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Single Point Mutation Detection in Living Cancer Cells by Far-red Emitting PNA-FIT Probes

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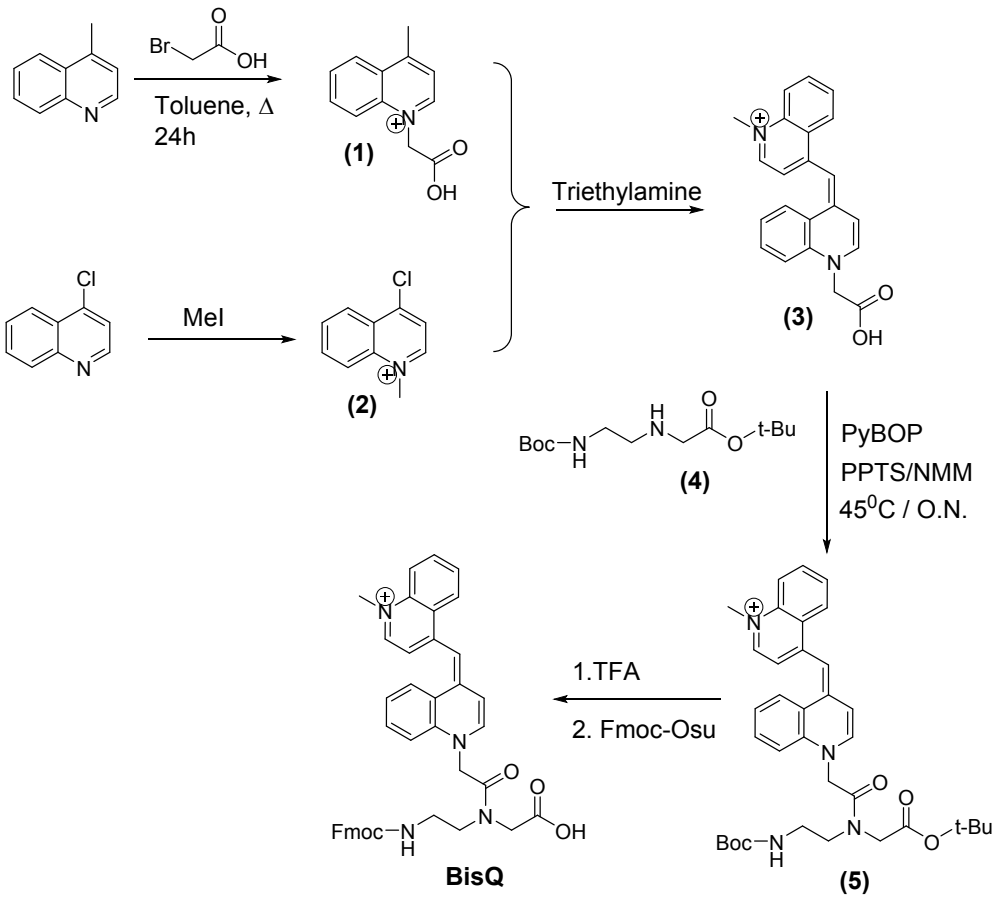
General procedures and materials

Manual solid-phase synthesis was performed by using 5 mL polyethylene syringe reactors (Phenomenex) that are equipped with a fritted disk. All column chromatography was performed using 60A, 0.04-0.063mm Silica gel (Biolab, Israel) and manual glass columns. TLC was performed using Merck Silica Gel 60 F254 plates. HPLC purifications and analysis were performed on a Shimadzu LC-1090 system using a semi-preparative C18 reversed-phase column (Jupiter C18, 5u, 300Å, 250x10mm, Phenomenex) at 50ºC. Eluents: A (0.1% TFA in water) and B (MeCN) were used in a linear gradient (11-40 %B in 38min) with a flow rate of 4mL/min. NMR spectra were recorded on a 300 and 600 MHz Bruker NMR using deuterated solvents as internal standards. MS measurements of compounds **1-5** and **BisQ** were measured on a ThermoQuest Finnigan LCQ-Duo ESI mass spectrometer. Mass analysis of PNAs was acquired on an Orbitrap MS (Voyager DePro, Applied Biosystems, CA, USA). DNA oligos were purchased from Sigma-Aldrich, Israel. Dry DMF was purchased from Acros and Fmoc/Bhoc protected PNA monomers from PolyOrg Inc. (USA). The Fmoc-protected amino acids and reagents for solid phase synthesis were purchased from Merck (Germany).

Synthesis of BisQ



Scheme S1: Synthetic scheme for the preparation of **BisQ**.

1-carboxymethyl-4-methylquinolinium bromide (1).

Compound **1**was synthesized as previously described[1] with some slight modifications: 4-methylquinoline (570mg, 4mmol) and bromoacetic acid (607mg, 4.4mmol) were suspended in 10ml of dry toluene and refluxed for 24h. The solvent was evaporated and the brown residue was dissolved in DCM and cooled to 0ºC. Acetone (30 ml) was added dropwise, and the solid was collected by filtration and washed with acetone

(3x10ml). The crude solid was suspended in chloroform and stirred for 1h. The solid was collected by filtration and washed with chloroform to afford **1** as a grey solid (825mg, 80%).

^{1}H NMR (CD_3OD): 1.99 (3H, s, CH_3), 3.79 (2H, s, CH_2), 6.95 (2H, t, J = 7.1, 2ArH), 7.13 (1H, t, J = 7.8, ArH), 7.22 (1H, d, J = 8.9, ArH), 7.48 (1H, d, J = 8.5, ArH), 8.15 (1H, d, J = 6.1, ArH). MS: M_{obt}=202.20, M_{calc}=202.09

1-methyl-chloroquinolinium iodide (2). Compound **2** was synthesized as previously described[2] with some slight modifications: 4-chloroquinoline (1g, 7 mmol) and iodomethane (4ml, 45 mmol) were combined and heated to 45ºC for 4h. The resulting solid was precipitated from a cold ether (60ml) and vacuum dried to afford **2** as a yellow solid (1g, 50%).

4-[[1-carboxymethyl-4(1H)-quinolinylidene]methyl]-1methyl quinoliniumbromide (3). A mixture of **1** (560mg, 2mmol), **2** (600mg, 2mmol) and triethylamine (TEA, 4mmol) in 6 ml dry DCM was stirred for one hour to produce a dark blue solution. As compound **3** decomposes rapidly, it was used in the next reaction without purification (see below).

Boc-Aeg-OtBu (4). Boc/t-Bu protected PNA-backbone was synthesized as previously reported. [3]

Boc-Aeg(Dye)-OtBu (5). To the stirring reaction mixture of Compound **3** from previous synthetic step, (2 mmol, 686 mg) equimolar amounts of PyBOP (1040mg),

PPTS (500mg), NMM (220ul) in 3ml dry DMF were added. The mixture was stirred for 10 minutes following by the addition of 350 mg (1.3mmol) of Boc-Aeg-OtBu. The reaction vessel was sealed and the reaction mixture was stirred under argon overnight at 45ºC. The volatiles were removed under reduced pressure. The crude product was purified by silica gel column chromatography (0 to 2% MeOH gradient in DCM) to yield a blue colored paste (360mg, 45%). $\mathbf{R_f}$ (2%MeOH in DCM) = 0.26

^{1}H NMR(CD_3Cl): 8.3(d, 2H, ArH), 7.8(d, 1H, ArH), 7.67(d, 2H, ArH), 7.53(m, 4H, ArH), 7.34(t, 2H, ArH), 7.0(s, 1H, CH), 5.78(d, 2H, CH_2), 4.38 (s, 1H, N-CH_2), 4.03 (s, 1H, N-CH_2), 3.96(s, 3H, N^+-CH_3), 3.9(s, 2H, Gly-CH_2), 3.7(t, 1H, N-CH_2), 3.28(t, 1H, N-CH_2), 1.42(s, 9H, t-Bu), 1.40(s, 9H, t-Bu)

HRMS: M_{obt}=599.322, M_{calc}=599.322

Fmoc-Aeg(Dy)-OH (BisQ). Compound **5** was dissolved in a 20 ml mixture of DCM/TFA (1:1). After two hours the solvents were evaporated and the resulting slurry was dissolved in 10 ml DCM. The pH was adjusted to ca. 10 by adding 10 equivalents (860ul) of TEA. Next, (242 mg, 0.7 mmol) Fmoc-OSu were added dropwise under continuous stirring. After 12h the solvent was evaporated and the crude mixture was purified by silica gel chromatography (20% MeOH in DCM) followed by further purification by preparative HPLC (Luna 10 microns, 100A, C-18 250x21.2mm, Phenomenex), using an acetonitrile gradient (12-60% in 60 min.) in 0.1% TFA in H_2O. $\mathbf{R_t}$=42min. Yield=40%.

^{1}H NMR(DMSO-d_6): 8.73 (d, 1H, ArH), 8.63 (d, 1H, ArH), 8.32 (m, 1H, ArH), 7.97-7.87(m, 5H, ArH), 7.71(m, 5H, ArH), 7.56(m, 3H, ArH), 7.41(t, 2H, ArH), 7.31(t, 2H, ArH), 7.26(s, 1H, CH), 5.55(s, 1H, CH_2), 5.33 (s, 1H, CH_2), 4.37(m, 1H, Fmoc-CH_2),

4.35 (s, 0.5H, Gly-CH_2), 4.30(d, 1H, Fmoc-CH_2), 4.24(t, 0.5H, Fmoc-CH), 4.20 (t, 0.5H, Fmoc-CH), 4.12(s, 3H, N^+-CH_3), 4.02 (s, 1.4H, Gly-CH_2), 3.60 (t, 1H, N-CH_2), 3.39(m, 2H, N-CH_2), 3.14(m, 1H, N-CH_2).

^{13}C-NMR (DMSO-d_6): (two rotamers) δ ppm: 37.7, 38.6 (N–CH_2), 41.8 (CH_3), 46.5 (Fmoc-CH), 46.8, 47 (2xN–CH_2), 47.6, 49 (2xGly-CH_2), 53.6, 54.2 (2xCH_2), 65.2, 65.4 (2xFmoc-CH_2), 96.6, 96.5 (2xArC), 107.4, 109.3, 109.5 (3xArC), 115.4, 113 (2xArCq), 117, 117.7 (2xArC), 119.9 (Fmoc-ArC), 120.1 (ArCq), 124.4 (ArCq), 124.9 (Fmoc-ArC), 125.1(ArCq), 125.4, 125.6 (2xArC), 126.3, 126.8, 127.4 (3xFmoc-ArC), 132, 132.7 (2xArC), 138.4, 140.5 (2xFmoc-ArCq), 142.9, 143.1, 143.9 (3xArC), 144(ArCq), 148, 149.7 (2xFmoc-ArCq), 155.9, 156.3 (2x Fmoc-COONH), 166.1, 166.5 (2xDye-CON), 170, 171.9 (Gly-COOH)

HRMS: M_{obt}=665.275, M_{calc}=665.275

NMR spectra of BisQ

Fig. S1

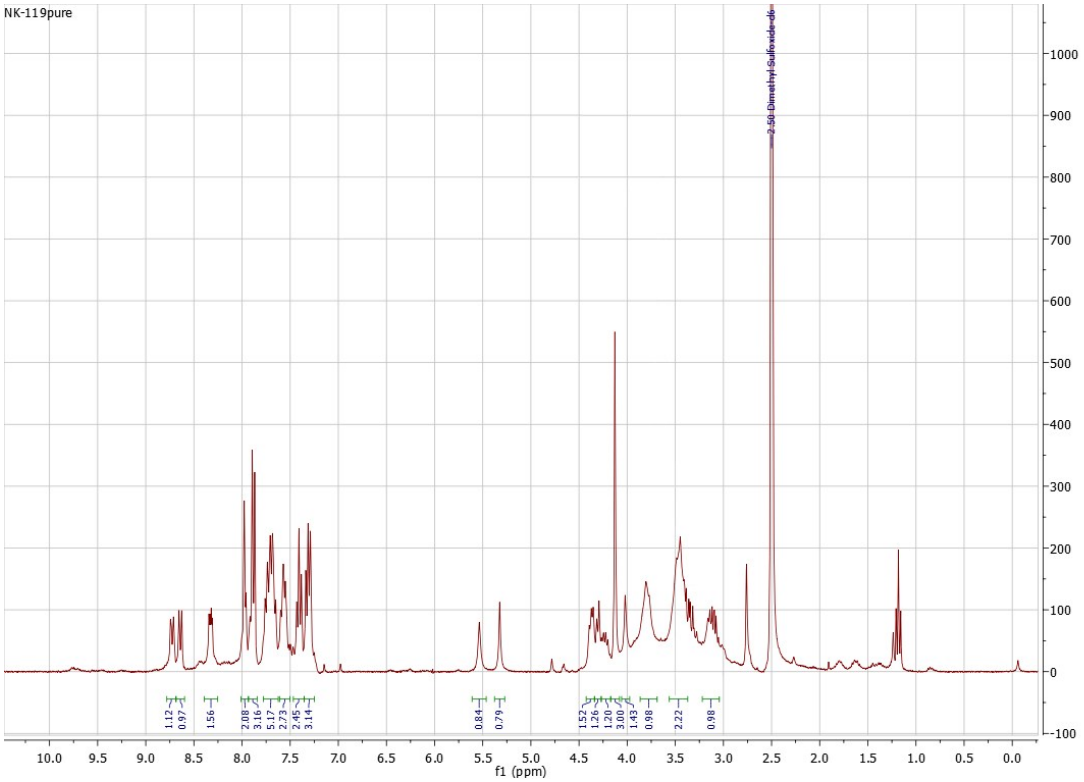


Fig. S1. ^{1}H NMR spectrum of BisQ in DMSO-d_6.

Solid phase synthesis of PNA1 and PNA2

Coupling of first amino onto Novasyn TGA Resin. The resin (250 mg, 0.2 mmol/g) was allowed to swell in 10 ml DMF for 30 min. For pre-activation, DIC (5 eq.) and DIMAP (0.1eq.) were added to a solution of Fmoc-protected glycine (10eq.) in DCM (15 ml) in an ice bath. After 15 min, the mixture was evaporated, re-dissolved in dry DMF and added to the resin. After 2.5 h, the resin was washed with DMF (5x2 mL), CH_2Cl_2 (5x2 mL) and the procedure was repeated.

Fmoc Cleavage. A solution of DMF/piperidine (4:1, 1 ml) was added to the resin. After 2 min the procedure was repeated. Finally the resin was washed with DMF (3x1 ml), DCM (3x1 ml).

Coupling of Fmoc-Bhoc-PNA-Monomers. 4eq. of PNA monomer, 4eq. HATU, 4eq. HOBt and 8eq. of dry DIPEA in DMF (1.5 ml) were mixed in a glass vial equipped with a screw cap. After 3 min of pre-activation, the solution was transferred to the resin. After 60 min, the reaction mixture was discarded and the resin was washed with DMF (2x1 ml) and DCM (2x1 ml).

Coupling of Fmoc-Aeg(Dye)-OH (BisQ). 4eq. of PNA monomer, 4eq. HATU, 4eq. HOBt and 8eq. of dry DIPEA in DMF (1.5 ml) were mixed in a glass vial equipped with screw cap. Following 3 min of pre-activation, the solution was transferred to the

resin. After 60 min, the procedure was repeated and finally the resin was washed with DMF (2x1 ml) and DCM (2x1 ml).

Cleavage of PNA from resin. 1ml TFA was added to the dry resin. After 2h another portion of TFA was added. The combined TFA solutions were concentrated in vacuo.

PNA Purification. PNAs were precipitated from the concentrated TFA solution by addition of cold diethyl ether (15 ml). The precipitate was collected by centrifugation and decantation of the supernatant. The residue was dissolved in water and purified by semi preparative HPLC. The purified PNAs were analysed by Orbitrap-MS.

HPLC and Maldi-TOF MS of PNA1 and PNA 2

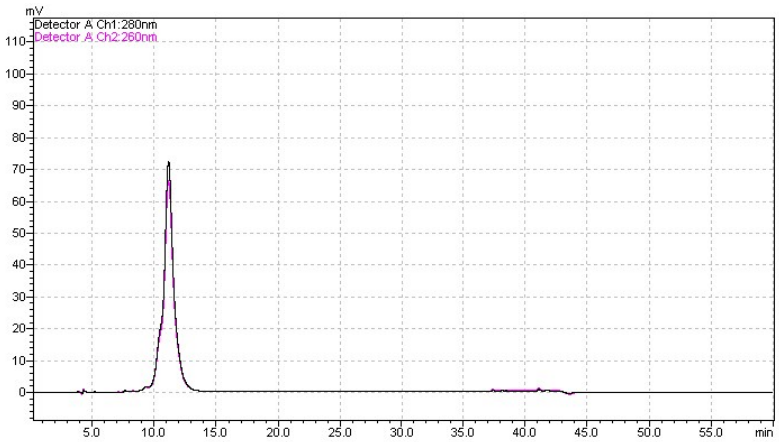


Fig. S2: HPLC chromatogram of **PNA1**. Eluents: A (0.1% TFA in water) and B (MeCN) were used in a linear gradient (11-40 %B in 38min) with a flow rate of 4mL/min.

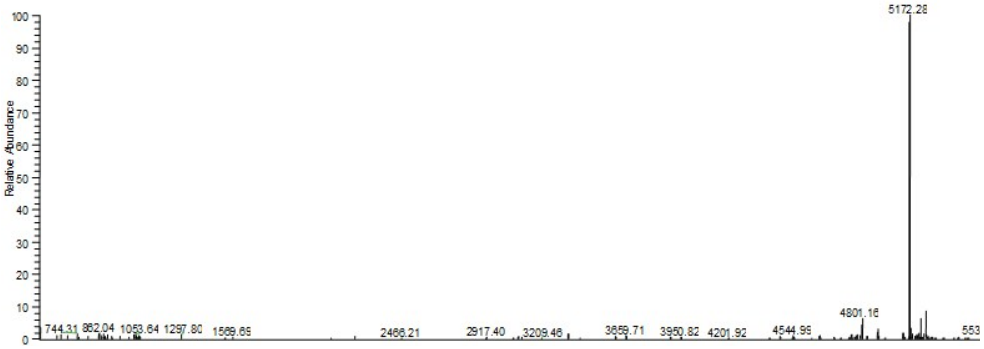


Fig. S3: Maldi-TOF MS of **PNA1**. M_{calc} = 5172.27, M_{obs} = 5172.28.

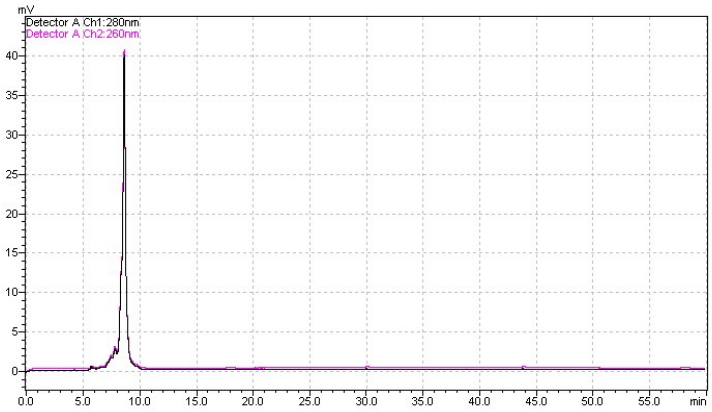


Fig. S4: HPLC chromatogram of **PNA2**. Eluents: A (0.1% TFA in water) and B (MeCN) were used in a linear gradient (11-40 %B in 38min) with a flow rate of 4mL/min.

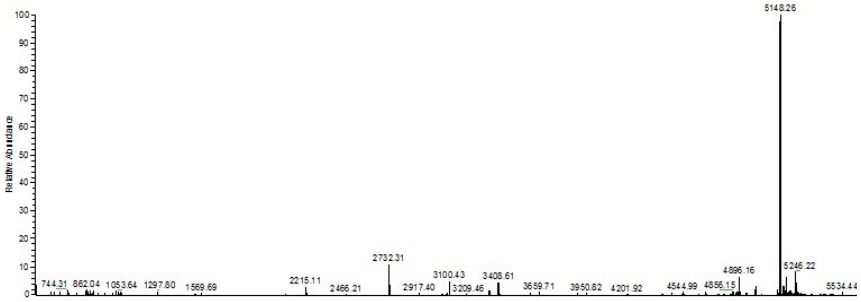


Fig. S5: Maldi-TOF MS of **PNA2**. M_{calc} = 5148.26, M_{obs} = 5148.26.

Fluorescence spectrometry

Fluorescence spectra were recorded by using a Jasco FT-6500 spectrometer. Measurements were carried out in fluorescence quartz cuvettes (10 mm) at 0.5-1.5 μM concentration in a PBS buffered solution (100 mM NaCl, 10 mM NaH_2PO_4, pH 7 Quantum yields were determined relative to fluorescein in PBS as described (doi:10.1016/j.tet.2013.03.005). PNAs were hybridized to complementary DNA or RNA by heating a 1:0.75 mixture of PNA:DNA and PNA:RNA (10-30 μM) to 95ºC for 5 min followed by slow cooling to 25ºC. Samples were excited at 588nm (for **PNA1**) and at 593nm (for **PNA2**) and emission spectra were recorded at 600-760 nm.

UV-Vis spectra of BisQ and TO monomers

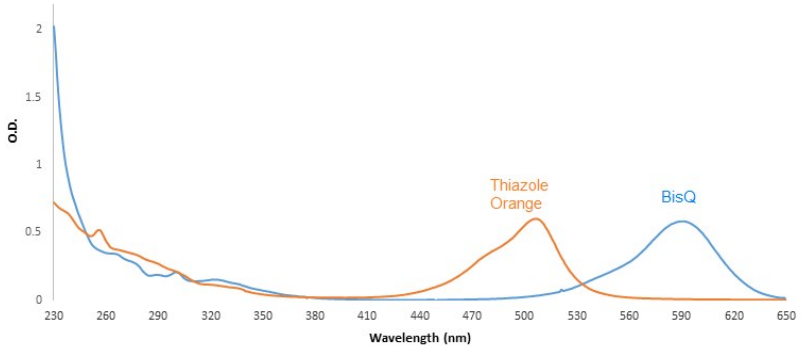


Fig. S6: UV-Vis spectra of **TO** and **BisQ** monomers**.**

UV-Vis spectra of **PNA1** and **PNA1**:DNA

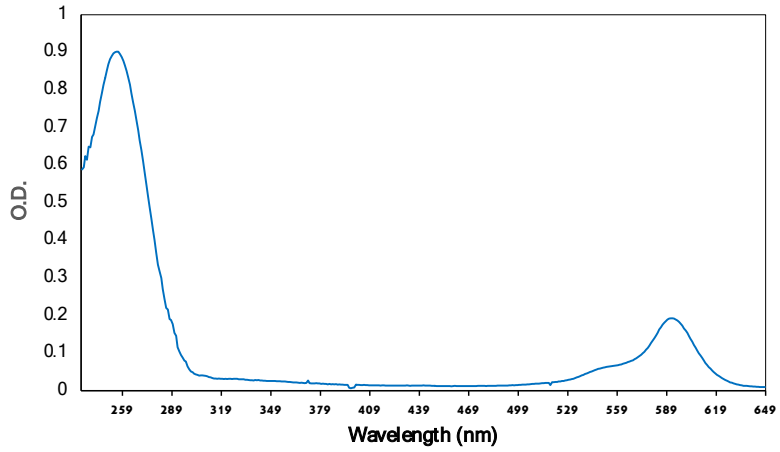


Fig. S7: UV-Vis spectrum of **PNA1**. Maximal absorption at 593 nm.

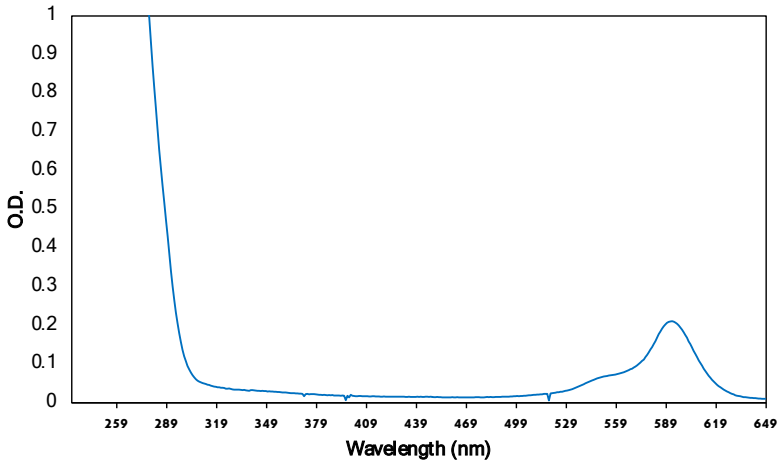


Fig. S8: UV-Vis spectrum of **PNA1**:DNA. Maximal absorption at 593 nm.

UV-Vis spectra of **PNA2** and **PNA2**:DNA

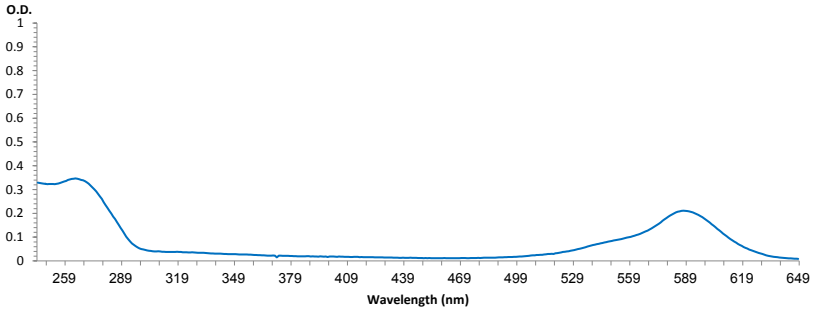


Fig. S9: UV-Vis spectrum of **PNA2**. Maximal absorption at 588 nm.

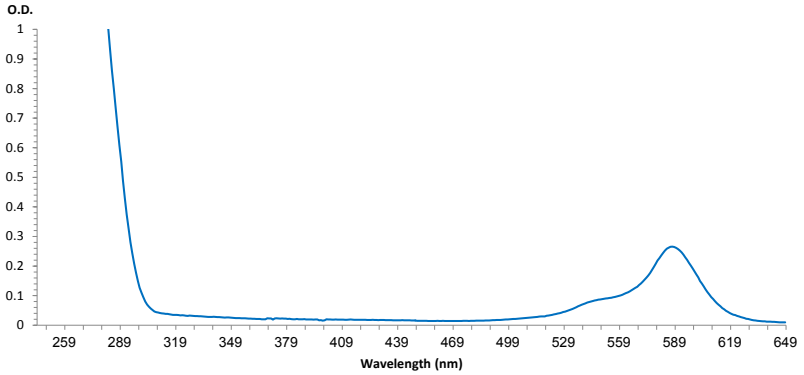


Fig. S10: UV-Vis spectrum of **PNA2**:DNA. Maximal absorption at 587 nm.

PNA1 stability in human serum as corroborated by HPLC.

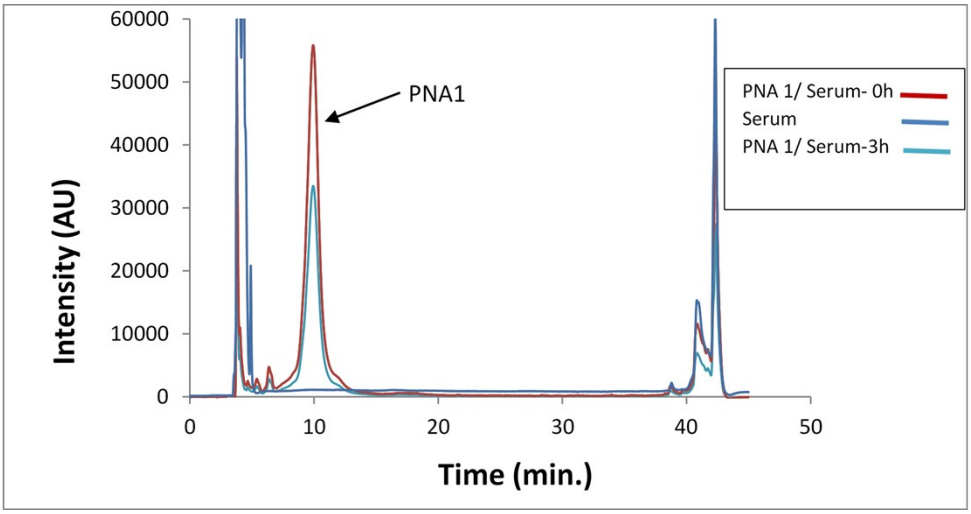


Fig. S11: HPLC chromatograms of **PNA1** incubated at 37°C in human serum after 0h (red) and 3h (light blue). Chromatogram of serum only is shown in blue. Eluents: A (0.1% TFA in water) and B (MeCN) were used in a linear gradient (11-40 %B in 38min) with a flow rate of 4mL/min.

Cell experiments

Cell lines and culture

Three cell lines were used: Panc-1, BxPC-3 (human pancreatic carcinoma, epithelial-like), and HT-29 (human colon adenocarcinoma grade II).

Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Panc-1 and HT-29 expressing mutated and wild type KRAS, respectively, were cultured (37 °C, 5% CO_2), in DMEM medium and supplemented with 10% fetal calf serum, 2mM L-glutamine, and 0.1 mg/mL Streptomycin (Beit Haemek Biological Industries, Israel).

BxPC-3 cells expressing wild type KRAS were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.1 mg/mL Streptomycin.

Cellular uptake analysis

Twenty four hours prior to PNA addition, Panc-1, HT-29, and BxPC-3 were plated separately on chamber slides (Ibidi GmbH, Munich, Germany) until reaching 70-80% confluence.

Hybridization and imaging in living cells

Before adding the PNAs, the medium was replaced and the cells were incubated (37 °C, humidified atmosphere containing 5% CO2) with 1 μM of **PNA1** and **PNA2** in complete medium. Cells were washed with PBS (×3) prior to cell imaging and the intracellular fluorescence was measured after 3 hours by confocal microscopy.

References:

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