64)

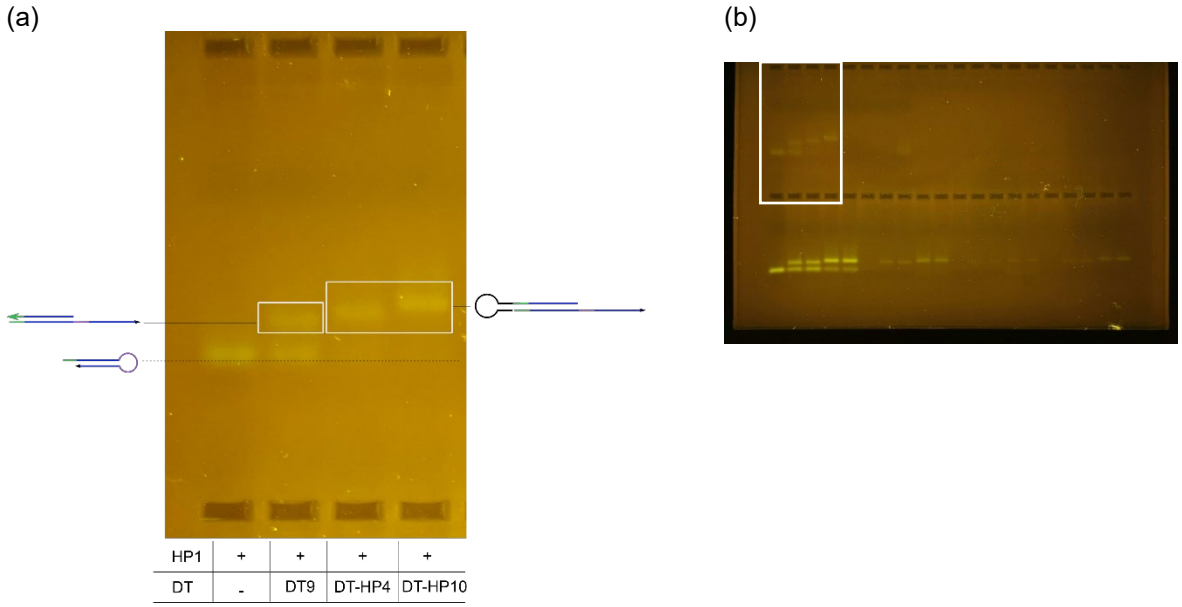


Figure S6. (a) The predominant species responsible for the melting temperature was characterized using gel electrophoresis (3% agarose gel, stained with 0.5X SYBR Gold, image taken under blue light illumination and orange filter using Huawei P30 smartphone camera) and identified as the hairpin-trigger (DT9, DT9-HP4 or DT9-HP10) duplex product. (b) Raw gel image with the relevant segment shown in (a) boxed up.