Supplementary Material (ESI) for Chemical Communications
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Electronic supplementary information (ESI) for *Chem. Commun.*

Optimized DNA-Targeting using Triplex Forming C5-Alkynyl Functionalized LNA†

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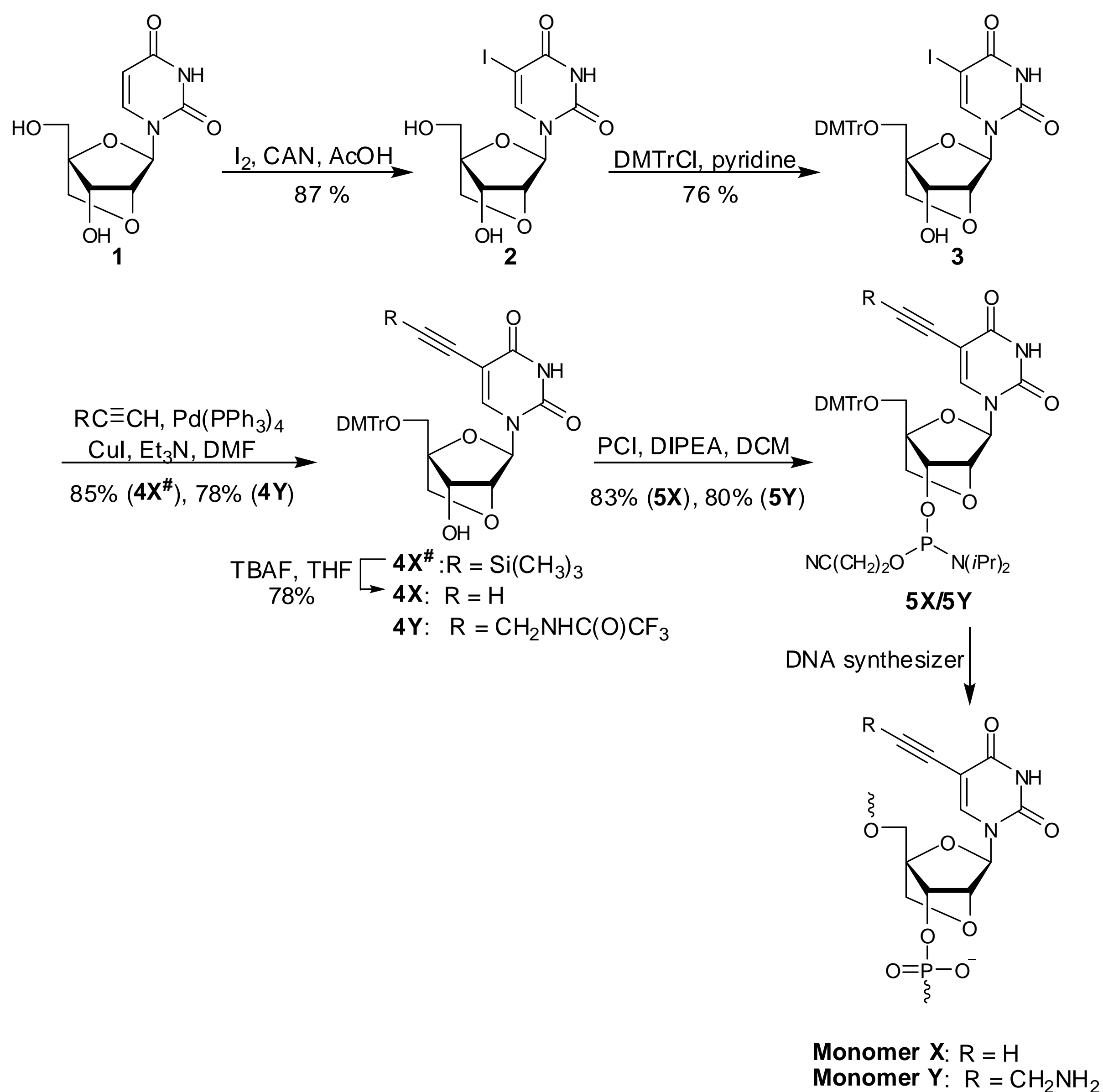

Scheme S1. Synthetic outline of phosphoramidites **5X** and **5Y**. CAN = ceric ammonium nitrate, DMTr = 4,4'-dimethoxytrityl, TBAF = tetrabutylammonium fluoride, PCl = 2-cyanoethyl *N*, *N*'-
5 diisopropylchlorophosphoramidite.

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Synthesis and purification of oligodeoxyribonucleotides: The 0.2 umol scale synthesis of oligodeoxyribo-nucleotides (ONs) containing LNA (**V**), C5-ethynyl (**X**) or C5-propargylamine (**Y**) functionalized LNA monomers was performed on an Expedite 8909 Synthesizer using succinyl linked LCAA-CPG (long chain alkyl amine controlled pore glass) columns with a pore size of 500 Å. Standard protocols for incorporation of DNA phosphoramidites were used, while extended coupling times (15 min) and oxidations (45 sec) were used for incorporation of all LNA-type building blocks. Cleavage from solid support, removal of protecting groups and conversion of O4-triazolyl-dT monomers to 5-methyl-dC monomers (^{m}C) was accomplished by treatment with 32% aq. ammonia (24-48 h, rt). All ONs were purified by RP-HPLC in DMT-on mode as described below. The DMT-group was subsequently cleaved using 80% aq. AcOH, followed by precipitation (acetone, -18 °C for 12-16 h).

Purification of the crude ONs was performed on a Varian Prostar HPLC system equipped with an XTerra MS C18 pre-column (10um, 7.8×10 mm) using the representative gradient protocol depicted in Table S1. The composition of all synthesized ONs was verified by MALDI-MS/MS analysis (Table S2) recorded in positive mode on a Quadrupole Time-Of-Flight tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U. K.) using anthranilic acid as a matrix, while purity (>80%) was verified by RP-HPLC (Table S1).

Table S1. Representative RP-HPLC gradient protocol.[a]

Time/min	Buffer A/(v%)	Buffer B/(v%)
0	100	0
2	100	0
50	30	70
64	0	100
69	0	100
71	100	0
80	100	0

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[a] Buffer A is 0.05 M TEAA (triethyl ammonium acetate) pH 7.4, while buffer B is 75% MeCN in H_2O v/v. A flow rate of 1.2 mL/ min was used.

Table S2. MALDI-TOF-MS data of TFOs.

Seq (5′→3′)	ON	Experimental m/z $[M+H]^+$	Calculated m/z $[M+H]^+$
TTT TT^{m}C TTT ^{m}CT^{m}C T^{m}CT	**T1**	4497	4496
TTT TT^{m}C T**B**T ^{m}CT^{m}C T^{m}CT	**V1**	4525	4524
	X1	4525	4534
	Y1	4564	4563
TTT T**B**^{m}C TT**B** ^{m}CT^{m}C **B**^{m}CT	**V2**	4581	4580
	X2	4611	4610
	Y2	4698	4697
TTT T**B**^{m}C T**B**T ^{m}C**B**^{m}C T^{m}CT	**V3**	4581	4580
	X3	4611	4610
	Y3	4698	4697
TT**B** T**B**^{m}C **B**T**B** ^{m}C**B**^{m}C **B**^{m}CT	**V4**	4665	4664
	X4	4725	4724
	Y4	4900	4898

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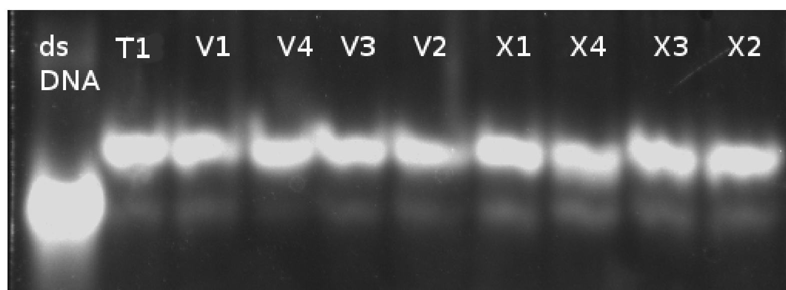

Fig. S1. Non-denaturing PAGE of complexes between reference DNA **T1**, LNA **V1**-**V4** or C5-ethynyl functionalized LNA **X1**-**X4** TFOs and dsDNA target. Left lane: dsDNA target without TFO probes. For sequences, see Table 1. Conditions: 1:1-ratio TFO:dsDNA (each ON at 1 μM), pH 7.2 HEPES buffer (50 mM HEPES, 10 mM $MgCl_2$, 150 mM NaCl, 10% sucrose and 1 mg/mL spermine tetra-HCl). Preparation: denaturation at 90 °C for 5 min followed by incubation at rt for 4h. Electrophoresis: 12% non-denaturing PAGE run at temperatures below rt. SYBR gold staining was used for visualization.

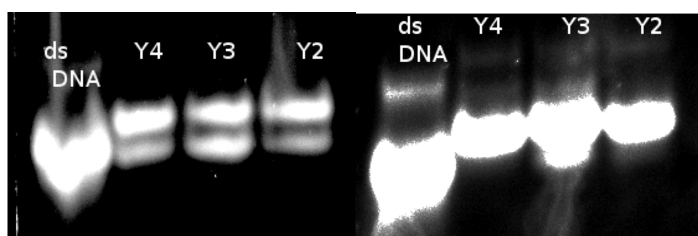

Fig. S2. Non-denaturing PAGE of complexes between C5-propargylamine functionalized LNA TFOs **Y2**-**Y4** and dsDNA target. For sequences, see Table 1. Conditions are as described in Fig. S1 except ratios. Left panel: 1:1 TFO:dsDNA target; Right panel: 2:1 TFO:dsDNA target.

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Thermal denaturation studies: Concentrations of all TFOs were determined using the extinction coefficient of 118500 L×(mol×cm)$^{-1}$. 1 mL solution containing 1.0 nmol of each strand was denatured by heating and subsequently cooled to the starting temperature of the experiment in quartz optical cells with a path-length of 1.0 cm. Thermal denaturation profiles were recorded on a Cary 100 UV/VIS spectrophotometer equipped with a 12-cell Peltier temperature controller using a ramp of 0.5 °C/min. Thermal denaturation temperatures (T_m) for triplex to duplex transitions were determined as the first derivative of difference thermal denaturation profiles (dA_{260} vs. T), which were obtained by subtracting the thermal denaturation profile of the dsDNA target (always included as one of the samples in multicell holder of UV-Vis spectrophotometer) from the raw thermal denaturation profile of the TFO+dsDNA (Fig. S3). Reported T_m-values are an average of at least two experiments within ±1.0 °C.

Association kinetics studies: Association rate constants (k_{on}) for triplex formation were determined by fitting second order rate equations to absorption (A_{260}) decay profiles upon association of TFOs with dsDNA target (Fig. S6) as previously described (L. E. Xodo, Eur. J. Biochem., 1995, 228, 918). More specifically, the A_{260} of 0.5 nmol TFO in 400 µL HEPES buffer (see below) at 20 °C was first monitored for at least 30 min to ascertain a horizontal baseline. Hereupon, 100 µL of a 5 µM dsDNA solution target in HEPES buffer (annealed and then pre-equilibrated at 20 °C) was rapidly added and mixed, and the decay curve recorded. A_{260}-profiles were recorded using a pH 7.2 HEPES buffer (50 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 10% sucrose, 1 mg/mL spermine tetra-HCl) and the same instrumentation as for thermal denaturation measurements. Reported rate constants are averages of at least three individual experiments.

3-Exonuclease studies: SVPDE (snake venom phosphordiesterase) was purchased from Worthington Biochemical Corporation. The increase in absorbance (hyperchromicity) at 260 nm as a function of exposure time to SVPDE was monitored for 2 nmol of TFO in 500 µL Tris buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, pH 9.0) at 37 °C, to which SVPDE dissolved in H_2O was added (0.43 µg in 10 µL). The absorbance at t = 0 min was normalized for all investigated TFOs, while the observed absorbance after exposing reference TFO **T1** to the abovementioned conditions for 2.5 h was assumed to indicate full degradation.

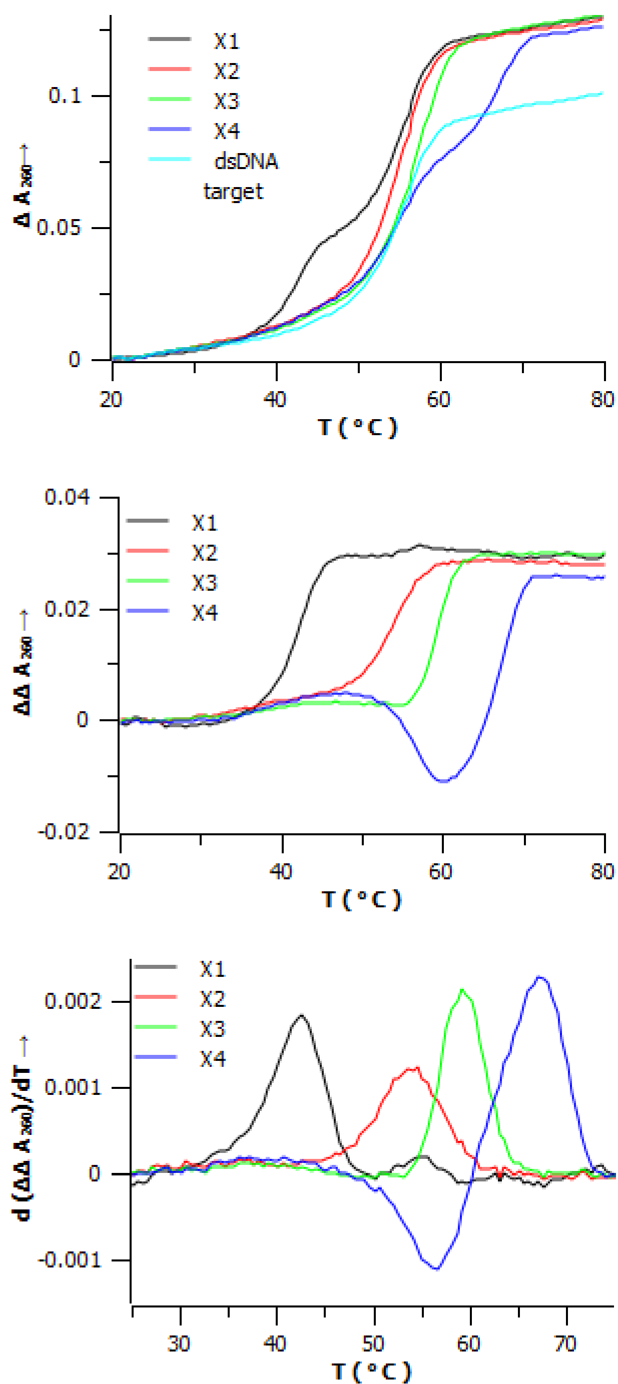
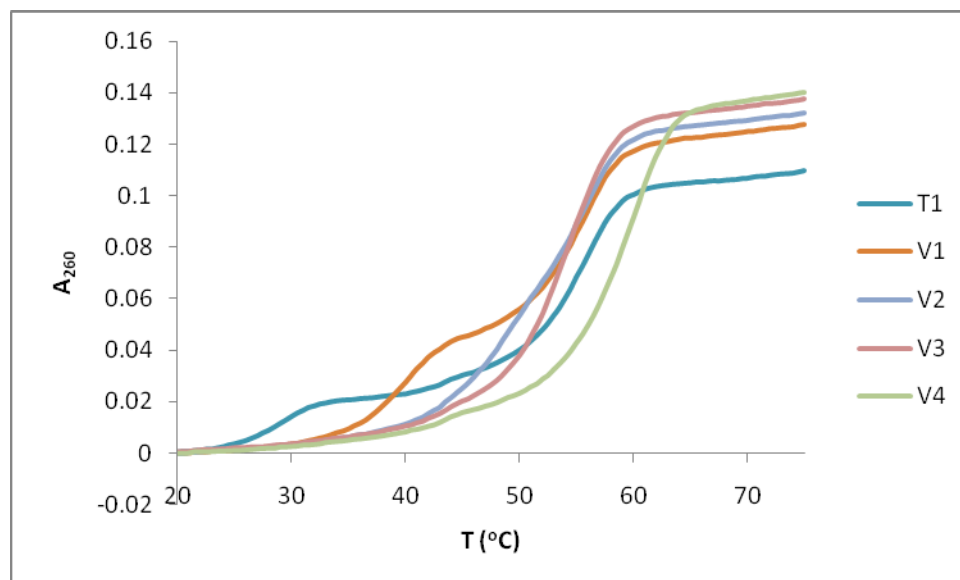


Fig. S3. Thermal denaturation profiles for **X**-series. Top panel: raw thermal denaturation profile; middle panel: differential thermal denaturation profile; lower panel: first derivative of differential thermal denaturation profile. For sequences and conditions, see Table 1.



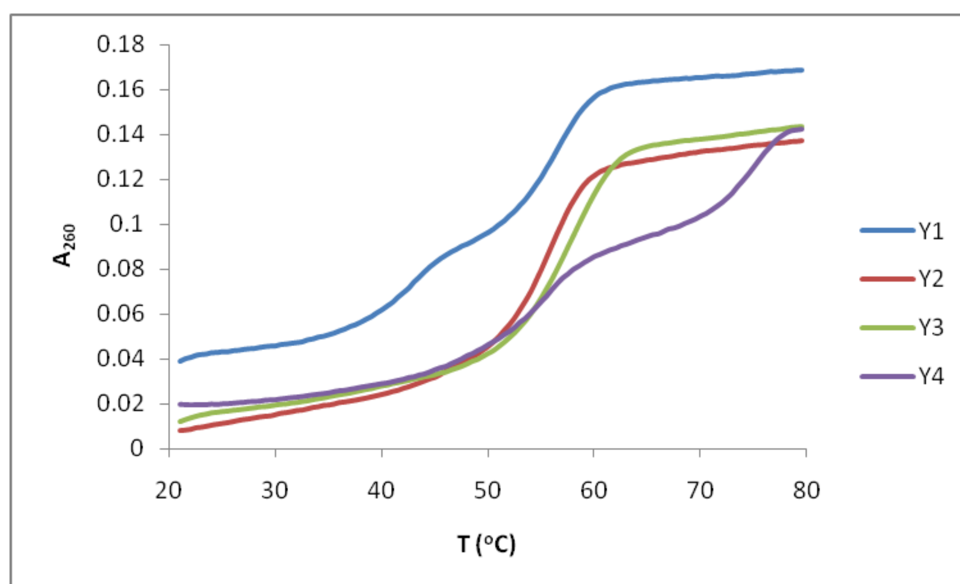


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Fig. S4. Raw thermal denaturation profiles of complexes between dsDNA target and unmodified TFO **T1**, **V**-series TFOs, or **Y**-series TFOs. For sequences and conditions, see Table 1.

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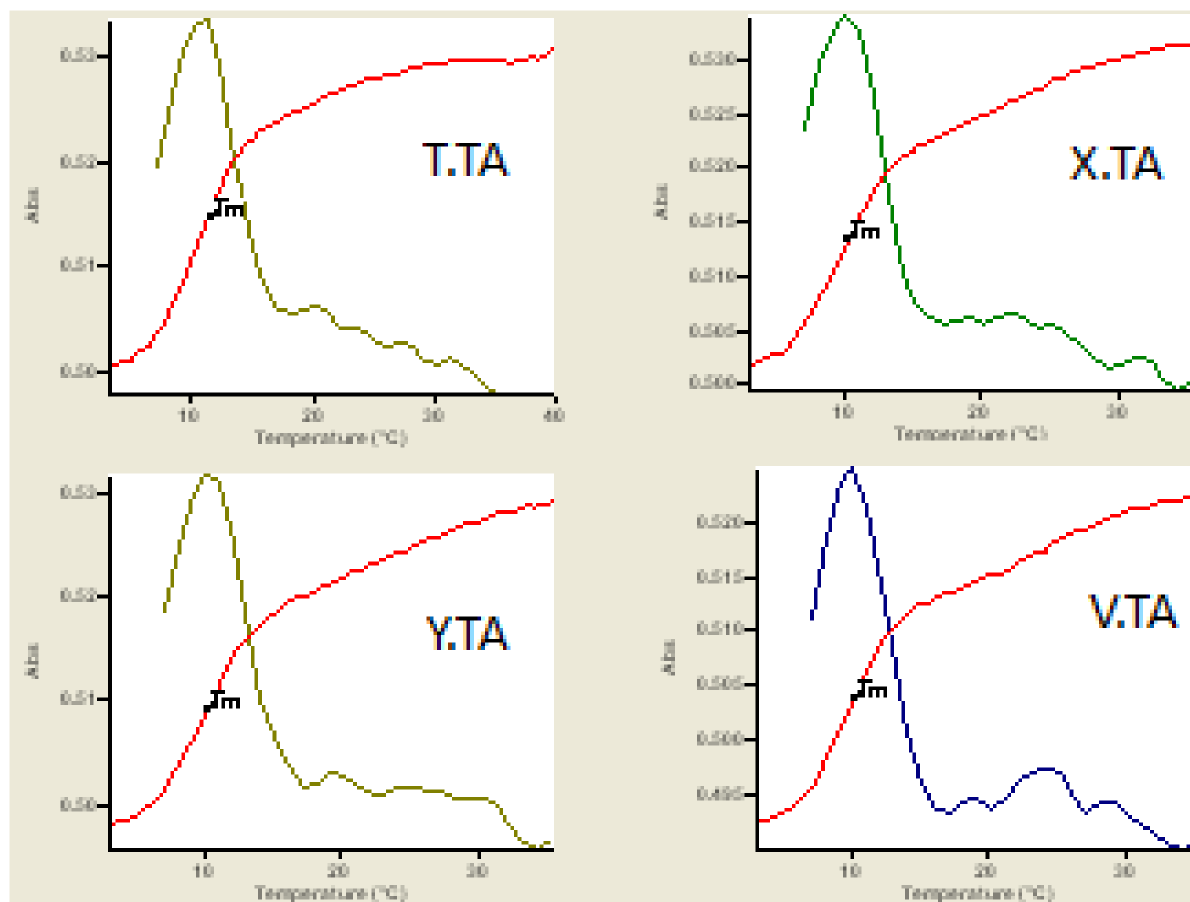

Fig. S5. Raw thermal denaturation profiles and first derivative plots of triplexes between TFOs **T1**, **V1**, **X1**, or **Y1** and the **B**:**TA** mismatched dsDNA target (5′- GCT AAA AAG ATA GAG AGA TCG-3′:3′- CGA TTT TTC TAT CTC TCT ACG-5′). For sequences and conditions, see Table 2.

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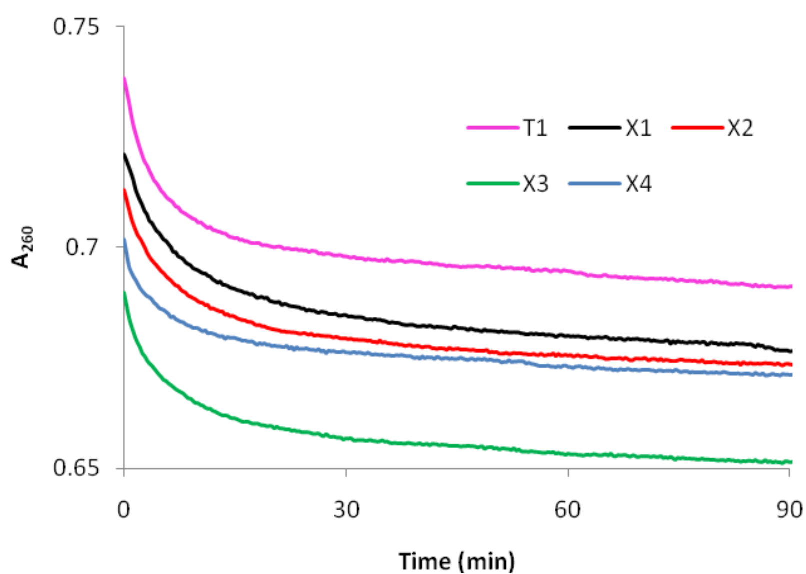

Fig. S6. Representative absorption decay profiles for triplex formation by DNA reference TFO **T1** and C5-ethynyl LNA TFOs (**X**-series).

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Additional discussion regarding thermal denaturation temperatures.

TFOs with six modifications of LNA and C5-alkynyl functionalized LNA monomers exhibit transitions at higher temperatures (T_m's > 61 °C) than the dsDNA target → single strands transition (T_m = 56 °C). Thus, the triplex transition cannot reflect dissociation of the TFO from the underlying Watson-Crick duplex (TFO-Pu-Py → TFO + Pu-Py → TFO + Pu + Py). To investigate if these high melting triplex transitions reflect denaturation of the parallel Hoogsteen duplex between the TFO and polypurine target strand (TFO-Pu-Py → Py + TFO-Pu → Py + TFO + Pu), we recorded thermal denaturation profiles of equimolar mixtures of TFOs + 5'- GCT AAA AAG AAA GAG AGA TCG-3' (purine-rich strand of dsDNA target) using the same denaturation buffer as employed in the triplex experiments (see Table 1). Interestingly, significant hysteresis was observed for parallel duplex involves denaturation of the triplex to three single strands as recently suggested in TFO-studies with LNA analogs (Fig. S7) [S. M. A. Rahman, S. Seki, S. Obika, S. Haitani, K. Miyashita and T. Imanishi, *Angew. Chem., Int. Ed.,* 2007, **46**, 4306].

Table S3. Apparent melting temperatures during heating (T_m) and cooling cycle (T_a) of parallel duplexes formed by TC-motif TFOs and the polypurine strand of the dsDNA target.[a]

ON	Seq 5'→3'	T_m/ T_a (°C)		
		B = V	**X**	**Y**
B1	TTT TT^{m}C T**B**T ^{m}CT^{m}C T^{m}CT	30.5/13.5	26.5/14.0	28.5/15.0
B2	TTT T**B**^{m}C TT**B** ^{m}CT^{m}C **B**^{m}CT	42.0/19.0	45.0/22.0	34.0/23.0
B3	TTT T**B**^{m}C T**B**T ^{m}C**B**^{m}C T^{m}CT	42.5/18.5	40.0/20.5	37.0/26.5
B4	TT**B** T**B**^{m}C **B**T**B** ^{m}C**B**^{m}C **B**^{m}CT	50.0/32.0	53.0/41.5	51.5/50.5

[a] The denaturation curves (A_{260} vs T) were recorded using 1.0 μM of each strand in a pH 7.0 phosphate buffer solution (adjusted with 10 mM NaH_2PO_4/5 mM Na_2HPO_4) containing 140 mM KCl at 0.5 °C/min heating and cooling rates. The T_m's and T_a's were determined by first derivative of denaturation curves and are averages of two independent measurements within 1 °C. For **T1** (**B=T**): T_m = 23.5 °C, and T_a = 12.0 °C, were observed.

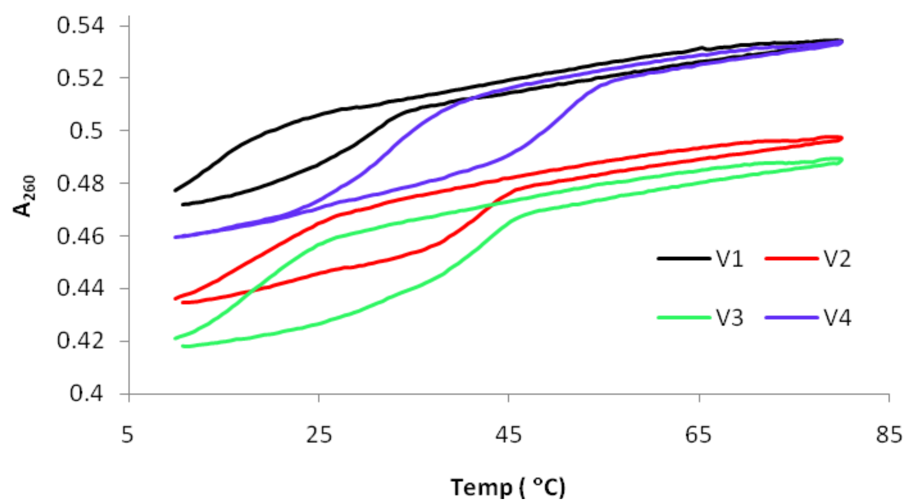

Fig. S7. Representative hysteresis profiles for parallel duplex formation by **V**-series TFOs. For conditions see footnote of Table S3.

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