**Supporting Information**

MALDI-MS of modified ONs (Table S1) S2

Representative thermal denaturation profiles (Figure S1) S3

DNA selectivity of X- and Y-modified 9-mer ONs (Table S2) S3

Discrimination of mismatched DNA targets by X2 and Y2 (Table S3) S4

Discrimination of mismatched DNA targets by X6 and Y6 (Table S4) S4

Absorption spectra of X1–X6 in absence or presence of cDNA or cRNA (Figure S2) S5

Absorption spectra of Y1–Y6 in absence or presence of cDNA or cRNA (Figure S3) S6

Absorbance of X-/Y-modified PS-DNA in absence/presence of cDNA/cRNA (Table S5) S7

Steady-state fluorescence emission spectra of X5 and Y5 in absence or presence of cDNA or cRNA (Figure S4) S7

Thermal advantage (TA) values for 13-mer PS-DNA/PO-DNA Invader probes (Table S6) S8

Recognition of dsDNA model target DH1 using X2:X5 or Y2:Y5 (Figure S5) S9

Recognition of dsDNA model target DH2 using different 13-mer Invader probes (Figure S6) S10

DNase I protocol and discussion S11

Nuclease susceptibility of PO-Invader probes in the presence of DNase I (Figure S7) S11

Supplementary references S12
Table S1. MALDI-MS of modified PS-DNA a.

| ON  | PS-DNA Sequence               | Calc. \(m/z\) \([M + H]^+\) | Found \(m/z\) \([M + H]^+\) |
|-----|------------------------------|------------------------------|------------------------------|
| X1  | 5'-GXG ATA TGC               | 3110.4                       | 3110.3                       |
| X2  | 5'-GTG AXA TGC               | 3110.4                       | 3110.4                       |
| X3  | 5'-GTG ATA XGC               | 3110.4                       | 3110.2                       |
| X4  | 3'-CAC XAT ACG               | 3039.4                       | 3039.2                       |
| X5  | 3'-CAC TAX ACG               | 3039.4                       | 3039.5                       |
| X6  | 3'-CAC XAX ACG               | 3211.5                       | 3212.0                       |
| Y1  | 5'-GYG ATA TGC               | 3097.5                       | 3097.2                       |
| Y2  | 5'-GTG AYA TGC               | 3097.5                       | 3097.4                       |
| Y3  | 5'-GTG ATA YGC               | 3097.5                       | 3097.2                       |
| Y4  | 3'-CAC YAT ACG               | 3026.5                       | 3026.3                       |
| Y5  | 3'-CAC TAY ACG               | 3026.5                       | 3026.4                       |
| Y6  | 3'-CAC YAY ACG               | 3242.5                       | 3242.3                       |
| X7  | 5'-GGX ATA TAT AGG C         | 4433.5                       | 4433.4                       |
| X8  | 3'-CCA XAT ATA TCC G         | 4313.5                       | 4313.4                       |
| X9  | 5'-GGX AXA TAT AGG C         | 4662.6                       | 4662.4                       |
| X10 | 3'-CCA XAX ATA TCC G         | 4542.6                       | 4542.4                       |
| X11 | 5'- GGT AXA XAT AGG C        | 4662.6                       | 4662.6                       |
| X12 | 3'-CCA TAX AXA TCC G         | 4542.6                       | 4542.4                       |
| X13 | 5'-GGX ATA TAX AGG C         | 4662.6                       | 4662.4                       |
| X14 | 3'-CCA XAT ATA XCC G         | 4542.6                       | 4542.5                       |
| Y7  | 5'-GGY ATA TAT AGG C         | 4420.5                       | 4420.5                       |
| Y8  | 3'-CCA YAT ATA TCC G         | 4300.5                       | 4300.5                       |
| Y9  | 5'-GGY AYA TAT AGG C         | 4636.6                       | 4636.6                       |
| Y10 | 3'-CCA YYA ATA TCC G         | 4516.5                       | 4516.6                       |
| Y11 | 5'- GTG AYA YAT AGG C        | 4636.6                       | 4636.6                       |
| Y12 | 3'-CCA TAY AYA TCC G         | 4516.5                       | 4516.5                       |
| Y13 | 5'-GGY ATA TAY AGG C         | 4636.6                       | 4636.8                       |
| Y14 | 3'-CCA YYAT ATA YCC G        | 4516.5                       | 4516.7                       |

a For structure of monomers X and Y see Figure 1 in the main manuscript.
**Figure S1.** Representative thermal denaturation profiles of duplexes between X-/Y-modified PS-DNA strands and cDNA. For experimental conditions, see Table 1.

**Table S2.** DNA selectivity of X- and Y-modified PS-DNA \(^a\).

| ON | PS-DNA Sequence | \(\Delta \Delta T_m\) (DNA-RNA) (°C) |
|----|-----------------|-----------------------------------|
| X1 | 5’-G\(\text{X}\)G ATA TGC | +9.0 |
| X2 | 5’-G\(\text{X}\)T G\(\text{A}\) TGC | +13.0 |
| X3 | 5’-G\(\text{X}\)T G\(\text{A}\) XGC | +12.0 |
| X4 | 3’-C\(\text{X}\) C\(\text{A}\)T ACG | +9.0 |
| X5 | 3’-C\(\text{X}\) C\(\text{A}\)X ACG | +9.5 |
| X6 | 3’-C\(\text{X}\) C\(\text{A}\)X ACG | +19.0 |
| Y1 | 5’-G\(\text{Y}\)G ATA TGC | +8.5 |
| Y2 | 5’-G\(\text{Y}\)T G\(\text{A}\) TGC | +12.0 |
| Y3 | 5’-G\(\text{Y}\)T G\(\text{A}\) YGC | +9.5 |
| Y4 | 3’-C\(\text{Y}\) C\(\text{A}\)T ACG | +7.5 |
| Y5 | 3’-C\(\text{Y}\) C\(\text{A}\)Y ACG | +9.5 |
| Y6 | 3’-C\(\text{Y}\) C\(\text{A}\)Y ACG | +17.0 |

\(^a\) DNA selectivity defined as \(\Delta \Delta T_m\) (DNA-RNA) = \(T_m\) (vs. cDNA) - \(T_m\) (vs. cRNA).
Binding Specificity of X-/Y-modified PS-DNA

The binding specificities of centrally modified 9-mer PS-DNA strands were studied using DNA targets with mismatched nucleotides opposite to the modification (Table S3). Excellent discrimination of the C-mismatched target is observed, while discrimination of G- or T-mismatched targets is much less efficient. On the other hand, doubly modified 9-mer PS-DNA discriminate DNA targets with a single mismatched nucleotide opposite of the central 2'-deoxyriboadenosine very efficiently (Table S4). These trends mirror our observations with X-/Y-modified PO-DNA strands, further suggesting that the pyrene moieties are intercalating upon cDNA hybridization [1,2].

Table S3. Discrimination of mismatched DNA targets by X2 and Y2 a.

| ON | PS-DNA Sequence | B = DNA: 3'-CAC TBT ACG | Tm (°C) | ΔTm (°C) |
|----|-----------------|---------------------------|---------|----------|
| X2 | 5'-GTG A XA TGC |                           | 32.5    | −16.0    |
| Y2 | 5'-GTG A YA TGC |                           | 31.5    | −13.0    |

a For conditions of thermal denaturation experiments, see Table 1. Tm’s of fully matched duplexes are shown in bold. ΔTm = change in Tm relative to fully matched DNA:DNA duplex.

Table S4. Discrimination of mismatched DNA targets by X6 and Y6 a.

| ON | PS-DNA Sequence | B = DNA: 5'-GTG ABA TGC | Tm (°C) | ΔTm (°C) |
|----|-----------------|---------------------------|---------|----------|
| X6 | 3'-CAC XAX ACG  |                           | 30.5    | −20.5    |
| Y6 | 3'-CAC YAY ACG  |                           | 30.0    | −18.5    |

a For conditions of thermal denaturation experiments, see Table 1. Tm’s of fully matched duplexes are shown in bold. ΔTm = change in Tm relative to fully matched DNA:DNA duplex.
Figure S2. Absorption spectra of X1–X6 in absence or presence of cDNA/cRNA. Spectra were recorded at $T = 5 \, ^\circ C$ using each strand at 1.0 $\mu$M concentration in $T_m$ buffer. Note, different axis scales are used.
Figure S3. Absorption spectra of Y1–Y6 in absence or presence of cDNA/cRNA. Spectra were recorded at $T = 5\, ^\circ\text{C}$ using each strand at 1.0 μM concentration in $T_m$ buffer. Note, different axis scales are used.
**Table S5.** Absorption maxima in the 300–500 nm region for X- and Y-modified PS-DNA and the corresponding duplexes with cDNA or cRNA.

| ON | PS-DNA Sequence | $\lambda_{\text{max}}$ [Å$\lambda_{\text{max}}$]/nm | SSP  | +cDNA | +cRNA |
|----|-----------------|--------------------------------------------|------|-------|-------|
| X1 | 5'-G$X$G ATA TGC | 350 351 [+1] 350 [+0] | 348 353 [+5] 351 [+3] | 349 352 [+3] 351 [+2] | 349 349 [+0] 349 [+2] |
| X2 | 5'-GTG A$X$A TGC | 350 351 [+1] 351 [+1] | 349 352 [+2] 351 [+1] | 349 352 [+3] 351 [+2] | 349 352 [+3] 350 [+1] |
| Y1 | 5'-GYG ATA TGC | 350 351 [+1] 351 [+1] | 349 352 [+2] 351 [+1] | 349 352 [+3] 351 [+2] | 349 352 [+3] 350 [+1] |
| Y2 | 5'-GTG A$Y$A TGC | 350 351 [+1] 351 [+1] | 349 352 [+2] 351 [+1] | 349 352 [+3] 351 [+2] | 349 352 [+3] 350 [+1] |

*a SSP = single-stranded probe. Measurements were performed at 5 °C using a spectrophotometer and quartz optical cells with 1.0 cm path lengths. For buffer conditions, see Table 1.

**Figure S4.** Representative fluorescence spectra of single-stranded X5 and Y5 and the corresponding duplexes with cDNA/cRNA. Spectra were recorded at $T = 10$ °C using $\lambda_{\text{ex}} = 350$ nm. Each strand was used at 1.0 μM concentration in $T_m$ buffer.
**Table S6.** Thermal advantage (TA) values for 13-mer PS-DNA/PO-DNA Invader probes.

| ON | Invader Probe | | BS | PS-DNA Backbone | PO-DNA Backbone |
|----|---------------|---|---|----------------|----------------|
|    |               |   | TA°C | TA°C |               | X | Y | X | Y |
| B7 | 5'-GGBATA TAT AGG C |                | 20 | 20 | 22 | 18 |
| B8 | 3'-CCA BAT ATA TCC G |                | 28.5 | 23 | 25 | 29.5 |
| B9 | 5'-GGB ABA TAT AGG C |                | 34.5 | 25.5 | 35 | 28.5 |
| B10| 3'-CCA BBA BATA TCC G |               | 27 | 23.5 | 32 | 21.5 |
| B11| 5'- GTG ABA BAT AGG C |               | 34.5 | 25.5 | 35 | 28.5 |
| B12| 3'-CCA TAB ABA TCC G |               | 27 | 23.5 | 32 | 21.5 |
| B13| 5'-GGGBATA TAB AGG C |               | 27 | 23.5 | 32 | 21.5 |
| B14| 3'-CCA BBAT ATA BCC G |               | 27 | 23.5 | 32 | 21.5 |

a The term thermal advantage (TA) = \( T_m (5'\text{-Inv:cDNA}) + T_m (3'\text{-Inv:cDNA}) - T_m (\text{Invader probe}) - T_m (\text{dsDNA target}) \), serves as a first approximation to describe the energy difference between the ‘products’ and ‘reactants’ of the prototypical recognition process, with more positive values signifying greater thermodynamic dsDNA recognition potential [3]. See Table 1 in main manuscript for \( T_m \)’s of 5'-Inv:cDNA and 3'-Inv:cDNA. See Table 2 in the main manuscript for \( T_m \)’s of Invader probes. \( T_m \) of the isosequential dsDNA target is 37.5 °C [3].

b Data from reference [3].
Figure S5. Recognition of dsDNA model target DH1 using X2:X5 or Y2:Y5. (a) Illustration of recognition process. Sequence of DH1: 5′-GTGATATGC-(T10)-GCTTATCAC-DIG-3′; (b) representative gel electrophoretograms from experiments in which DH1 (34.4 nM) was incubated with X2:X5 or Y2:Y5 at ambient temperature for 12-16 h; (c) representative gel electrophoretograms from experiments in which DH1 (34.4 nM) was annealed in the presence of X2:X5 or Y2:Y5 at 85 °C for 15 min, followed by cooling to room temperature over ~30 min and incubation at ambient temperature for 12–16 h. Experiments were conducted in 1X HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 10% sucrose, 1.4 mM spermine tetrahydrochloride, pH 7.2) and then run on 16% non-denaturing PAGE (performed at 70 V, 2.5 h, ~4 °C) using 0.5× TBE as a running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA); DIG: digoxigenin.
Figure S6. Recognition of dsDNA model target DH2 using different Invader probes. (a) Representative electrophoretograms for recognition of DH2 using 1- to 500-fold excess of the PO-DNA versions of X11:X12, X13:X14, Y11:Y12, Y13:Y14; (b) dose-response curves (average of at least three independent experiments, error bars represent standard deviation) relative to the corresponding PS-DNA Invaders. The sequence of DNA hairpin DH2 and experimental are given in Figure 3.
Protocol—Stability of Invader Probes against DNase 1

An aqueous solution of DNase I (Worthington Biochemical Corporation—0.61 μL of a 2 μg/mL solution) was added to a 6.3 μM solution of a specific pre-annealed Invader in TE buffer (100 μL, 10 mM Tris-HCl, 0.1 mM EDTA, 10 mM MgCl₂, pH 8.0) and the mixture was incubated at 20 °C in a water bath. Aliquots (10 μL) were removed at specific times (0.5, 1, 2, 5, 10, 20 and 30 min) and degradation was quenched by addition of ethidium bromide buffer (2.0 mL, 5 mM Tris-HCl, 0.5 mM EDTA, 0.5 μg/mL EtBr, pH 8.0). The fluorescence intensity of the solution was measured (λ<sub>ex</sub> = 525 nm; λ<sub>em</sub> = 600 nm) using the same instrumentation employed for the steady-state fluorescence experiments. Intensities were averaged over 15 minutes. Experiments were performed in duplicates and representative graphs are shown.

Discussion—Stability of Invader Probes against DNase 1

The stability of Invader probes against DNase I was evaluated using an ethidium bromide based assay. In this assay, high levels of fluorescence are observed when the studied duplex is intact due to intercalating ethidium bromide, while low levels of fluorescence are expected if a duplex has been degraded to single strands (or shortened to a level where the duplex dissociates) [4,5].

Similar assays have been used to show that long PS-DNA duplexes exhibit excellent stability against DNase I [6,7]. Figure S7 shows the fluorescence intensity profiles of representative 13-mer Invaders in the presence of DNase I. The PO-DNA analogs of X13:X14 and Y13:Y14 are moderately resistant to DNase I degradation with half-lives of 15 min and >30 min, respectively, whereas the unmodified PO-DNA duplex is rapidly degraded.

PS-DNA Invaders X13:X14 and Y13:Y14 did not show any change in fluorescence emission relative to background EtBr buffer (data not shown), presumably because the probe duplexes are dissociated into single strands at the experimental conditions used for this assay (T<sub>m</sub> of X13:X14 and Y13:Y14 are <25 °C, Table 2).

![Figure S7. DNase I stability of PO-DNA Invader probes X13:X14, Y13:Y14 and the corresponding unmodified PO-DNA duplex, as assessed by ethidium bromide assay. Curves are average of two experiments.](image)
**Supplementary References**

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