# Oligonucleotides containing a piperazino-modified 2'-amino-LNA monomer exhibit very high duplex stability and remarkable nuclease resistance

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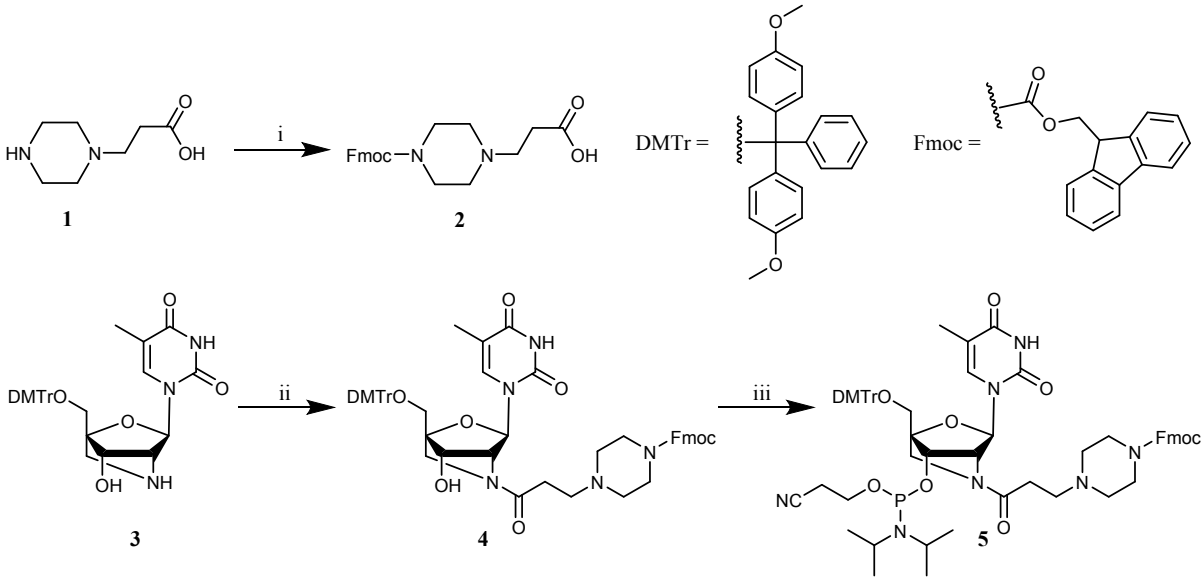
## S1. General Experimental

All reagents used were purchased from Sigma-Aldrich, Fluka and used without purification. DNA phosphoramidite monomers, solid supports and additional reagents were purchased from Sigma-Aldrich or Glen Research. Dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIPEA), *N,N*-dimethylformamide (DMF) and pyridine were dried over activated molecular sieve (3 Å, 8-12 mesh) and their dryness was determined on Karl Fischer titrator (< 15 ppm). All reactions were carried out under nitrogen or argon atmosphere using glassware that had been dried at 120 ˚C overnight. Column chromatography was carried out under pressure using Merck Millipore silica gel 60 (0.040-0.063 mm). Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 $F_{254}$ (0.22 mm thickness, aluminium backed). Compounds were visualized at 254 nm or stained with 10 % sulfuric acid in EtOH. $^{1}$H-NMR spectra were measured at 400 MHz on a Bruker AVANCE III 400 spectrometer. $^{13}$C-NMR spectra were measured at 101 MHz on the same spectrometer. Chemical shifts are given in ppm and *J* values are given in Hz. All assignments for $^{1}$H-NMR and $^{13}$C-NMR have been confirmed by H-H COSY, HMQC and HMBC. $^{31}$P-NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer at 162 MHz. $CD_3CN$ was used as solvents. High resolution mass spectra were recorded in acetonitrile or methanol using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. HPLC grade $CH_3CN$ or methanol were used as the solvent.

## S2. Synthesis of PipLNA-T phosphoramidite




**Scheme S1.** *Reagents and conditions:* (i) Fmoc-OSu, DIPEA, pyridine, RT, 12 h, 88%; (ii) **2**, HATU, DIPEA, DMF, RT, 1 h, 84%; (iii) bis(diisopropylamino)(2-cyanoethoxy)phosphine, diisopropylammonium tetrazolide, DCM, 18 h, RT, 67%.

### 3-[1-*N*-(4-*N*-Fluorenylmethyloxycarbonyl)piperazino]propionic acid (2)

3-(1-*N*-Piperazinyl)-propionic acid (0.304 g, 1.92 mmol) was suspended in anhydrous pyridine (10 mL) and *N*,*N*-diisopropylethylamine (0.67 mL, 3.85 mmol) was added. To this Fmoc-OSu (0.906 g, 2.84 mmol, dissolved in 2 mL anhydrous pyridine) was added dropwise. The resulting mixture was stirred at room temperature for 16 h. After most of the solvents was removed *in vacuo*, the residue was partitioned between DCM (50 mL) and water (30 mL). The organic layer was separated and washed with water (3 × 30 mL), brine (50 mL), then dried (on sodium sulfate), filtered and the solvent removed *in vacuo* to give a white foam (0.642 g, 88%) upon purification by silica gel column chromatography (3.2% methanol in DCM) $R_f$ = 0.25 (methanol/DCM, 1:10). $^1$H NMR: (400 MHz, $CDCl_3$) δ ppm 11.0 (br, s, 1H, COOH), 7.76 (d, *J* = 7.5 Hz, 2H, CH-Ar), 7.55 (d, *J* = 7.5 Hz, 2H, CH-Ar), 7.40 (t, *J* = 7.4 Hz, 2H, CH-Ar), 7.32 (td, *J* = 7.4, 1.1 Hz, 2H, CH-Ar), 4.51 (d, *J* = 6.3 Hz, 2H, $CH_2$), 4.23 (t, *J* = 6.3 Hz, 1H, CH), 3.64-3.40 (m, 4H, $CH_2$), 2.77 (t, *J* = 6.3 Hz, 2H, $CH_2$), 2.68-2.42 (m, 6H, $CH_2$). $^{13}$C NMR: (101 MHz, $CDCl_3$) δ ppm 173.4 (C=O), 154.9 (C-Ar), 143.8(C-Ar), 141.4(C-Ar), 127.8 (CH-Ar), 127.1 (CH-Ar), 124.9 (CH-Ar), 120.0 (CH-Ar), 67.3 ($CH_2$), 53.2 ($CH_2$), 51.8 ($CH_2$), 47.4 (CH), 43.0 ($CH_2$), 30.2 ($CH_2$). HRMS [ESI]: $C_{22}H_{25}N_2O_4$ calculated 381.1809 found 381.1812. This compound has the following CAS-number: [1339485-97-0] but no reference and no data could be found.

**(1*R*,3*R*,4*R*,7*S*)-1-(4,4'-Dimethoxytrityloxymethoxyl)-7-hydroxyl-5-*N*-{3-[1-*N*-(4-*N*-fluorenylmethyloxycarbonyl)piperazino]propanoyl}-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2,2,1]heptane (4)**

Compound **2** (0.213 g, 0.560 mmol) and HATU (0.213 g, 0.560 mmol) were dissolved in anhydrous DMF (8 mL) before *N*,*N*-diisopropylethylamine (0.30 mL, 1.72 mmol) was added. The resulting solution was stirred at room temperature for 10 min. **3** (0.267 g, 0.467 mmol, dissolved in 2 mL anhydrous DMF) was then added dropwise and the reaction mixture was further stirred at room temperature for 1 h before methanol (1 mL) was added to quench the reaction. After all the solvent was removed *in vacuo*, the residue was partitioned between DCM (30 mL) and water (20 mL). The organic layer was washed with water (2 × 15 mL), brine (15 mL), and then dried over anhydrous sodium sulfate. After filtration, the solution was evaporated *in vacuo* to give a white foam (0.366 g, 84%, 2:1 ratio of rotamers according to $^1$H-NMR) upon purification by silica gel column chromatography (3.2% methanol in DCM with 0.5% DIPEA). $R_f$ = 0.52 (methanol/DCM, 1:8). $^1$H NMR: (400 MHz, $CDCl_3$) δ ppm 7.74 (d, *J* = 7.5 Hz, 2H, C<u>H</u>-Ar), 7.67 (d, *J* = 0.8 Hz, 1H$_{\mathbf{I}}$, C<u>H</u>$^{\mathbf{6}}$), 7.65 (d, *J* = 1.0 Hz, 1H$_{\mathbf{II}}$, C<u>H</u>$^{\mathbf{6}}$), 7.56-7.49 (m, 2H, C<u>H</u>-Ar), 7.46 (d, *J* = 7.4 Hz, 2H, C<u>H</u>-Ar), 7.41-7.18 (m, 11H, C<u>H</u>-Ar), 6.87-6.79 (m, 4H, C<u>H</u>-Ar), 5.55 (s, 1H$_{\mathbf{I}}$, C<u>H</u>$^{\mathbf{3'}}$), 5.47 (s, 1H$_{\mathbf{II}}$, C<u>H</u>$^{\mathbf{3'}}$), 5.10 (s, 1H$_{\mathbf{I}}$, C<u>H</u>$^{\mathbf{4'}}$), 4.63 (s, 1H$_{\mathbf{II}}$, C<u>H</u>$^{\mathbf{4'}}$), 4.47-4.44 (m, 1H$_{\mathbf{I}}$, C<u>H</u>$^{\mathbf{7'}}$), 4.44-4.36 (m, 2H, C<u>H</u>$_2$), 4.34 (s, 1H$_{\mathbf{II}}$, C<u>H</u>$^{\mathbf{7'}}$), 4.23-4.16 (m, 1H, C<u>H</u>), 3.76 (d, *J* = 2.6 Hz, 6H$_{\mathbf{II}}$, OC<u>H</u>$_3$), 3.74 (d, *J* = 2.6 Hz, 6H$_{\mathbf{I}}$, OC<u>H</u>$_3$), 3.60-3.22 (m, 6H, DMTrOC<u>H</u>$_2$, C<u>H</u>$_2$, C<u>H</u>$_2$), 2.87-2.62 (m, 4H, C<u>H</u>$_2$, C<u>H</u>$_2$), 2.57-2.20 (m, 6H, C<u>H</u>$_2$, C<u>H</u>$_2$, C<u>H</u>$_2$), 1.63 (s, 3H$_{\mathbf{II}}$, C<u>H</u>$_3$), 1.59 (s, 3H$_{\mathbf{I}}$, C<u>H</u>$_3$). $^{13}$C NMR: (101 MHz, $CDCl_3$) δ ppm 171.3 (<u>C</u>=O), 164.0 (<u>C</u>=O), 158.7 (<u>C</u>-Ar), 155.1 (<u>C</u>-Ar), 150.5 (<u>C</u>-Ar), 144.5 (<u>C</u>-Ar), 143.9 (<u>C</u>-Ar), 141.3 (<u>C</u>-Ar), 135.5 (<u>C</u>-Ar), 135.3 (<u>C</u>-Ar), 134.6 (<u>C</u>H$^{\mathbf{6}}$), 130.1 (<u>C</u>H-Ar), 128.1 (<u>C</u>H-Ar), 127.7 (<u>C</u>H-Ar), 127.1 (<u>C</u>H-Ar), 124.9 (<u>C</u>H-Ar), 120.0 (<u>C</u>H-Ar), 113.3 (<u>C</u>H-Ar), 110.6 (<u>C</u>-Ar), 88.4 (<u>C</u>$^{\mathbf{1'}}$), 87.3 (**II**, <u>C</u>H$^{\mathbf{3'}}$), 87.0 (**I**, <u>C</u>H$^{\mathbf{3'}}$), 69.8 (**II**, <u>C</u>H$^{\mathbf{7'}}$), 68.5 (**I**, <u>C</u>H$^{\mathbf{7'}}$), 67.4 (<u>C</u>H$_2$), 64.0 (**II**, <u>C</u>H$^{\mathbf{4'}}$), 61.8 (**I**, <u>C</u>H$^{\mathbf{4'}}$), 59.3 (DMTrO<u>C</u>H$_2$), 55.2 (O<u>C</u>H$_3$), 54.2 (<u>C</u>H$_2$), 52.9(<u>C</u>H$_2$), 52.4 (<u>C</u>H$_2$), 51.4 (<u>C</u>H$_2$), 47.3 (<u>C</u>H), 43.4 (<u>C</u>H$_2$), 31.5 (<u>C</u>H$_2$), 12.6 (<u>C</u>H$_3$). HRMS [ES$^+$]: $C_{54}H_{56}N_5O_{10}$ calculated 934.4022 found 934.3981.

**(1*R*,3*R*,4*R*,7*S*)-1-(4,4'-Dimethoxytrityloxymethoxyl)-7-[2-cyanoethyl(*N*,*N*-diisopropyl-amino)phosphanyloxyl]-5-*N*-{3-[1-*N*-(4-*N*-fluorenylmethyloxycarbonyl)piperazino]-propanoyl}-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2,2,1]heptane (5)**

*N*,*N*-Diisopropylamino tetrazolide (0.330 g, 1.93 mmol) and bis(diisopropylamino)(2-

cyanoethoxy)phosphine (0.813 mL, 2.56 mmol) was added to a solution of **4** (0.400 g, 0.428 mmol) in anhydrous DCM (10 mL). The mixture was stirred under argon for 18 h at room temperature. The solvent was removed *in vacuo* and the residue was purified under nitrogen atmosphere by silica gel column chromatography (25% acetone in 1:1 DCM/ethyl acetate) to yield the diastereomeric product as a white foam (0.326 g, 67%). $R_f$ = 0.52, 0.58 (methanol/ethyl acetate/DCM, 1:6:6). $^{31}$P NMR (162 MHz, $CD_3CN$) δ ppm 149.9, 149.8, 149.4, 148.0. HRMS [$ES^+$]: $C_{63}H_{73}N_7O_{11}P$ calculated 1134.5047 found 1134.5100.

## S3. Oligonucleotide synthesis, purification and analysis

Oligonucleotide synthesis was carried out on a PerSeptive Biosystems expedite 8909 automated DNA/RNA synthesizer in 0.2 μmol scale (CPG support) using the phosphoramidite approach and following manufacturer's standard protocols. The coupling time for standard and LNA monomers was 144 s and stepwise coupling efficiencies were determined by the absorbance of trityl cation at 495 nm on UV-VIS spectrophotometer and in all cases were >98.0 %. 2'-amino-LNA-T and PipLNA-T phosphoramidite monomer were incorporated via hand-coupling using 5-[3,5-bis(trifluoromethyl)phenyl]-*H*-tetrazole (0.25 M, in anhydrous acetonitrile) as activator and extended coupling time (15 min), resulting in stepwise coupling yields of 96 % and 80 % respectively. The oligonucleotides attached to the solid support were treated with 20 % diethylamine in acetonitrile (5 mL) for 20 min then washed with acetonitrile extensively. This procedure removes cyanoethyl groups from the phosphotriesters and scavenges the resultant acrylonitrile, preventing cyanoethyl adducts being formed at the secondary amines of PipLNA-T. Cleavage from solid support and removal of nucleobase protecting groups were performed using 28 % aqueous ammonia 16 h at 55 °C. The resulting oligonucleotides were purified by DMTr-ON RP-HPLC using the Waters System 600 equipped with a Waters XBridge BEH C18-column (5 μm, 100 mm × 19 mm). Elution was performed starting with an isocratic hold of A-buffer for 5 min followed by a linear gradient to 70 % B-buffer over 16.5 min at a flow rate of 5.0 mL/min (A-buffer: 0.05 M triethylammonium acetate in Milli-Q water, pH 7.4; B-buffer: 25 % A-buffer, 75 % acetonitrile). After all the solvents were removed under nitrogen flow, oligonucleotides were detritylated using an 80 % aqueous solution of acetic acid for 20 min, then desalted with an aqueous solution of sodium acetate (3 M, 15 μL) and sodium perchlorate (5 M, 15 μL) followed by cold acetone (1 mL). The resulting suspension was stored at -20 °C for 1 h. After centrifugation (13200 rpm, 5 min, 4 °C), the supernatant was removed and the pellet further washed with cold acetone (2 × 1 mL), dried for 30 min under nitrogen flow, and dissolved in

Milli Q water (1.0 mL). The PipLNA-T containing oligonucleotides were further purified by anion-exchange HPLC (IE-HPLC) using the DIONEX Ultimate 3000 system equipped with a DNAPac PA100 semipreparative column (13 μm, 250 mm × 9 mm) heated to 60 °C. Elution was performed with an isocratic hold of buffer B (10 %), starting from 2 min hold on 2 % Buffer A in Milli-Q water (solvent A), followed by a linear gradient to 25 % buffer A in 20 min at a flow rate of 2.0 mL/min (buffer A: 1.0 M sodium perchlorate; buffer B: 0.25 M Tris-Cl, pH 8.0, solvent A: Milli-Q water). After IE-HPLC purification, oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare) according to the manufacturer's instructions.

Mass spectra of oligonucleotides were recorded on a Bruker Daltonics Microflex LT MAIDI-TOF MS instrument in $ES^+$ mode (representative MS in Figure S1, S2 S4 & S5). Analytical IE-HPLC traces were recorded on a Merck-Hitachi Lachrom system equipped with a DNAPac PA100 analytical column (13 μm, 250 mm × 4 mm) heated to 60 °C. Elution was performed with an isocratic hold of buffer B (10 %), starting from 2 min hold on 2 % Buffer A in Milli-Q water (solvent A), followed by a linear gradient to 30 % buffer A in 23 min at a flow rate of 1.1 mL/min (buffer A: 1.0 M sodium perchlorate; buffer B: 0.25 M Tris-Cl, pH 8.0; solvent A: Milli-Q water) (representative IE-HPLC traces in Figure S1, S2 S4 & S5). Concentrations of purified oligonucleotides were determined by UV at 260 nm, assuming identical molar absorptivities for T, amino-LNA-T and PipLNA-T nucleotides.

## S4. Ultraviolet duplex melting studies

The representative UV absorption curves of synthetic oligonucleotides are shown in Figure S6, Figure S7, Figure S8, Figure S9, Figure S10 and Figure S11. All the duplex melting curves were recorded at 260 nm.

To determine duplex melting temperatures ($T_m$), UV melting studies were carried out on a Perkin Elmer Lambda 35 UV/Vis Spectrometer using Hellma SUPRASIL synthetic quartz 10 mm path length cuvettes, monitoring at 260 nm with a complementary DNA/RNA strand concentration of 2.5 μM and a volume of 1.0 mL. Samples were prepared as follows: The modified sequences and their corresponding complementary strand were mixed in a 1:1 ratio in 2 mL Eppendorf tubes before medium salt buffer (2 times, 11.7 mM sodium phosphate, 200 mM NaCl, 0.20 mM EDTA, pH 7.0, 500 μL) was added, which was completed in 1.0 mL using Milli-Q water. Thus, all oligonucleotide samples were dissolved in 1× buffer

condition (5.8 mM sodium phosphate, 100 mM NaCl and 0.10 mM EDTA). The samples were denatured by heating to 90 ˚C in water-bath followed by slowly cooling to room temperature before they were transferred into the cuvettes. UV absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 10 °C to 75 °C at a rate of 1.0 °C/min programmed by a Peltier temperature controller. Two separate melting curves were measured and $T_m$ values were calculated using UV-WinLab software, taking an average of the two melting curves with a deviation no more than 0.5 °C.

## S5. Ultraviolet triplex melting studies

The UV-scan curves of duplex and triplex are shown in Figure S3. 270 nm were used in the triplex UV-melting studies (Figure S12).

To determine triplex melting temperatures ($T_m$), UV melting studies were carried out on a Perkin Elmer Lambda 35 UV/Vis Spectrometer using Hellma SUPRASIL synthetic quartz 10 mm path length cuvettes, monitoring at 270 nm with a DNA duplex concentration of 1.0 μM and a volume of 1.0 mL. Samples were prepared as follows: The third strand and the duplex were mixed in a 1.5:1 ratio in 2 mL Eppendorf tubes before cacodylate buffer (2 times, 20 mM sodium cacodylate, 300 mM NaCl, 20 mM $MgCl_2$, pH 6.0 or 7.0, 500 μL) was added, which was completed in 1.0 mL using Milli-Q water. Thus, all oligonucleotide samples were dissolved in 1 × buffer condition (10 mM sodium cacodylate, 150 mM NaCl and 10 mM $MgCl_2$). The samples were denatured by heating to 90 ˚C in water-bath followed by slowly cooling to room temperature and stored in fridge for overnight before they were transferred into the cuvettes. UV absorbance at 270 nm as a function of time was recorded while the temperature was increased linearly from 10 °C to 80 °C at a rate of 0.5 °C/min programmed by a Peltier temperature controller. Two separate melting curves were measured and $T_m$ values were calculated using UV-WinLab software, taking an average of the two melting curves with a deviation no more than 0.5 °C.

## S6. Nuclease Resistance Assays[1]

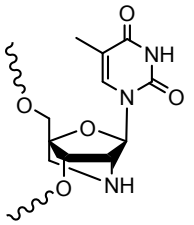
Oligonucleotide stability against snake venom phosphodiesterase I from *Crotalus adamanteus* (Pharmacia Biotech) was evaluated by incubating 3 μM of 5′-$^{32}$P-labeled oligonucleotide with 6.7 ng/μL phosphodiesterase I in 100 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$ (20 μL) at 21 °C. Initial aliquots (3 μL, 0 min) were drawn immediately prior to adding the enzyme. At time points 5, 10, 15, 30 and 60 min aliquots (3 μL) were taken and

added to tubes containing 2 μL ice-cold loading buffer (95 % formamide, 20 mM EDTA, xylene cyanol, and bromophenol blue). All samples were heated to 80 °C for 2 min to stop nucleolytic activity and were then resolved on 20 % denaturing polyacrylamide electrophoresis gels with 7 M urea and visualized by autoradiography on a Typhoon Trio Variable Mode Imager.

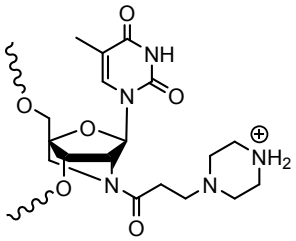
## S7. Molecular modelling

The initial oligonucleotides were built and modified with LNA monomers in the B-type duplex conformation in the MacroModel V9.1 suite of programs.[2] The minimization was performed using the AMBER force field as implemented by MacroModel.[3] The global energy structure obtained was subjected to a 5 ns MD simulation (simulation temperature 300 K, time step 1.5 fs, SHAKE all bonds to hydrogen and the molecular dynamics setting) during which 250 individual structures were sampled. These sample structures were subsequently minimized to obtain a converged global minimum. The global minimum was used for analysis. The distances between protonated piperazino group and four neighbouring negative-charged phosphate backbone were monitored in the modelling process. Non-bonded interactions were treated with extended cut-offs (van der Waals 8.0 Å and electrostatics 20.0 Å). Resulting structure was further processed in the PyMOL Molecular Graphics System.

| | One substitution | Three substitutions |
|---|---|---|
| **DNA/DNA** | 3'-CACTATACG<br>5'-GTGA**X**ATGC | 3'-CACTATACG<br>5'-G**X**GA**X**A**X**GC |
| | **D1** | **D2** |
| **DNA/RNA** | 3'-CACUAUACG<br>5'-GTGA**X**ATGC | 3'-CACUAUACG<br>5'-G**X**GA**X**A**X**GC |
| | **D3** | **D4** |



2'-amnio-LNA-T



PipLNA-T

**X** = T, 2'-amino-LNA-T or PipLNA-T

**Table S1.** Oligonucleotides used in duplex biophysical studies.

| Sequence | Code | X | Duplex | Type | Complementary Strands |
|---|---|---|---|---|---|
| 5′-GTGA**X**ATGC | **ON1** | T | D1&D2 | DNA/DNA | 5′-GCATATCAC |
| | | | D3&D4 | DNA/RNA | 5’-GCAUAUCAC |
| | **ON2** | 2’-amino-LNA-T | D1 | DNA/DNA | 5′-GCATATCAC |
| | | | D3 | DNA/RNA | 5’-GCAUAUCAC |
| | **ON3** | PipLNA-T | D1 | DNA/DNA | 5′-GCATATCAC |
| | | | D3 | DNA/RNA | 5’-GCAUAUCAC |
| 5′-G**X**GA**X**A**X**GC | **ON4** | 2’-amino-LNA-T | D2 | DNA/DNA | 5′-GCATATCAC |
| | | | D4 | DNA/RNA | 5’-GCAUAUCAC |
| | **ON5** | PipLNA-T | D2 | DNA/DNA | 5′-GCATATCAC |
| | | | D4 | DNA/RNA | 5’-GCAUAUCAC |

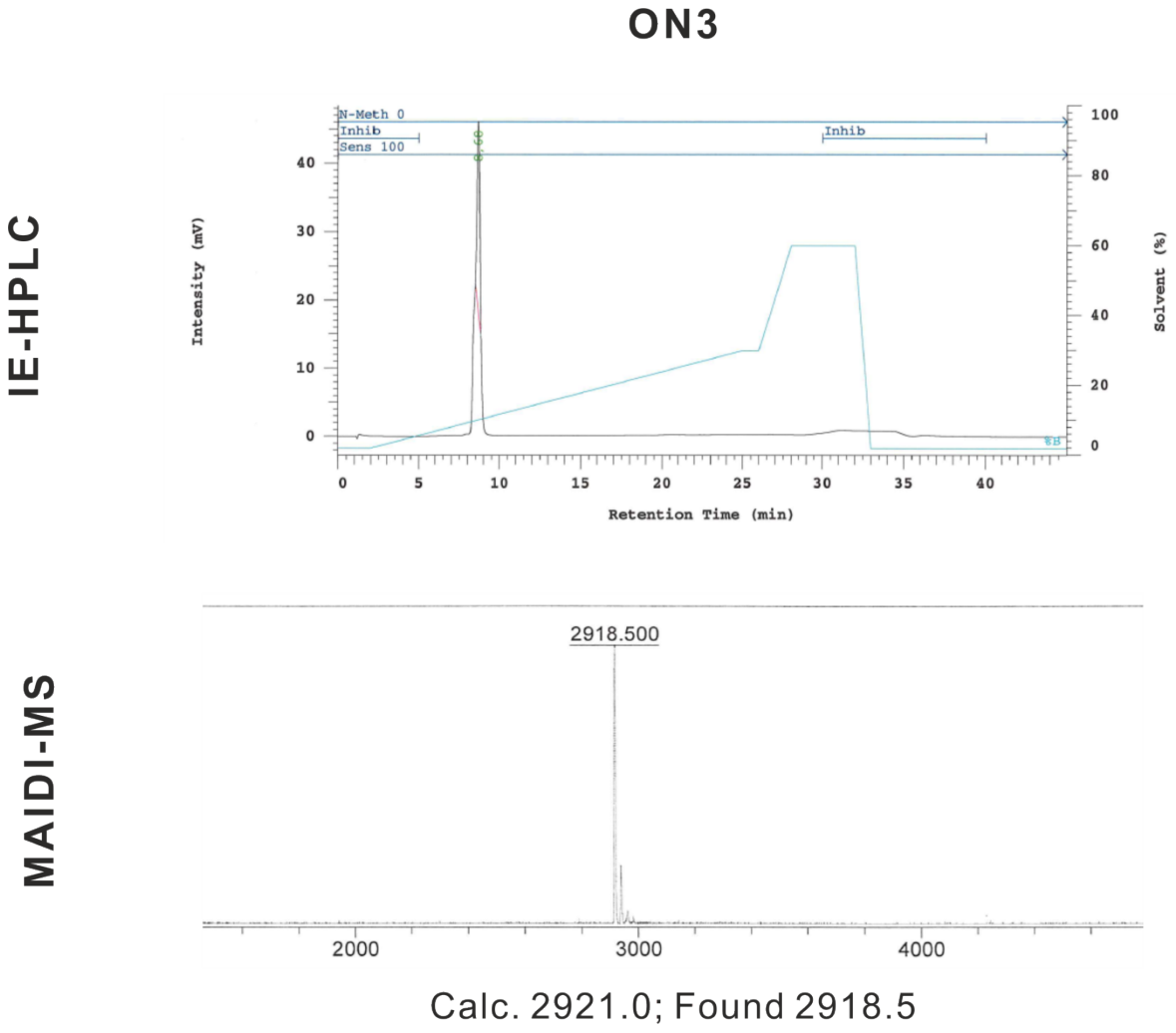



**Figure S1:** representative IE-HPLC trace and MAIDI-TOF mass spectra on ON3.

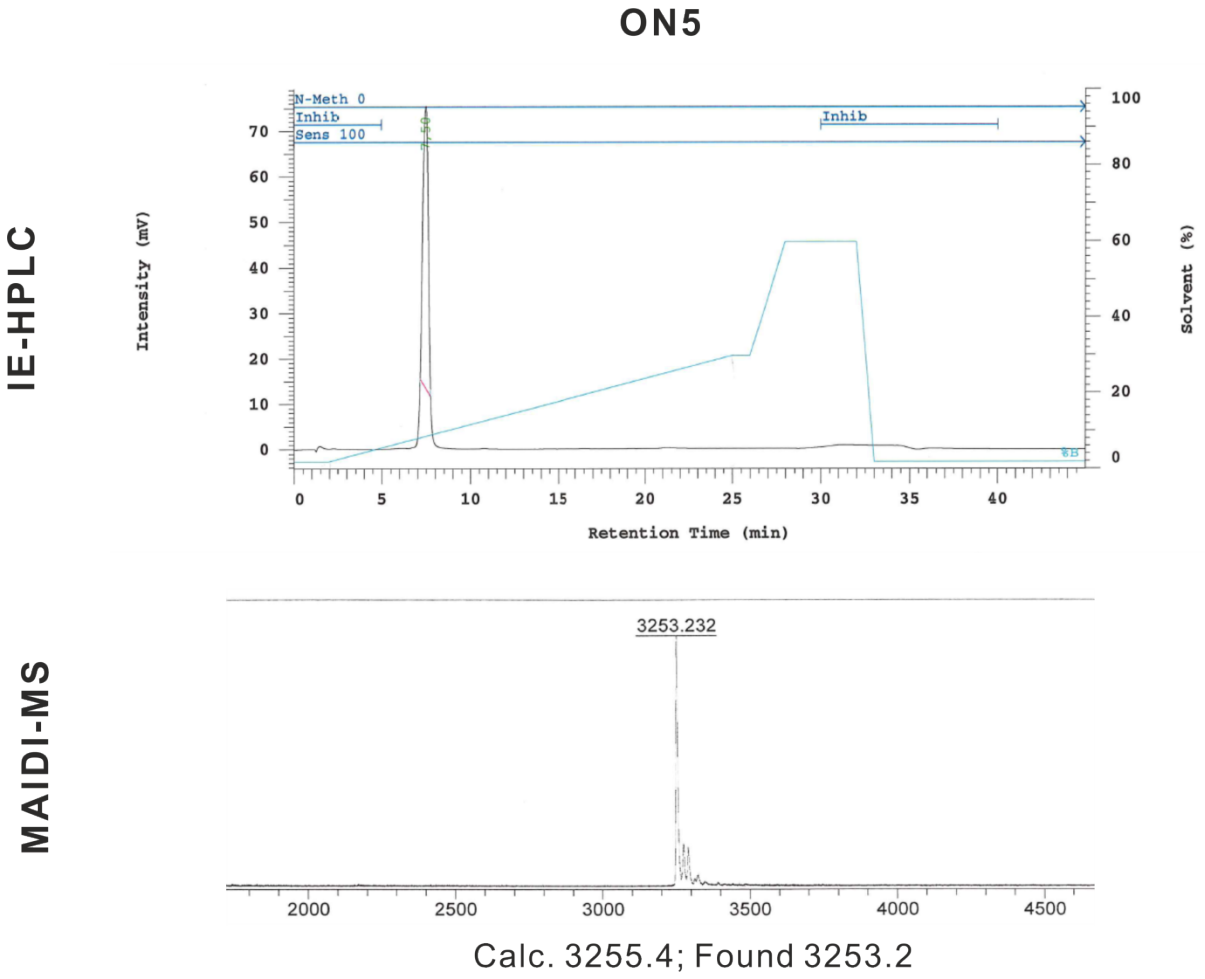



**Figure S2:** representative IE-HPLC trace and MAIDI-TOF mass spectra on ON5.

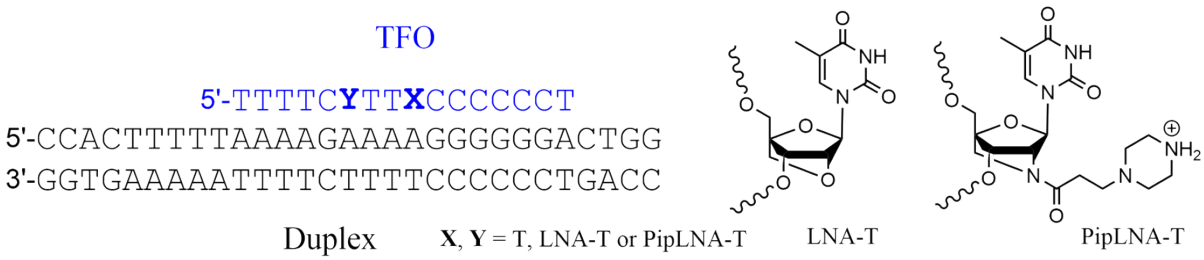



**Table S2.** Oligonucleotides used in triplex biophysical studies.

| TFO | Code | X | Y | Underlying duplex |
|---|---|---|---|---|
| 5′-TTTTCYTTXCCCCCCT | TFO-1 | T | T | 5′-CCACTTTTTAAAAGAAAAGGGGGGACTGG<br>3’-GGTGAAAAATTTTCTTTTCCCCCCTGACC |
| | TFO-2 | LNA-T | T | |
| | TFO-3 | PipLNA-T | T | |
| | TFO-4 | LNA-T | LNA-T | |
| | TFO-5 | PipLNA-T | PipLNA-T | |

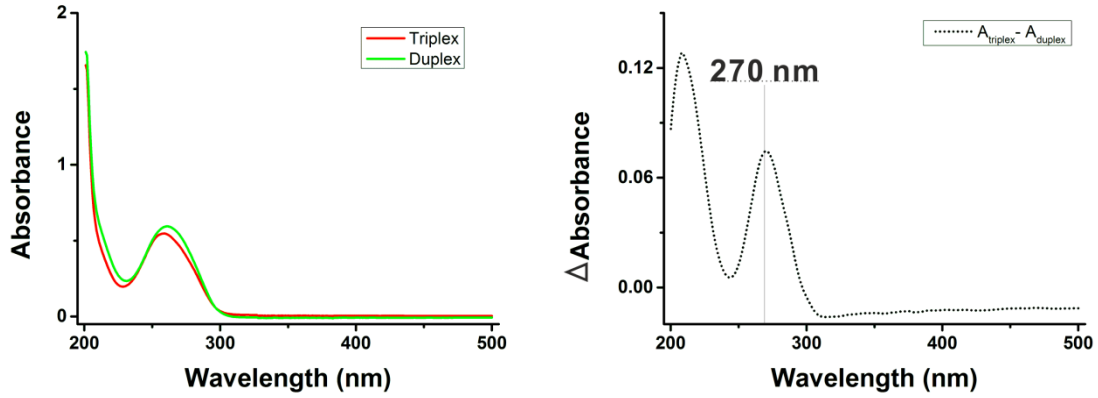



**Figure S3.** UV-scan curves (left) and the absorption difference (right) of triplex and underlying duplex. As the maximum difference, 270 nm was chosen for following triplex melting study.

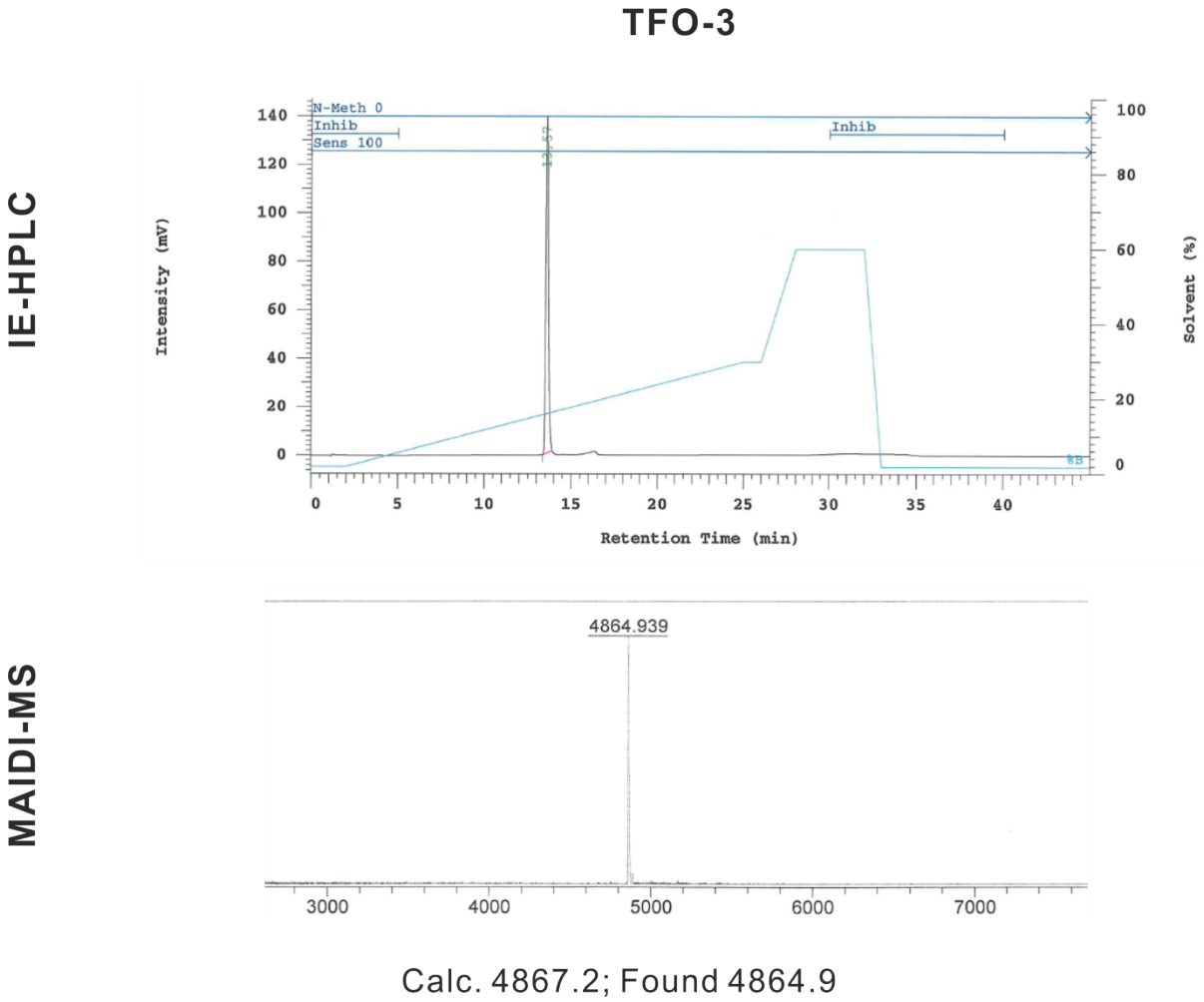



**Figure S4:** representative IE-HPLC trace and MAIDI-TOF mass spectra on TFO-3.

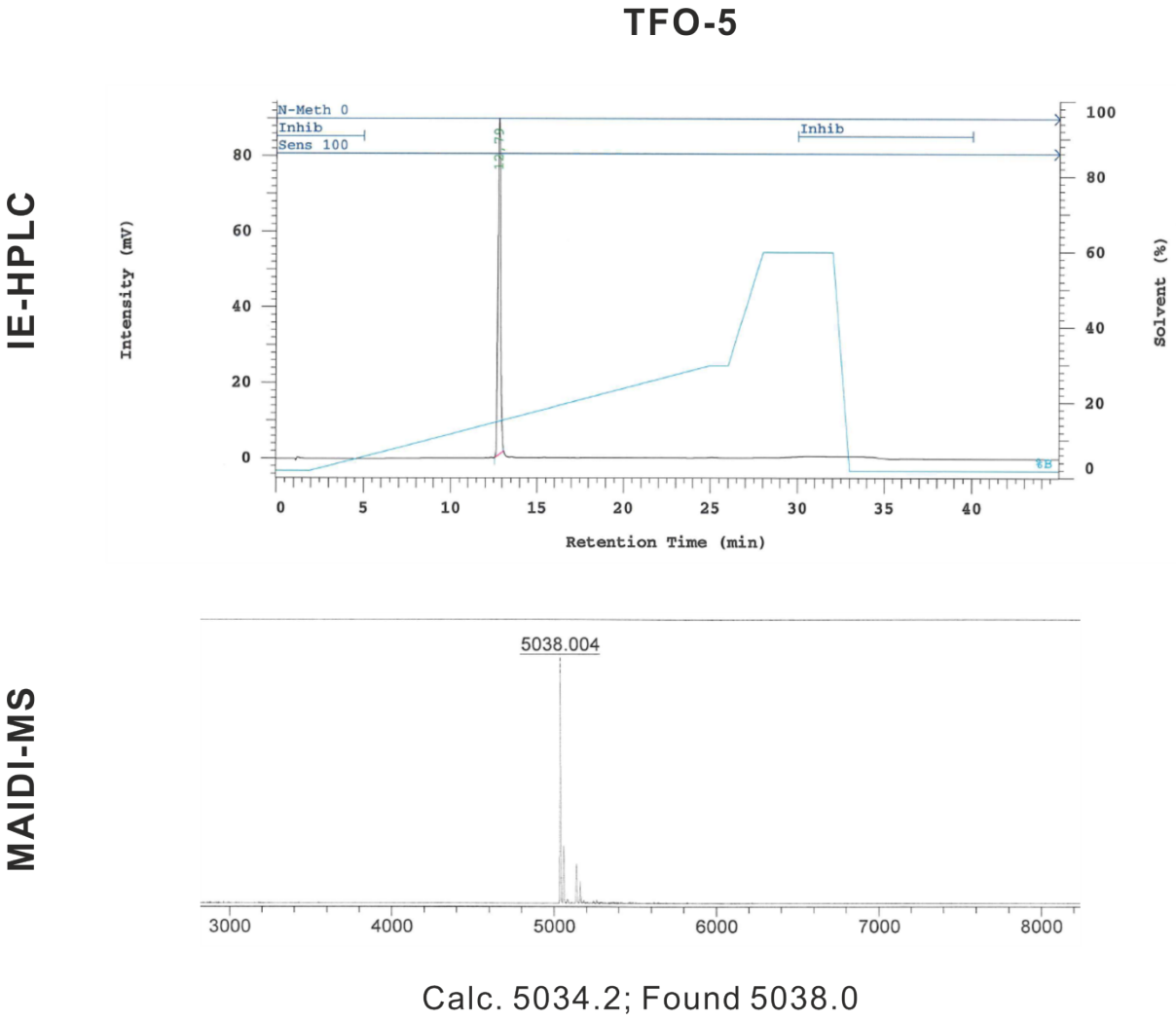
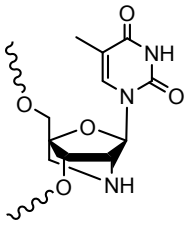
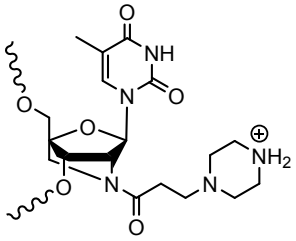



**Figure S5:** representative IE-HPLC trace and MAIDI-TOF mass spectra on TFO-5.

| | One substitution | Three substitutions |
|---|---|---|
| **DNA/DNA** | 3'-CACTATACG<br>5'-GTGA**X**ATGC<br>**D1** | 3'-CACTATACG<br>5'-G**X**GA**X**A**X**GC<br>**D2** |
| **DNA/RNA** | 3'-CACUAUACG<br>5'-GTGA**X**ATGC<br>**D3** | 3'-CACUAUACG<br>5'-G**X**GA**X**A**X**GC<br>**D4** |



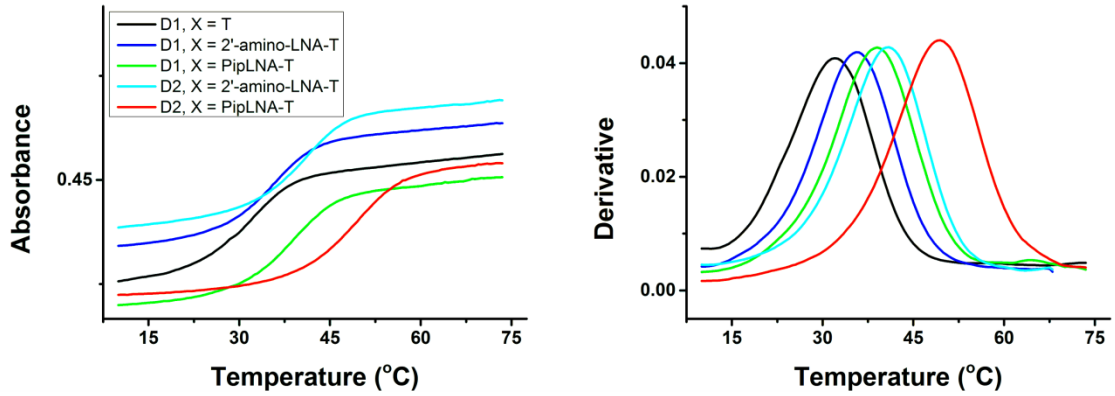

2'-amnio-LNA-T




PipLNA-T

**X** = T, 2'-amino-LNA-T or PipLNA-T

**A**




**B**

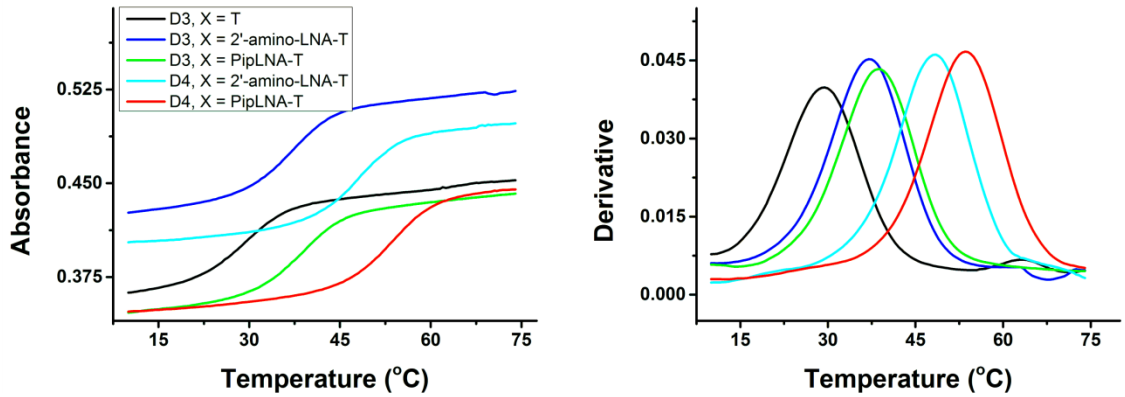



**Figure S6.** UV melting curves (left) and derivatives (right) of DNA/DNA and DNA/RNA duplexes with single or triple additions (**X** = T, black; **X** = 2'-amino-LNA-T, blue & cyan; **X** = PipLNA-T, green & red). The experiments were recorded on 260 nm in 5.8 mM $NaH_2PO_4/Na_2HPO_4$ buffer (pH 7.0, containing 100 mM NaCl and 0.10 mM EDTA). The concentration of modified oligo: Watson-Crick complementary strand = 2.5 μM : 2.5 μM. A) D1 and D2, DNA/DNA duplexes; B) D3 and D4, DNA/RNA hybrids.

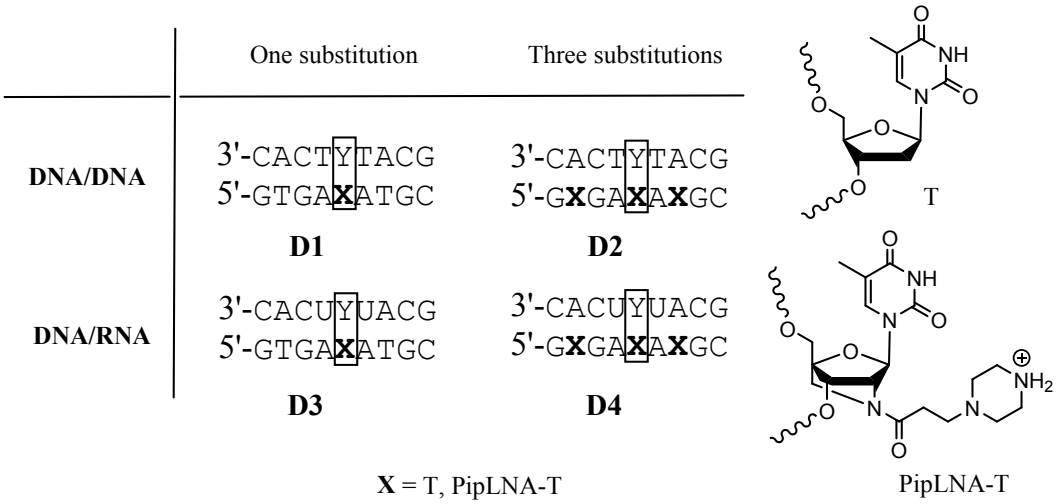



**Table S3.** UV-melting analysis on above duplexes[a]

| Duplex | Y | A | G | T/U | C |
|---|---|---|---|---|---|
| **D1** | **X** = T | 32.0 | 22.5 (-9.5) | 16.5 (-15.5) | 15.5 (-16.5) |
| | **X** = PipNA-T | 39.0 | 24.5 (-14.5) | 23.0 (-16.0) | 20.0 (-19.0) |
| **D3** | **X** = T | 29.5 | 25.0 (-4.5) | 14.0 (-15.5) | 9.5 (-20.0) |
| | **X** = PipNA-T | 38.5 | 28.0 (-10.5) | 21.5 (-17.5) | 22.0 (-16.5) |
| **D2** | **X** = PipNA-T | 49.5 | 33.5 (-16.0) | 34.0 (-15.5) | 32.0 (-17.5) |
| **D4** | **X** = PipNA-T | 54.0 | 42.0 (-12.0) | 38.5 (-15.5) | 40.0 (-14.0) |

[a] $T_m$ values (°C) are an average of two independent melting temperatures with a deviation < 0.5 ˚C. DNA/DNA and DNA/RNA duplexes varied its nucleobase partner near the middle with one or three additions of T or PipLNA-T. Values in brackets are $\Delta T_m$ = $T_m$ (Y = G, T/U or C) - $T_m$ (Y = A). The experiments were carried out at pH 7.0 under the same condition as in Figure S6.

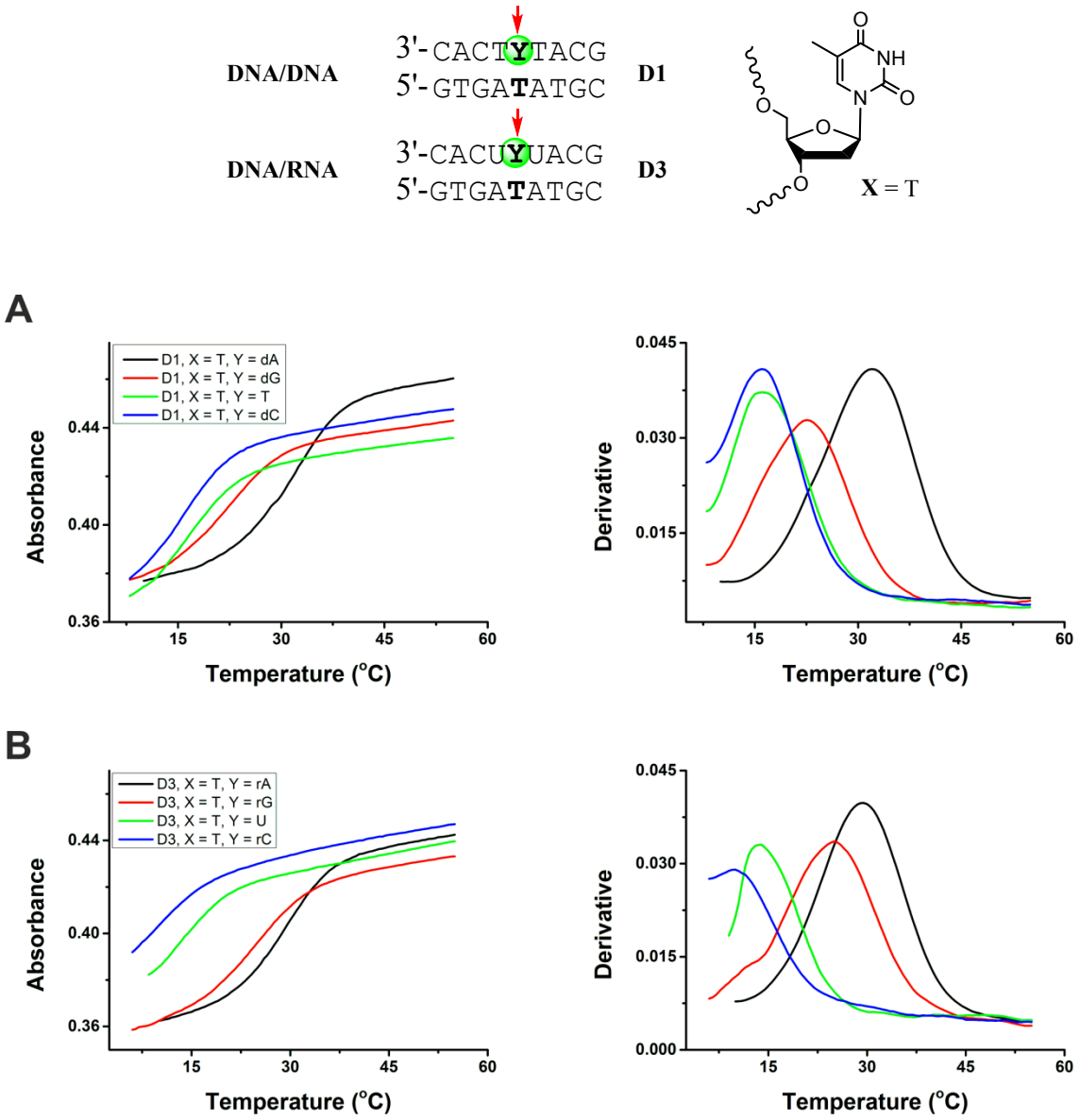



**Figure S7.** UV melting curves (left) and derivatives (right) of the above DNA/DNA and DNA/RNA duplexes (**Y** = dA or rA, black, match; **Y** = dG or rG, red; **Y** = T or U, green; **Y** = dC or rC, blue). The experiments were carried out at pH 7.0 under the same condition as in Figure S6. A) D1, DNA/DNA duplexes; B) D3, DNA/RNA hybrids.

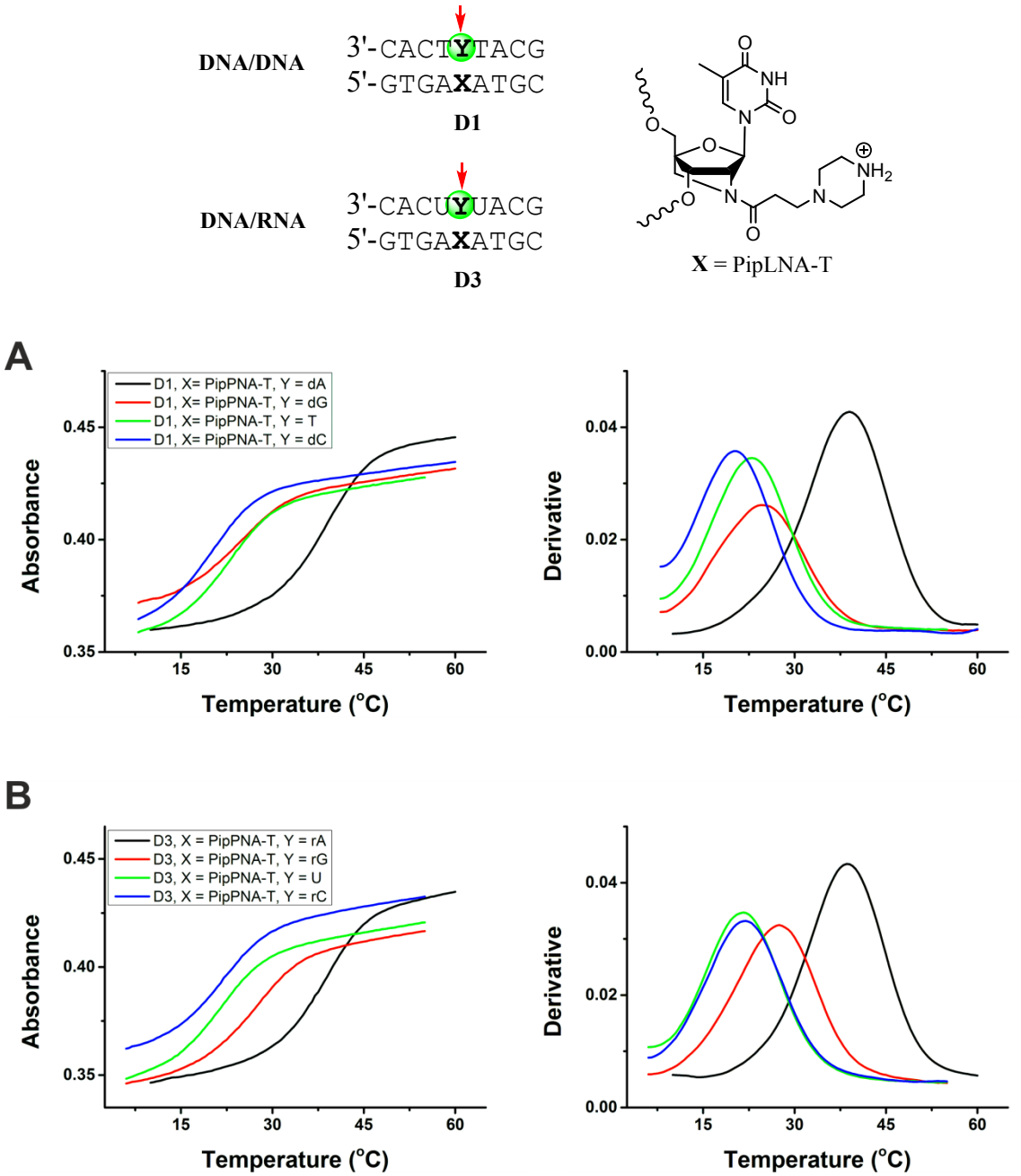



**Figure S8.** UV melting curves (left) and derivatives (right) of the above DNA/DNA and DNA/RNA duplexes with one PipLNA-T insertion (**X** = PipLNA-T; **Y** = dA or rA, black, match; **Y** = dG or rG, red; **Y** = T or U, green; **Y** = dC or rC, blue). The experiments were carried out at pH 7.0 under the same condition as in Figure S6. A) D1, DNA/DNA duplexes; B) D3, DNA/RNA hybrids.

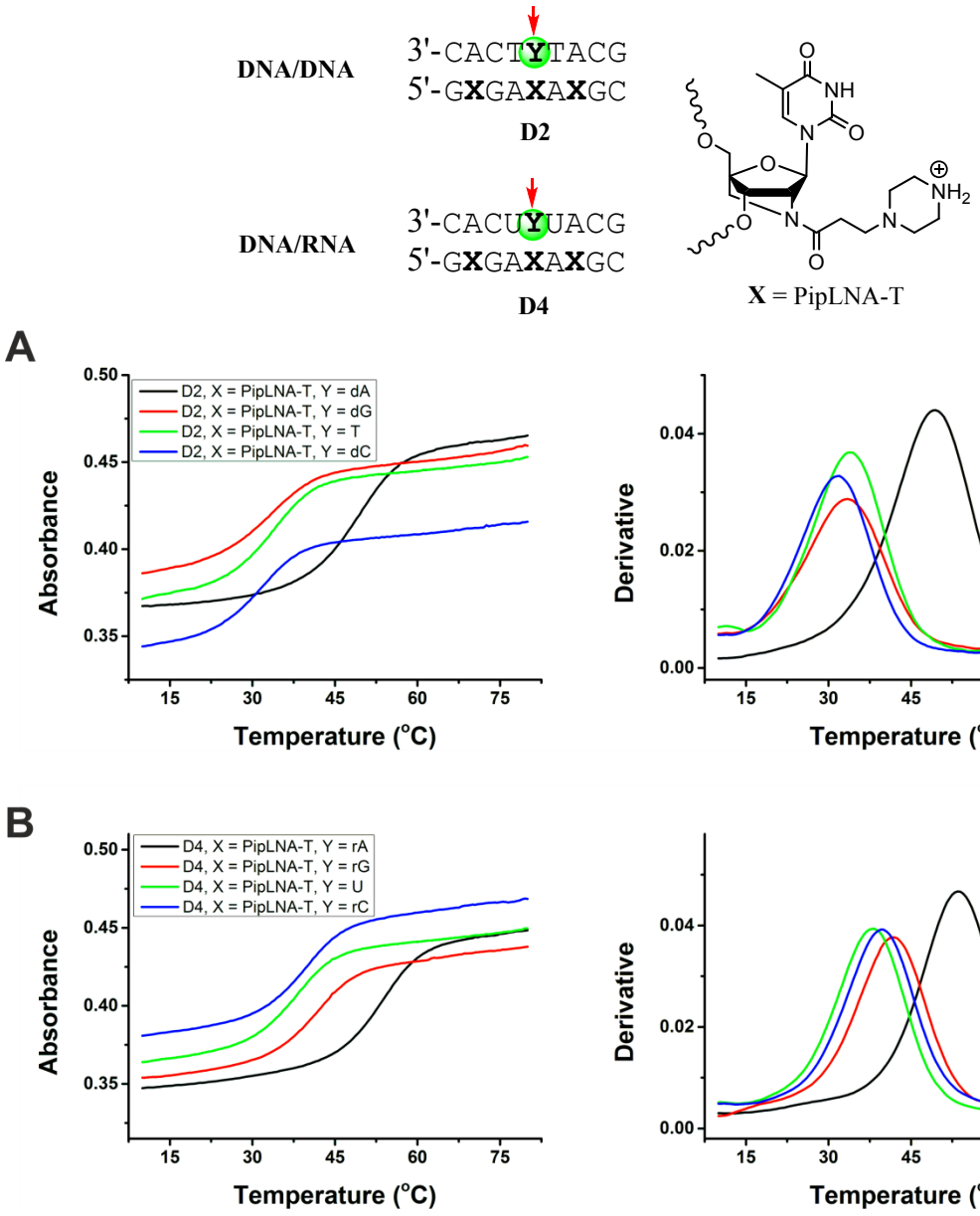



**Figure S9.** UV melting curves (left) and derivatives (right) of the above DNA/DNA duplex and DNA/RNA hybrid with three PipLNA-T insertions (**X** = PipLNA-T; **Y** = dA or rA, black, match; **Y** = dG or rG, red; **Y** = T or U, green; **Y** = dC or rC, blue). The experiments were carried out at pH 7.0 under the same condition as in Figure S6. A) D2, DNA/DNA duplexes; B) D4, DNA/RNA hybrids.

Three substitutions

**X** = T, 2'-amino-LNA-T or PipLNA-T

**DNA/DNA**

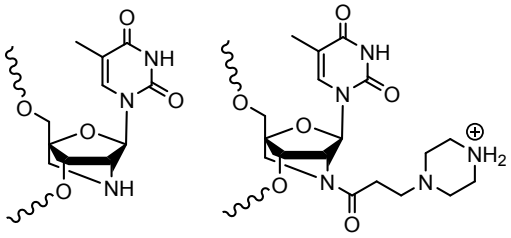
3'-CACTATACG
5'-G**X**GA**X**A**X**GC

**D2**

**DNA/RNA**

3'-CACUAUACG
5'-G**X**GA**X**A**X**GC

**D4**




2'-amnio-LNA-T PipLNA-T

**Table S4.** UV-melting study under low buffer condition[a]

| **Three additions** | | Medium Salt | Low Salt |
|---|---|---|---|
| DNA/DNA (D2) | **X** = PipLNA-T | 49.5 | 39.0 (-10.5) |
| | **X** = 2'-amino-LNA-T | 41.0 | 27.5 (-13.5) |
| | **X** = T | 32.0 | 18.0 (-14.0) |
| DNA/RNA (D4) | **X** = PipLNA-T | 54.0 | 42.5 (-11.5) |
| | **X** = 2'-amino-LNA-T | 49.0 | 36.0 (-13.0) |
| | **X** = T | 29.5 | 16.0 (-13.5) |

[a] $T_m$ values (°C) of DNA/DNA and DNA/RNA duplexes with three additions of T, 2'-amino-LNA-T or PipLNA are an average of two independent melting temperatures with deviation < 0.5 ˚C. Values in parentheses are $\Delta T_m = T_m$ (low salt) - $T_m$ (medium salt). The experiments were carried out at pH 7.0 under the same condition in medium salt buffer (5.8 mM $NaH_2PO_4/Na_2HPO_4$ buffer, containing 100 mM NaCl and 0.10 mM EDTA) or low salt buffer (6.7 mM $NaH_2PO_4/Na_2HPO_4$ buffer, containing 0.10 mM EDTA) as in Figure S6.

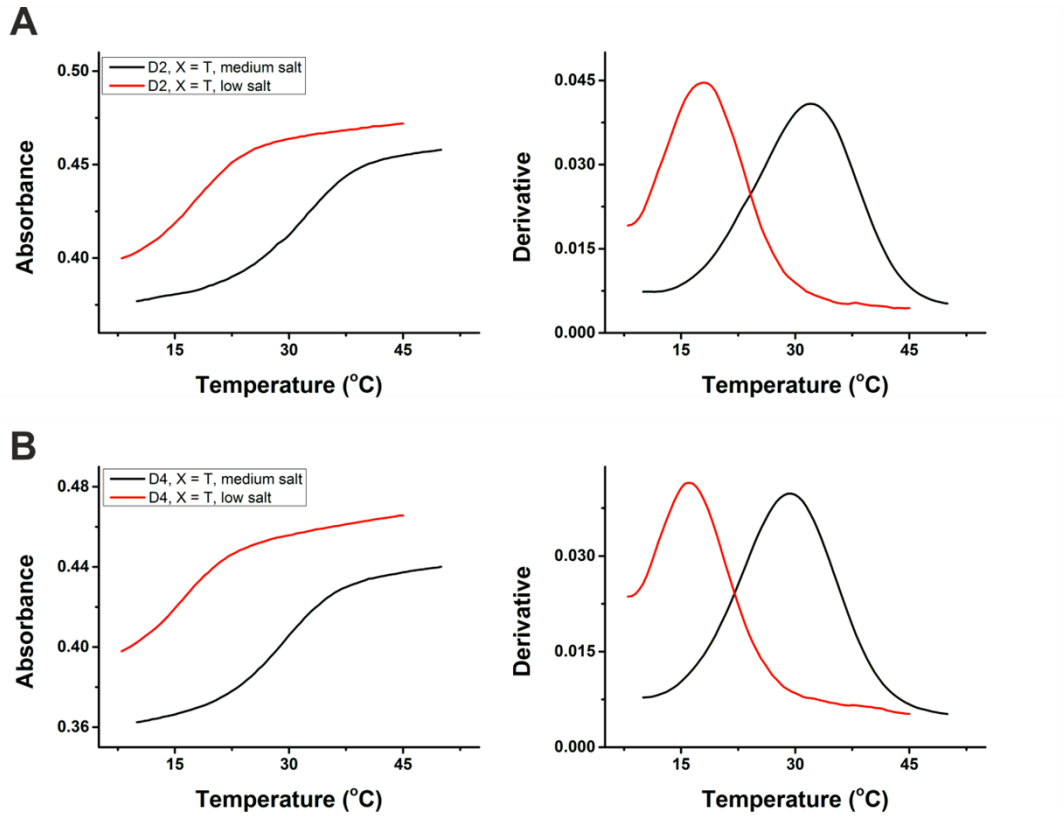



**Figure S10.** UV melting curves (left) and derivatives (right) of the above DNA/DNA duplex and DNA/RNA hybrid in Table S4 (**X** = T). The experiments were carried out at pH 7.0 under the same condition as in Table S4. A) D2, DNA/DNA duplex; B) D4, DNA/RNA hybrid.

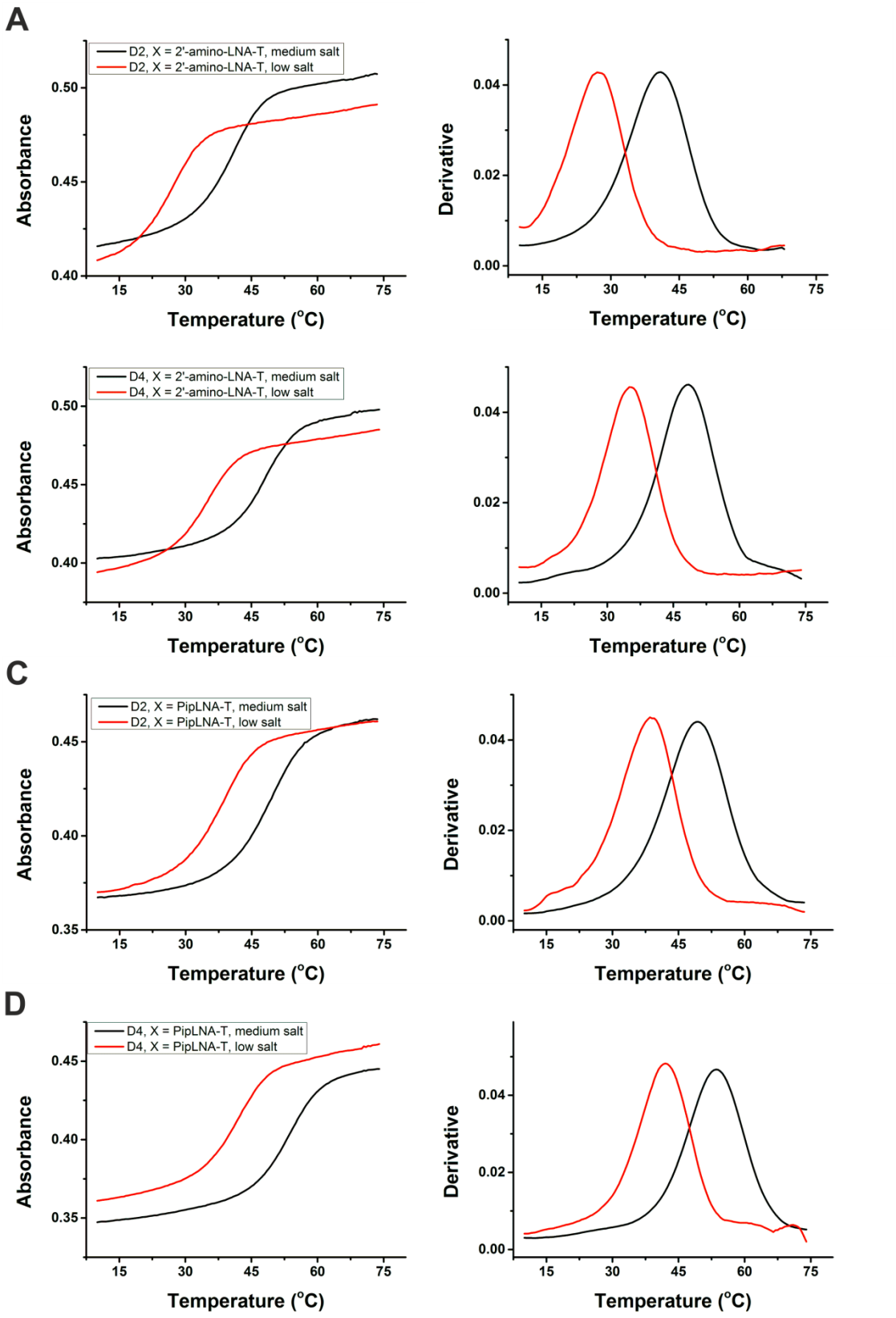



**Figure S11.** UV melting curves (left) and derivatives (right) of the above DNA/DNA and DNA/RNA duplexes in Table S4 (**X** = 2'-amino-LNA-T or PipLNA-T). The experiments were carried out at pH 7.0 under the same condition as in Table S4. A) D2, DNA/DNA duplex, **X** = 2'-amino-LNA-T; B) D4, DNA/RNA hybrid, **X** = 2'-amino-LNA-T; C) D2, DNA/DNA duplex, **X** = PipLNA-T; D) D4, DNA/RNA hybrid, **X** = PipLNA-T.

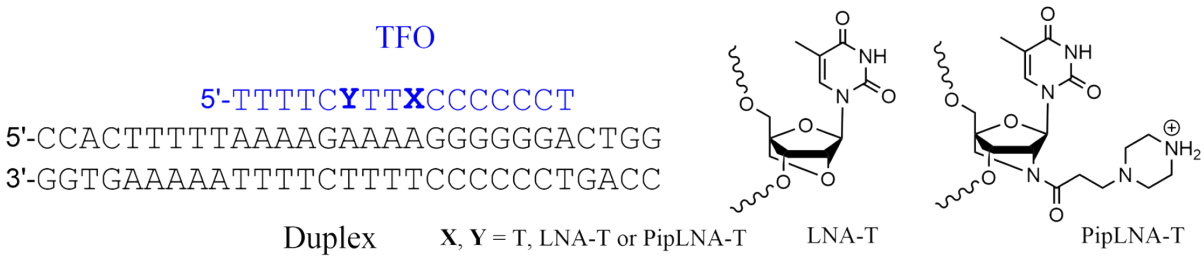



**Table S5.** Triplex melting analysis[a]

| TFO | X | Y | pH 6.0 | pH 7.0 |
|---|---|---|---|---|
| 5′-TTTTCYTTXCCCCCCT | T | T | 24.0 | n.d. |
| | LNA-T | T | 32.0 (+8.0) | 12.5 |
| | PipLNA-T | T | 32.0 (+8.0) | 13.0 |
| | LNA-T | LNA-T | 36.5 (+12.5) | 18.0 |
| | PipLNA-T | PipPNA | 33.5 (+9.5) | 15.0 |

[a]$T_m$ values (˚C) are an average of two independent melting temperatures with deviation no more than 0.5 ˚C. Values in brackets are $\Delta T_m$ = $T_m$ (LNA-T or PipLNA-T) - $T_m$ (T) at pH 6.0. The experiments were performed in 10 mM sodium cacodylate, 150 mM NaCl and 10 mM $MgCl_2$ under pH 6.0 and pH 7.0. The concentration of TFO: target DNA was 1.5 μM:1.0 μM. n.d. = not detected.

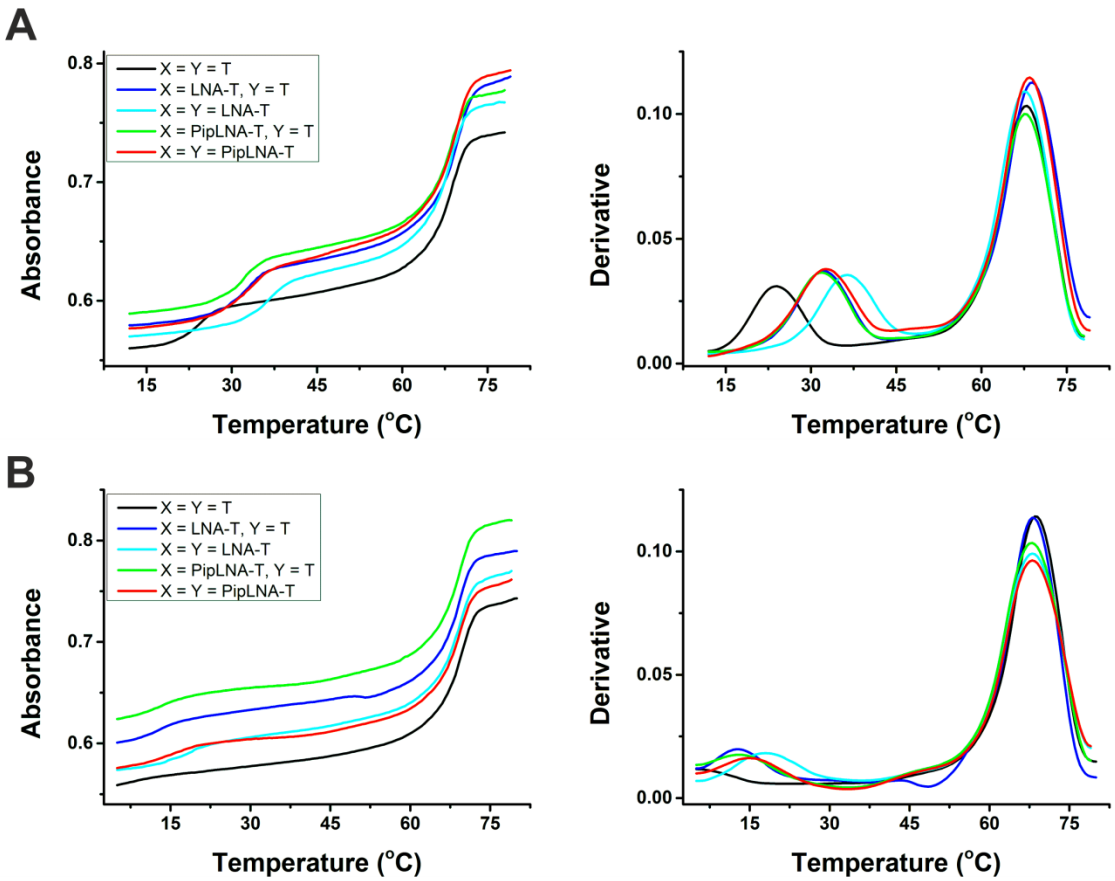
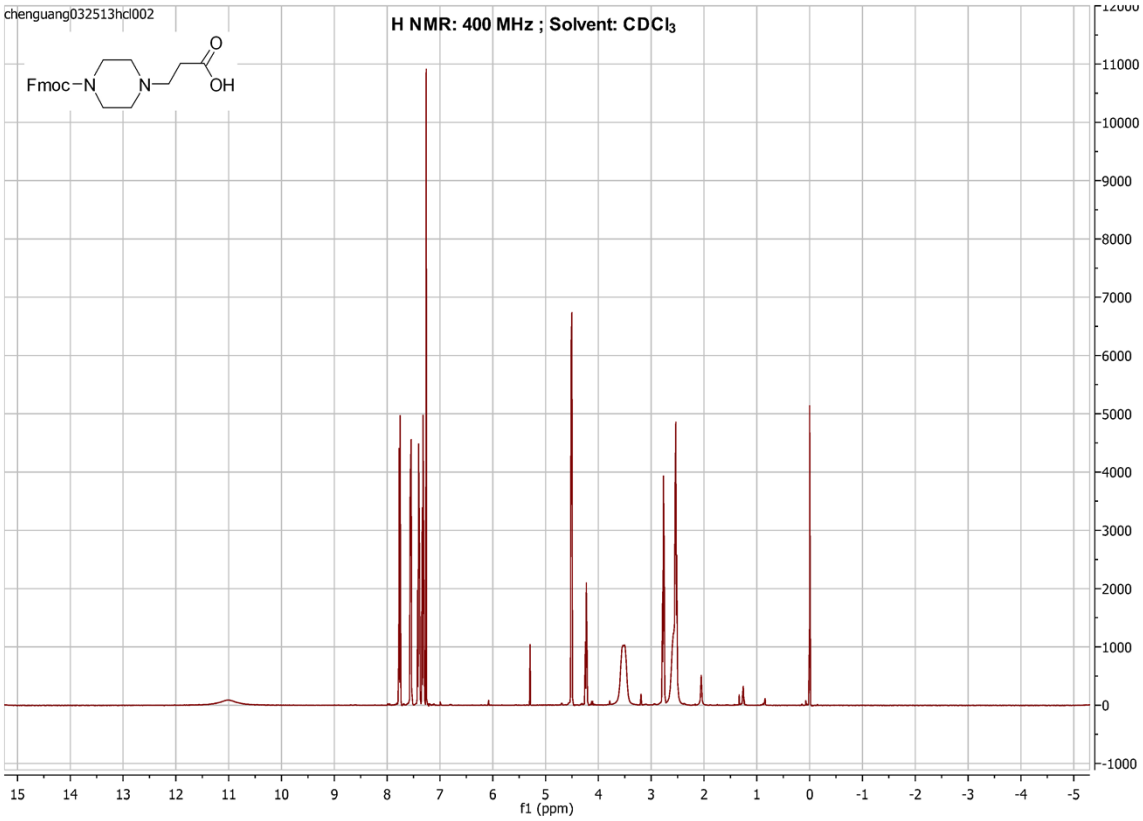



**Figure S12.** UV melting curves (left) and derivatives (right) of TFOs with target hairpin duplex (Table S5) at pH 6.0 and pH 7.0: **X** = **Y** = T, TFO-1; **X** = LNA-T, **Y** = T, TFO-2; **X** = PipLNA-T, **Y** = T, TFO-3; **X** = **Y** = LNA-T, TFO-4; **X** = **Y** = PipLNA-T, TFO-5. A) pH 6.0 ; B) pH 7.0.

## References:


1. N. K. Andersen, H. Dossing, F. Jensen, B. Vester and P. Nielsen, *J. Org. Chem.*, 2011, 76, 6177-6187.
2. MacroModel, version 9.1; Schrödinger, LLC, New York, NY, 2005.
3. S. J. Weiner, P. A. Kollman, D. T. Nguyen and D. A. Case, *J. Comput. Chem.*, 1986, 7, 230-252.

## Appendix: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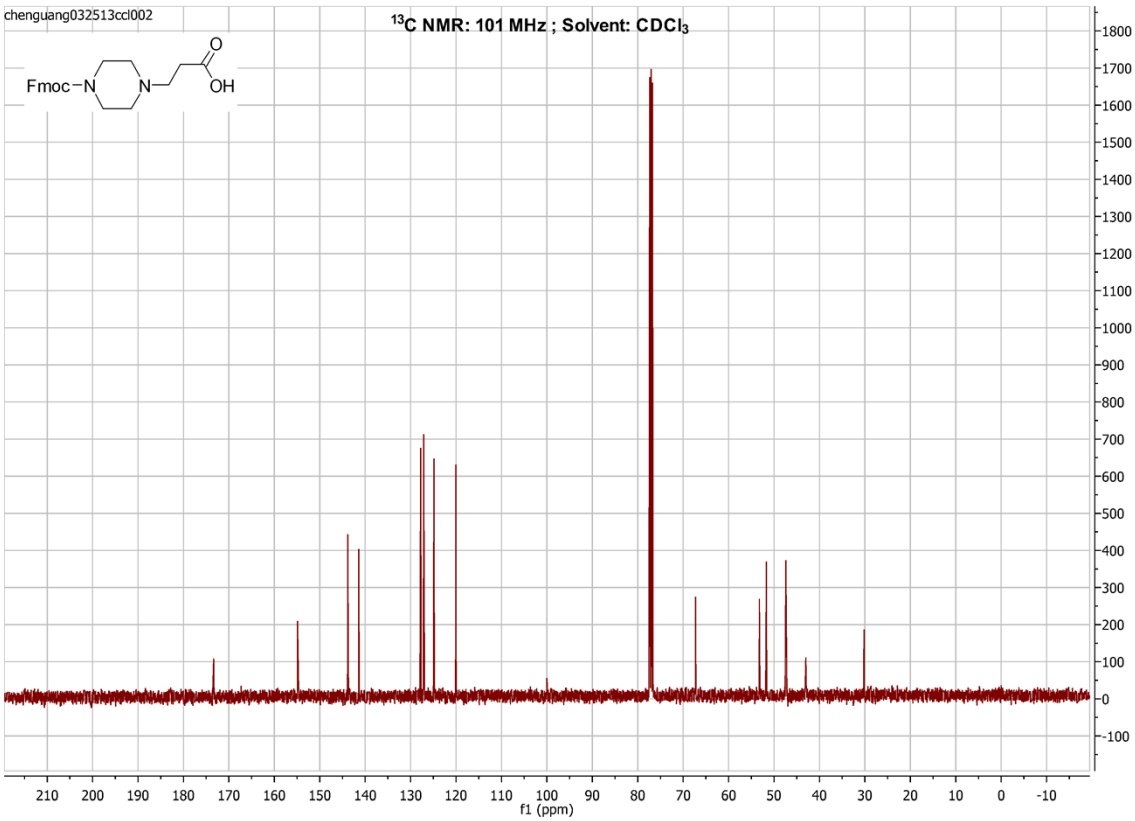
N
O
OH
f1 (ppm)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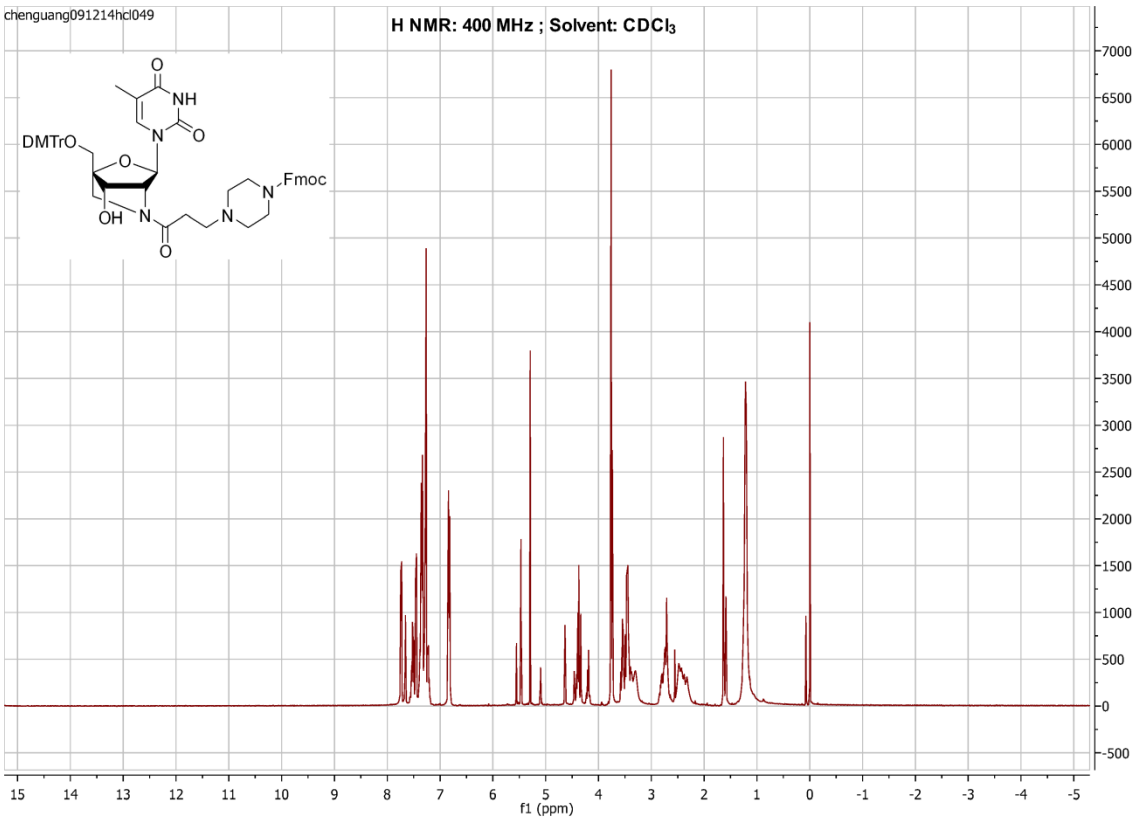
N
O
OH
f1 (ppm)


chenguang091214hcl049
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O
O
OH
N
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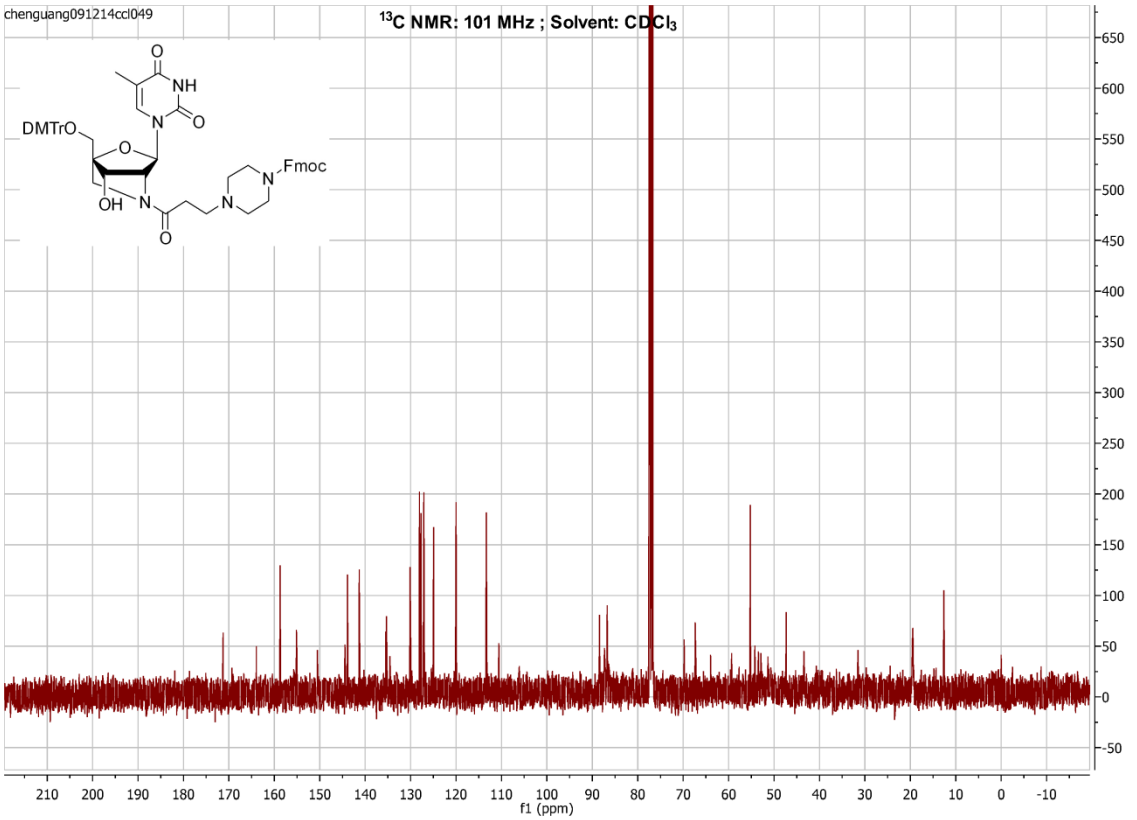
f1 (ppm)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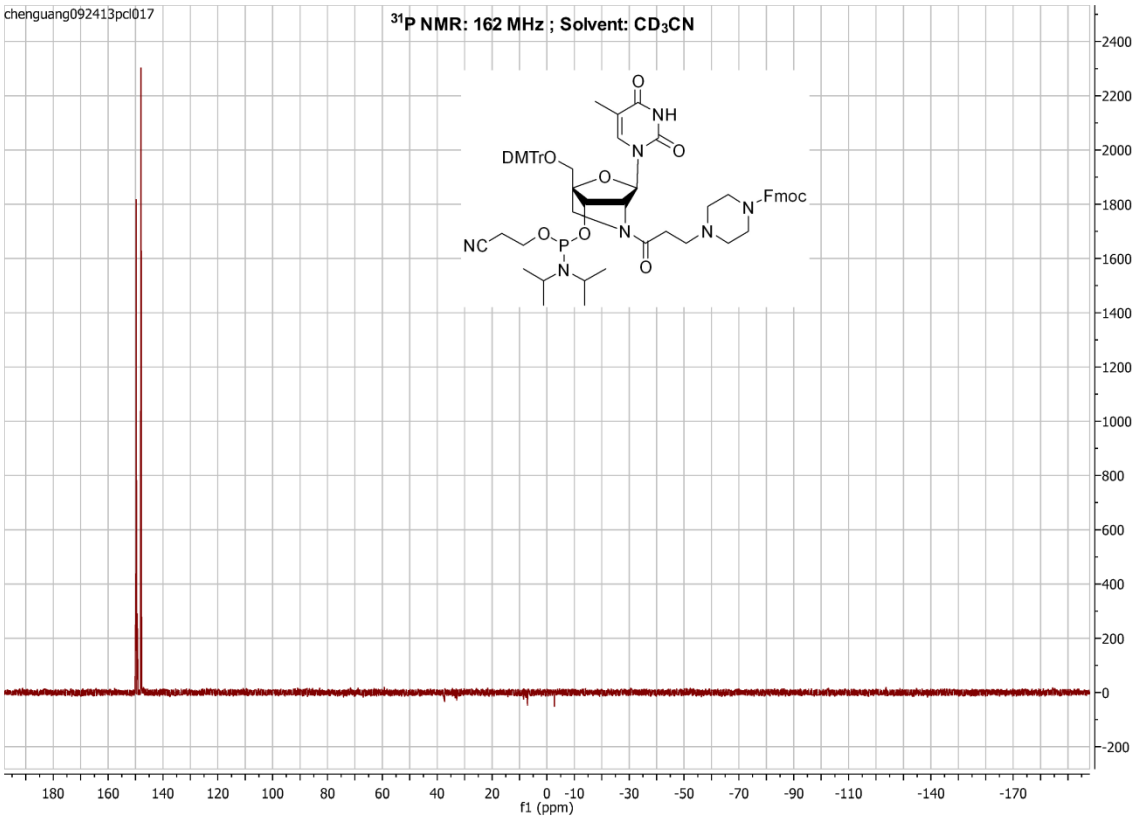
f1 (ppm)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Fmoc
f1 (ppm)