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Supplementary Information

Gamma-FIT-PNAs as Sensitive RNA Probes

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Material and Methods

All RNA oligomers were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). Fmoc/Bhoc-protected PNA monomers were sourced from PolyOrg Inc. (Leominster, MA, USA), while γ-L-serine PNA monomers ($A^{boc}/C^{boc}/G^{boc}$ and T) were obtained by Dr. N. S. (Hyderabad, India). The synthesis of the BisQ monomer and FIT-PNA **1** was carried out as previously reported.[1] Solid-phase synthesis was conducted in 5 mL polyethylene syringe reactors (Phenomenex, Torrance, CA, USA) fitted with fritted disks, utilizing NovaSyn TGA resin (90 μm; Sigma-Aldrich). Fmoc-protected amino acids and O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU) were purchased from Chem-Impex, Illinois, USA. Reagents for solid-phase synthesis: 1-hydroxybenzotriazole (HOBt), Dichloromethane (DCM), Dimethylformamide (DMF), N, N-diisopropylethylamine (DIPEA), Trifluoroacetic acid (TFA), diethyl ether, and Acetonitrile were obtained from Bio-Lab LTD, Jerusalem, Israel. Diisopropylcarbodiimide (DIC), 4-Dimethylaminopyridine (DMAP), Pyridine (Py), Piperidine, Acetic anhydride (Ac_2O), meta-Cresol, 1xPBS buffer salt, and Sinapinic acid were purchased from Merck (USA).

Solid Phase PNA Synthesis (SPPS): All FIT–PNA oligomers were manually synthesized on NovaSyn TGA resin (90 μm, 0.25 mmol/g; Sigma-Aldrich). Following resin swelling, the first coupling was for Fmoc-D-Lys(tBOC)OH (Fluka) using a coupling solution of diisopropylcarbodiimide (DIC, 5 equivalents), 4-dimethylaminopyridine (DMAP, 0.1 equivalents), and anhydrous DMF. The reaction mixture was stirred for 6–7 hours, after which unreacted amine sites were capped with acetic-anhydride (Sigma-Aldrich). Fmoc was deprotected with 20% piperidine/DMF, and the resin was rinsed three times with DMF and DCM. The qualitative Kaiser test revealed successful deprotection. Subsequent couplings of Fmoc-D-Lys(tBOC)OH and γ-L-serine PNA monomers ($T/A^{Boc}/C^{Boc}/G^{Boc}$) were carried out using coupling solution of HATU (4 equivalents), HOBt (4 equivalents), and DIEA (4 equivalents) in anhydrous DMF. Each coupling step was carried out with shaking for 1 hour, and the cycle was repeated for each monomer until the desired oligomer length was achieved.

Upon completion, the FIT–PNA oligomers were detached from the solid support utilizing a cleavage solution of m-cresol/trifluoroacetic acid (1:9, v/v) with agitation for 2 hours. The filtrate

was collected, and the product was precipitated using cold diethyl ether. The FIT–PNA oligomers were purified by high-performance liquid chromatography (HPLC) and characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Purification and Characterization: FIT-PNA oligomers were purified by using a Dionex UltiMate 3000 HPLC system (ThermoFisher Scientific, Waltham, MA, USA) using a semi-preparative C18 reversed-phase column (Jupiter C18, 5 μm, 300 Å, 250 × 10 mm, Phenomenex, Torrance, USA). Elution was carried out by using eluent A (0.1% TFA in water) and B (Acetonitrile) in a linear gradient between 5-30% acetonitrile in water, over 30 minutes, at a flow rate of 4 mL/min, monitoring absorptions at 260 nm and 590 nm. The purified peak of each FIT-PNA oligomer (Fig. S1-S10) was characterized by MALDI-TOF mass spectrometry (Brucker, USA) using sinapinic acid as matrix, prepared in acetonitrile/water (70:30 ratio) solution containing 0.1% TFA (Fig. S11-S15).

Table S1. RNA Sequences used for studies

ID	RNA and mismatches RNA sequences (5' – 3')
RNA	UAUGUA U GUUG
RNA-TG	UAUGU**G** U GUUG
RNA-TC	UAUGU**C** U GUUG
RNA-TU	UAUGU**U** U GUUG

bold letters – mismatches in RNA sequence

HPLC Chromatograms of FIT-PNAs

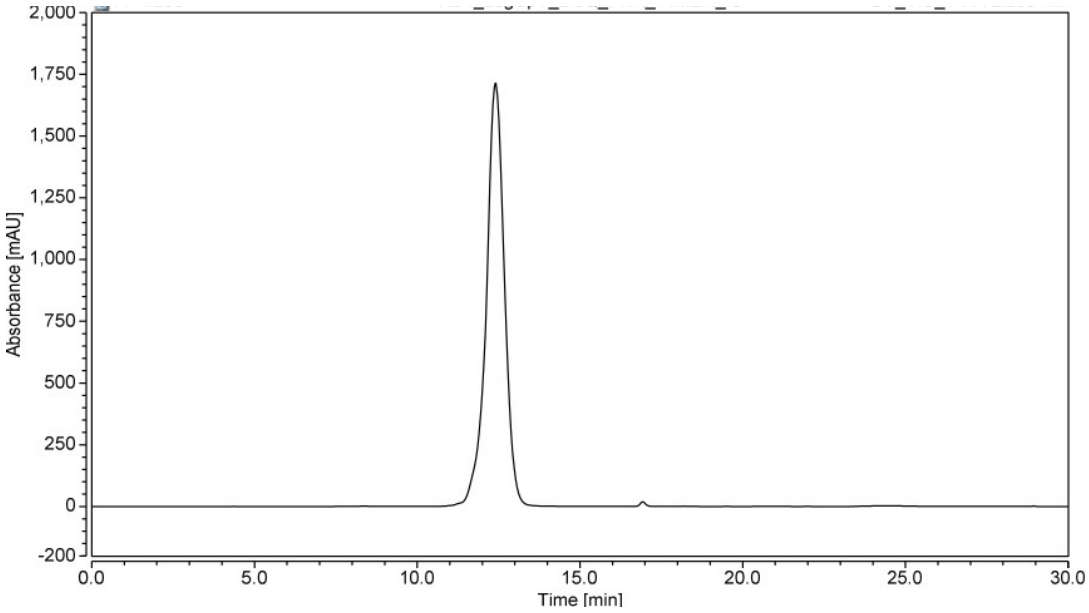


Fig. S1. HPLC chromatogram of FIT-PNA 2 at 260 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

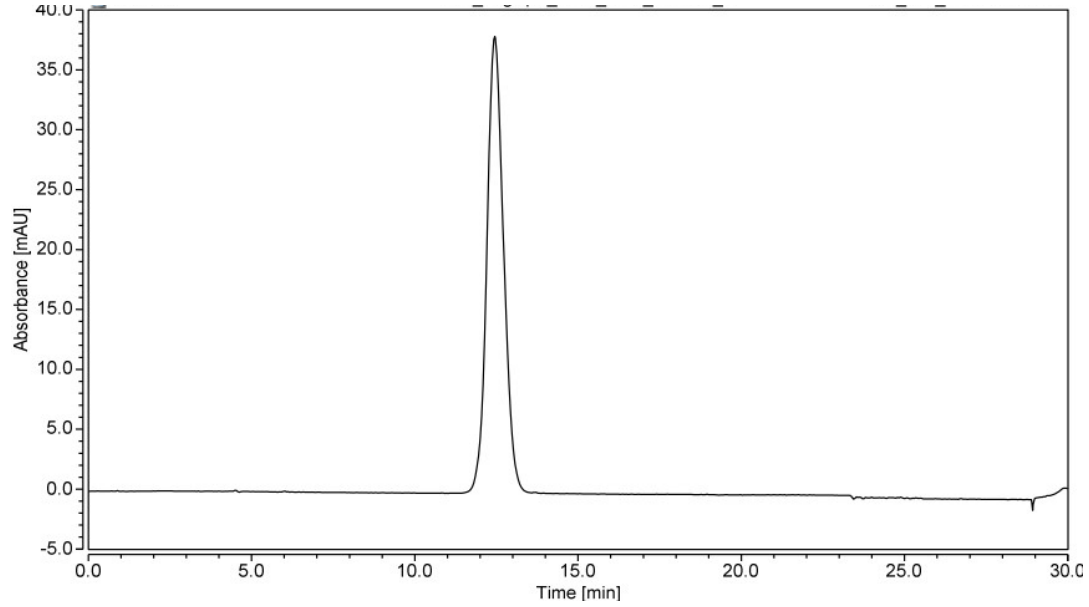


Fig. S2. HPLC chromatogram of FIT-PNA 2 at 590 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

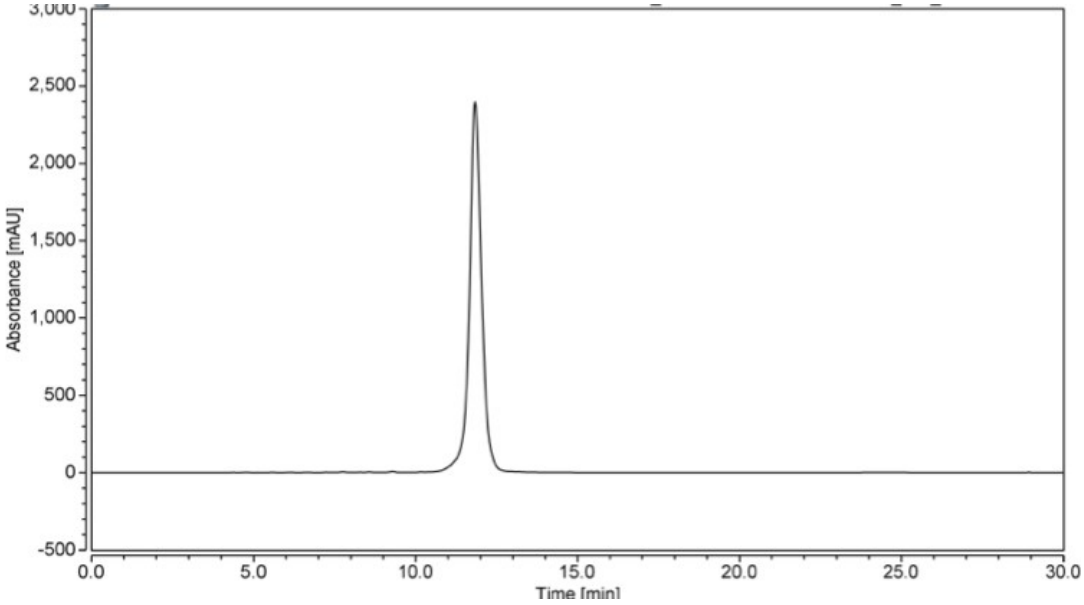


Fig. S3. HPLC chromatogram of FIT-PNA 3 at 260 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

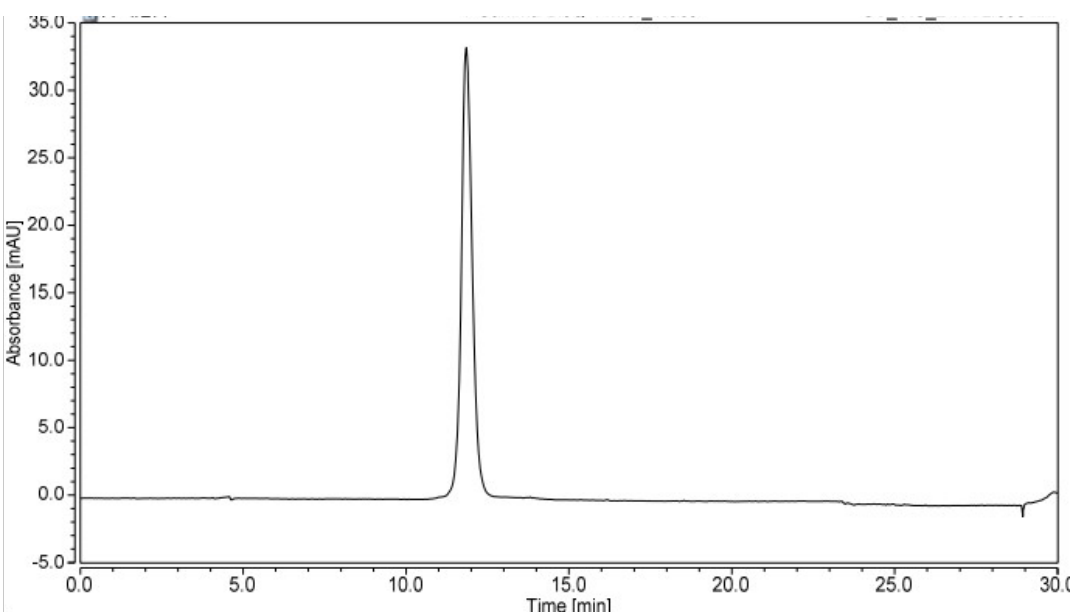


Fig. S4. HPLC chromatogram of FIT-PNA 3 at 590 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

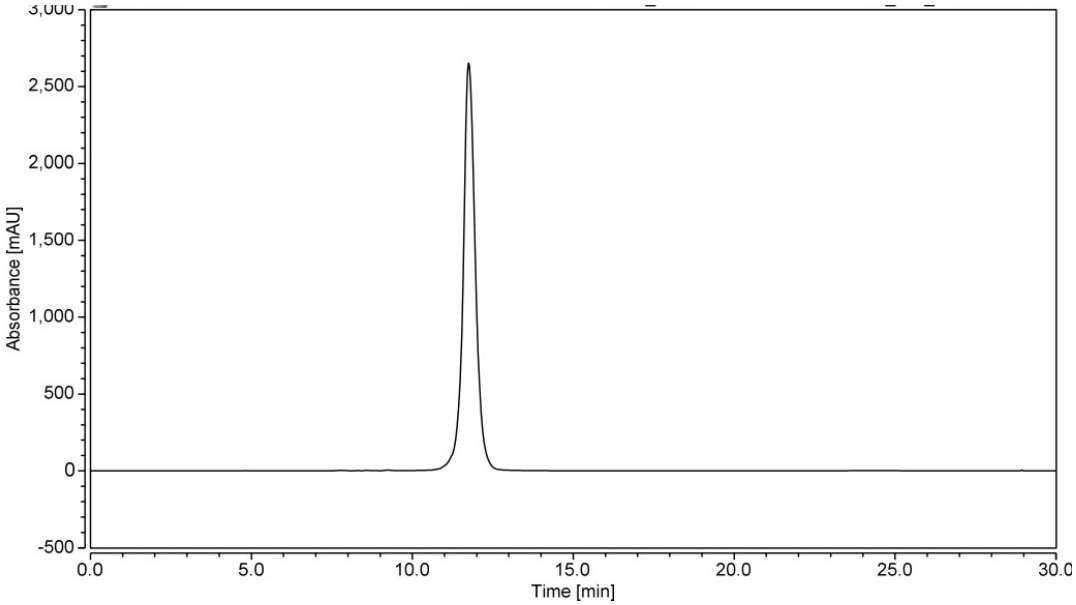


Fig. S5. HPLC chromatogram of FIT-PNA 4 at 260 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

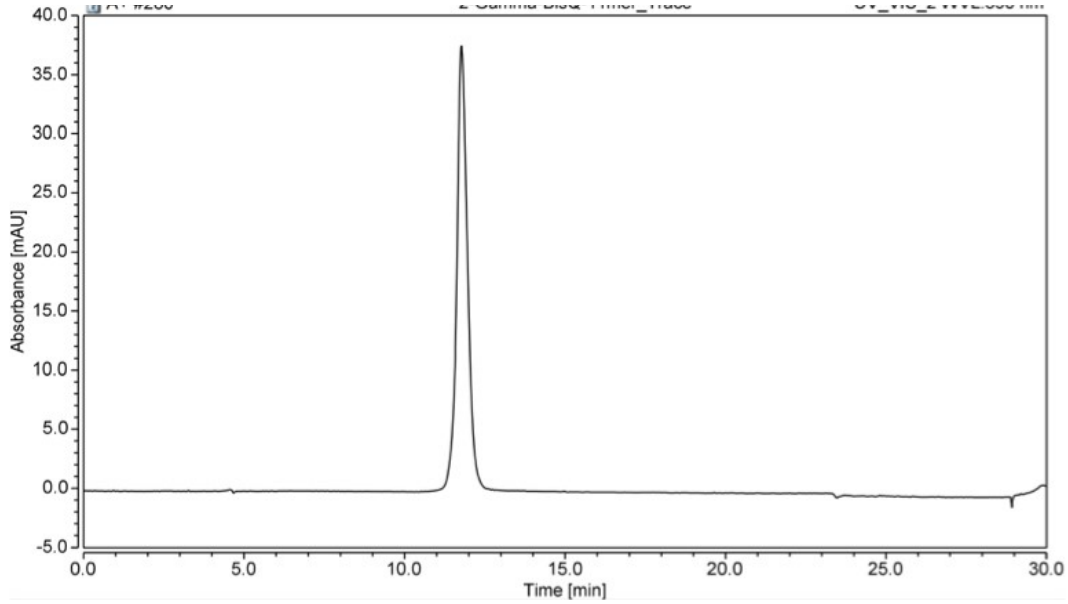


Fig. S6. HPLC chromatogram of FIT-PNA 4 at 590 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

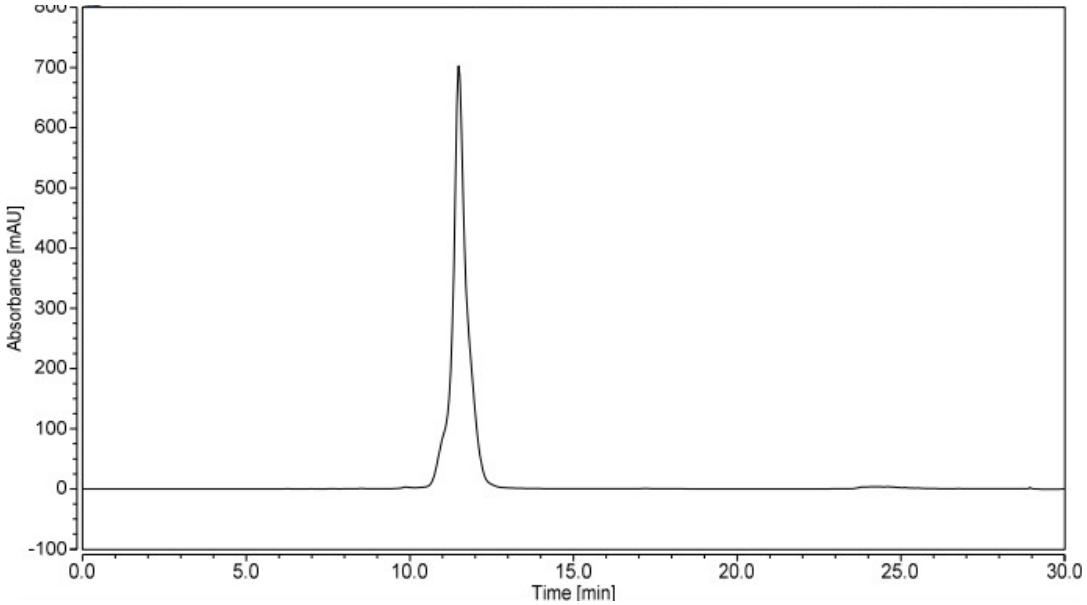


Fig. S7. HPLC chromatogram of FIT-PNA 5 at 260 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

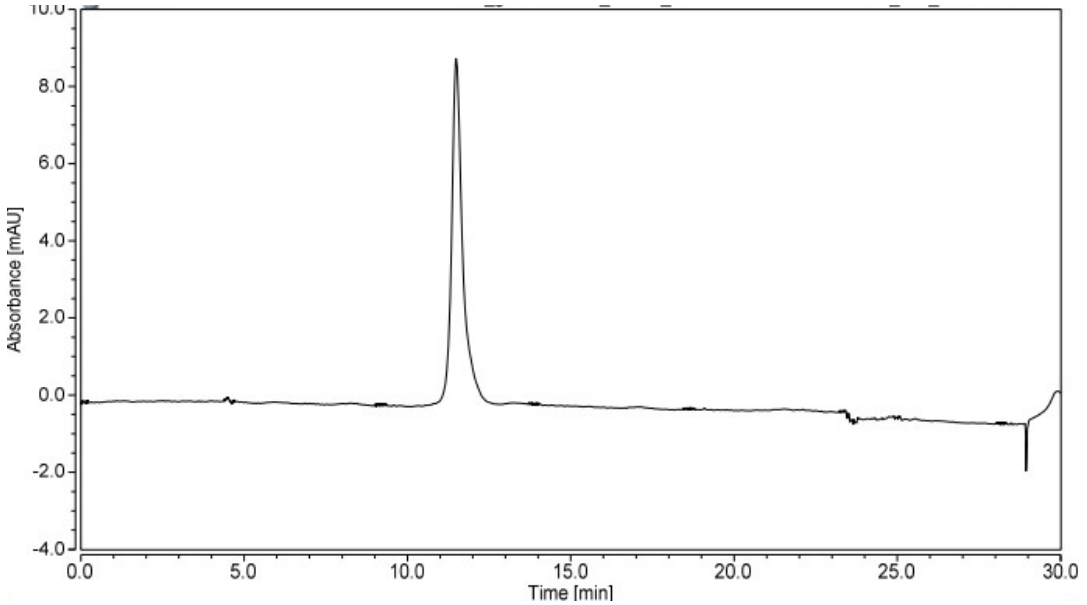


Fig. S8. HPLC chromatogram of FIT-PNA 5 at 590 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

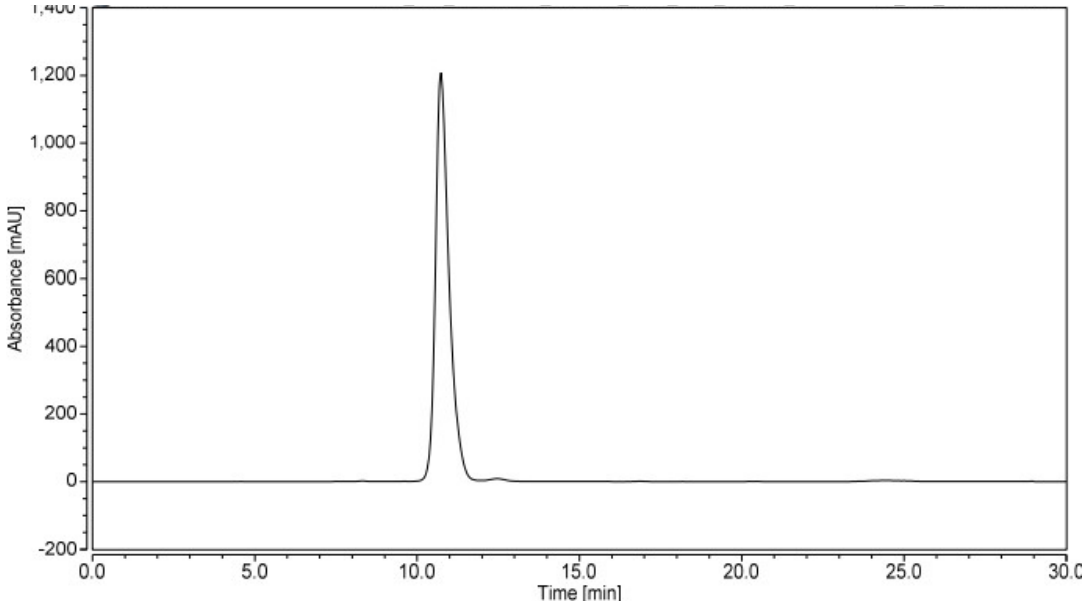


Fig. S9. HPLC chromatogram of FIT-PNA 6 at 260 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

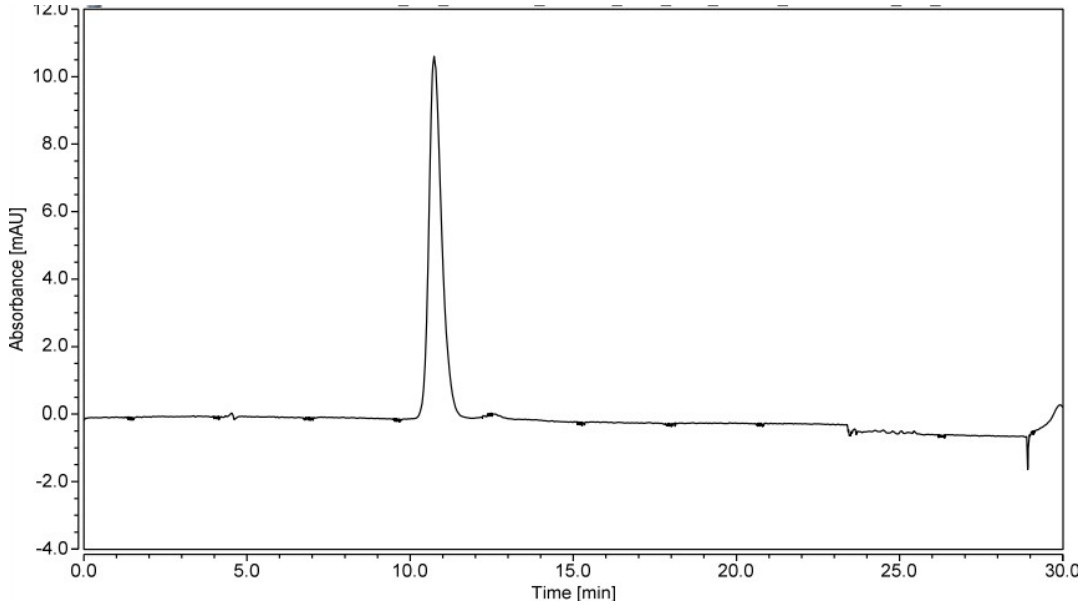


Fig. S10. HPLC chromatogram of FIT-PNA 6 at 590 nm. Eluents: A (0.1% TFA in water) and B (ACN) were used in a linear gradient (5-30% B in 20 min) with a flow rate of 4 mL/min.

Maldi-TOF MS for FIT-PNAs

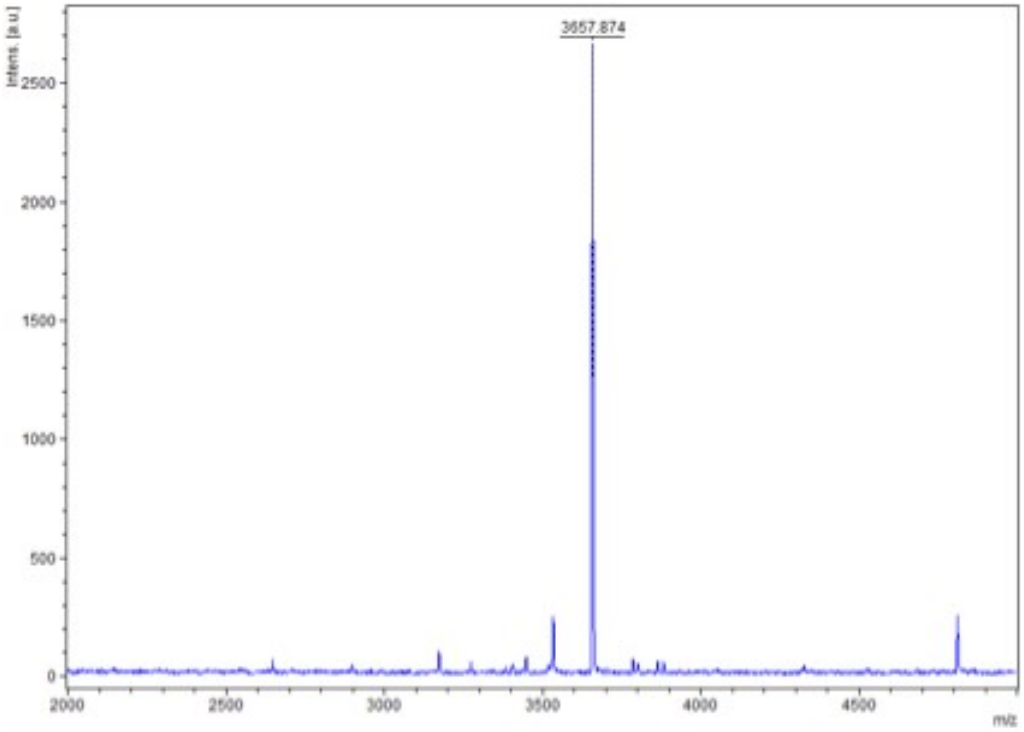


Fig. S11. Maldi-TOF MS for FIT-PNA 2. Calculated mass: 3657.7 $[M]^+$. Observed Mass: 3667.9

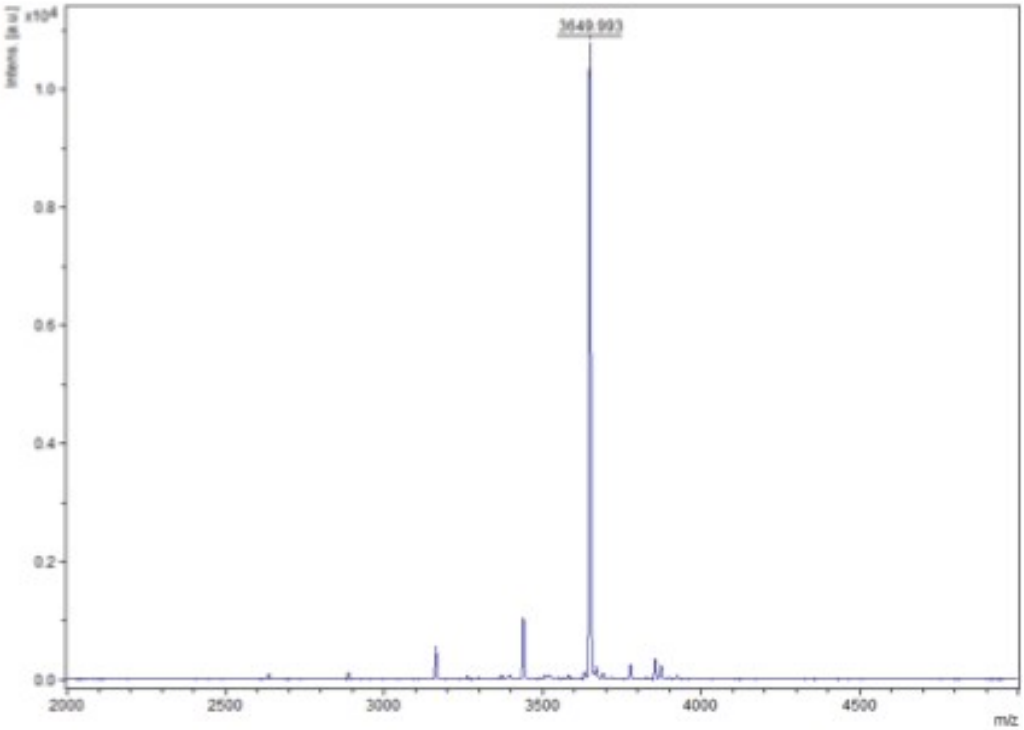


Fig. S12. Maldi-TOF MS for FIT-PNA 3. Calculated mass: 3649.8 $[M+H]^+$. Observed Mass: 3650.0

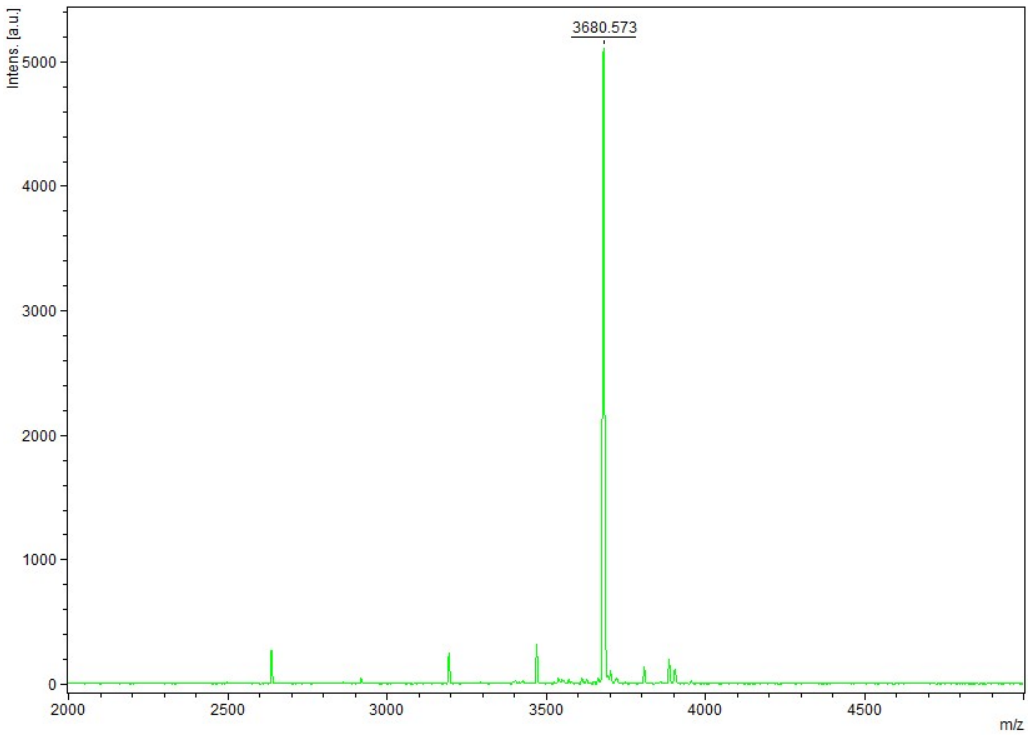


Fig. S13. Maldi-TOF MS for FIT-PNA 4. Calculated mass: 3679.8 $[M+H]^+$. Observed Mass: 3680.6.

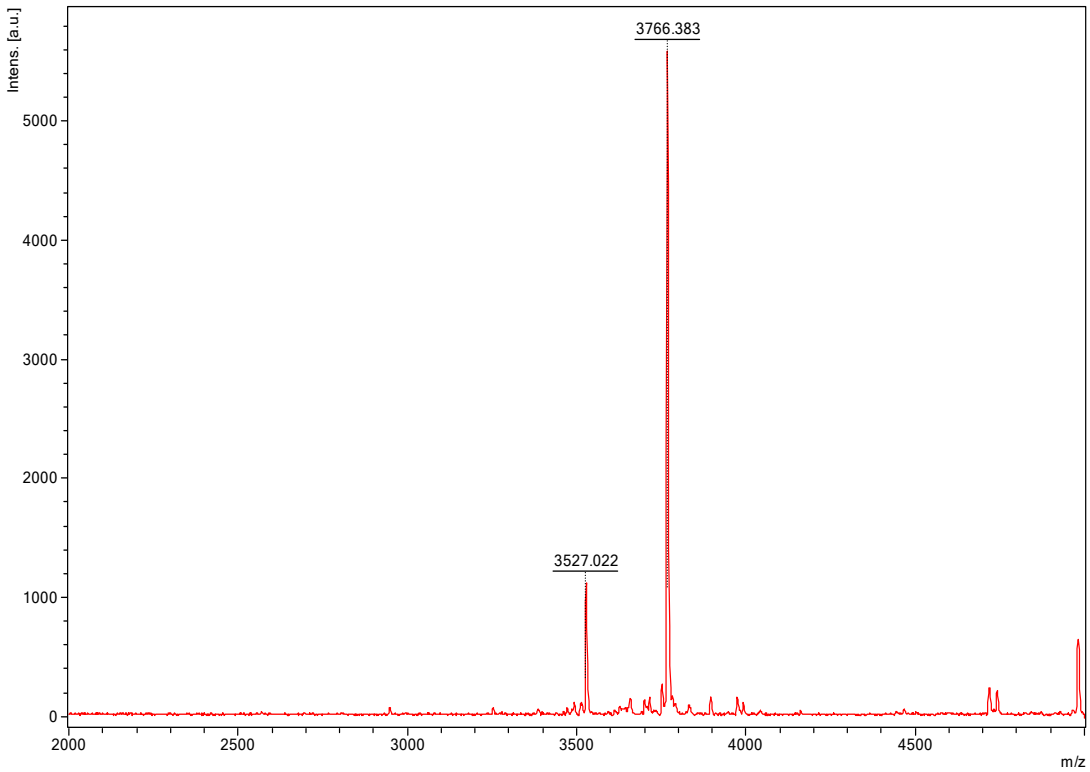


Fig. S14. Maldi-TOF MS for FIT-PNA 5. Calculated mass: 3768.9 $[M]^+$. Observed Mass: 3766.4.

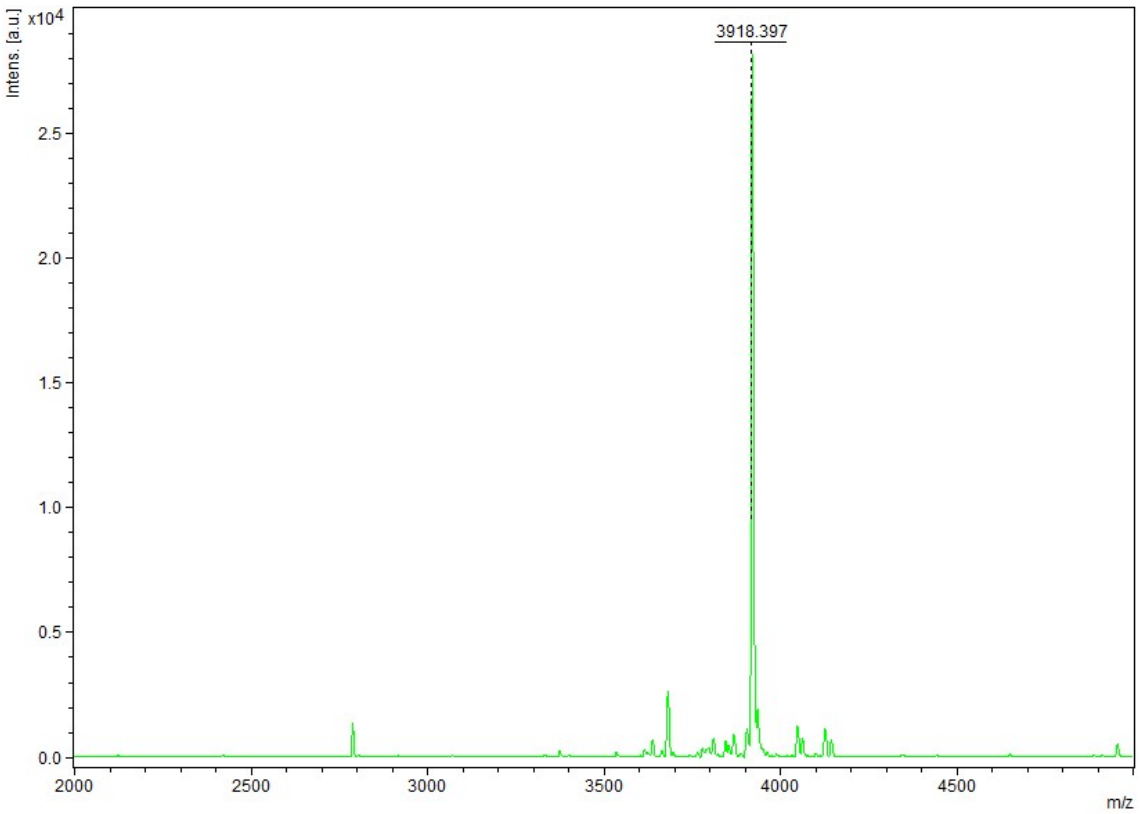


Fig. S15. Maldi-TOF MS for FIT-PNA 6. Calculated mass: 3919.0 $[M]^+$. Observed Mass: 3918.4.

Table S2. Melting Temperatures of RNA:FIT-PNA Duplexes Measured at 260 nm

S.No.	RNA with complementary	T_m (°C)			Average T_m (°C)	Std. Dev.
		1st	2nd	3rd		
1	aeg-BisQ-PNA	42.52	43.89	42.49	43.0	0.6
2	aeg-cpT-BisQ-PNA	43.72	46.08	45.77	45.2	1.0
3	$(\gamma\text{-ser})_1$-BisQ-PNA	44.39	43.42	44.34	44.0	0.4
4	$(\gamma\text{-ser})_2$-BisQ-PNA	46.70	45.96	44.47	45.7	0.9
5	$(\gamma\text{-ser})_5$-BisQ-PNA	44.27	44.75	44.92	44.6	0.3
6	$(\gamma\text{-ser})_{10}$-BisQ-PNA	51.41	51.07	50.44	51.0	0.4

Thermal Melting Profiles for RNA:FIT-PNA Duplexes

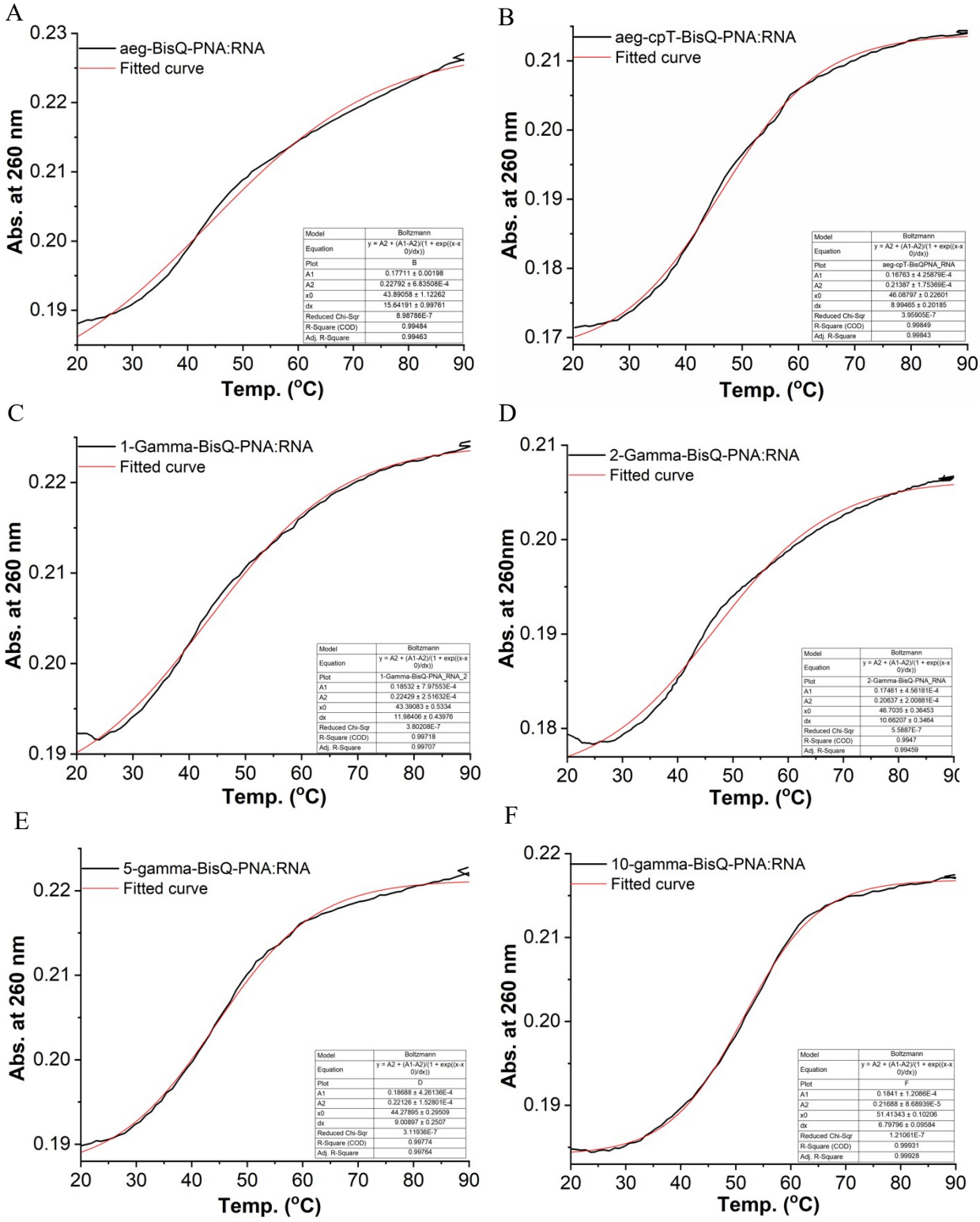
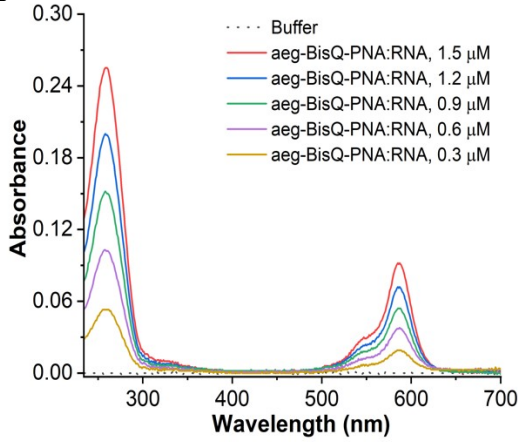


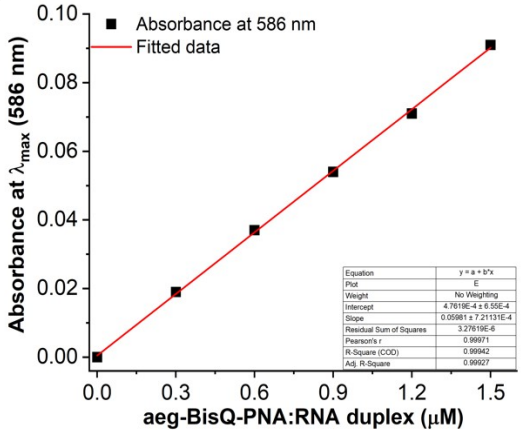
Fig. S16. Representative melting curve profiles for RNA:FIT-PNA duplexes (1 - 6). [FIT-PNA] = 1 μM, [RNA] = 1 μM.

UV-Visible Titrations for FIT-PNA:RNA Duplexes

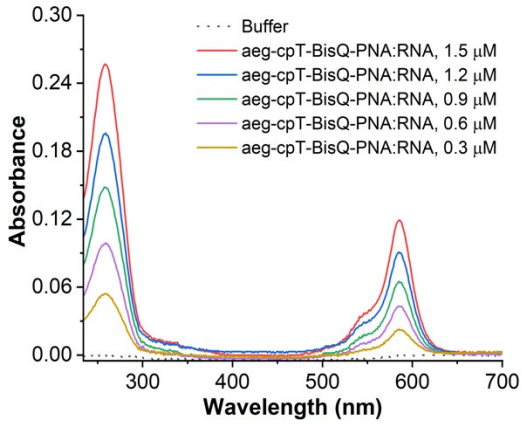
A



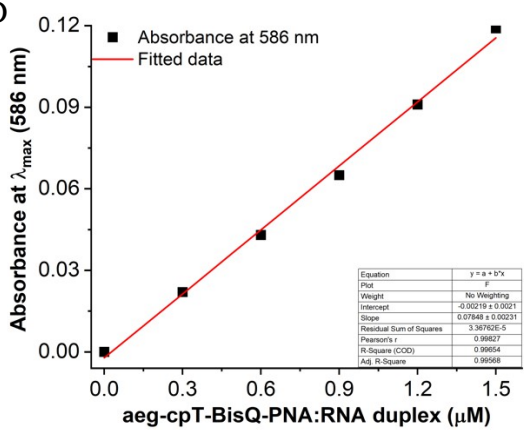
B



C



D



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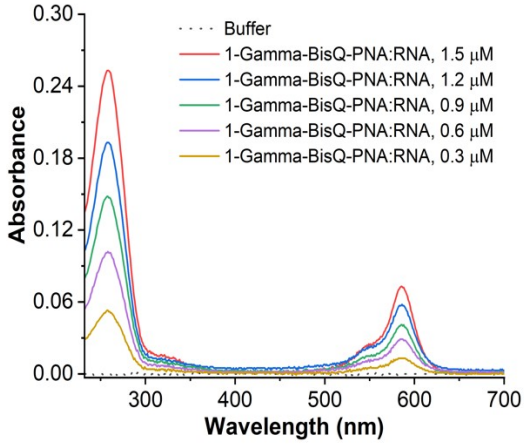
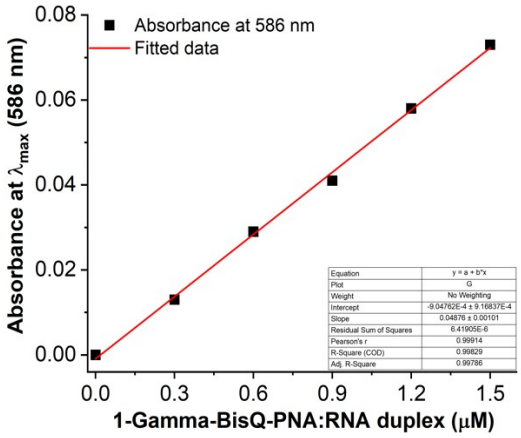


F



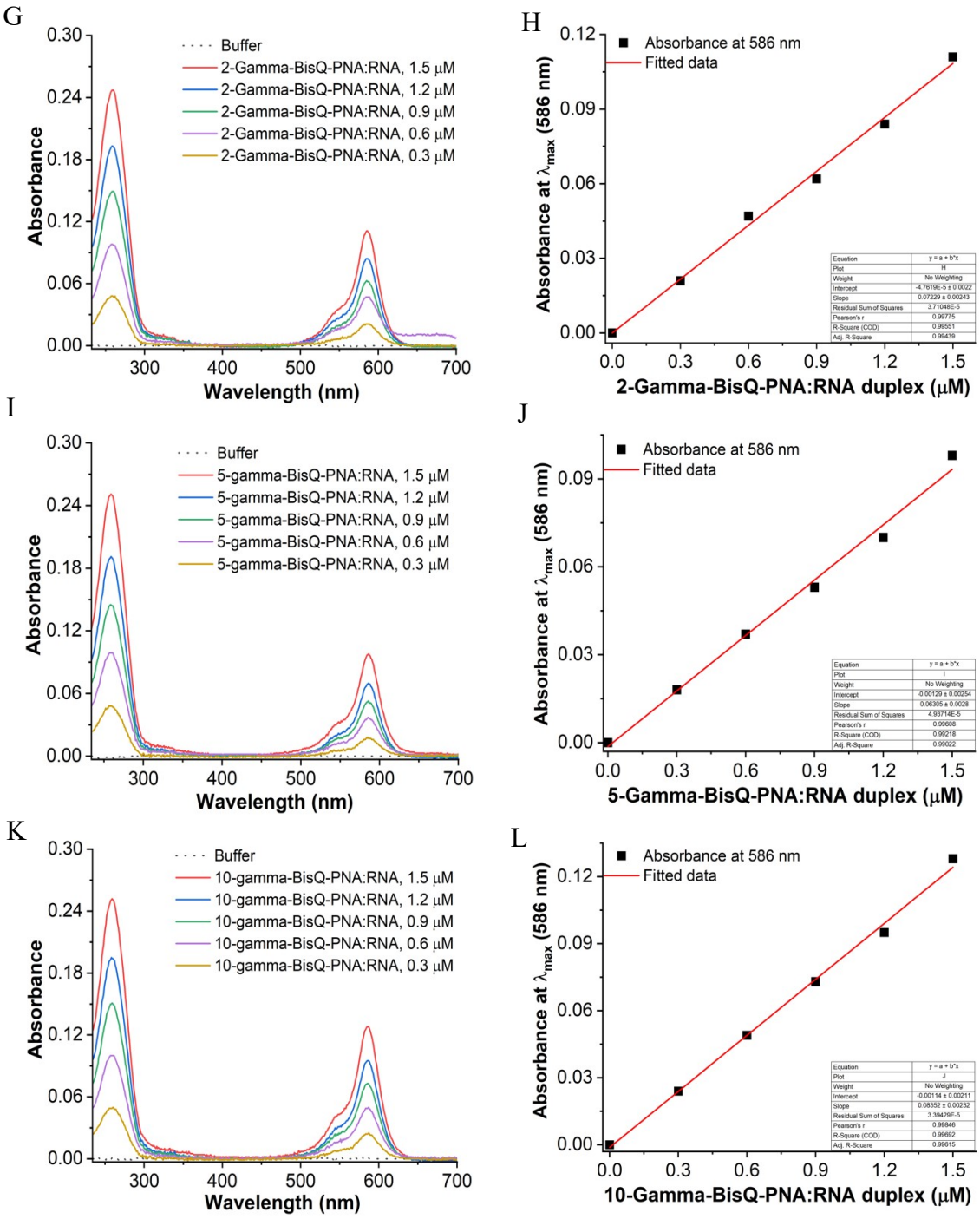


Fig. S17. UV-Vis titrations for complementary RNA annealed to FIT-PNAs (1–6). [FIT–PNA] = 1.5 μM and [RNA] = 1.5 μM in 1xPBS (pH 7.0). UV-Vis spectra of the respective duplexes are given in panels A, C, E, G, I, and K, and the corresponding linear plots at the absorbance maximum (λ_{max} = 586 nm) are shown in panels B, D, F, H, J, and L.

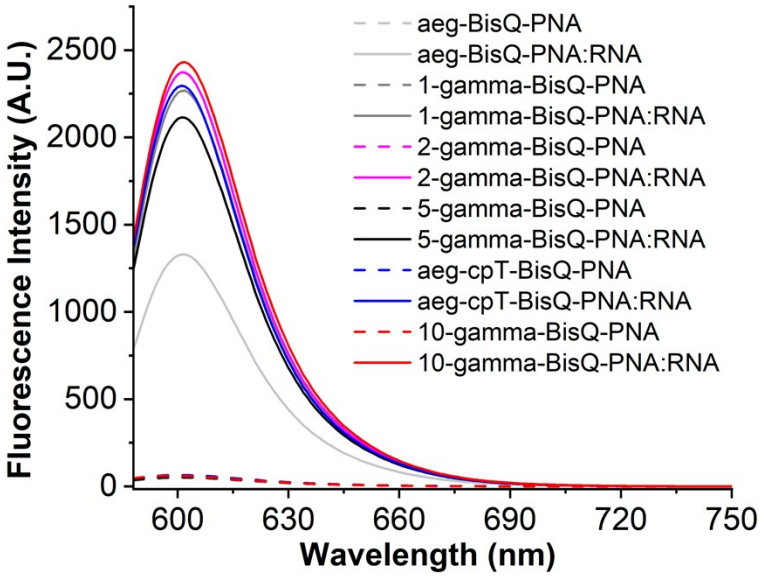


Fig. S18. Fluorescence emission of FIT-PNA:RNA duplexes (1-6). [FIT-PNA] = 3μM, [RNA] = 4.5μM, in 1xPBS buffer, pH=7. λ_{ex} = 580 nm and λ_{em} = 588 nm (n = 3).

FACS results of FIT-PNAs in OVCA433

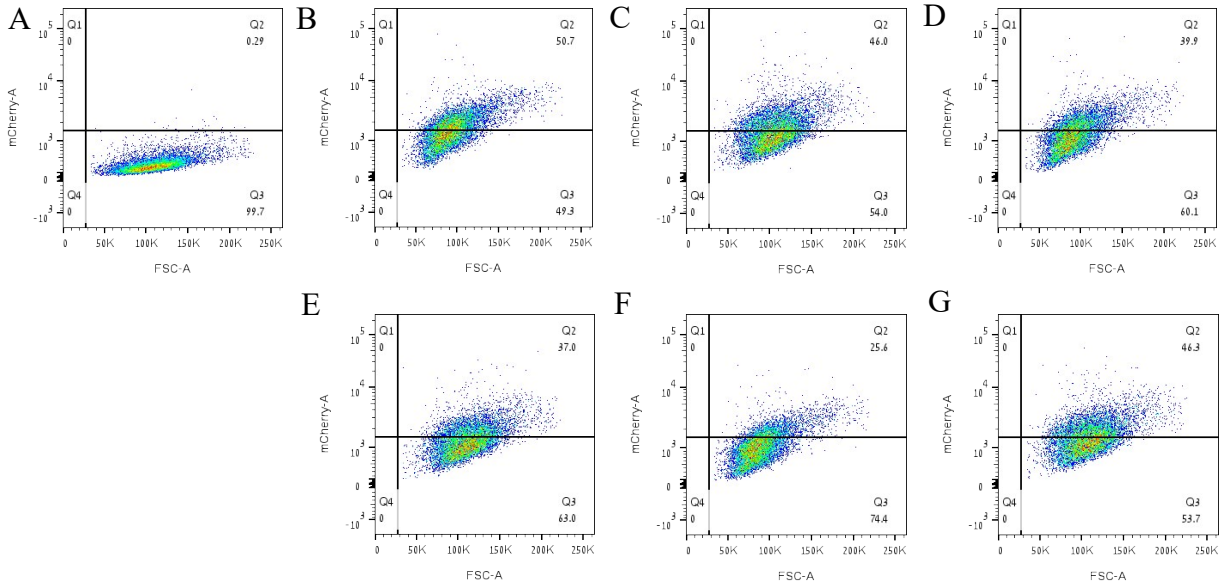


Fig. S19. FACS results for FIT-PNAs (1-6) in OVCA433 cells. OVCA433 cells were incubated in cell culture media with 5 μM of FIT-PNAs for 5h, washed 3x with 1xPBS, trypsinized and collected in 1xPBS. (A) untreated OVCA433 cells (as control) (B) aeg-BisQ-PNA (C) aeg-cpT-BisQ-PNA (D) $(\gamma\text{-Ser})_1$-BisQ-PNA (E) $(\gamma\text{-Ser})_2$-BisQ-PNA (F) $(\gamma\text{-Ser})_5$-BisQ-PNA, and (G) $(\gamma\text{-Ser})_{10}$-BisQ-PNA

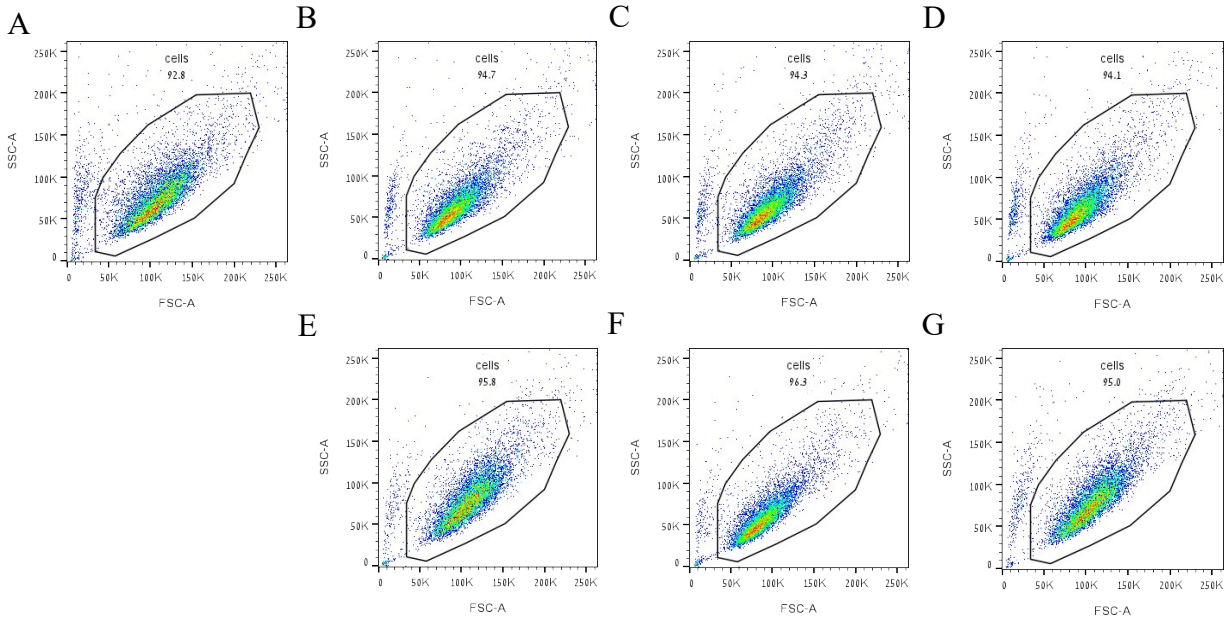


Fig. S20. Forward and sideward scattered plots of all FIT-PNAs in the OVCA433 cells. (A) untreated OVCA433 cells (as control) (B) aeg-BisQ-PNA (C) aeg-cpT-BisQ-PNA (D) $(\gamma\text{-Ser})_1$-BisQ-PNA (E) $(\gamma\text{-Ser})_2$-BisQ-PNA (F) $(\gamma\text{-Ser})_5$-BisQ-PNA, and (G) $(\gamma\text{-Ser})_{10}$-BisQ-PNA

Reference:

1. O. Tepper, H. C. Zheng, D. H. Appella and E. Yavin, *Chem. Commun.*, 2021, **57**, 540-543. Erratum: 2023, **59**, 11593.