Bulged Invader probes: Activated duplexes for mixed-sequence dsDNA recognition with improved thermodynamic and kinetic profiles

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ELECTRONIC SUPPLEMENTARY INFORMATION

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Protocol - synthesis and purification of ONs. Modified ONs were synthesized on an automated DNA synthesizer (0.2 μmol scale) using a long chain alkyl amine controlled pore glass (LCAA-CPG) solid support with a pore size of 500 Å. The corresponding phosphoramidite of monomer **X** was prepared as previously described[S1] and incorporated into ONs via hand-couplings (0.05 M in acetonitrile, using 0.01 M 4,5-dicanoimidazole as the activators (15 min)) with extended oxidation (45 s). The nonanyl linker was incorporated in a similar manner using the commercially available DMT-nonane diol phosphoramidite (ChemGenes). Treatment with 32% ammonia (55 °C, 17 h) facilitated deprotection and cleavage from solid support. DMT-protected ONs were purified via ion-pair reverse phase HPLC (XTerra MS C18 column: 0.05 M triethyl ammonium acetate and acetonitrile gradient) followed by detritylation (80% acetic acid, 20 min) and precipitation (NaOAc, $NaClO_4$, acetone, -18 °C, 16 h). The purity and identity of synthesized ONs were verified using analytical HPLC (>85% purity) and MALDI-MS analysis (Tables S1) recorded on a Quadrupole Time-of-Flight (Q-TOF) mass spectrometer with anthranilic acid or 2,4,6-trihydoxyacetophenone matrix.

Protocol - thermal denaturation experiments. The concentrations of ONs were estimated using the following extinction coefficients (OD_{260}/μmol): G (12.01), A (15.20), T (8.40), C (7.05) and pyrene (22.4). Thermal denaturation temperatures were calculated as the first-derivative maximum of A_{260} vs T curves. ONs (0.5 μM) were annealed (85 °C, 2 min) in medium salt buffer ($[Na^+]$ = 110 mM, $[Cl^-]$ = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4), [EDTA] = 0.2 mM) and subsequent cooling to the starting temperature. The experimental temperature ranged from 5 °C to at least 15 °C above the T_m, with the T_m determined as the average of two experiments within ±1.0 °C.

Protocol - electrophoretic mobility shift assay. DNA hairpins were obtained from commercial sources and used without further purification. Hairpins were labeled using the 2nd generation DIG Gel Shift Kit (Roche Applied Bioscience). Briefly, 11-digoxigenin-ddUTP was incorporated at the 3′-end of the hairpin (100 pmol) using a recombinant DNA terminal transferase. The reaction mixture was quenched through addition of EDTA (0.05 M), diluted to 68.8 nM, and used without further processing. The recognition experiments were conducted essentially as previously reported.[S1] Thus, Invader probes (variable concentration) were annealed (90 °C for 2 min, followed by cooling to room temperature) and subsequently incubated with DIG-labeled DNA hairpins (34.4 nM final concentration) in HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.2, 10% sucrose, 1.44 mM spermine tetrahydrochloride) at either 8 °C ± 2 °C or ambient temperature 22 °C ± 2 °C for a specified period. For time-course experiments (Figure 4 and S6), aliquots were taken at specific time points, flash frozen in liquid N_2, and stored at -76 °C until analysis. Loading dye (6X) was added and the

reaction mixtures were loaded onto 12% non-denaturing TBE-PAGE (45 mM tris-borate, 1 mM EDTA; acrylamide:bisacrylamide (19:1)). Electrophoresis was performed using constant voltage (70 V) at ~4 °C for 1.5 h. Bands were blotted onto positively charged nylon membranes (100 V, 30 min, ~4 °C) and cross-linked through exposure to UV light (254 nm, 5 × 15 watt bulbs, 3 min). Membranes were incubated with anti-digoxigenin-alkaline phosphatase F_{ab} fragments as recommended by manufacturer, and transferred to a hybridization jacket. Membranes were incubated with the chemiluminescence substrate (CSPD) for 10 min at 37 °C, and chemiluminescence was captured on X-ray films. Digital images of developed X-ray films were obtained using a Fluor-S MultiImager and quantified using appropriate software (Quantity One). The percentage of dsDNA recognition was calculated as the intensity ratio between the recognition complex band and the total lane. Unless otherwise noted, an average of three independent experiments is reported along with standard deviations (±).

Definition - interstrand zipper arrangement. The following nomenclature describes the relative arrangement between two monomers positioned on opposing strands in a duplex. The number *n* describes the distance measured in number of base-pairs and has a positive value if a monomer is shifted toward the 5'-side of its own strand relative to a second reference monomer on the other strand. Conversely, *n* has a negative value if a monomer is shifted toward the 3'-side of its own strand relative to a second reference monomer on the other strand.

Table S1. MALDI-MS of modified ONs.

ON	Sequence	Observed *m/z* $[M+H]^+$	Calculated *m/z* $[M+H]^+$
ON3	5′- GG **9** TA**T**A**T**ATAGGC	4667.5	4667.6
ON4	3′- CC **9** ATA**T**A**T**ATCCG	4547.4	4547.5
ON5	5′- GGTA**T**A**T**ATAG **9** GC	4666.7	4667.6
ON6	3′- CCATA**T**A**T**ATC **9** CG	4547.4	4547.5
ON7	5′- GG **9** TA**T**A**T**ATAG **9** GC	4889.2	4889.7
ON8	3′- CC **9** ATA**T**A**T**ATC **9** CG	4769.1	4769.6

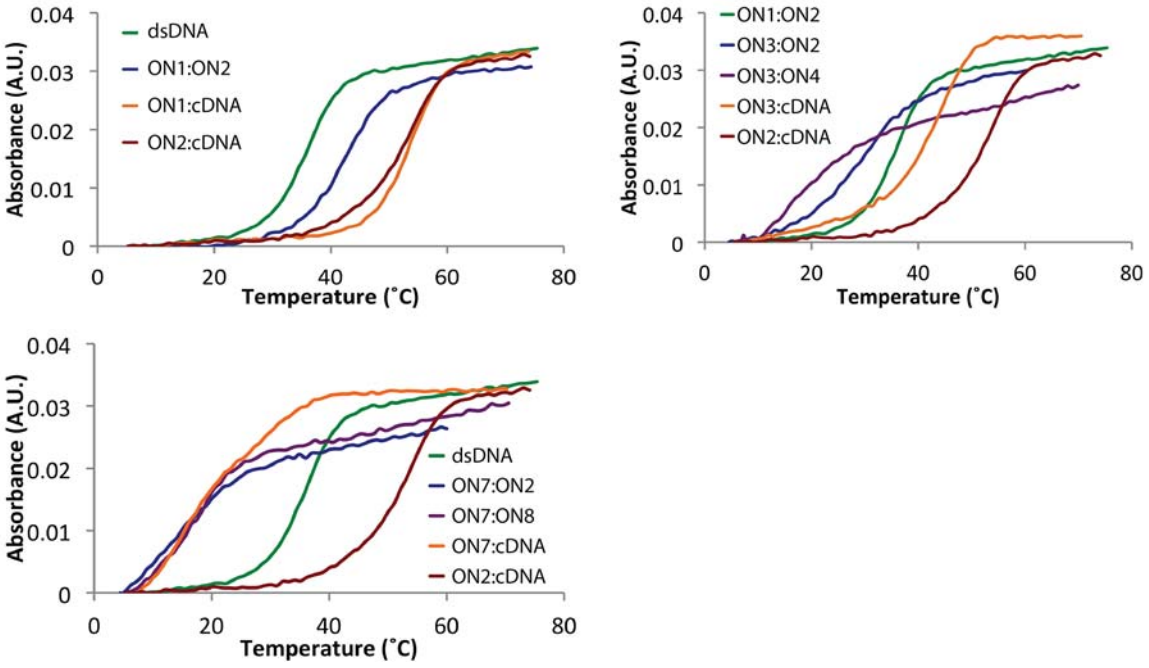

Figure S1. Representative thermal denaturation curves of Invader probes, duplexes between individual probe strands and cDNA, and unmodified reference duplexes. For experimental conditions, see Table 1.

Table S2. Degree of recognition of DNA hairpin **DH1** using a 200-fold molar excess of different Invader probes at two different temperatures.

ON	Sequence	Rec_{200} (%)	
		8 °C[a]	22 °C[b]
1 2		22	>90
3 2		38	>90
5 2		37	>90
1 4		30	>90
1 6		34	>90
3 4		51	>90
5 6		42	>90
7 2		55	>90
1 8		43	>90
3 6		<10	20
5 4		<10	43

[a] Data shown in Figure 2.

[b] Experiments performed in duplicate.

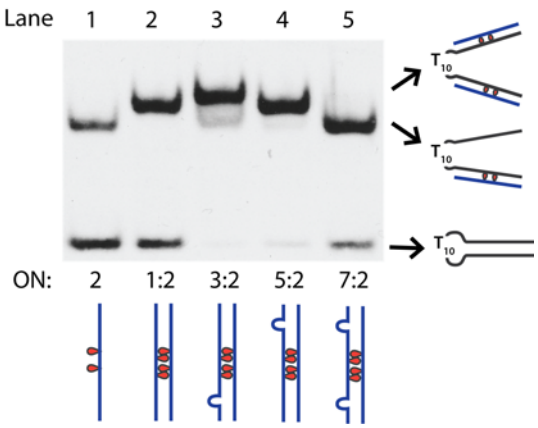

Figure S2. The structure of recognition complexes formed upon incubation of different Invaders strands/probes and DNA hairpin **DH1** at 22 °C. Invader probes used at 100-fold excess (3.44 μM). Experimental conditions are otherwise as described in Figure 2. The greater mobility of the recognition complex between **ON7**:**ON2** and **DH1** relative to the complexes involving **ON1**:**ON2**, **ON3**:**ON2** and **ON5**:**ON2**, and the similar mobility relative to the complex involving **ON2**, strongly suggests that a binary complex is formed. Nonetheless, recognition of **DH1** is more efficient with **ON7**:**ON2** than **ON2**, indicating that **ON7** – despite its low cDNA affinity – plays a role in the recognition process, possibly through weak/transient binding to the binary complex, thus preventing re-formation of **DH1**.

Table S3. Thermal denaturation temperatures (T_m's) of additional Invader probes.[a]

ON	Sequence	T_m [ΔT_m] (°C) Invader	5'-Inv:cDNA	3'-Inv:cDNA
1 2		45	55.5	55.5
7 4		<15	<15	46.5
7 6		<15	<15	43.5
3 8		<15	44	<15
5 8		<15	44.5	<15
7 8		<15	<15	<15

[a] The corresponding unmodified dsDNA has a T_m = 37.5 °C.

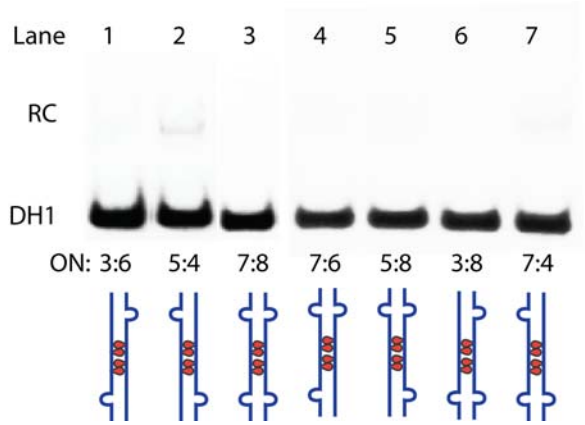

Figure S3. Recognition of model dsDNA target **DH1** using 200-fold molar excess (6.88 μM) of different Invader probes at 8 °C. Image is a composite of two electrophoretograms (lanes 1-3 and lanes 4-7). Experiments were performed in duplicate. Very similar results (not shown) were observed at 22 °C.

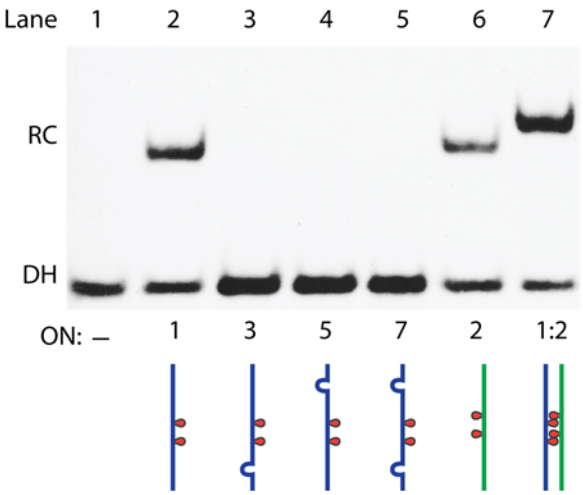

Figure S4. Recognition of **DH1** by individual Invader probe strands. Each lane includes **DH1** incubated with 200-fold molar excess (6.88 μM) of single-stranded probes or conventional Invader probe **ON1**:**ON2** at 22 °C. Experiments were performed in duplicate. Single-stranded probes, **ON1** and **ON2,** result in ~50% recognition of **DH1**, while **ON1**:**ON2** results in ~70% recognition, emphasizing the need for both strands for maximal recognition.

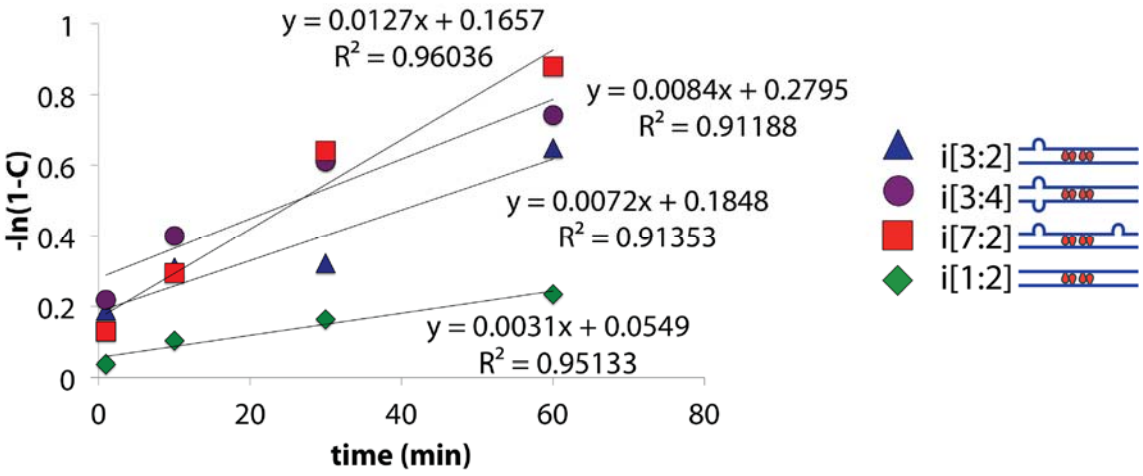

Figure S5. Pseudo-first order rate plots for dsDNA recognition by Invader probes at 22 °C. Raw time profiles shown in Figure 4.

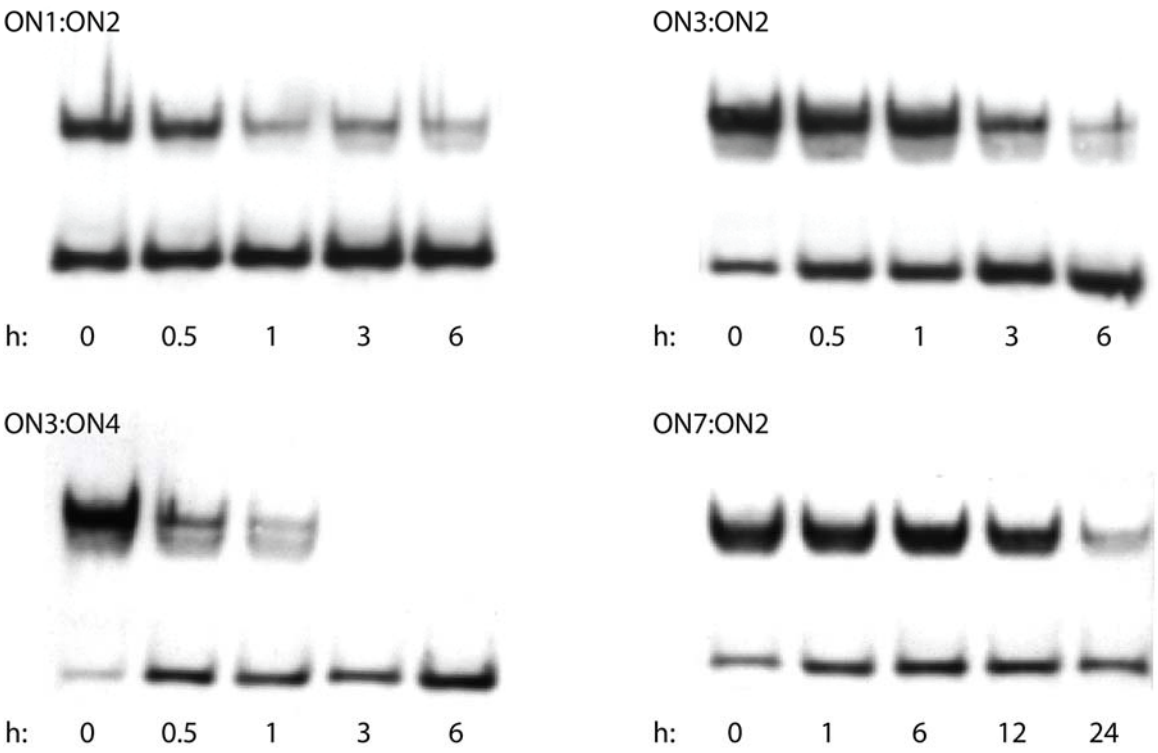

Figure S6. Representative electrophoretograms from the competitive dissociation assay shown in Figure 4.

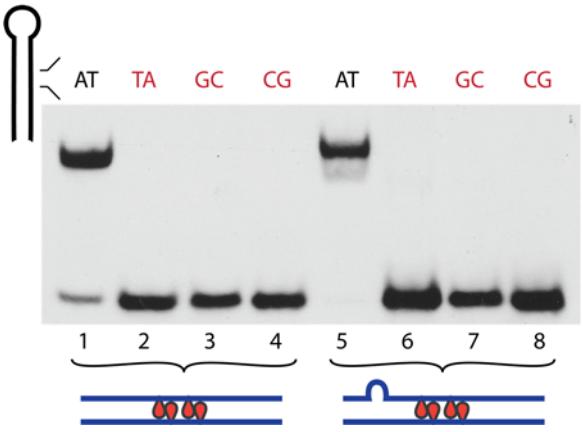

Figure S7. Specificity of Invader-mediated dsDNA recognition. Fully base-paired DNA hairpins containing a single base-pair mismatch (red) relative to Invader probes **ON1**:**ON2** (left) and **ON3**:**ON2** (right) were used. Base-pairs above electrophoretograms correspond to **B**:**B′** in: 5'-GGTAT**B**TATAGGC-T_{10}-GCCTATA**B′**ATACC. A 200-fold molar excess of Invader probes (6.88 μM) were used. Experiments were performed in duplicate at 22 °C (shown) or 37 °C (results not shown – identical observations). Excellent discrimination of the singly mismatched DNA hairpins was observed with both Invader probes.

References

S1) S. Karmakar, B. A. Anderson, R. L. Rathje, S. Andersen, T. Jensen, P. Nielsen and P. J. Hrdlicka, *J. Org. Chem.*, 2011, **76**, 7119-7131.