Supplementary Material (ESI) for Organic and Biomolecular Chemistry

Supporting information

End-capped HyBeacon Probes for the Analysis of Human Genetic Polymorphisms Related to Warfarin Metabolism

Nouha Ben Gaied[a], James A. Richardson[a], Daniel G. Singleton[a], Zhengyun Zhao[a], David French[b], Tom Brown[a]*

[a]School of Chemistry, University of Southampton, Highfield, SO17 1BJ, Southampton, UK

[b]Innovation and Development, LGC, Queens Road, Teddington, TW11 0LY. UK.

*To whom correspondence should be sent:
Tel: +44(0)2380 592974, fax: +44(0)2380 592991, E-mail: tb2@soton.ac.uk

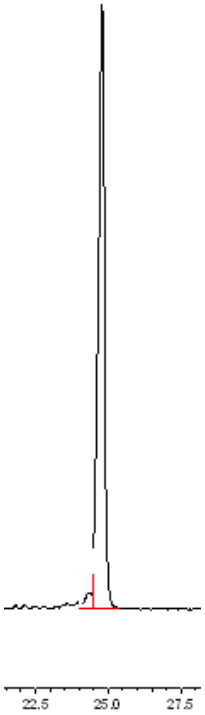
	Modification	Oligonucleotide sequence
1	Matched sequence	3'-CCTCTTGAACACGGTCCTCAATGCTCC-5'
2	Mismatched sequence	3'-CCTCTTGAACACAGTCCTCAATGCTCC-5'
3	3'-P	5'-CGATFGAGGACCGFGTTCAAG-P-3'
4	5'-TMS-3'-P	5'-TMS-CGATFGAGGACCGFGTTCAAG-P-3'
5	3'-AnthdR	5'-CGATFGAGGACCGFGTTCAAG-AnthdR-3'
6	5'-TMS-3'-AnthdR	5'-TMS-CGATFGAGGACCGFGTTCAAG-AnthdR-3'
7	3'-AnthdRNH$_2$	5'-CGATFGAGGACCGFGTTCAAG-AnthdRNH$_2$-3'
8	5'-TMS-3'-AnthdRNH$_2$	5'-TMS-CGATFGAGGACCGFGTTCAAG-AnthdRNH$_2$-3'
9	3'-AmBuPyr	5'-CGATFGAGGACCGFGTTCAAG-AmBuPyr-3'
10	5'-TMS-3'-AmBuPyr	5'-TMS-CGATFGAGGACCGFGTTCAAG-AmBuPyr-3'
11	3'-ThrPyr	5'-CGATFGAGGACCGFGTTCAAG-ThrPyr-3'
12	5'-TMS-3'-ThrPyr	5'-TMS-CGATFGAGGACCGFGTTCAAG-ThrPyr-3'

Table S1: List of oligonucleotides. Twelve oligonucleotides were prepared: two targets and ten probes labelled with two internal fluorescein-dT residues (F), a 3'-modification and/or a 5'-trimethoxystilbene (TMS). P: phosphate.

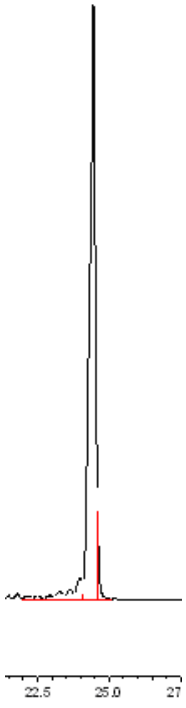
Sequence	Modification	MW calc.	MW found
ODN1	Matched sequence	8128	8135
ODN2	Mismatched sequence	8113	8125
ODN3	3'-P	7589	7600
ODN4	5'-TMS/ 3'-P	8021	8031
ODN5	AnthdR	8067	8099
ODN6	5'-TMS-AnthdR	8489	8494
ODN7	AnthdRNH$_2$	8168	8169
ODN8	5'-TMS-3'-AnthdRNH$_2$	8603	8606
ODN9	3'-AmBuPyr	8079	8078/8100
ODN10	5'-TMS-3'-AmBuPyr	8513	8514
ODN11	3'-ThrPyr	7948	7950
ODN12	5'-TMS-3'-ThrPyr	8383	8386

Table S2: Mass spectra. Mass spectra of all targets and HyBeacons® were recorded by negative mode electrospray on a Fisons VG platform spectrometer in water with Triisopropylamine (2%).

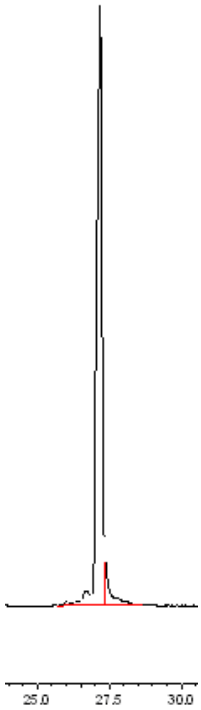
ODN1



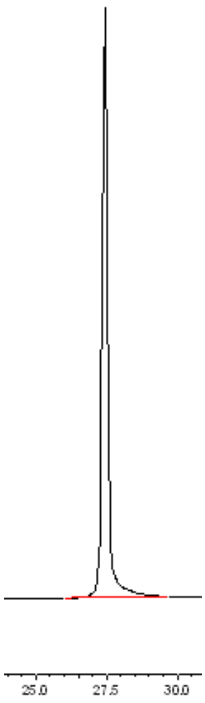
ODN2



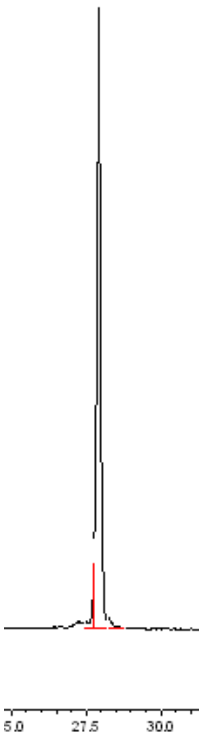
ODN3



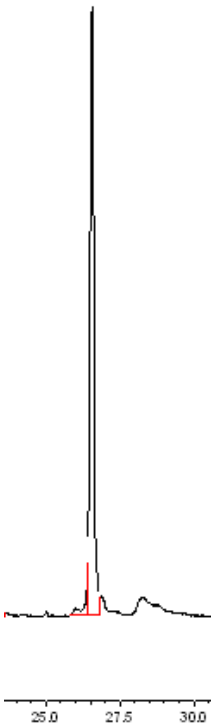
ODN4



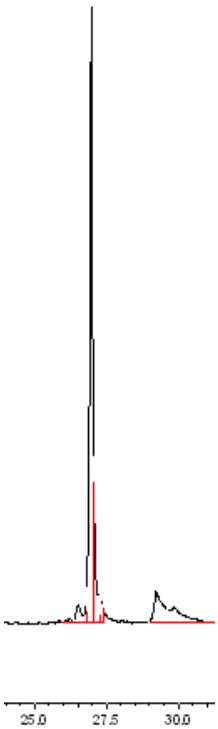
ODN5



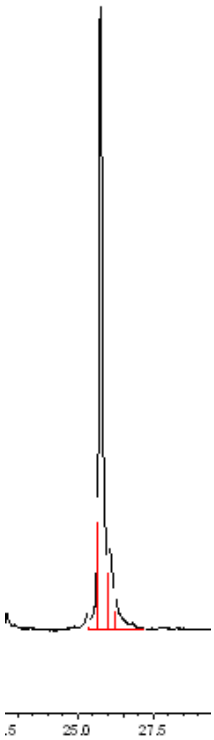
ODN6



ODN7



ODN8



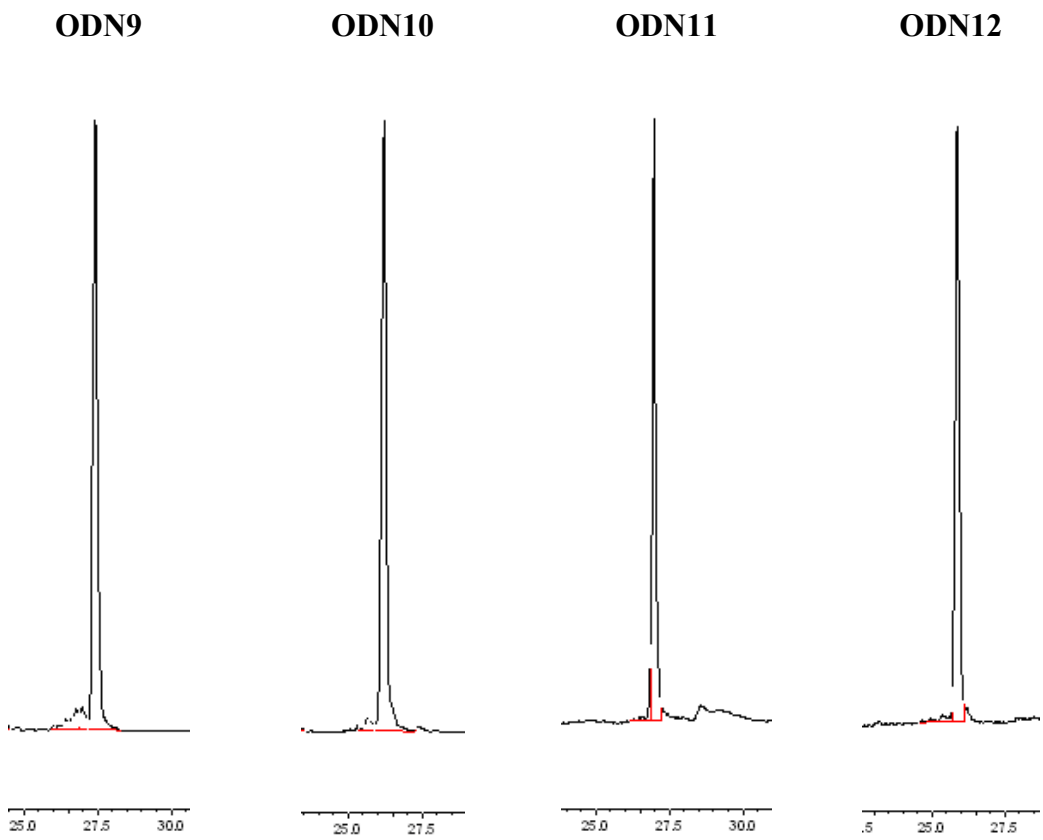
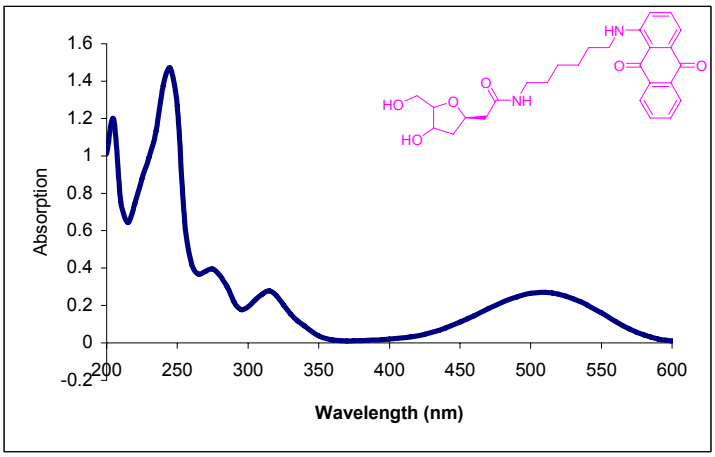


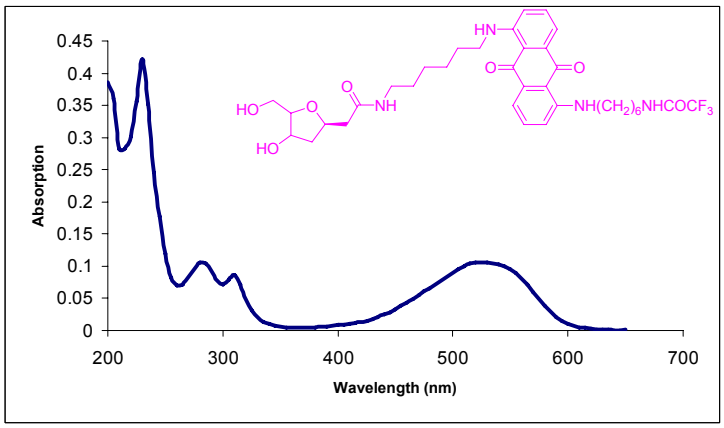
Figure S1: Capillary electrophoresis (CE) analysis of modified oligonucleotides after HPLC purification.

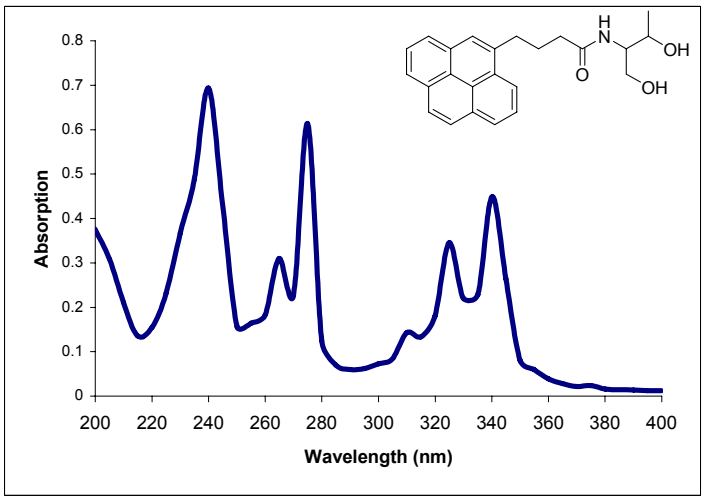
The purity of oligonucleotides was confirmed by injection (0.4 OD/100 µL) of each sample individually on ssDNA 100-R Gel. Tris-Borate 7M Urea were used (kit N° 477480) on a Beckman coulter P/ACE™ MDQ Capillary Electrophoresis system using 32 Karat software. UV-254, inject voltage 10.0 kV and separate voltage 9.0 kV (45.0 min duration). The x-axis shows time in min and the y-axis is UV absorbance at 254 nm.

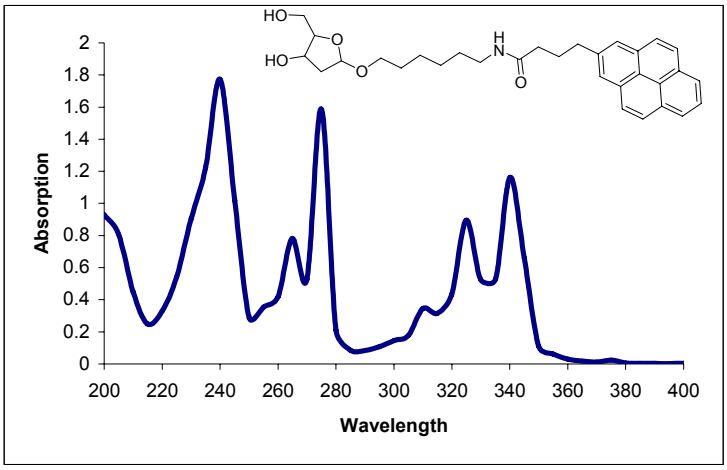
Figure S2: UV spectra

A) UV spectra of 3'-caps

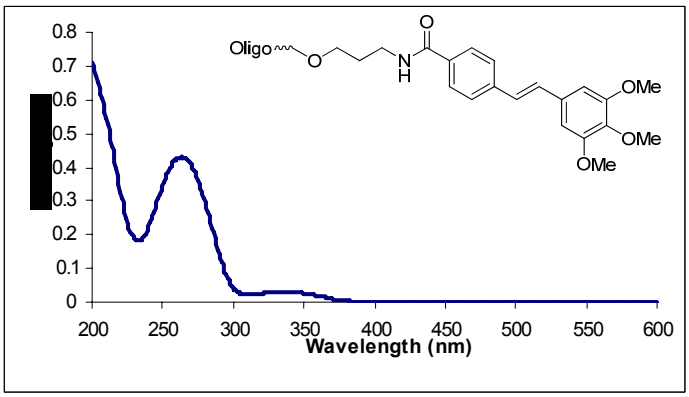








B) UV/vis spectra of 5'-Trimethoxystilbene oligonucleotide.



Fluorescence properties of end-caps.

The above end-caps and 5'-modified TMS-oligonucleotide were excited by irradiation at 460 nm. None of them yielded a fluorescence emission signal.

Quantum yield determination of 3'-end caps (350 nm excitation)

Quantum yields (Φ) of the 3'-modifications were calculated at an optical density of 0.01 at the excitation wavelength of 350 nm for the various 3'- modifications using a solution of quinine sulphate in 1 M H_2SO_4 as a standard while compounds were dissolved in MeOH.

Determination of extinction coefficients of 3'-modifications

General Experimental

Flash chromatography was performed on silica (40-63μm) purchased from Fisher Scientific and thin layer chromatography was performed on Merck Kieselgel $60F_{254}$

coated plates (0.22 mm thickness, aluminum backed). Compounds were visualized by staining with vanillin (2g of vanillin in 100 mL of EtOH/ H_2SO_4 98:2) and by ultraviolet absorbance at 254nm. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV300 or a Bruker DPX 400 spectrometer. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Chemical shifts are given in ppm relative to tetramethylsilane. *J* values are given in Hz and corrected to within 0.5 Hz. Multiplicities of ^{13}C signals were determined using the DEPT spectral editing technique. High-resolution mass spectra were recorded in acetonitrile, methanol or water (HPLC grade) using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer.

3'-Oligonucleotide modifications

Anthraquinone and Pyrene analogues for 3'-oligonucleotide modification and the corresponding oligonucleotide synthesis resins were prepared as described previously[1] These compounds were detritylated by the general procedure outlined below to obtain extinction coefficients for calculating oligonucleotide concentrations. DMTr protected compounds (**1-4**) were dissolved in a solution of 3% trichloroacetic acid (TCA) in CH_2Cl_2 (oligonucleotide grade) at a concentration of 0.03 M. The mixture was stirred at room temperature until detritylation was complete (TLC CH_2Cl_2: MeOH 90:10). The reaction mixture was then quenched with a saturated solution of $NaHCO_3$ and CH_2Cl_2 was added. The organic layer was washed with H_2O and brine, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by silica gel flash chromatography using an Isolute cartridge (Biotage) eluting with a gradient of MeOH in CH_2Cl_2.

N-(6-(9,10-Dihydro-9,10-dioxoanthracen-1-ylamino)hexyl)-2'-deoxy-D-ribofuranose-1-β-acetamide (1). The reaction was performed on 84 mg (0.107 mmol) in 3.5 mL of TCA 3% in CH_2Cl_2. 50 mg, 97%.

R_f CH_2Cl_2: MeOH (90:10) 0.31. **NMR ^1H** (400 MHz, DMSO-d6) δ_H 9.73-9.71 (2H, t, *J*= 5 Hz, NH), 8.26-8.18 (2H, dd, *J*= 7.5 Hz, H-Ar), 7.97-7.88 (3H, m, H-Ar), 7.71-7.67 (1H, t, *J*= 8.5 Hz, H-Ar), 7.29-7.27 (1H, d, *J*= 8.5 Hz, H-Ar), 4.99-4.98 (1H, d, *J*= 4 Hz, OH), 4.74-4.71 (1H, m, OH), 4.43-4.36 (1H, m, H1'), 4.16 (1H, d, *J*= 2.48 Hz, H3'), 3.72-3.69 (1H, m, H4'), 3.46-3.37 (4H, m, CH_2), 3.21-3.10 (2H, m, CH_2), 2.48-2.29 (2H, ddd, J_1= 7 Hz, J_2= 6.5 Hz, J_3= 33.1 Hz, H5'), 1.92-1.88 (1H, m, H2'),

1.77-1.69 (3H, m, CH_2, H2'), 1.54-1.44 (6H, m, CH_2). **NMR ^{13}C** (100 MHz, DMSO-d6) δ_C 183.77, 182.66 (CO), 180.24 (CO-NH), 169.49 (C-Ar), 151.18 (C-Ar), 135.41, 134.26 (CH-Ar), 134.20 (C-Ar), 133.72 (CH-Ar), 133.21 (C-Ar), 128.19, 126.25 (CH-Ar), 126.06 (C-Ar), 118.35, 114.79 (CH-Ar), 111.70 (C-Ar), 87.15 (C1'), 74.72 (C4'), 71.88 (C3'), 62.26 (CH_2-NH), 42.6 (CH_2-CO), 40.74 (C2'), 38.85 (C5'), 29.53, 29.01, 26.76, 26.62 (CH_2). UV-Vis λ_{max} (MeOH)/nm 510 (ε/dm^3.mol^{-1}.cm^{-1} 2360). Φ (MeOH, 350 nm) 0.04. **m/z LRMS** [ES$^+$, MeOH] 503.3 (M+Na$^+$, 100%), 983.8 (2M+Na$^+$, 50%). **m/z HRMS** (M+Na$^+$) ($C_{27}H_{32}N_2O_6Na$): calc. 503.2158 found 503.2153.

N-(6-(9,10-Dihydro-9,10-dioxoanthracen-1-ylamino)-5-yl-(6-aminohexyl)hexyl)-2-deoxy-D-ribofuranose-1-β-acetamide (2). The reaction was performed on 0.114 g (0.115 mmol) in 4 mL of TCA 3% in CH_2Cl_2. Purple solid 75 mg, 95%.

R_f CH_2Cl_2: MeOH (90:10) 0.33. **NMR ^{1}H (400 MHz, DMSO-d$_6$)** δ_H 9.77-9.74 (2H, t, *J*= 5 Hz, NH), 9.51 (1H, bs, NH), 7.89-7.86 (1H, t, *J*= 5.2 Hz, NH), 7.73-7.69 (2H, t, *J*= 7.7 Hz, H-Ar), 7.53-7.51 (2H, d, *J*= 7.5 Hz, H-Ar), 7.24-7.22 (2H, d, *J*= 8.5 Hz, H-Ar), 4.97 (1H, bs, OH), 4.72 (1H, bs, OH), 4.42-4.35 (1H, m, H1'), 4.15-4.14 (1H, m, H4'), 3.71-3.68 (1H, m, H3'), 3.58-3.42 (12H, m, CH_2), 3.33-3.28 (2H, q, *J*= 6.5 Hz, CH_2-CO), 2.47-2.28 (2H, ddd, J_1= 6.5 Hz, J_2= 14.0 Hz, J_3= 33.1 Hz, H5'), 1.91-1.86 (1H, m, H2'), 1.78-1.45 (13H, m, CH_2, H2'). **NMR ^{13}C (100 MHz, DMSO-d6)** δ_C 185.2 (CO), 181.2 ($COCF_3$), 170.5 (C-CO), 152.0 (C-NH), 136.5 (CH-Ar), 117.9 (CH-Ar), 115.2 (CH-Ar), 112.8 (CF_3), 88.2 (C1'), 75.8 (C4'), 72.9 (C3'), 63.3 (CH_2-CO), 43.0 (C2'), 39.3 (C5'), 29.4, 29.0, 28.9, 28.6, 26.7, 26.6, 26.3 (CH_2). UV-Vis λ_{max} (MeOH)/nm 525 (ε/ dm^3.mol^{-1}.cm^{-1} 1077). Φ (MeOH, 350 nm) 0.04. **m/z LRMS** [ES$^+$, MeOH] 713.5 (M+Na$^+$, 100%). **m/z HRMS** (M+Na$^+$) ($C_{35}H_{45}N_4O_7F_3Na$): calc. 713.3138. found 713.3133.

1-β-*O*-(hexylamidopropylpyrene-1-yl)-2-deoxy-D-ribofuranose (3). The reaction was performed on 0.200 g (0.25 mmol) in 8 mL TCA 3% in CH_2Cl_2. Colourless foam, 30 mg, 24 %.

R_f CH_2Cl_2: MeOH (90:10) 0.51. **NMR ^{1}H** (400 MHz, DMSO-d$_6$) δ_H 8.39-8.36 (1H, d, *J*= 9.1 Hz, NH), 8.28-8.19 (4H, m, H-Ar), 8.15-8.09 (2H, dd, J_1= 8.8 Hz, J_2= 10.2 Hz, H-Ar), 8.07-8.02 (1H, t, *J*= 7.7 Hz, H-Ar), 7.94-7.91 (1H, d, *J*= 8.0 Hz, H-Ar), 7.83-

7.79 (1H, t, *J*= 5.8 Hz, H-Ar), 4.97-4.95 (1H, dd, J_1= 2.6 Hz, J_2= 5.8 Hz, H1'), 4.84-4.82 (1H, d, *J*= 5.1 Hz, H4'), 4.65-4.62 (1H, t, *J*= 5.5 Hz, H3'), 3.95-3.86 (1H, m, OH), 3.71-3.66 (1H, m, OH), 3.60-3.47 (2H, m, CH_2), 3.41-3.24 (6H, m, CH_2), 3.09-3.02 (2H, q, *J*= 6.3 Hz, CH_2), 2.29-2.20 (3H, m, CH_2, H2'), 2.06-1.96 (2H, q, *J*= 7.4 Hz, H5'), 1.66-1.58 (1H, m, H2'), 1.47-1.37 (6H, m, CH_2). **NMR ^{13}C** (100 MHz, DMSO-d6) δ_C 172.1 (CO), 137.0, 131.3, 130.9, 129.7 (C-Ar), 128.8, 127.9, 127.6, 126.9, 126.6, 125.4, 125.2 (CH-Ar), 124.6 (C-Ar), 123.9 (C-Ar), 103.3 (C1'), 85.1 (C4'), 70.5 (C3'), 67.2 (CH_2), 61.7 (C5'), 41.5 (C2'), 38.8, 35.5, 32.7, 29.6, 28.0, 26.7, 25.9 (CH_2). UV-Vis λ_{max} (MeOH)/nm 340 (ε/ $dm^3.mol^{-1}.cm^{-1}$ 161523), 324 nm (124267), 274 (219444) and 264 (108333). Φ (MeOH, 350 nm) 0.12. **m/z LRMS** [ES^+, MeOH] 526.3 ($M+Na^+$, 100%), 1029.7 ($2M+Na^+$, 10%). **m/z HRMS** ($M+Na^+$) ($C_{31}H_{37}NO_5Na$): calc. 526.2569 found 526.2564.

L-threoninolamidoprop-1-yl pyrene (4). The reaction was performed on 200 mg (0.295 mmol) in 10 mL of TCA 3% in CH_2Cl_2. Colourless solid 75 mg, 68%.
R_f CH_2Cl_2: MeOH (90:10) 0.46. **NMR ^{1}H** (400 MHz, DMSO-d6) δ_H 8.51-8.47 (1H, dd, J_1= 4.3 Hz, J_2= 9.3 Hz, NH), 8.37-8.11 (7H, m, H-Ar), 8.04-8.01 (1H, dd, J_1=4.5 Hz, J_2= 8 Hz, H-Ar), 7.56-7.53 (1H, m, H-Ar), 4.73 (1H, bs, OH), 4.05 (1H, bs, CH-Thr), 3.89 (1H, bs, CH-Thr), 3.66 (1H, bs, OH), 3.53 (2H, bs, CH_2), 3.46-3.41 (2H, m, CH_2), 2.47-2.46 (2H, m, CH_2), 2.15 (2H, bs, CH_2), 1.20-1.17 (3H, t, *J*= 5.2 Hz, CH_3-Thr). **NMR ^{13}C** (100 MHz, DMSO-d6) δ_C 180.2 (CO), 172.2 (C-Ar), 136.6, 130.8, 130.3, 129.2, 128.1 (C-Ar), 127.4, 127.3, 127.1, 126.3, 126.0, 124.8, 124.7, 124.6 (CH-Ar), 124.0 (C-Ar), 123.4 (CH-Ar), 64.4 (CH-Thr), 60.6 (CH_2), 55.6 (CH-Thr), 35.1, 32.2, 27.8 (CH_2), 20.1 (CH_3). UV-Vis λ_{max} (MeOH)/nm 340 (ε/$dm^3.mol^{-1}.cm^{-1}$ 29864), 324 (23000), 274 (40889) and 264 (20665). Φ (MeOH, 350 nm) 0.1. **m/z LRMS** [ES^+, MeOH] 398.3 ($M+Na^+$, 100%), 773.6 ($2M+Na^+$, 50%). **m/z HRMS** ($M+Na^+$) ($C_{24}H_{25}NO_3Na$): calc. 398.1732 found 398.1729.

All compounds were detritylated efficiently except 1-β-*O*-(hexylamidopropylpyrene-1-yl)-2-deoxy-D-ribofuranose **3** for which an impurity corresponding to 6-hydroxyhexylamidopropylpyrene was identified by MS and NMR analysis. The same side-reaction was also observed during oligonucleotide synthesis to yield modest amounts of oligonucleotide products without 3'-pyrene. These impurities were easily removed by reversed-phase HPLC.

DMTr protected compounds **1-4** were attached *via* a succinyl linkage to long chain aminoalkyl silica resin (92 μmol/g amino-loading, Link Technologies Ltd) and used in oligonucleotide synthesis.[1]

Fluorescence melting curves

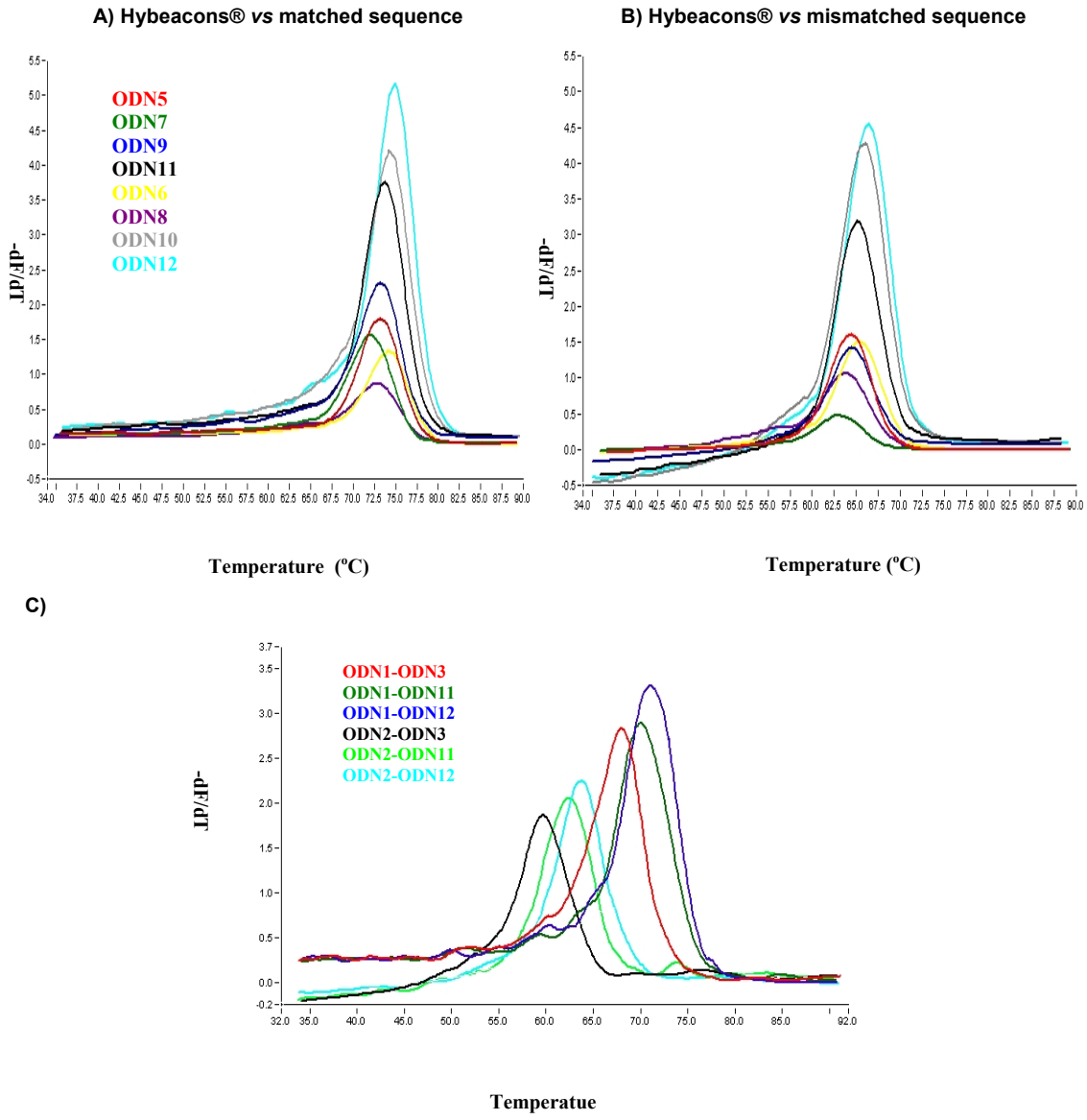
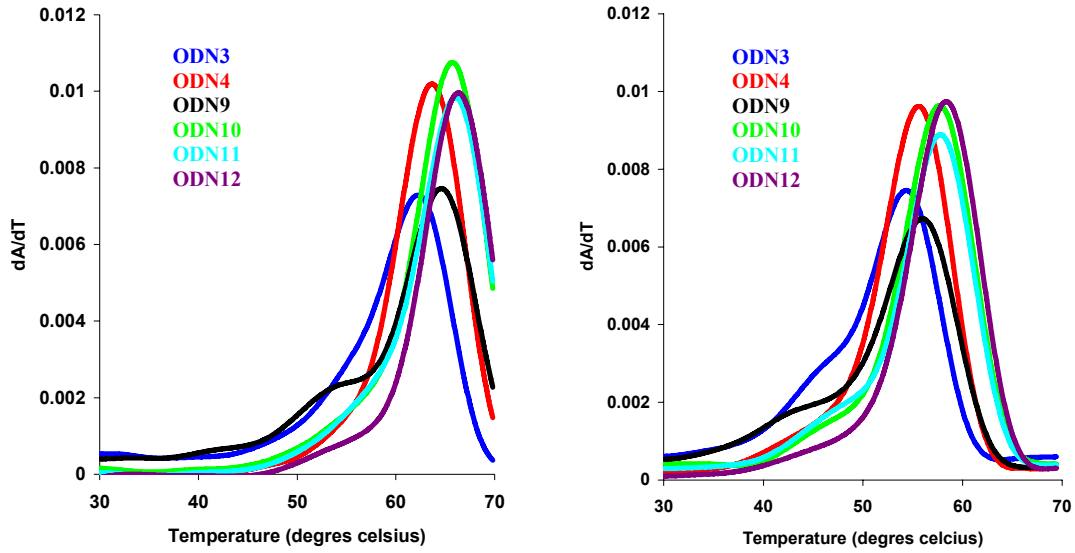


Figure S3: A) Fluorescence melting curves of all HyBeacon® analogues against matched sequence. B) Fluorescence melting curves of all HyBeacon® analogues against mismatch sequence. C) Fluorescence melting curves of ThrPyr analogue in presence and in absence of 5'-TMS in comparison to control HyBeacons®. Fluorescence melting was recorded in TaKaRa buffer 1x, 1M NaCl.

UV melting curves



C)

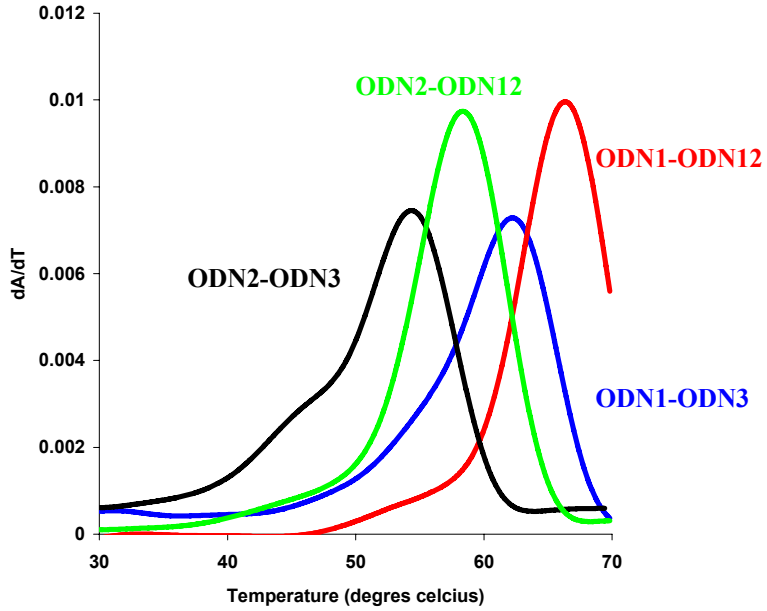


Figure S4: UV melting analysis. A) Pyrene analogues and control HyBeacons® against matched sequence (ODN1). B) Pyrene analogues and control HyBeacons® against mismatched sequence (ODN2). C) Direct comparison of 5'-TMS-3'ThrPyr (ODN12) and 3'-P HyBeacons® (ODN3) towards matched and mismatched sequence. UV melting recorded in phosphate buffer, 200 mM NaCl, pH 7.0.

1. N. Ben Gaied, Z. Y. Zhao, S. R. Gerrard, K. R. Fox and T. Brown, *Chembiochem*, 2009, **10**, 1839-1851.

Supplementary Material (ESI) for Organic and Biomolecular Chemistry