Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Triple Helical Recognition of Pyrimidine Inversions in Polypurine Tracts of RNA by Nucleobase-modified PNA

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Benzyl 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(2-oxopyrimidin-1(2*H*)-yl)acetamido)acetate (3a). To a stirring solution of **1**[1] (1.57 g, 3.64 mmol), (2-oxo-1(2H)-pyrimidinyl)acetic acid **2a**[2] (0.90 g, 5.83 mmol), and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (1.19 g, 7.29 mmol) in DMF (100 mL) at 40 °C was added *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (1.50 g, 7.84 mmol). After stirring overnight at 40 °C, H_2O (150 mL) was added and the product was extracted with EtOAc (3 × 300 mL). The combined organic layers were washed with 1 M aqueous HCl (400 mL), saturated aqueous $NaHCO_3$ (400 mL), H_2O (400 mL), and brine (400 mL). The organic layer was dried over Na_2SO_4, evaporated under reduced pressure and purified by silica gel column chromatography using 0-7% MeOH in CH_2Cl_2 to afford **3a** as a white solid material (1.38 g, yield 67%). R_f = 0.36 (MeOH:CH_2Cl_2, 7:93, v/v). Compound **3a** exists in solution as a pair of slowly exchanging rotamers; the signals due to the major (ma.) and minor (mi.) rotamers are designated: ^{1}H NMR (DMSO-d6, 360 MHz) δ: 8.58 (t, 1H, *J* = 3.6 Hz), 8.01 and 7.96 (ddd, 1H, *J* = 3.6 Hz and 3.6 Hz), 7.88 (d, 2H, *J* = 7.6 Hz), 7.68 (m, 1H), 7.45-7.30 (m, 10H), 6.45 (t, 1H, *J* = 3.6 Hz), 5.22 (s, 0.5H, mi.), 5.13 (s, 1.5H, ma.), 4.92 (s, 1.5H, ma.), 4.74 (s, 0.5H, mi.), 4.45-4.23 (m, 3H), 4.16 (s, 1H), 3.51-3.31 (m, 5H). ^{13}C NMR (DMSO-d6, 90.5 MHz): δ: 169.3, 168.8, 167.2, 167.0, 166.6, 166.5, 156.3, 155.5, 150.7, 143.8, 140.7, 135.7, 128.4, 128.2, 128.1, 128.0, 127.9, 127.6, 127.0, 125.1, 120.1, 103.6, 66.6, 66.0, 65.5, 65.4, 50.6, 50.4, 49.2, 48.0, 47.1 46.9, 46.7, 40.2, 37.9. ESI-MS found *m/z* 566.9 $[M+H]^+$, calculated for $C_{32}H_{30}N_4O_6$ 566.2.

2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(2-oxopyrimidin-1(2*H*)-yl)acetamido)acetic acid (4a). Compound **3a** (1.20 g, 2.11 mmol) was dissolved in MeOH:CH_2Cl_2 (1:1, 140 mL) and purged with N_2. 10% Pd/C (420 mg) was added and the solution was saturated with H_2 by slow bubbling over 3 h. The reaction mixture was filtered through a short pad of Celite and the pad was washed with MeOH (50 mL). The solvent was removed in vacuum to give 910 mg (91%) of **4a** as a white solid material. Compound **4a** was used for PNA synthesis without further purification. R_f = 0.24 (MeOH:CH_2Cl_2, 4:6, v/v). ^{1}H NMR (DMSO-d6, 360MHz) δ: 7.87 (d, 2H, *J* = 7.2 Hz), 7.67 (d, 2H, *J* = 7.2 Hz), 7.43-7.31(m, 6H), 6.25 (d, 1H, *J* = 3.6 Hz), 4.32-4.10 (m, 4H), 3.95 (d, 2H, *J* = 10.8 Hz), 3.36-3.11(m, 6H), 1.76 (s, 1H). ^{13}C NMR (DMSO-d6, 90.5 MHz) δ: 171.1, 170.8, 169.5, 169.2, 156.3, 156.1, 155.6, 155.4, 148.6, 143.9, 140.7, 139.9, 127.6, 127.1, 125.1, 124.2, 120.1, 119.9, 65.5, 65.4, 54.9, 49.2, 48.2,

[1] F. Wojciechowski, R. H. E. Hudson *J. Org. Chem.* 2008, **73**, 3807.
[2] Commercially available from ChemBridge.

47.7, 47.4, 46.7, 46.0, 45.8, 41.8, 38.1, 21.9, 21.8. ESI-MS found *m/z* 476.8 $[M+H]^+$, calculated for $C_{25}H_{24}N_4O_6$ 476.1).

Benzyl 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-3-(2-oxopyrimidin-1(2*H*)-yl)propanamido)acetate (3b) To a stirring solution of **1**[1] (0.81 g, 1.88 mmol), 3-(2-oxo-1,2-dihydropyrimidin-1-yl)propanoic acid **2b**[3] (0.61 g, 3.01 mmol), and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (0.61 g, 3.76 mmol) in DMF (50 mL) at 40 °C was added *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (0.77 g, 4.04 mmol). After stirring overnight at 40 °C, H_2O (100 mL) was added and the product was extracted with EtOAc (3 × 200 mL). The combined organic layers were washed with 1 M aqueous HCl (250 mL), saturated aqueous $NaHCO_3$ (250 mL), H_2O (250 mL), and brine (250 mL). The organic layer was dried over Na_2SO_4, evaporated under reduced pressure and purified by silica gel column chromatography using 0-6% MeOH in CH_2Cl_2 to afford **3b** as a white solid material (0.71 g, yield 65%). R_f = 0.52 (MeOH:CH_2Cl_2, 7:93, v/v). Compound **3b** exists in solution as a pair of slowly exchanging rotamers; the signals due to the major (ma.) and minor (mi.) rotamers are designated: ^{1}H NMR ($CDCl_3$, 360 MHz, major rotamer) δ: 8.41 (br s, 1H), 7.82 (br s, 1H), 7.73 (d, 2H, *J* = 7.2 Hz), 7.57 (d, 2H, *J* = 7.2 Hz), 7.38-7.25 (m, 11H), 6.11 (br s, 1H), 5.27 (s, 0.5H, mi.), 5.14 (d, 2H, *J* = 3.6 Hz, ma.), 4.37 (d, 2H, *J* = 7.2 Hz), 4.19-4.02 (m, 5H), 3.45 (br s, 2H), 3.28 (br s, 2H), 2.94 (br s, 2H), 2.76 (br s, 0.5H). ^{13}C NMR ($CDCl_3$, 90.5 MHz): δ: 171.7, 171.1, 169.6, 169.3, 165.9, 156.4, 150.2, 143.8, 141.2, 135.1, 134.7, 128.7, 128.6, 128.5, 128.4, 128.1, 127.6, 127.0, 125.0, 119.9, 103.6, 67.8, 67.1, 66.7, 66.4, 53.4, 50.6, 49.0, 48.8, 48.3, 48.1, 47.2, 47.1, 39.2, 39.1, 30.6, 30.5. ESI-MS found *m/z* 581.1 $[M+H]^+$, calculated for $C_{33}H_{32}N_4O_6$ 580.2).

2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-3-(2-oxopyrimidin-1(2*H*)-yl) propanamido)acetic acid (4b). Compound **3b** (0.67 g, 1.15 mmol) was dissolved in MeOH (60 mL) and purged with N_2. 10% Pd/C (260 mg) was added and the solution was saturated with H_2 by slow bubbling over 2 h. The reaction mixture was filtered through a short pad of Celite and the pad was washed with MeOH (50 mL). The solvent was removed in vacuum to give 480 mg (84%) of **4b** as a white solid material. Compound **4b** was used for PNA synthesis without further purification. R_f = 0.25 (MeOH:CH_2Cl_2, 2.5:7.5, v/v). ^{1}H NMR (DMSO-d6, 360MHz) δ: 7.87 (m, 3H), 7.67 (br s, 1H), 7.58 (d, 1H, *J* = 3.6 Hz), 7.44-7.33 (m, 5H), 6.11 (br s, 1H), 4.27 (m, 2H),

[3] Commercially available from Ukrorgsyntez Ltd.

3.93 (m, 1H), 3.34 (m, 4H), 3.17 (d, 2H, J = 3.6 Hz), 3.06 (br s, 1H), 2.37 (br s, 1H), 1.73 (m, 2H), 1.46 (d, 2H, J = 7.2 Hz). ^{13}C NMR (DMSO-d6, 90.5 MHz) δ: 171.3, 170.8, 156.2, 155.2, 148.6, 143.9, 140.7, 139.9, 127.6, 127.0, 125.2, 125.1, 124.2, 120.1, 119.9, 65.5, 65.4, 47.6, 46.7, 45.5, 43.8, 41.8, 37.0, 31.2, 30.6, 22.1, 18.1. ESI-MS found *m/z* 491.1 $[M+H]^+$, calculated for $C_{26}H_{26}N_4O_6$ 490.1.

Benzyl 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-3-(6-(benzyloxy)pyridazin-3-ylamino)propanamido)acetate (6). Compound **1**[1] (1.90 g, 4.41 mmol) was dissolved in anhydrous DMF (50 mL) and 3-(6-(benzyloxy)pyridazin-3-ylamino)propanoic acid[4] **5** (1.53 g, 5.59 mmol) and 3-hydroxy-1,2,3-benzotriazine-4(3H)-one (0.921 g, 5.66 mmol) were added. The mixture was cooled on ice bath and dicyclohexylcarbodiimide (1.29 g, 6.22 mmol) was added. After 1h the ice bath was removed and the mixture was stirred overnight at room temperature. The mixture was evaporated in vacuum, redissolved in dichloromethane (100 mL) and washed with 5% aqueous $NaHCO_3$ and 20ml of acetonitrile was added. The sample was evaporated, dried *in vacuo* and purified on silica gel column using 3% MeOH in CH_2Cl_2 to afford **6** as a white solid material (2.7 g, yield 89%). ^{1}H NMR ($DMSO_{d6}$, 360MHz, TMS reference) δ 8.59-7.30 (m, 15H), 6.44 (m, 1H), 5.13 (s, 2H), 4.92 (s, 1H), 4.74-4.16 (m, 5H), 3.51-3.13 (m, 6H). ^{13}C NMR (90.5 MHz, $CDCl_3$): d 169.3, 168.8, 167.2, 167.0, 166.6, 166.5, 156.3, 155.5, 150.7, 143.8, 140.7, 135.7, 128.4, 128.2, 128.1, 128.0, 127.9, 127.6, 127.0, 125.1, 120.1, 103.6, 66.6, 66.0, 65.5, 65.4, 50.6, 50.4, 49.2, 48.0, 47.1 46.9, 46.7, 40.2, 37.9. ESI-MS found *m/z* 686.2 $[M]^+$, calculated for $C_{40}H_{39}N_5O_6$ 685.3).

2-(N-(2-(((9H-fluoren-9-yl)methoxy)carbonylamino)ethyl)-3-(6-oxo-1,6-dihydropyridazin-3-ylamino)propanamido)acetic acid (7). Compound **6** (1.20 g, 2.11 mmol) was dissolved in absolute ethanol (100 mL) and purged with N_2. 10% Pd/C (960 mg) was added and the solution was saturated with H_2 by slow bubbling over 3 h. The reaction mixture was filtered through a short pad of Celite and the pad was washed with methanol (50 mL). The solvent was removed in vacuum to give **7** as a white solid material (650 mg, 73%). R_f = 0.08 in (20% MeOH:CH_2Cl_2, v/v).

[4] A. B. Eldrup, O. Dahl, P. E. Nielsen *J. Am. Chem. Soc.* **1997**, *119*, 11116.

Synthesis of PNA was done on Expedite 8909 synthesizer following the standard manufacturers protocol (2 μmol scale) and using NovaSyn TG Sieber resin (Novabiochem) as a support, HATU as an activator and Fmoc-PNA-A(Bhoc)-OH, Fmoc-PNA-C(Bhoc)-OH, Fmoc-PNA-G(Bhoc)-OH and Fmoc-PNA-T-OH as monomers (purchased from Link Technologies Ltd, UK). L-lysine was coupled to N-terminus of PNA on Expedite 8909 (using standard PNA coupling protocol) using Fmoc-L-lys(Boc)-OH and HATU. Chain extension followed a three-step cycle: (i) removal of the Fmoc-protecting group from the terminal amine with 20% piperidine in DMF, (ii) coupling of the next monomer onto the N-terminus of the growing chain with HATU, and (iii) capping of the unreacted amines with acetic anhydride. Treating the solid resin with m-cresol/ TFA (2:8) mixture for 2h. resulted in simultaneous removal of the protecting groups and cleavage of the oligomers from the resin. The crude PNA samples were precipitated from anhydrous ether. The solid was collected, dried, dissolved in HPLC grade water and purified by RP-HPLC on Xbridge Prep C-18 column (5 μm, 10 mm × 150 mm) at 60 °C eluting with a linear gradient 3%-25% of acetonitrile in water containing 0.1 % of TFA over 40 min, flow rate of 5 mL/min. Absorbency was monitored at 254 nm and 280 nm, and the fraction containing the major peak was collected, lyophilized to dryness to afford pure PNA samples. The PNA was quantified following procedure described for DNA and RNA.[5] The molecular weight of the synthesized PNAs was confirmed by ESI or MALDI TOF mass spectrometry:

PNA1. ESI-TOF (high acc.) found *m/2z* 1226.0 $[M+2H]^{2+}$, calculated for $C_{100}H_{136}N_{44}O_{31}$ 2450.0.

PNA2. MALDI TOF found *m/z* 2466 $[M+H]^{+}$, calculated for $C_{100}H_{136}N_{44}O_{31}$ 2465.

PNA3. ESI found *m/z* 2436.5 $[M+H]^{+}$, calculated for $C_{99}H_{134}N_{44}O_{31}$ 2435.0.

PNA4. ESI-TOF (high acc.) found *m/2z* 1227.5 $[M+2H]^{2+}$, calculated for $C_{100}H_{136}N_{44}O_{31}$ 2449.0.

PNA5. ESI found *m/2z* 1233 $[M+2H]^{2+}$, calculated for $C_{100}H_{136}N_{44}O_{31}$ 2464.

PNA6. ESI-TOF (high acc.) found *m/2z* 1225.6 $[M+2H]^{2+}$, calculated for $C_{99}H_{136}N_{46}O_{30}$ 2449.1.

RNA was purchased from Dharmacon Inc. and deprotected according to manufacturers recommendations. After deprotection RNA samples were purified using RP-HPLC on Xbridge Prep C-18 column (5 μm, 10 mm × 150 mm) at 60 °C eluting with a linear gradient (5%-20%) of mobile phase B in mobile phase A over 40 min, flow rate 5 ml/min. Mobile phase A was 0.1 M of triethylammonium acetate (pH = 7.0) in HPLC water and mobile phase B was a mixture of 0.1 M of triethylammonium acetate (pH = 7.0) in HPLC water and HPLC grade acetonitrile (60/40, v/v). Absorbency was monitored at a wavelength of 254 nm and 280 nm, and the fraction containing

[5] Puglisi, J. D.; Tinoco, I., Jr., Absorbance melting curves of RNA. *Methods Enzymol.* **1989**, *180*, 304-325.

the major peak was collected, lyophilized to dryness to afford pure RNA samples. RNA was quantified using the extinction coefficient provided by Dharmacon.

ITC Experiments were done on a Nano ITC G2 (TA Instruments). RNA stock solution (17.5 µL, 0.24 mM) was evaporated to dryness and the solid was dissolved in 1.6 mL of acetate buffer (100 mM of sodium acetate, 1.0 mM of EDTA, pH = 5.5, 6.25 or 7.0). After degassing, the RNA solution (0.95 mL, 0.002625 mM) was loaded into ITC reaction cell and the reference cell was loaded with degassed HPLC water. PNA stock solution (70 µL, 0.24 mM) was evaporated to dryness and the solid was dissolved in 350 µL of acetate buffer. After degassing the PNA solution (250 µL, 0.048 mM) was loaded in titration syringe. The syringe was inserted into reaction cell and the instrument was equilibrated at 25 °C until the baseline was flat and stable. The following parameters were used:

- Experiment type: Incremental titration
- Stirring rate = 250 rpm
- Temperature set point = 25 °C
- Equilibration time = 300 sec
- Interval of individual injection = 260-800 sec
- Number of injections = 50
- Volume of individual injection = 5 µl

The titration data (Figures S1-S36) were analyzed using NanoAnalyze software (TA Instruments) and independent model to obtain the fitting graph and thermodynamic data of the experiments.

UV melting of each RNA (2.5 µM) and PNA (12.5 µM) complexes was done in 100 mM of sodium acetate, 1.0 mM of EDTA, pH = 6.25. Absorbance vs. temperature profiles were measured at 260 nm on Shimadzu 800 UV-visible spectrometers equipped with a six or eight position Peltier temperature controllers, respectively. The temperature was increased at a rate of 0.5 °C per minute. The melting temperatures were obtained using Shimadzu LabSolutions Tm Analysis (Version 1.2.1.0) software. The experimental absorbance vs. temperature curves were converted into a fraction of strands remaining hybridized (α) vs. temperature curves by fitting the melting profile to a two-state transition model, with linearly sloping lower and upper base lines. The melting temperatures (t_m) were obtained directly from the temperature at $\alpha = 0.5$.

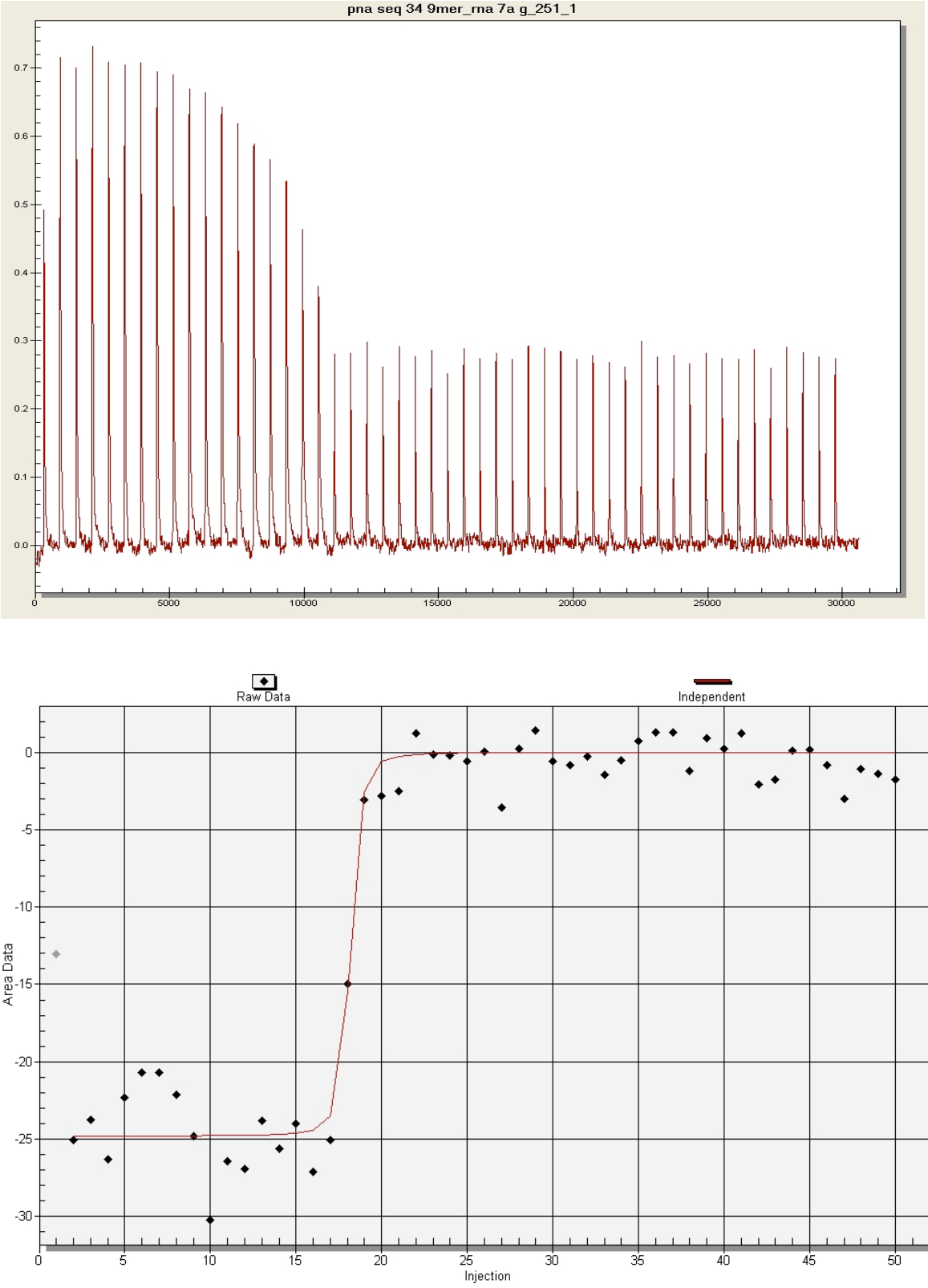


Figure S1. ITC data for binding of **PNA1 (C)** to **HRP1 (G-C)** pH 5.5.

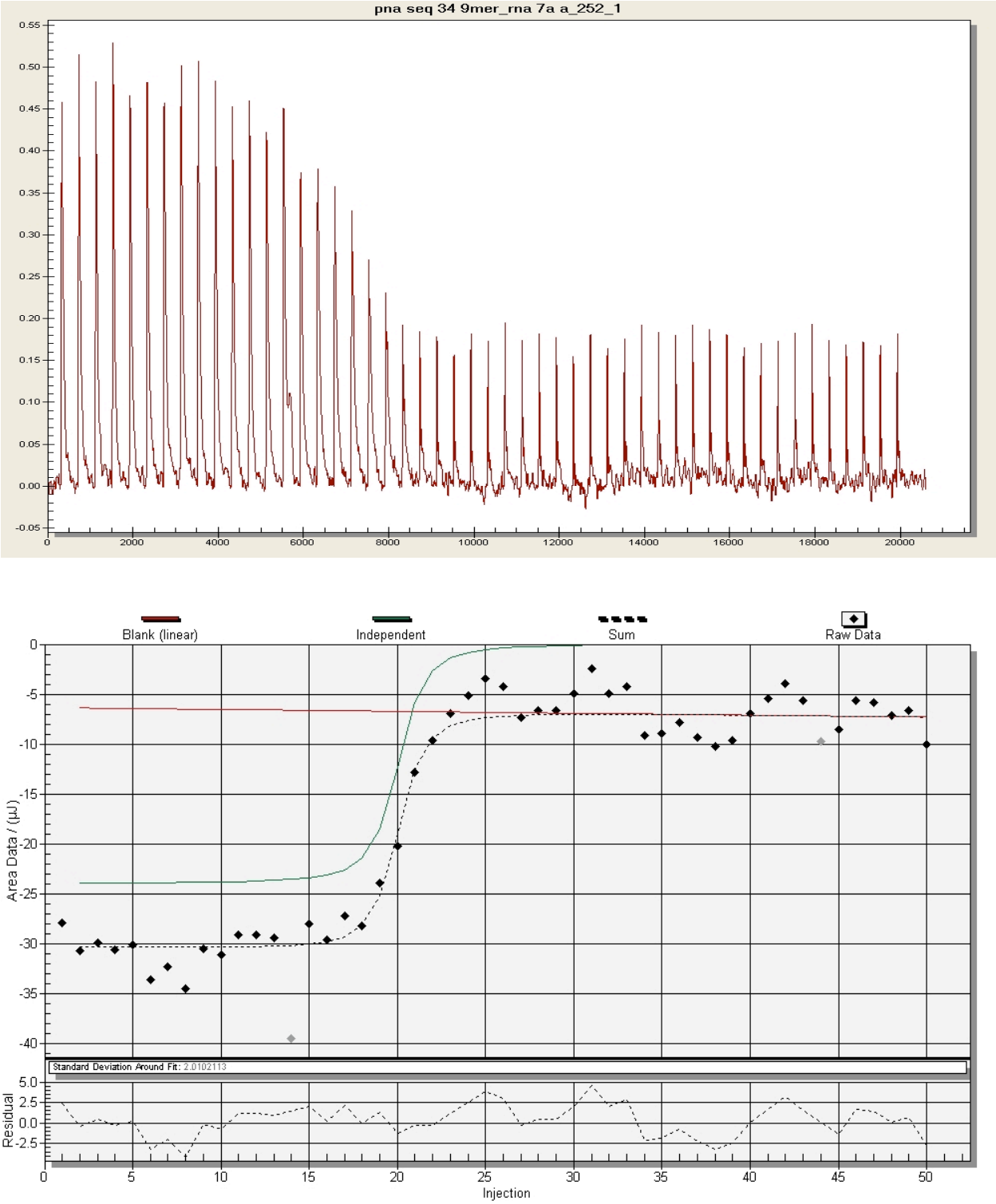


Figure S2. ITC data for binding of **PNA1 (C)** to **HRP2 (A-U)** pH 5.5.

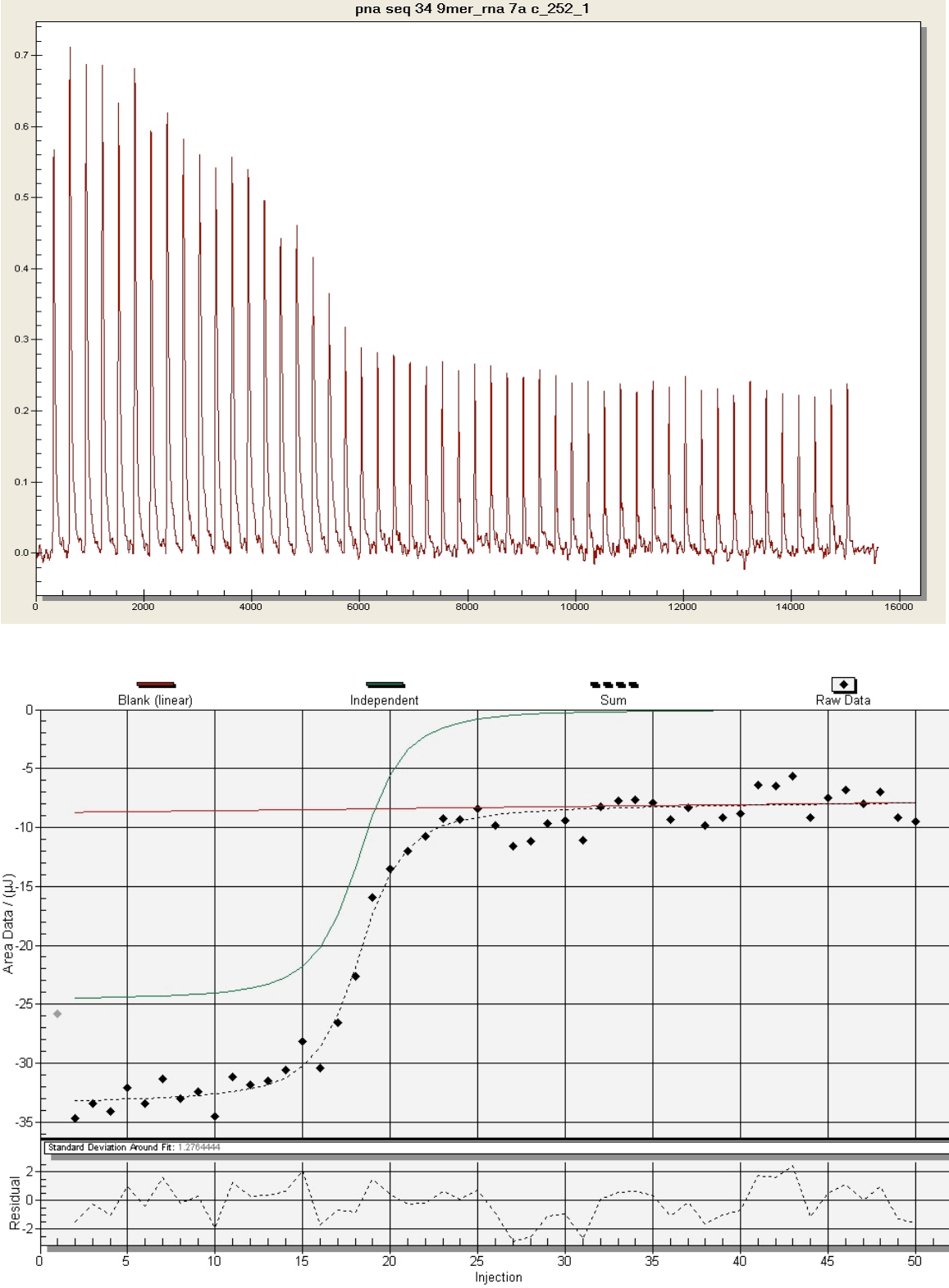


Figure S3. ITC data for binding of **PNA1 (C)** to **HRP3 (C-G)** pH 5.5.

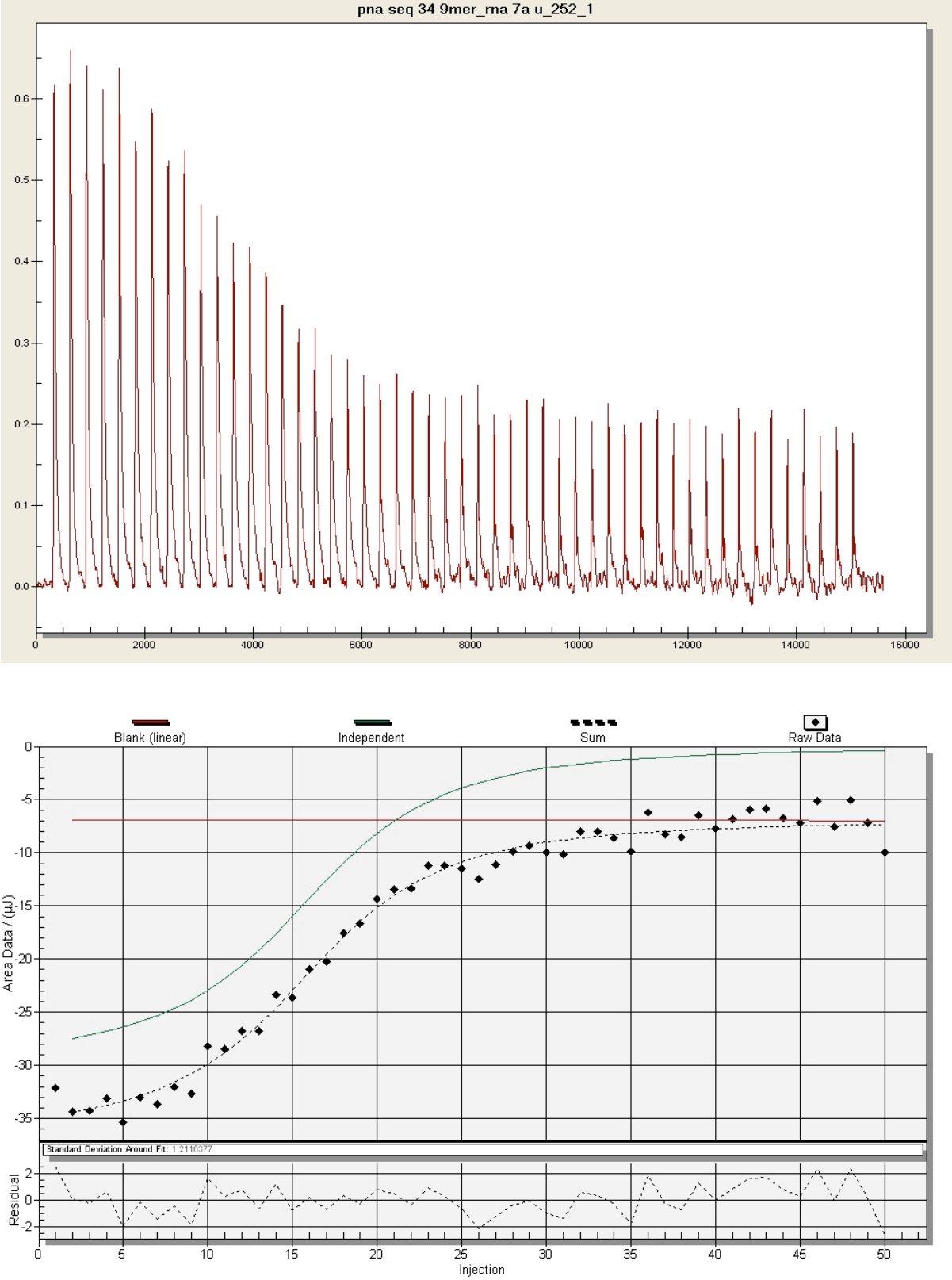


Figure S4. ITC data for binding of **PNA1 (C)** to **HRP4 (U-A)** pH 5.5.

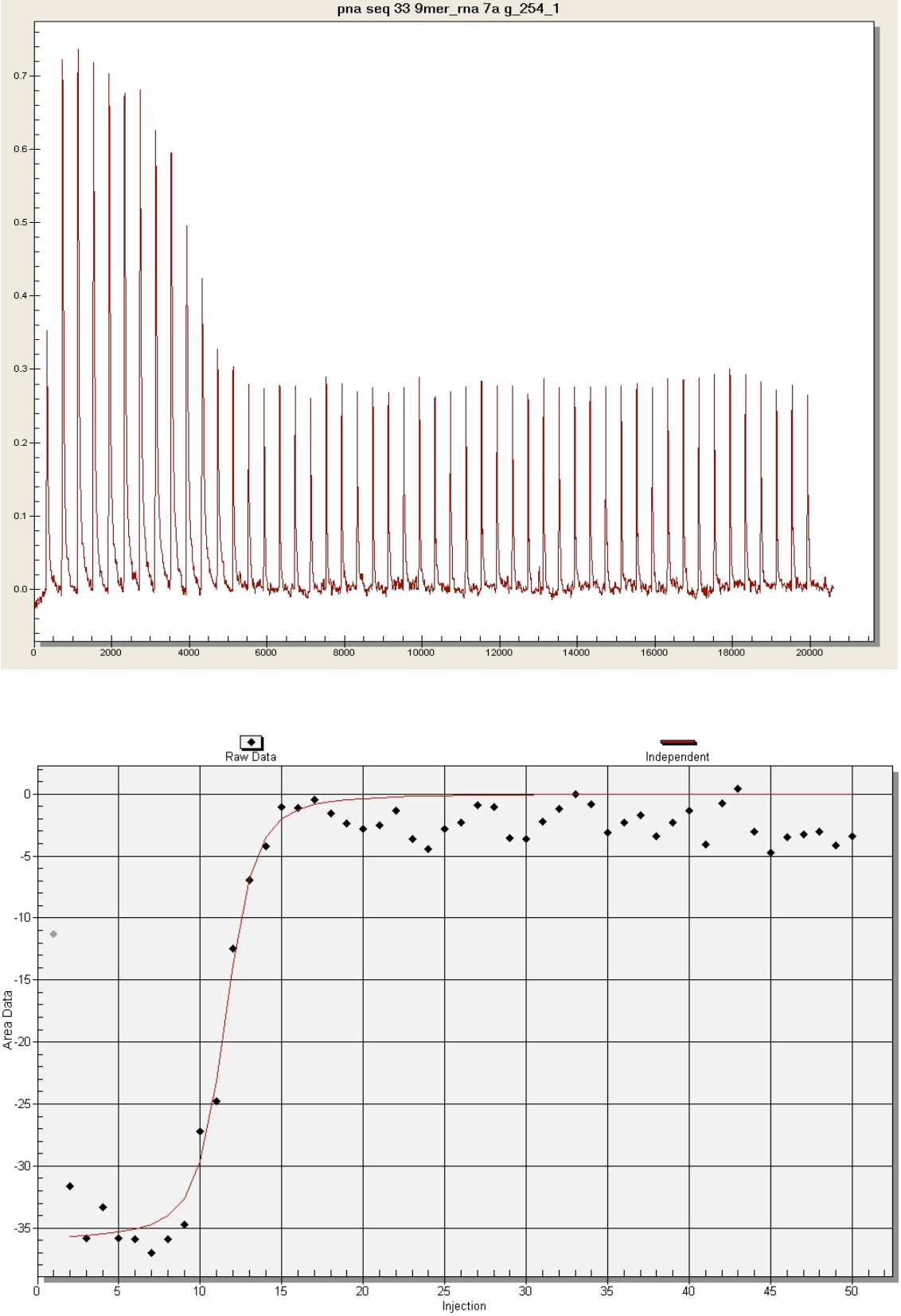


Figure S5. ITC data for binding of **PNA2 (T)** to **HRP1 (G-C)** pH 5.5.

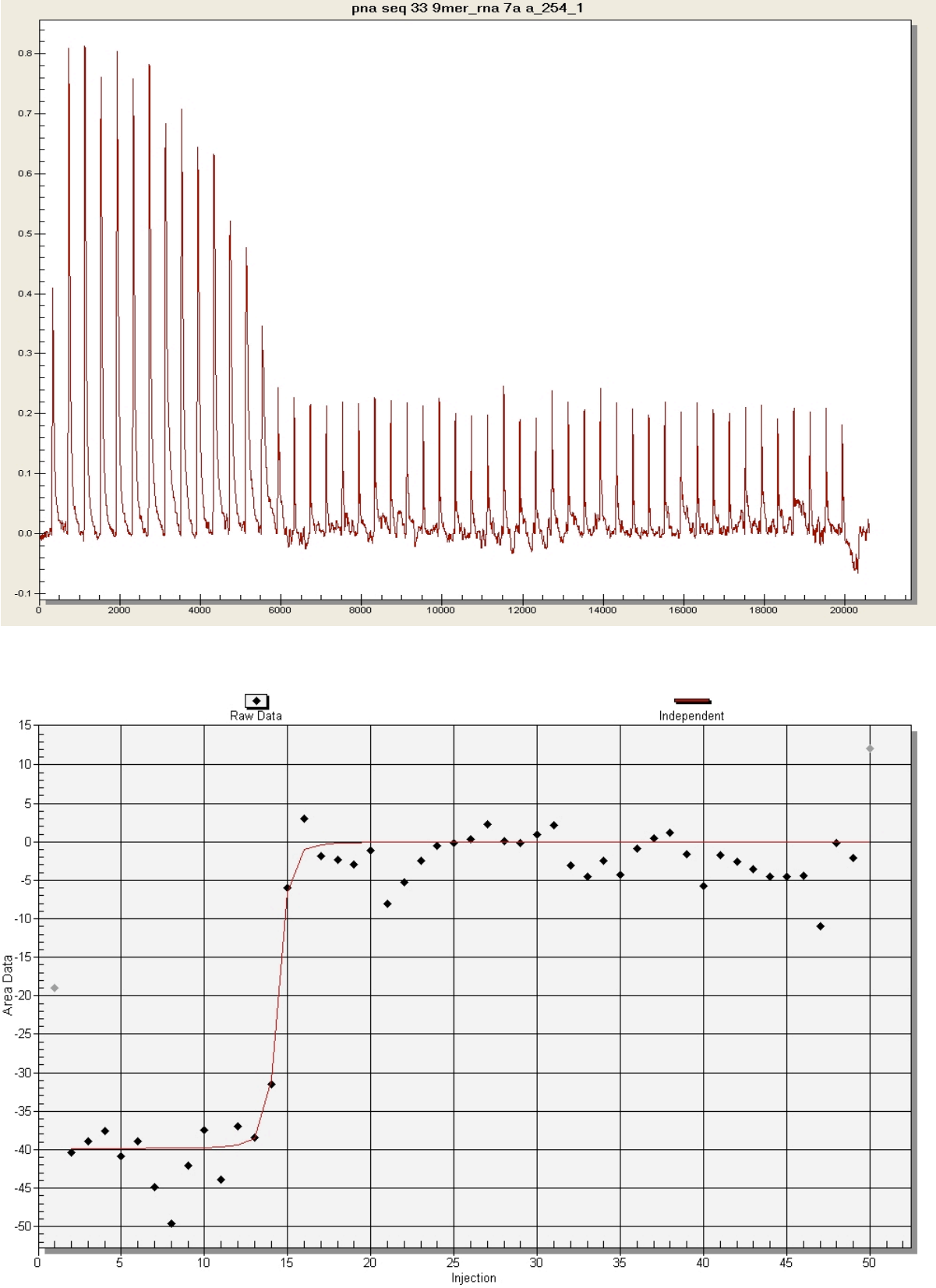


Figure S6. ITC data for binding of **PNA2 (T)** to **HRP2 (A-U)** pH 5.5.

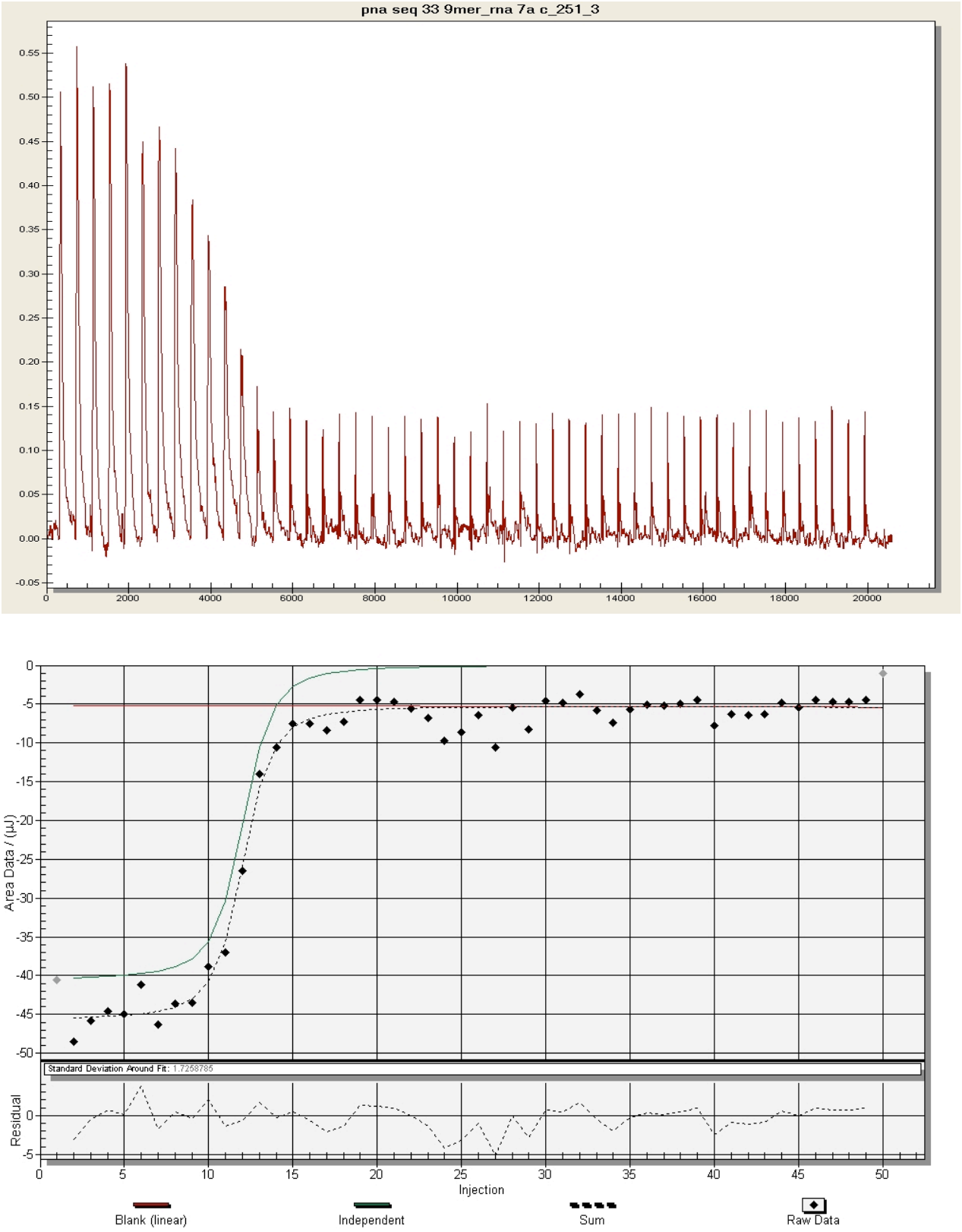


Figure S7. ITC data for binding of **PNA2 (T)** to **HRP3 (C-G)** pH 5.5.

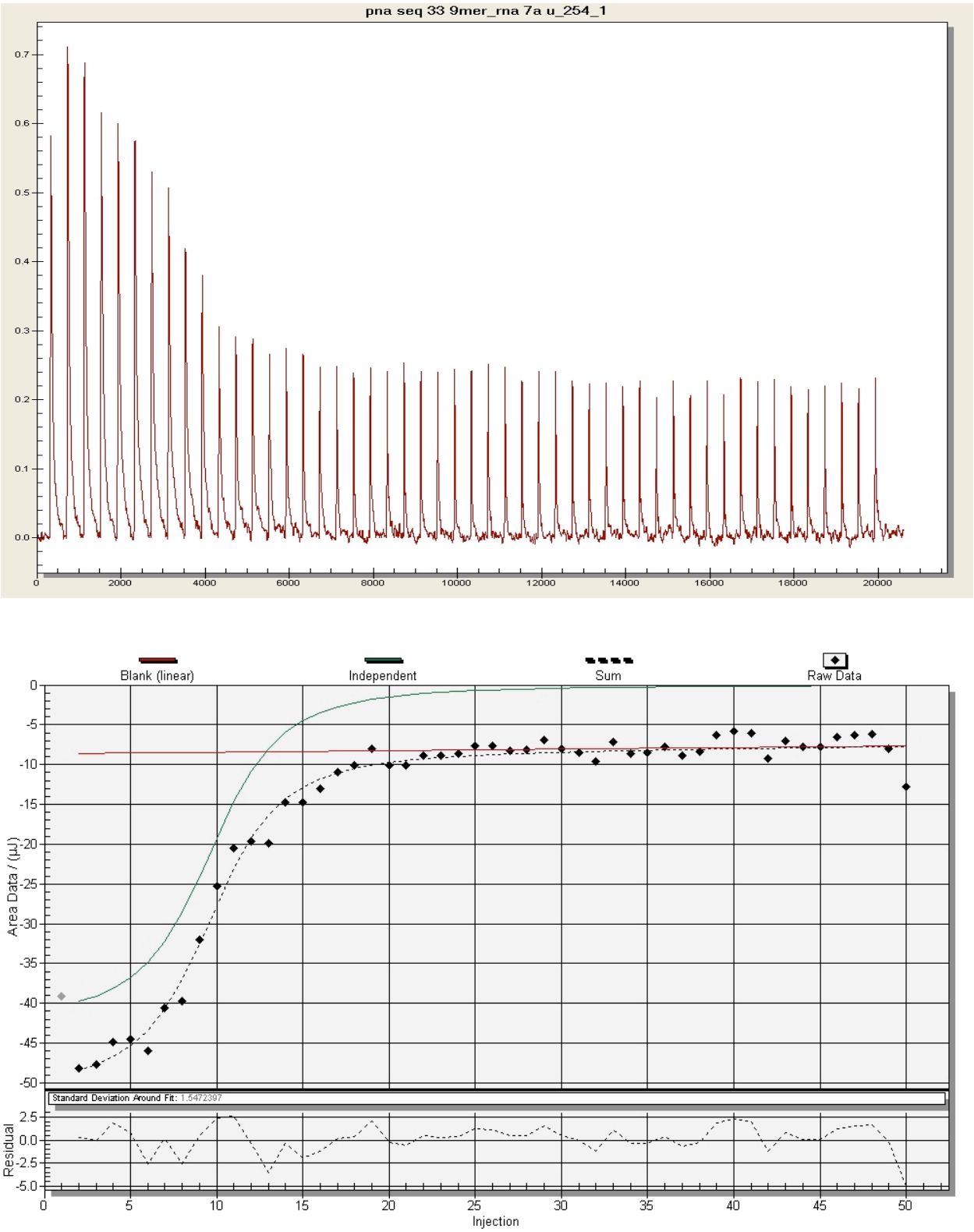


Figure S8. ITC data for binding of **PNA2 (T)** to **HRP4 (U-A)** pH 5.5.

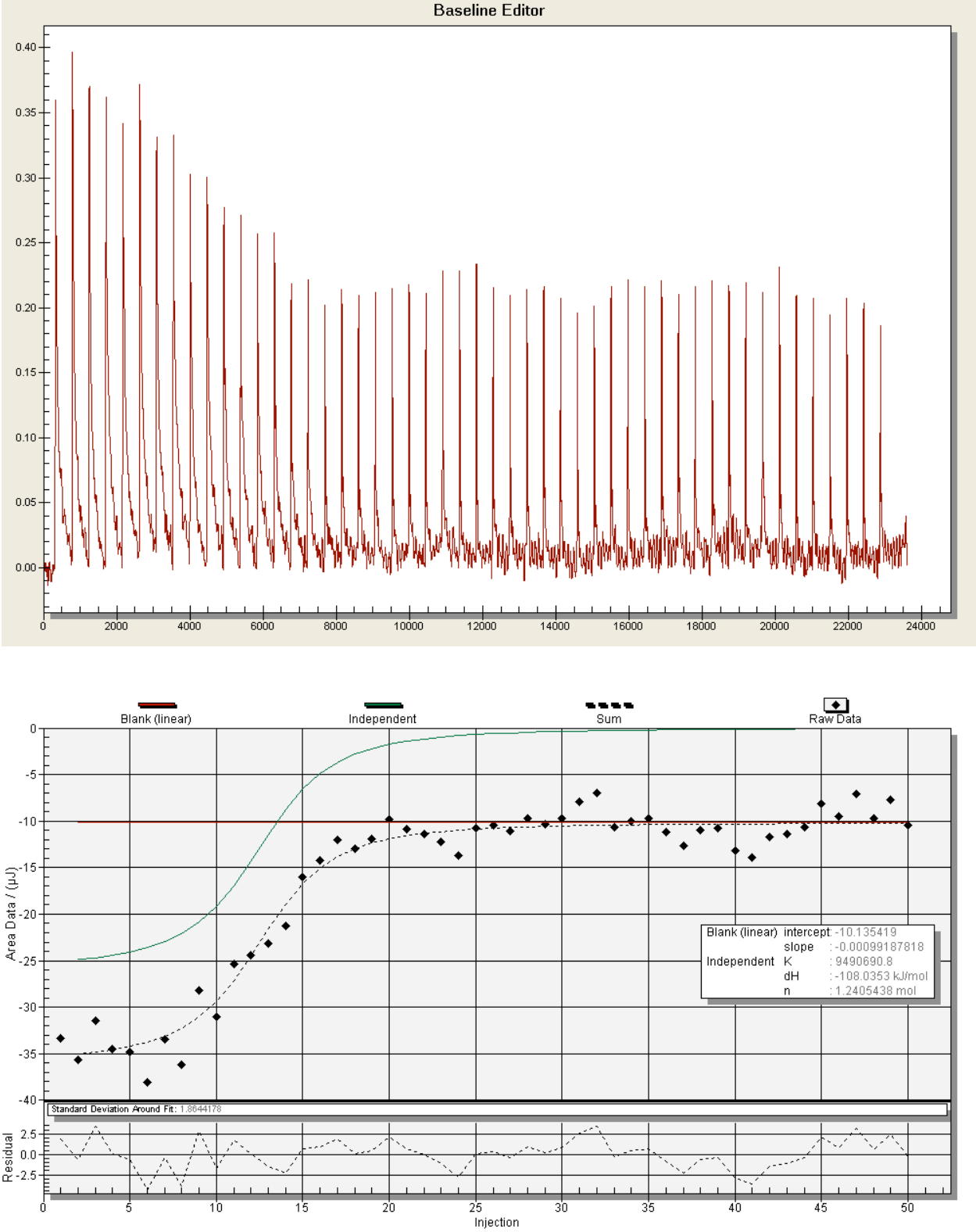


Figure S9. ITC data for binding of **PNA3 (P)** to **HRP1 (G-C)** pH 5.5.

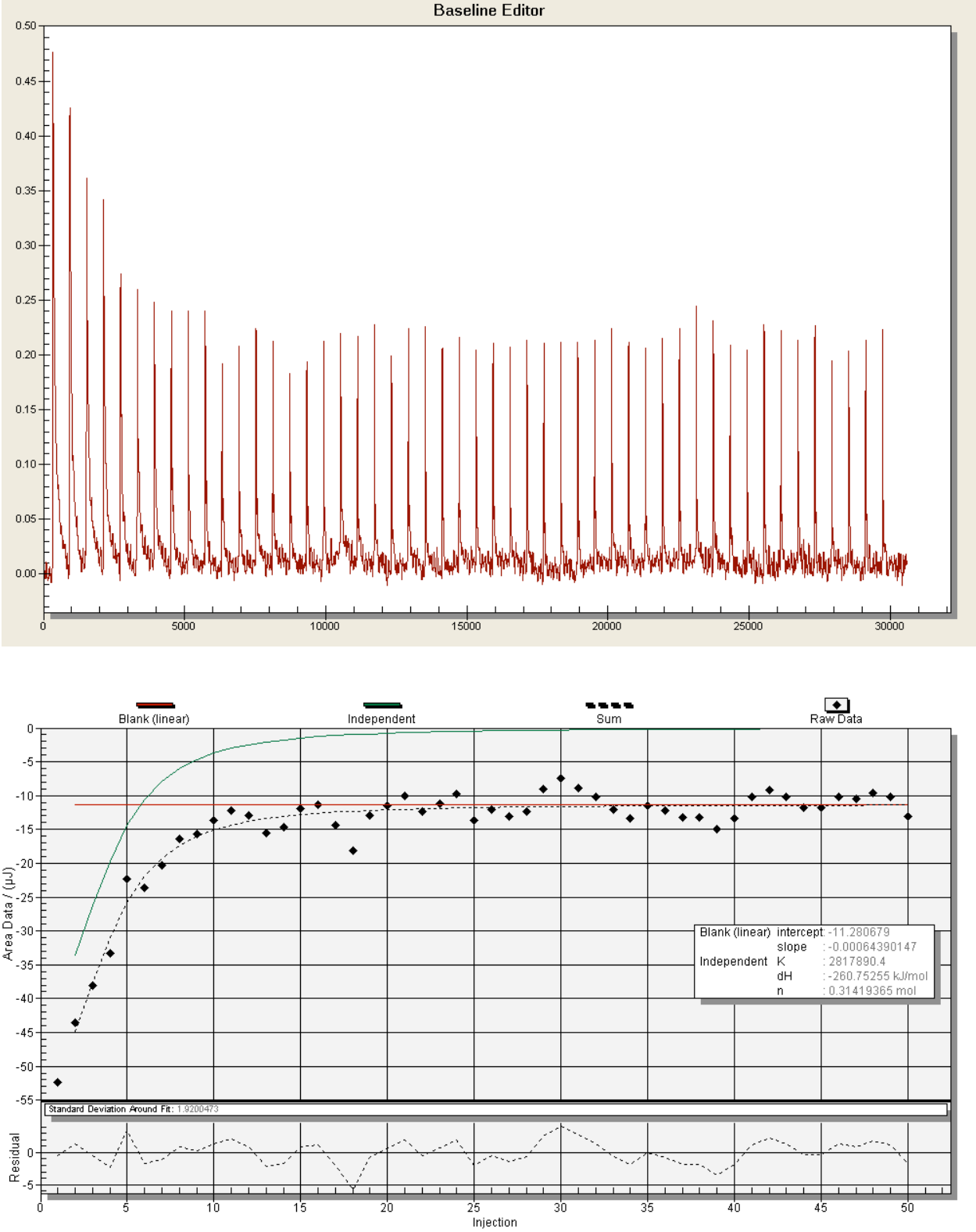


Figure S10. ITC data for binding of **PNA3 (P)** to **HRP2 (A-U)** pH 5.5.

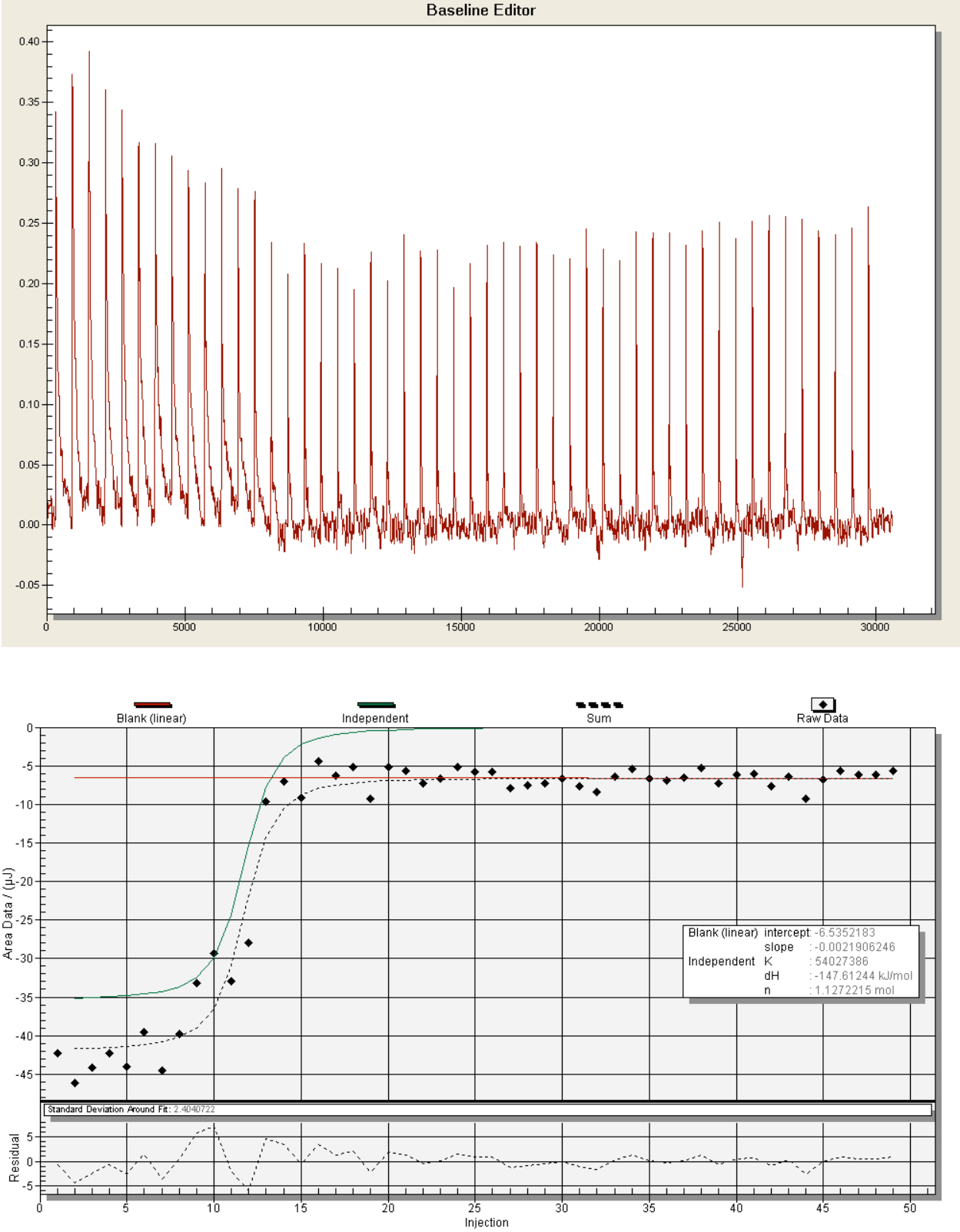


Figure S11. ITC data for binding of **PNA3 (P)** to **HRP3 (C-G)** pH 5.5.

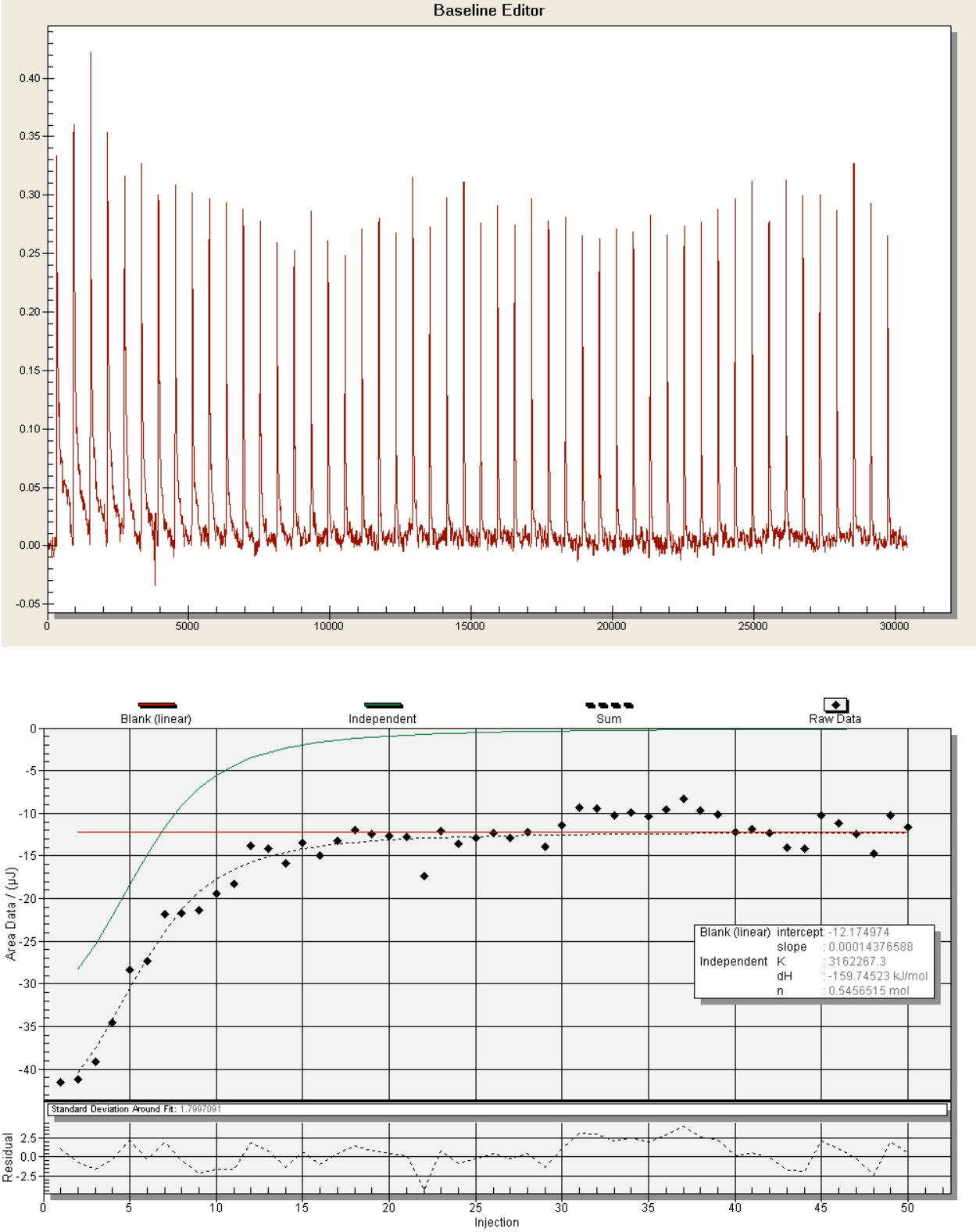


Figure S12. ITC data for binding of **PNA3 (P)** to **HRP4 (U-A)** pH 5.5.

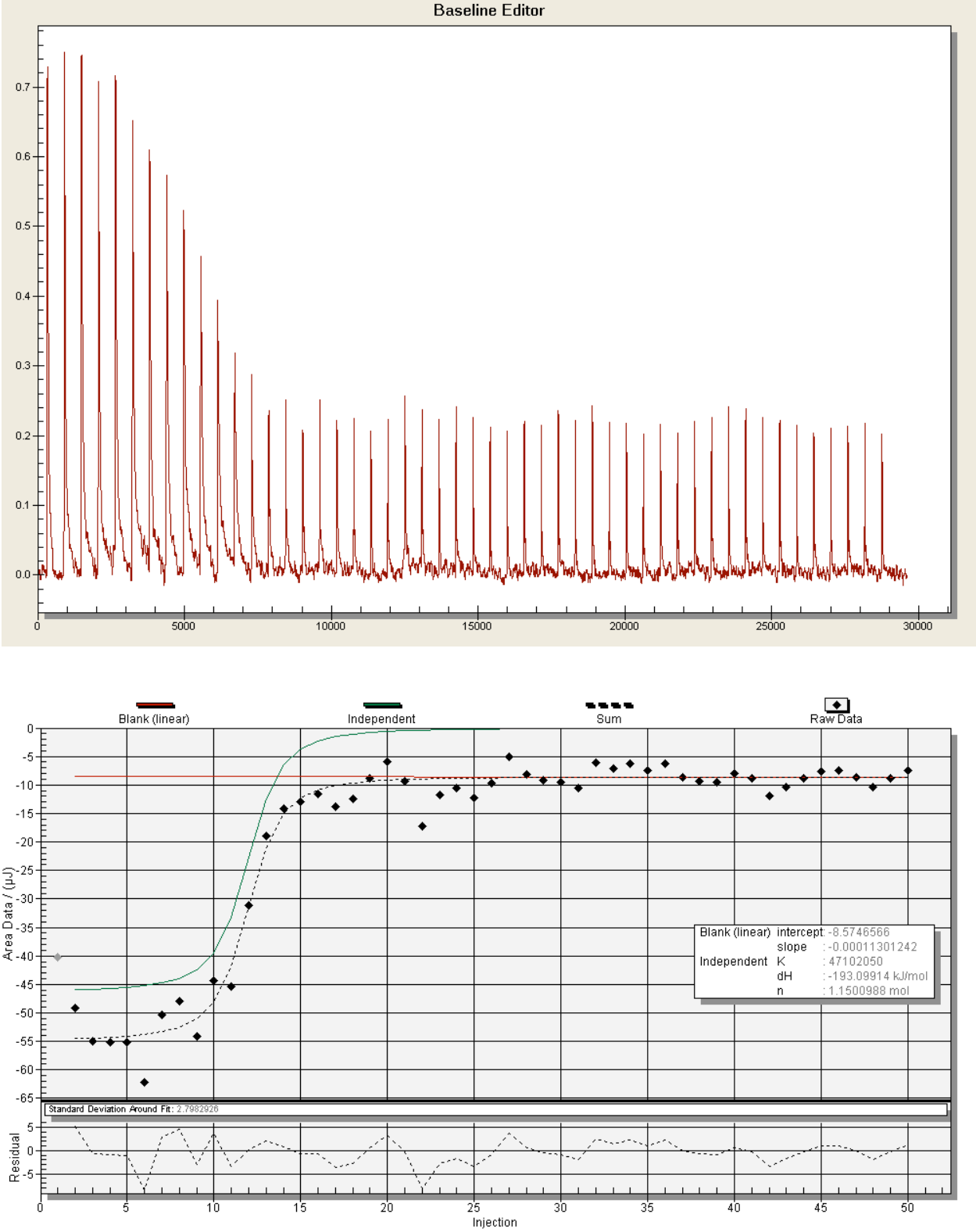


Figure S13. ITC data for binding of **PNA4 (P_{ex})** to **HRP1 (G-C)** pH 5.5.

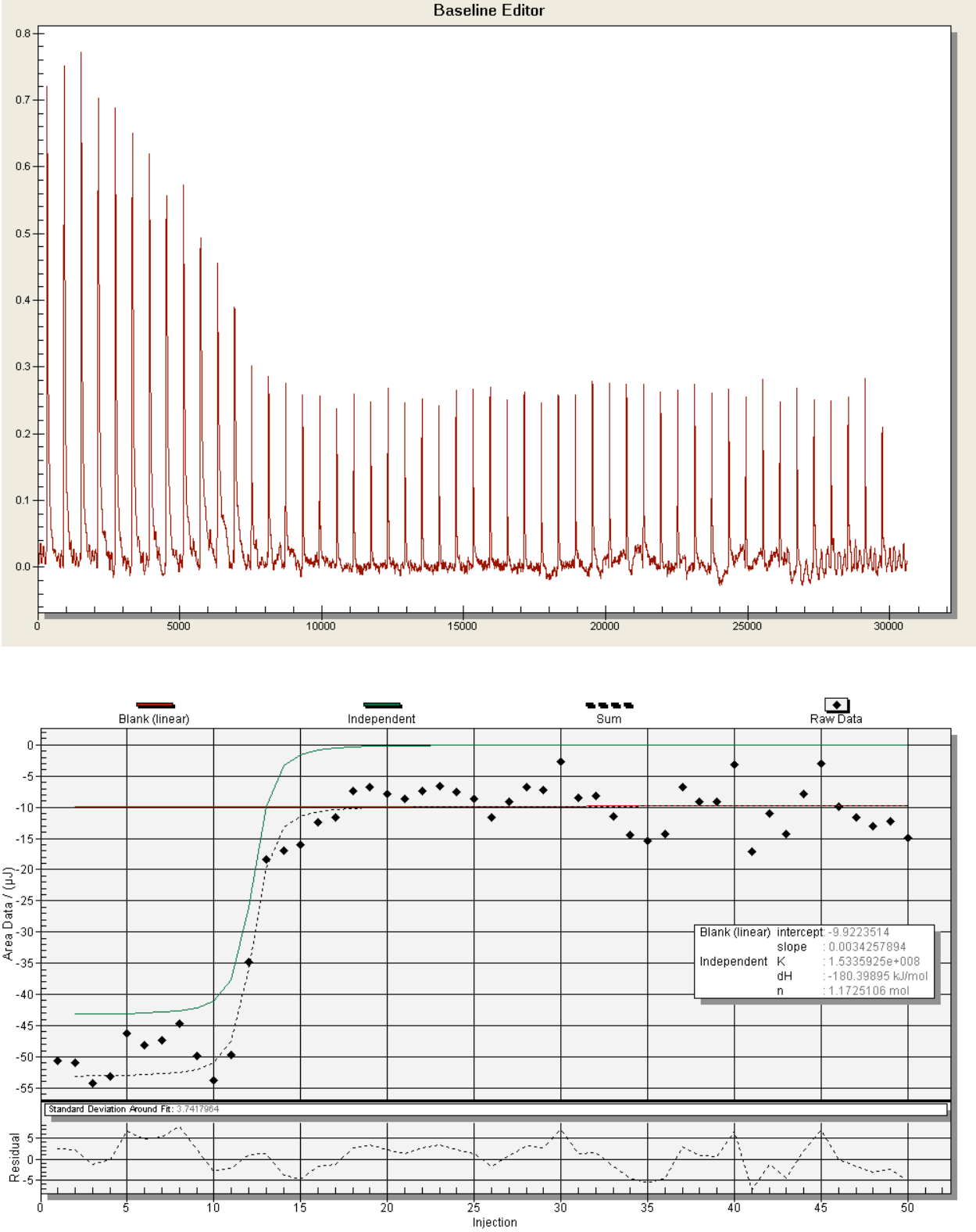


Figure S14. ITC data for binding of **PNA4 (P_{ex})** to **HRP2 (A-U)** pH 5.5.

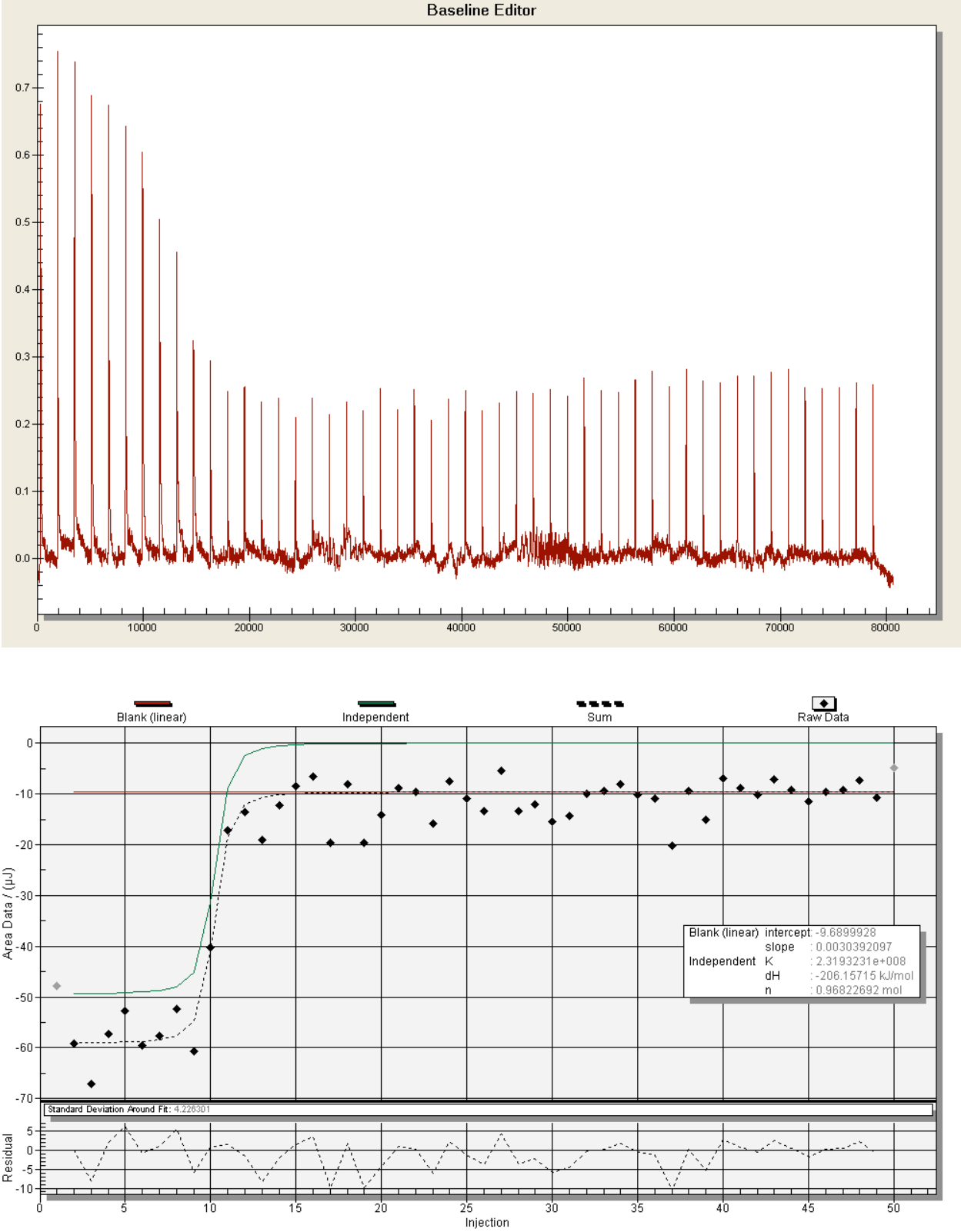


Figure S15. ITC data for binding of **PNA4 (P_{ex})** to **HRP3 (C-G)** pH 5.5.

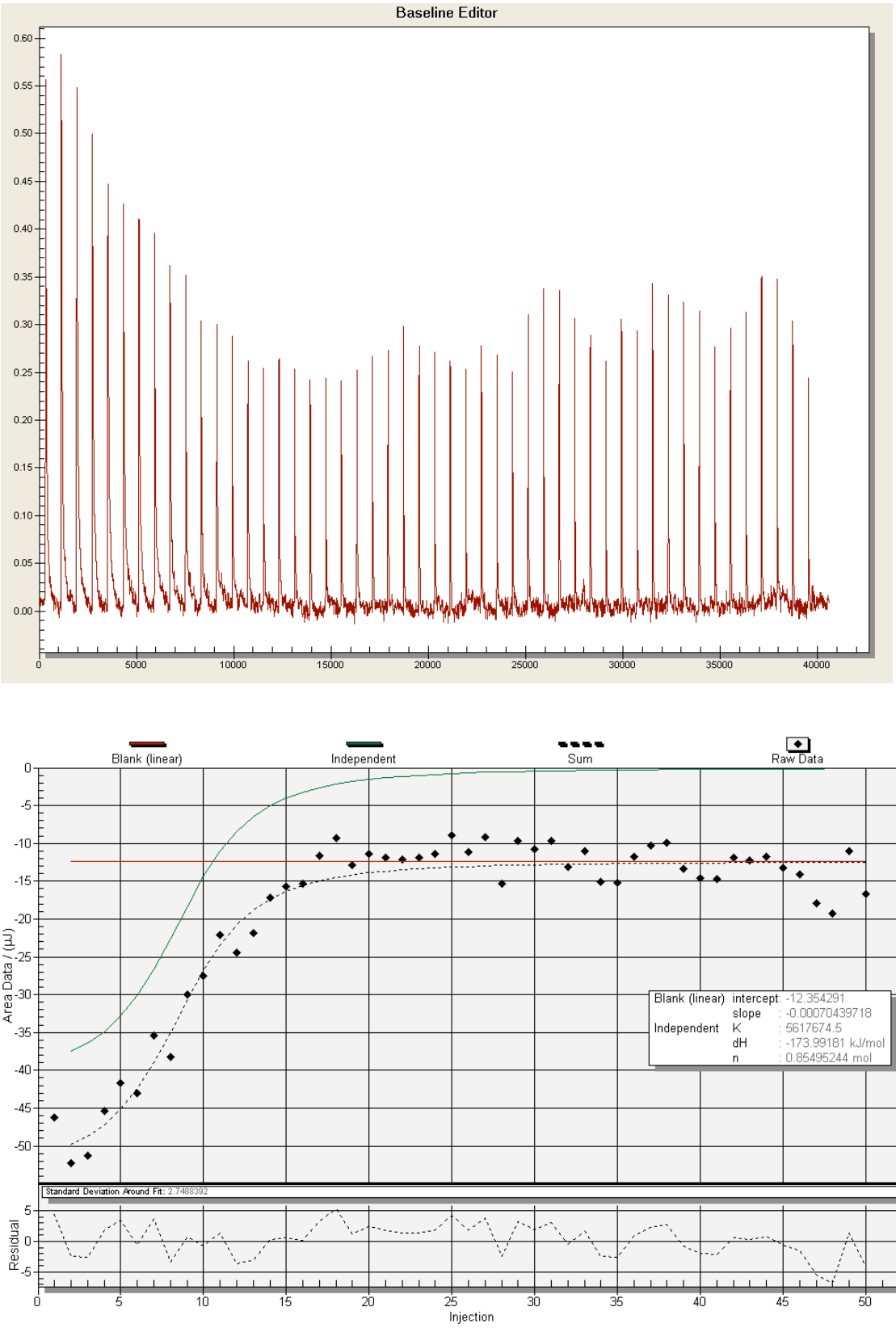


Figure S16. ITC data for binding of **PNA4 (P_{ex})** to **HRP4 (U-A)** pH 5.5.

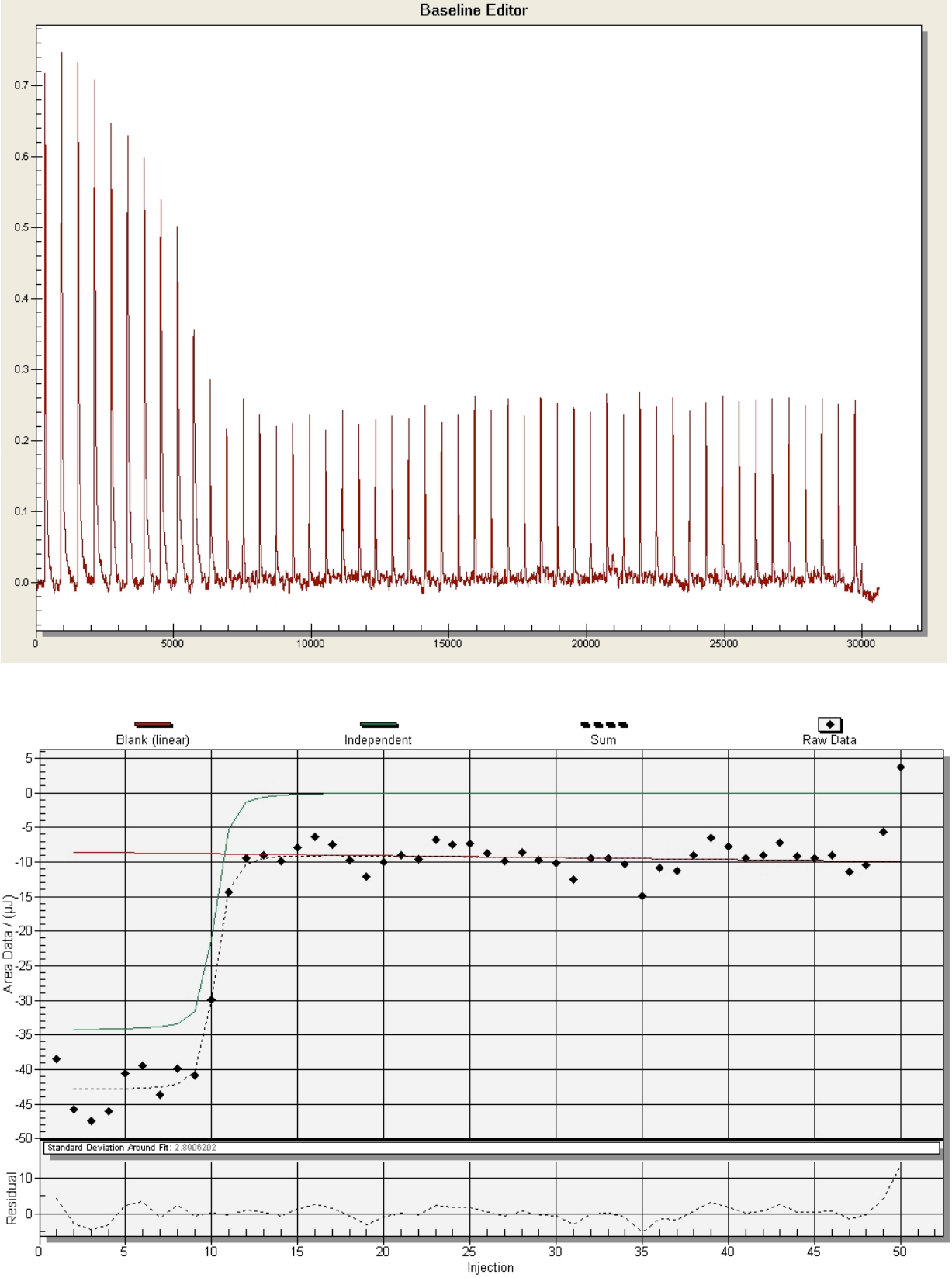
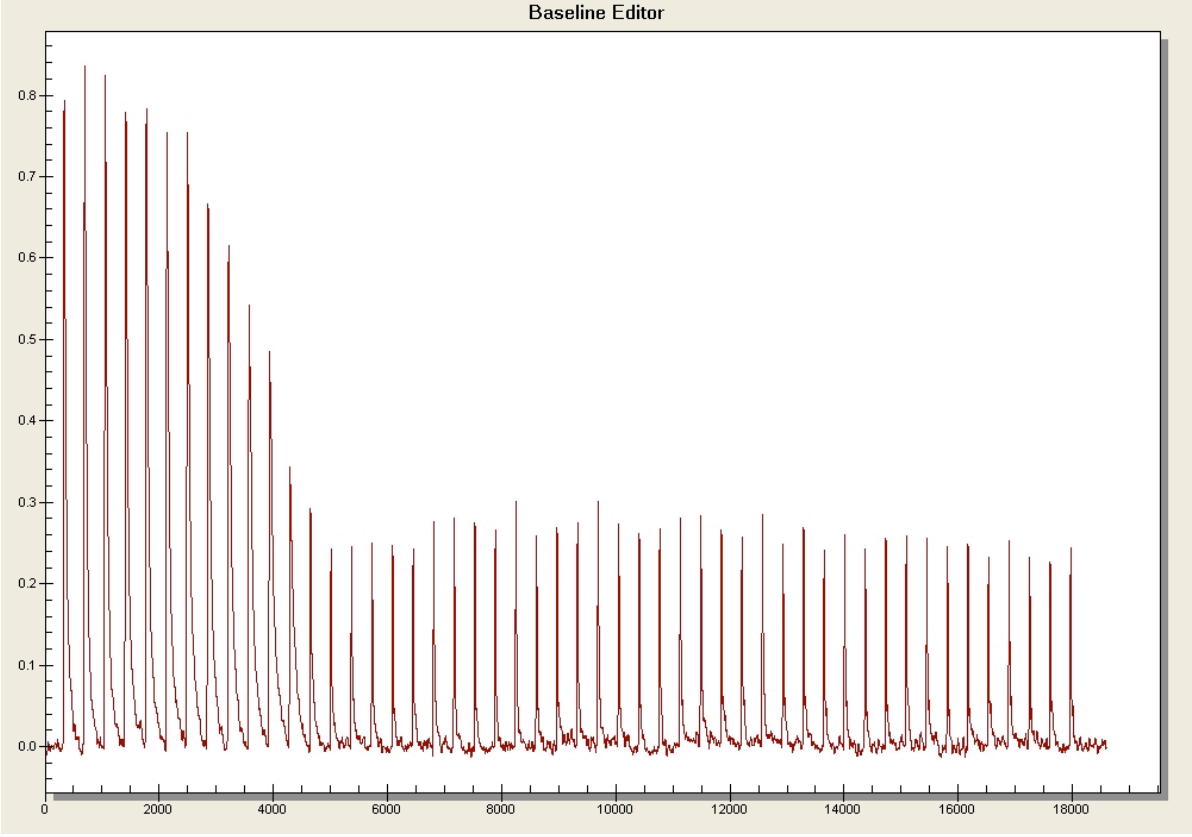


Figure S17. ITC data for binding of **PNA5 (E)** to **HRP1 (G-C)** pH 5.5.



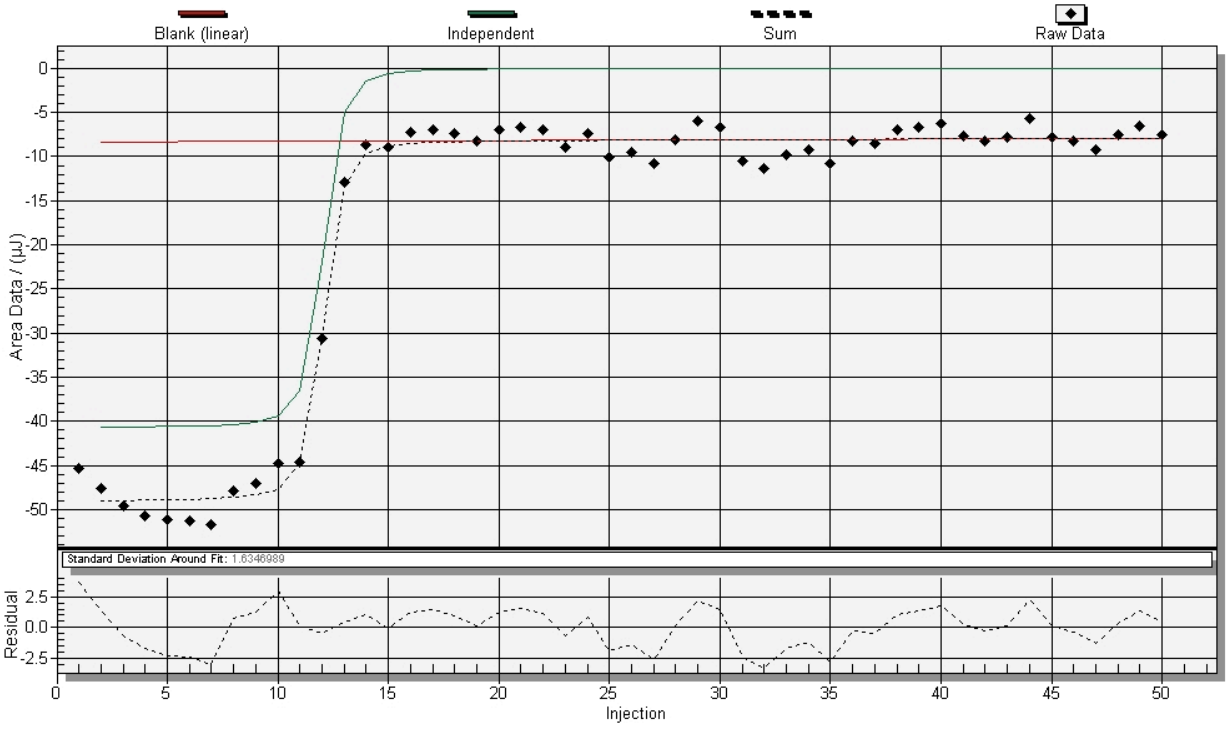


Figure S18. ITC data for binding of **PNA5 (E)** to **HRP2 (A-U)** pH 5.5.

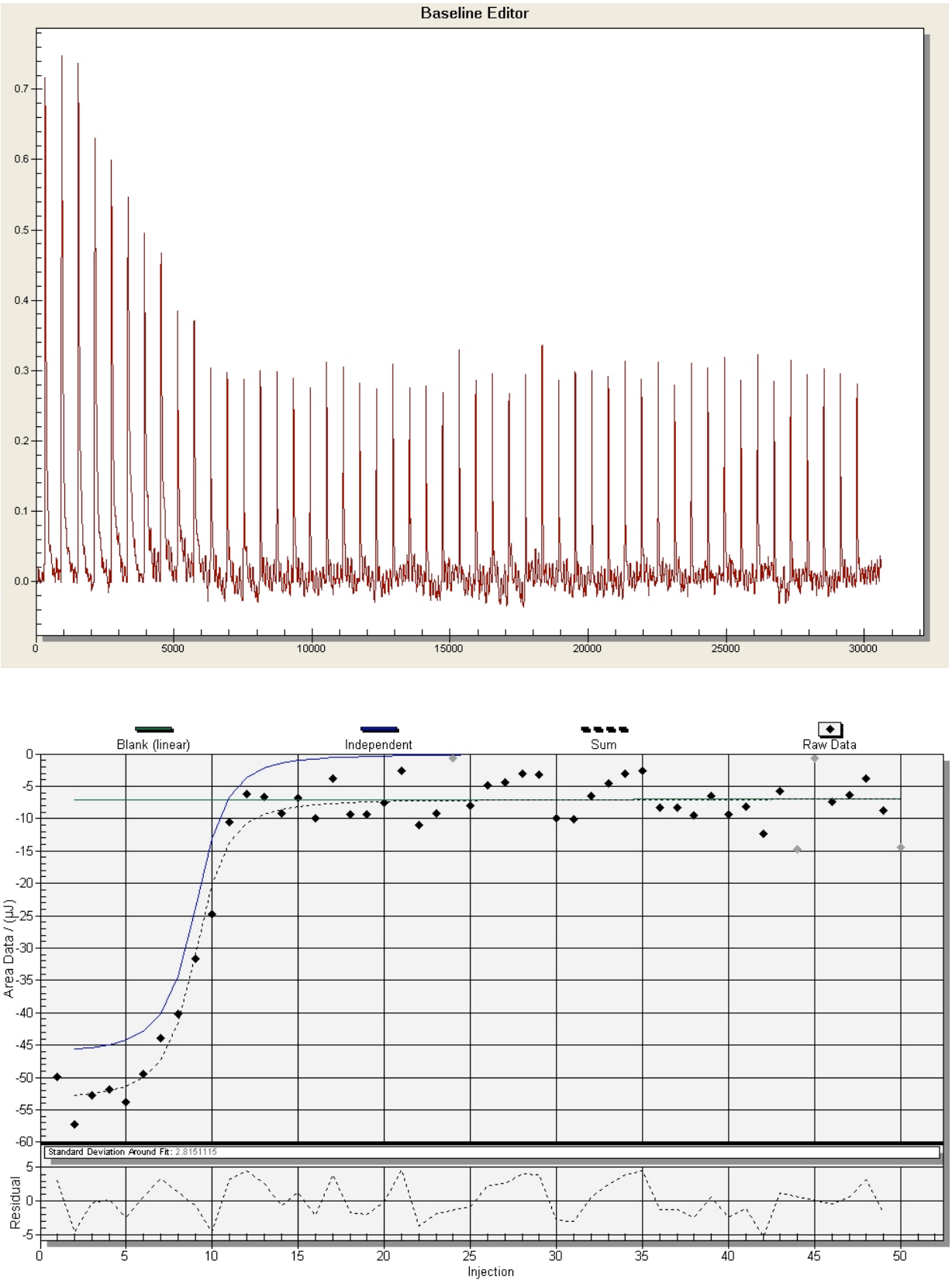
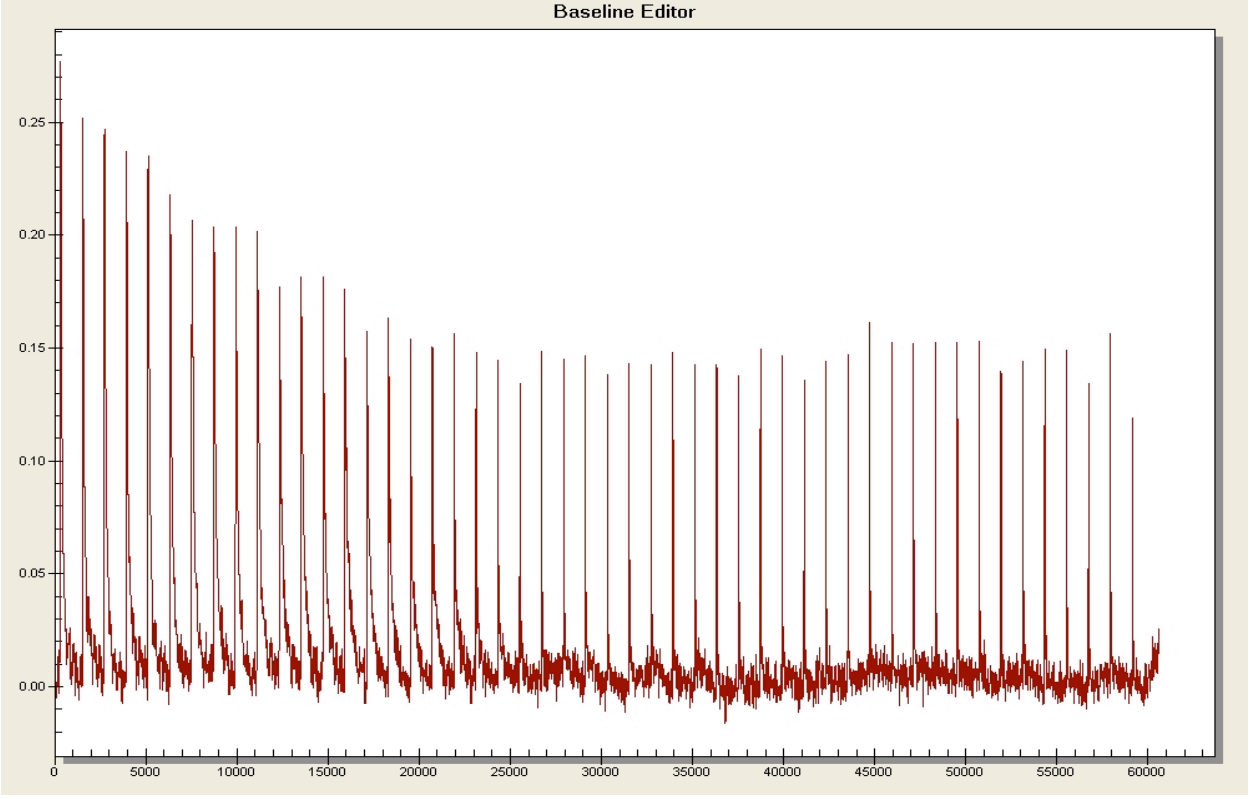


Figure S19. ITC data for binding of **PNA5 (E)** to **HRP4 (U-A)** pH 5.5.



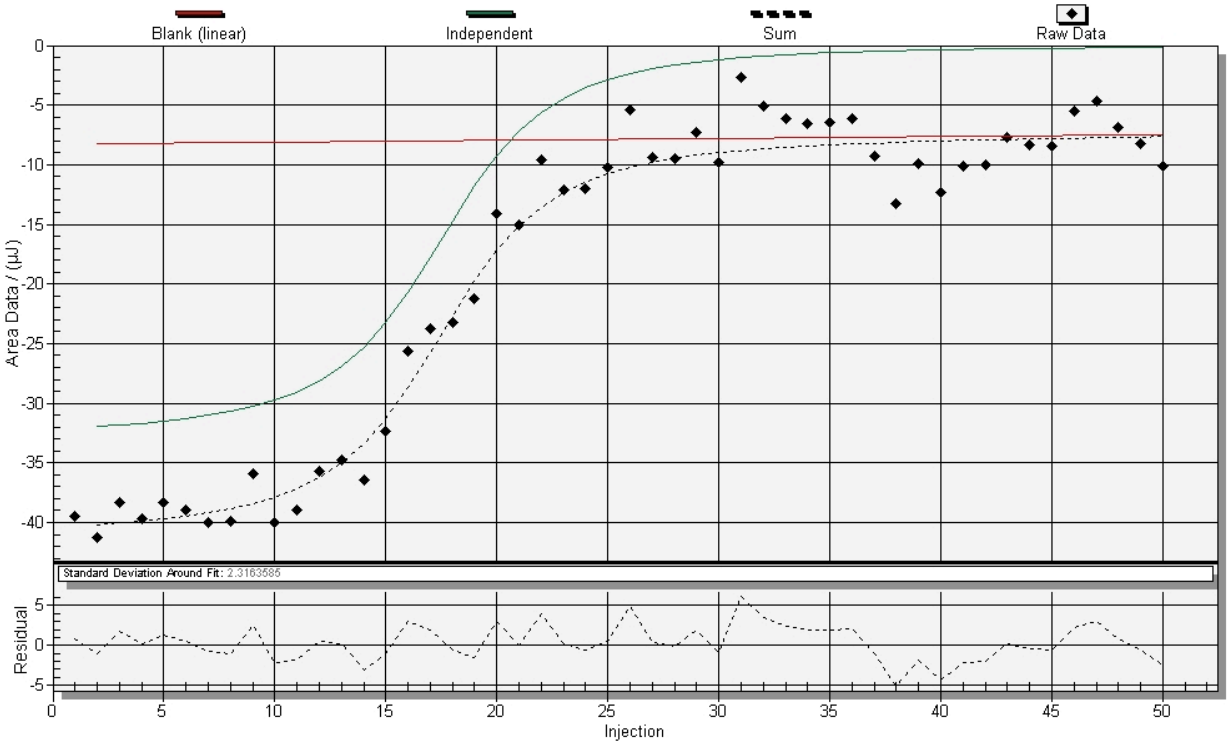


Figure S20. ITC data for binding of **PNA1 (C)** to **HRP1 (G-C)** pH 6.25.

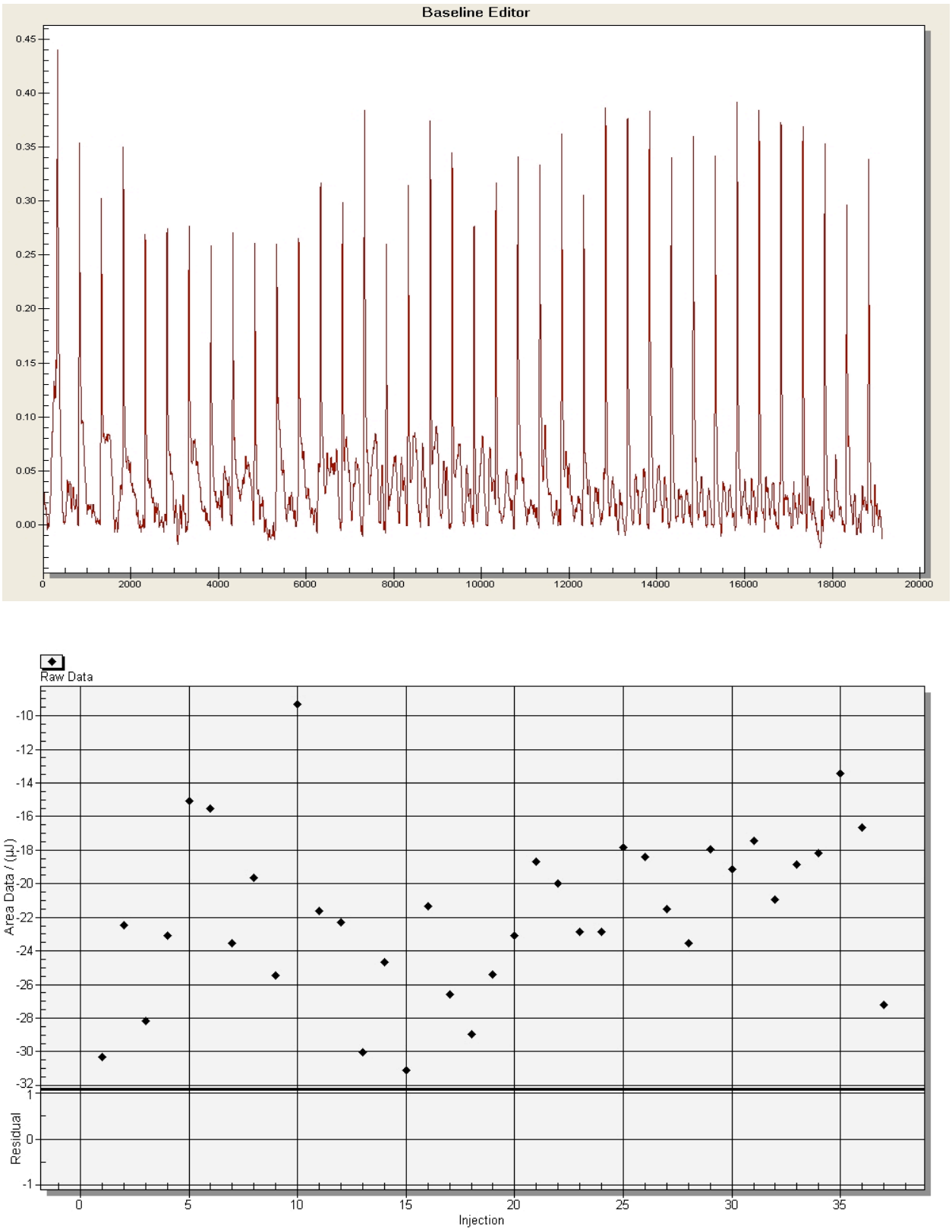


Figure S21. ITC data for binding of **PNA1 (C)** to **HRP2 (A-U)** pH 6.25.

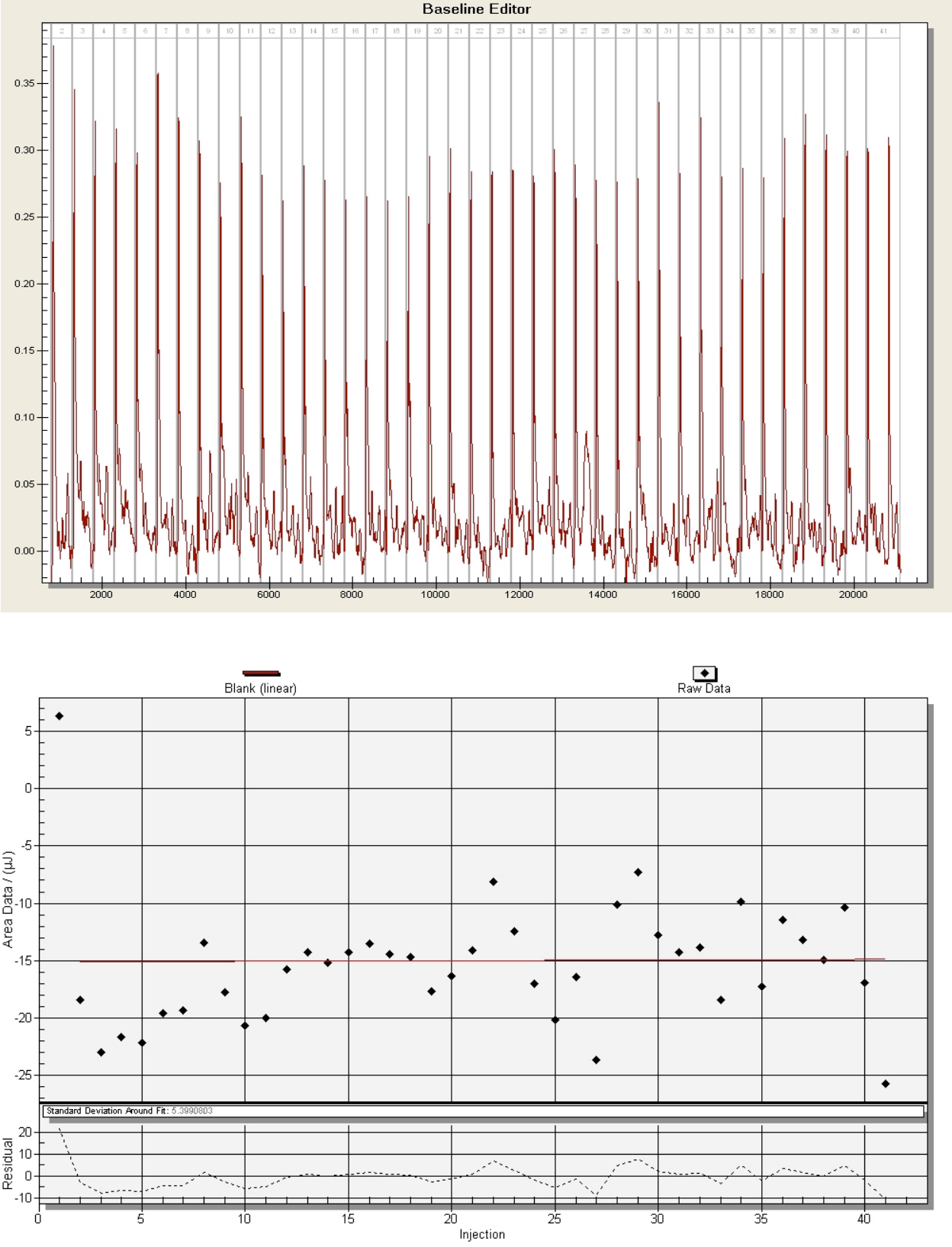


Figure S22. ITC data for binding of **PNA1 (C)** to **HRP3 (C-G)** pH 6.25.

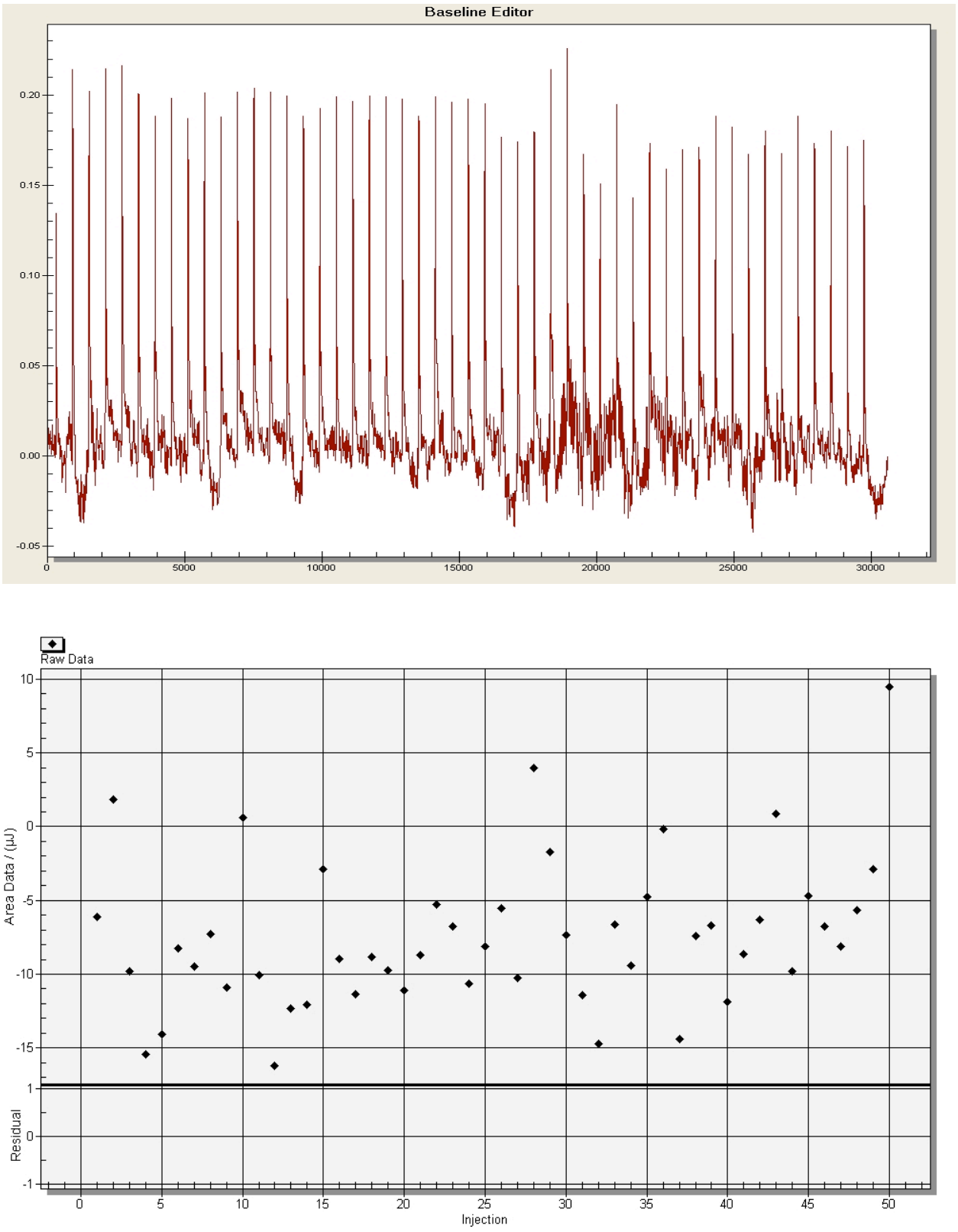


Figure S23. ITC data for binding of **PNA1 (C)** to **HRP4 (U-A)** pH 6.25.

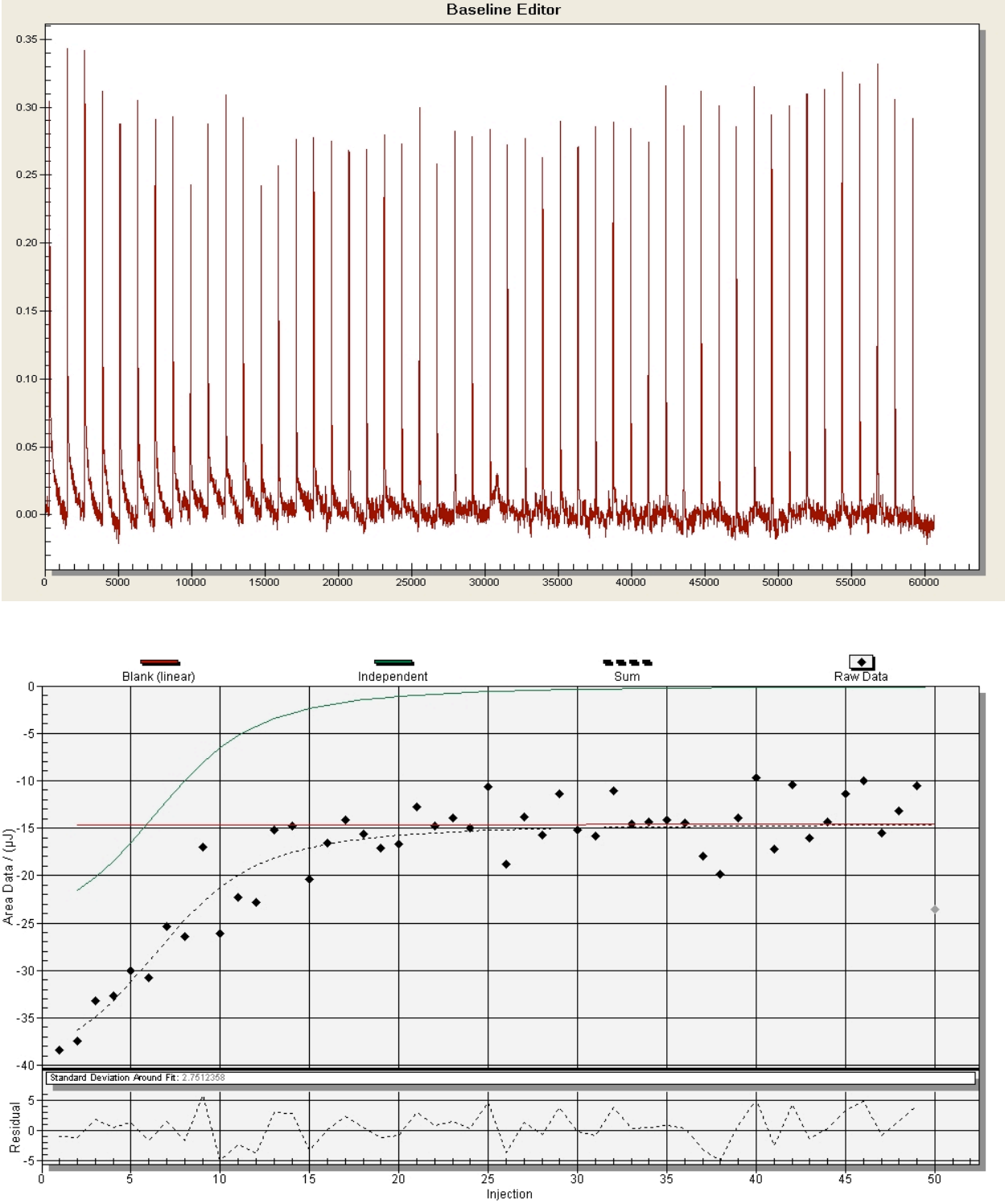


Figure S24. ITC data for binding of **PNA2 (T)** to **HRP1 (G-C)** pH 6.25.

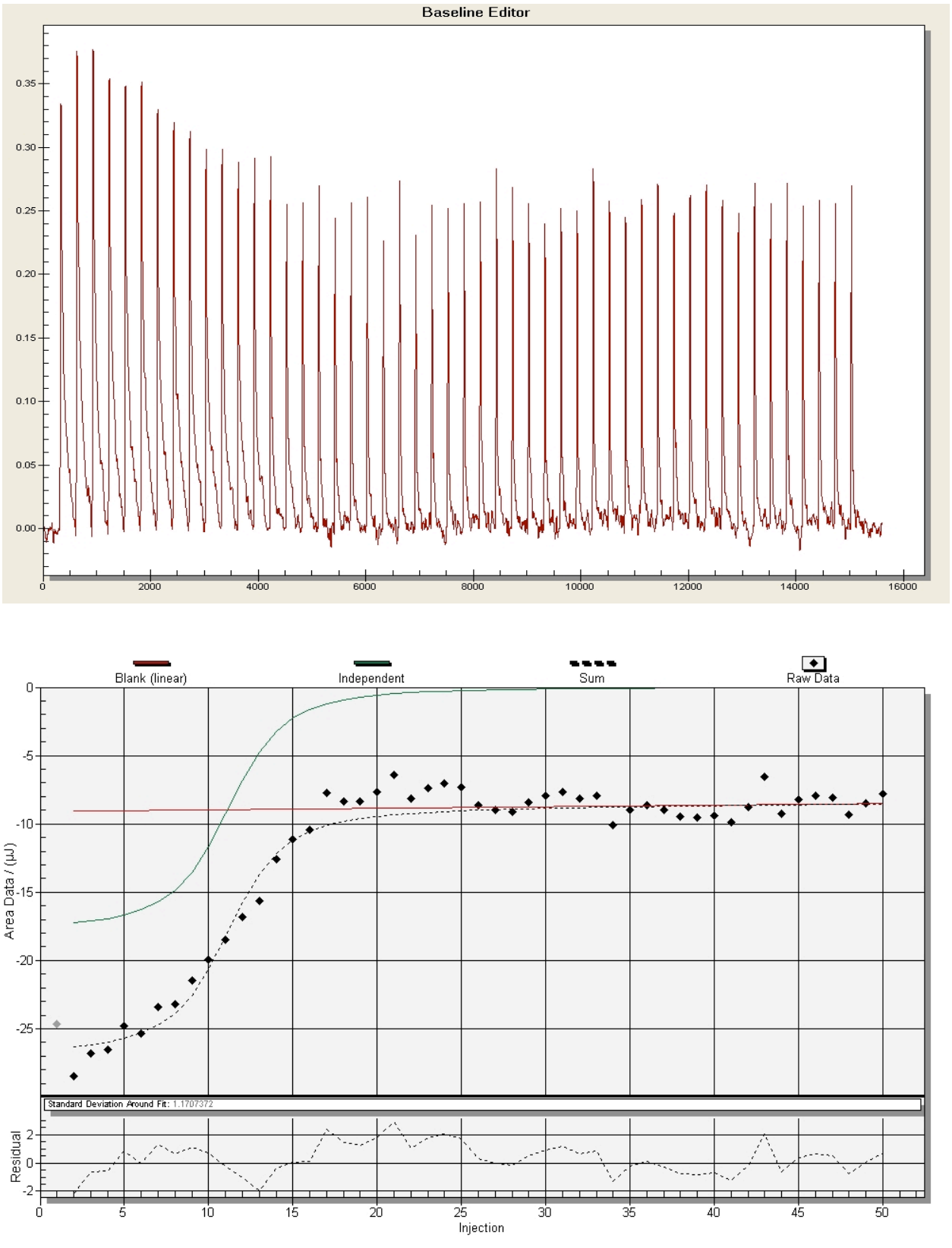


Figure S25. ITC data for binding of **PNA2 (T)** to **HRP2 (A-U)** pH 6.25.

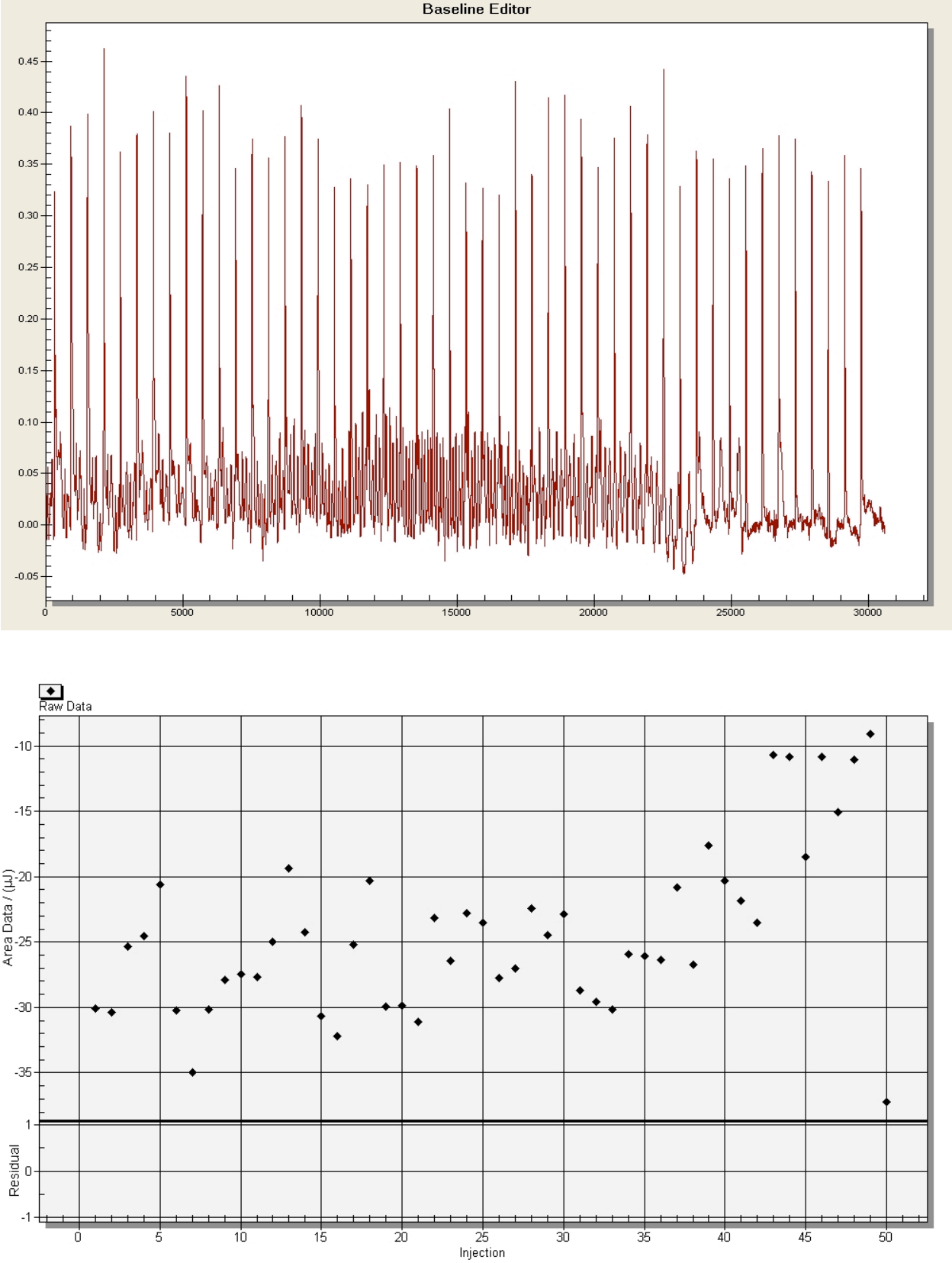
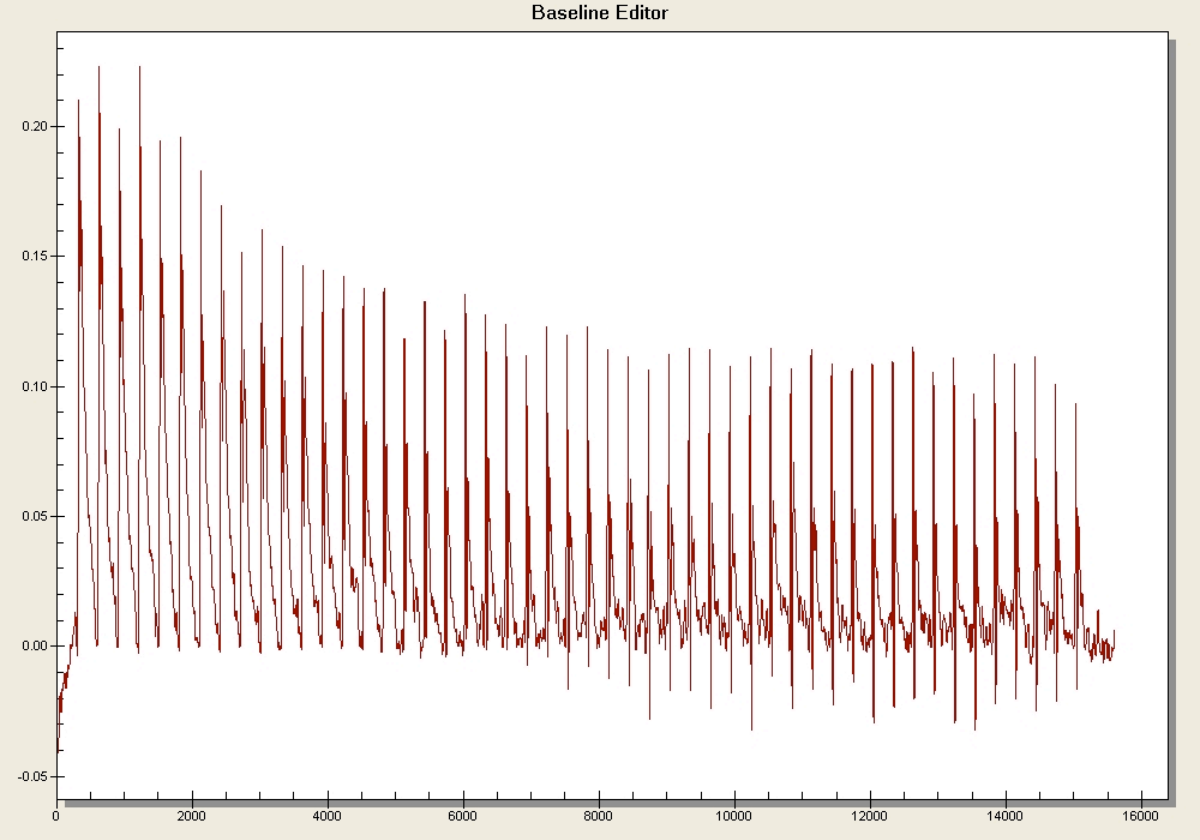


Figure S26. ITC data for binding of **PNA2 (C)** to **HRP3 (C-G)** pH 6.25.



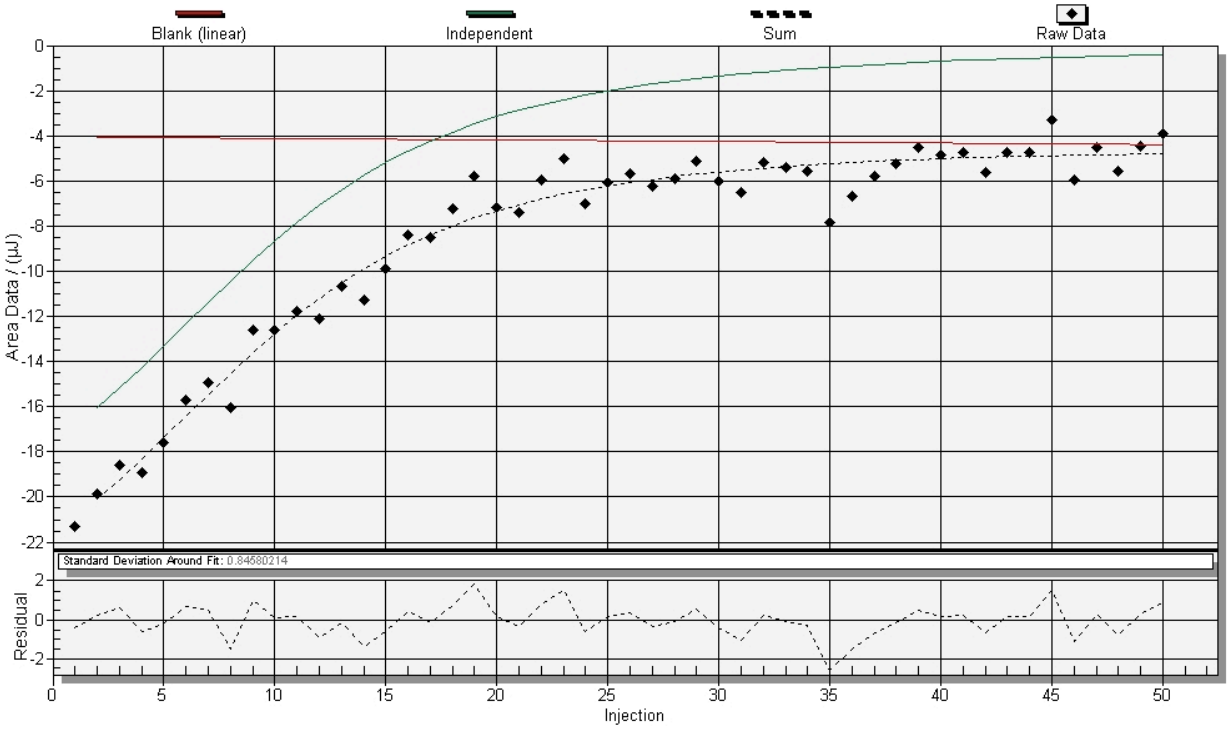


Figure S27. ITC data for binding of **PNA2 (T)** to **HRP4 (U-A)** pH 6.25.

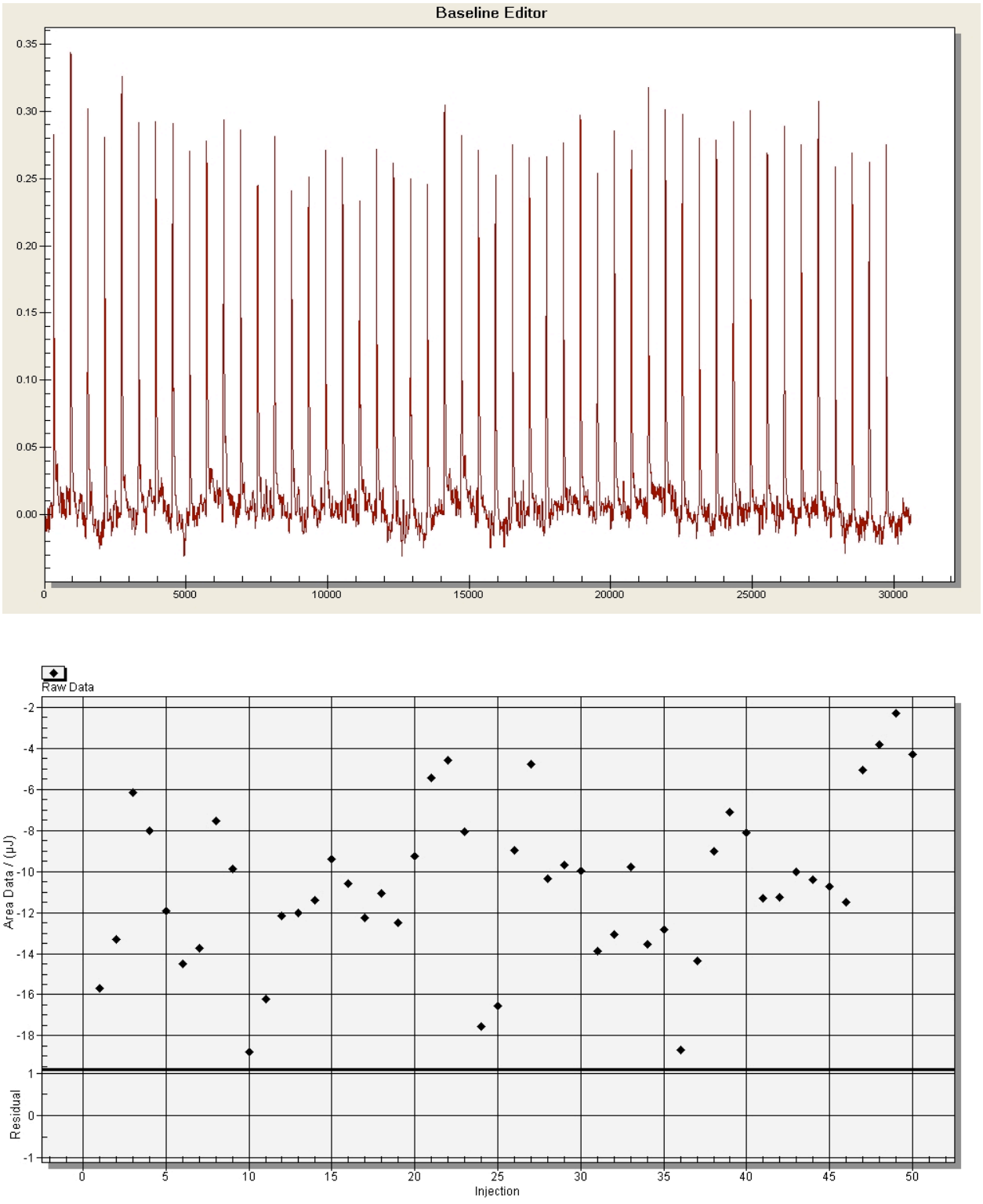


Figure S28. ITC data for binding of **PNA4 (P_{ex})** to **HRP1 (G-C)** pH 6.25.

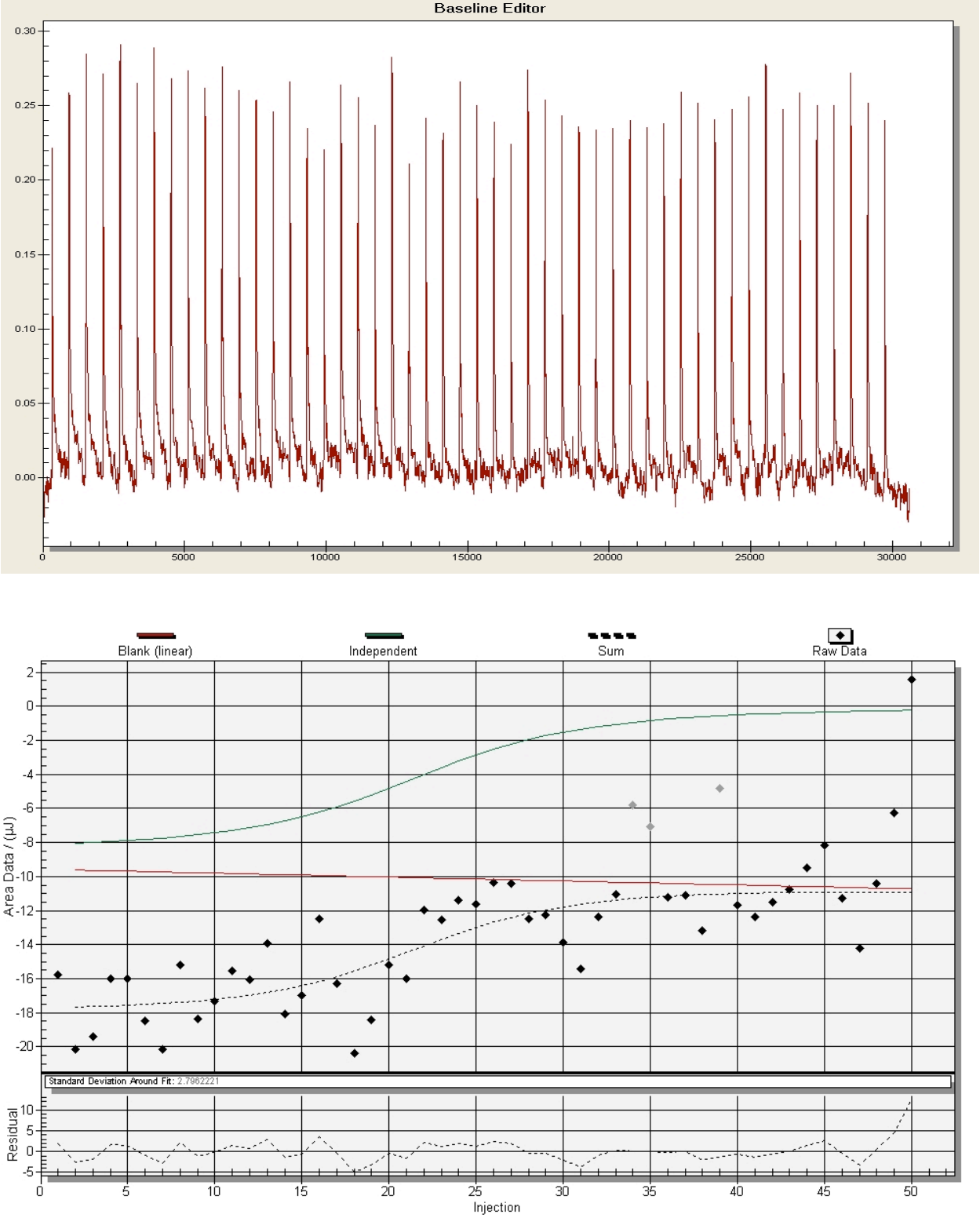


Figure S29. ITC data for binding of **PNA4 (P_{ex})** to **HRP2 (A-U)** pH 6.25.

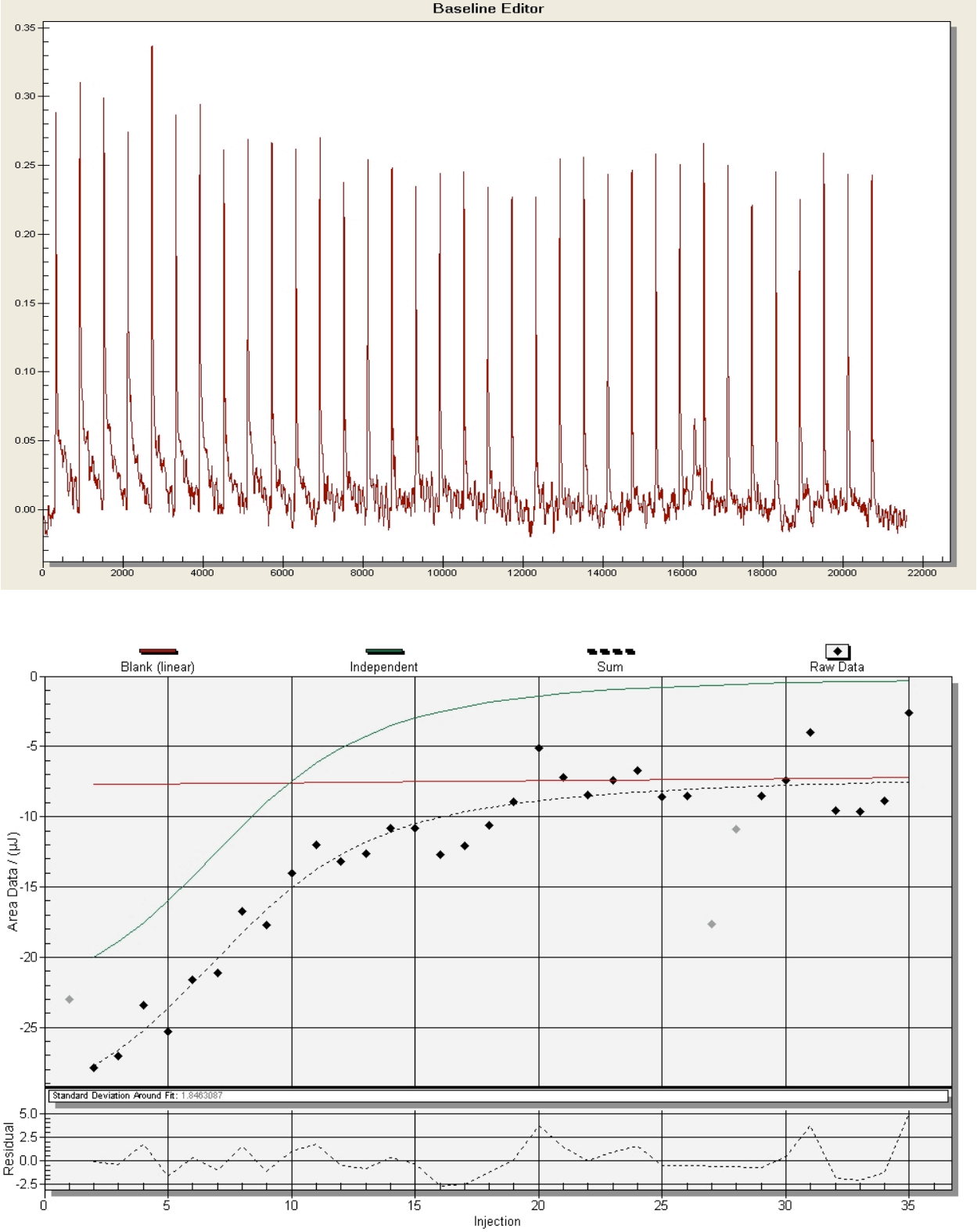


Figure S30. ITC data for binding of **PNA4 (P_{ex})** to **HRP3 (C-G)** pH 6.25.

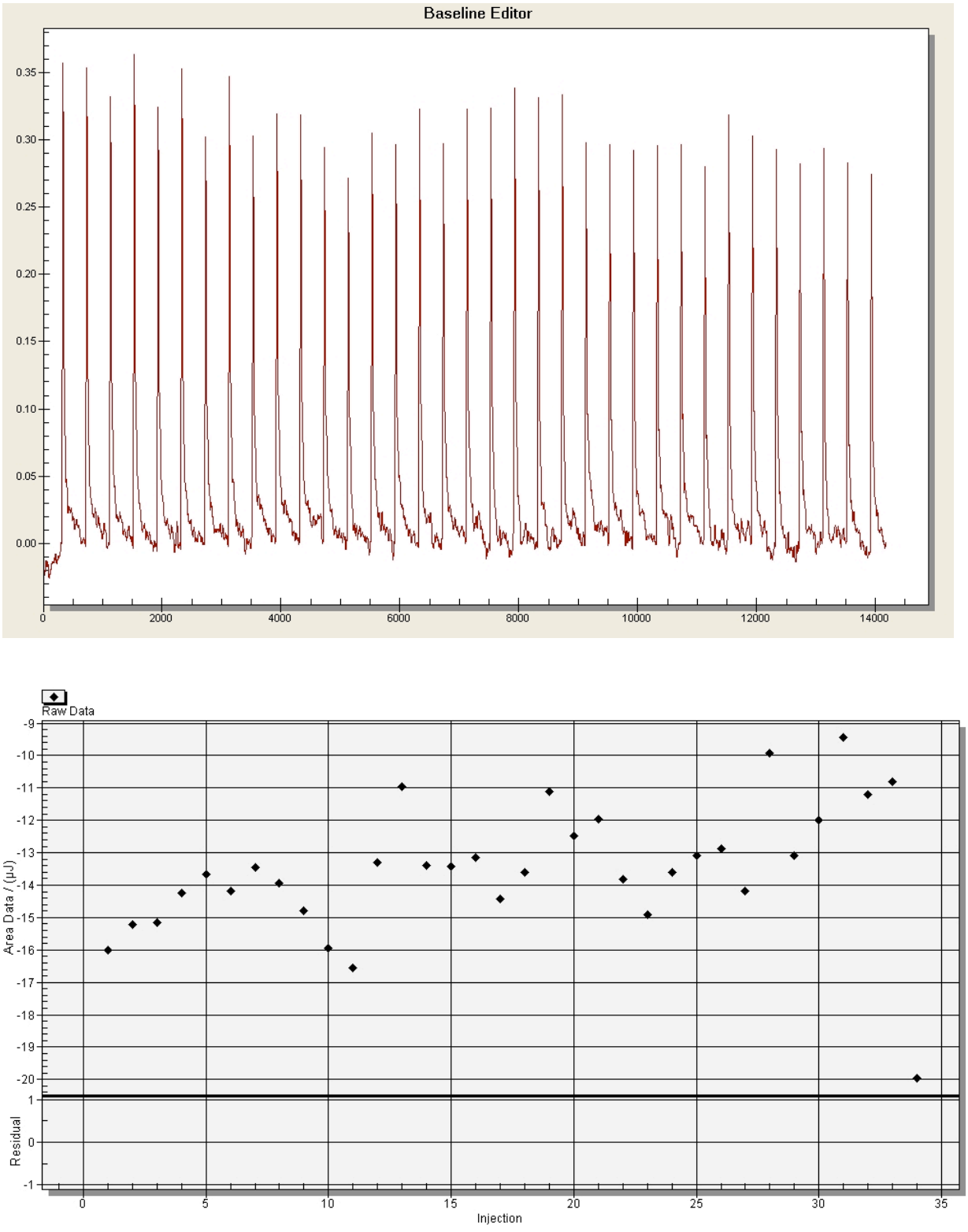


Figure S31. ITC data for binding of **PNA4 (P_{ex})** to **HRP4 (U-A)** pH 6.25.

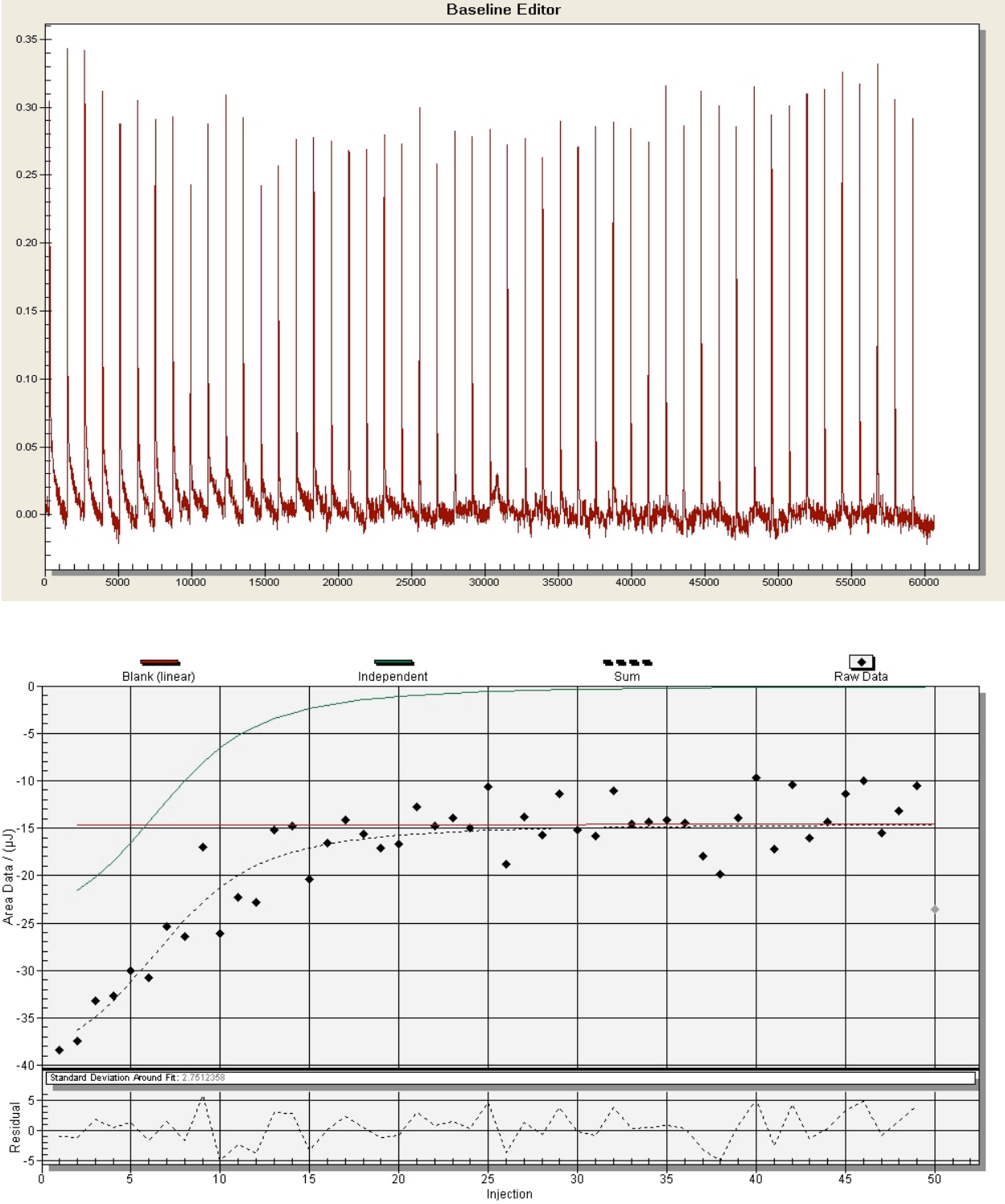


Figure S32. ITC data for binding of **PNA5 (E)** to **HRP1 (G-C)** pH 6.25.

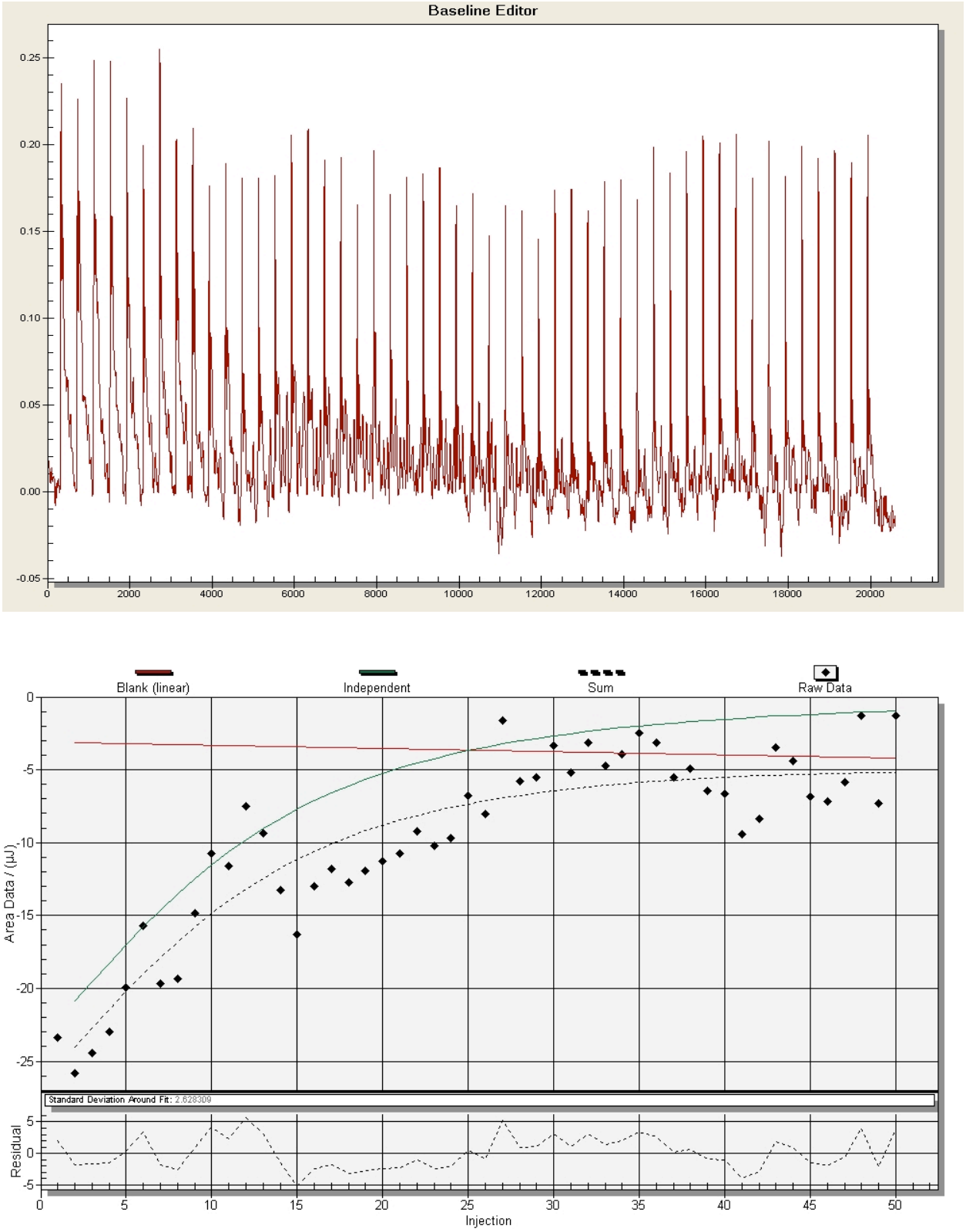


Figure S33. ITC data for binding of **PNA5 (E)** to **HRP2 (A-U)** pH 6.25.

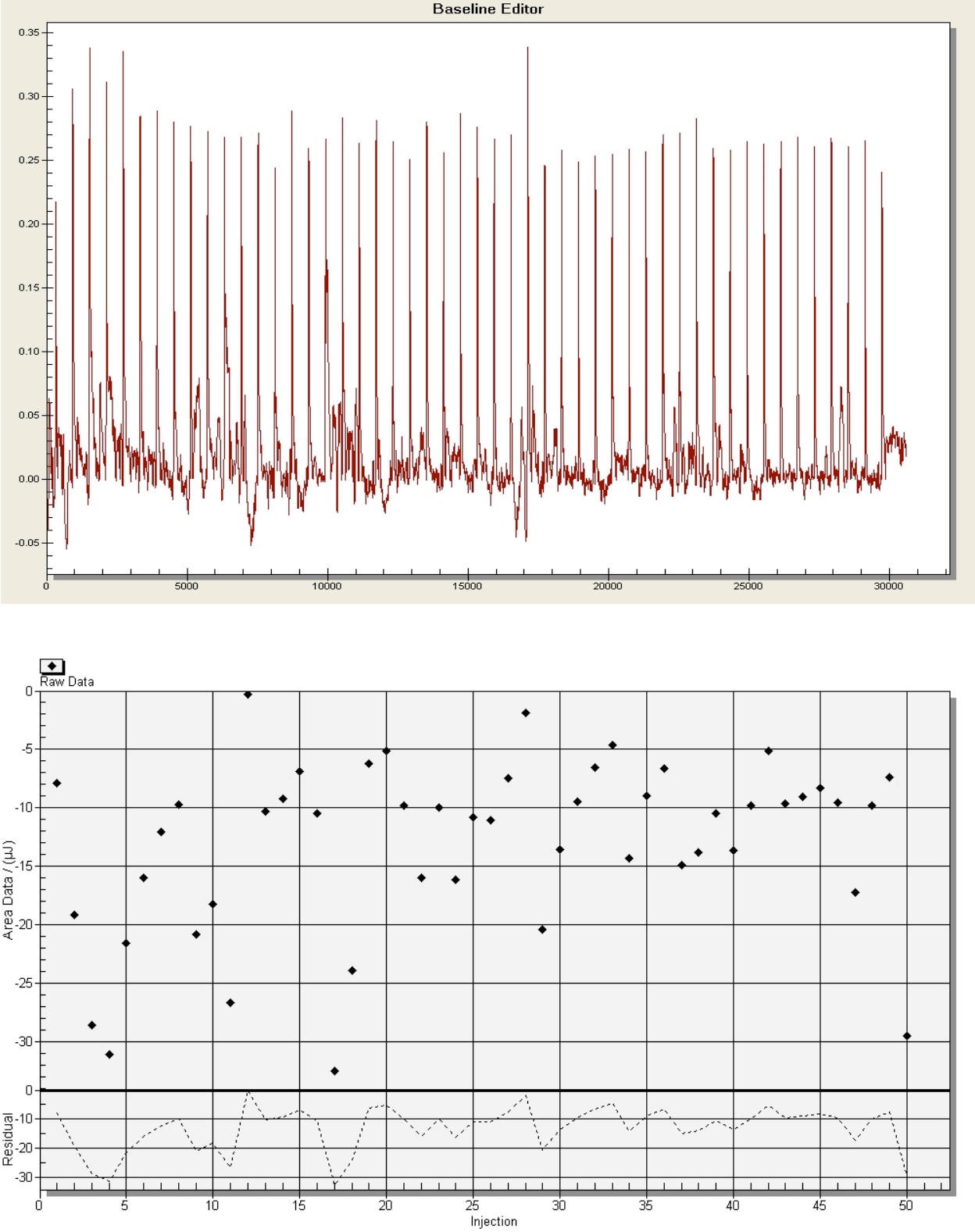


Figure S34. ITC data for binding of **PNA5 (E)** to **HRP3 (C-G)** pH 6.25.

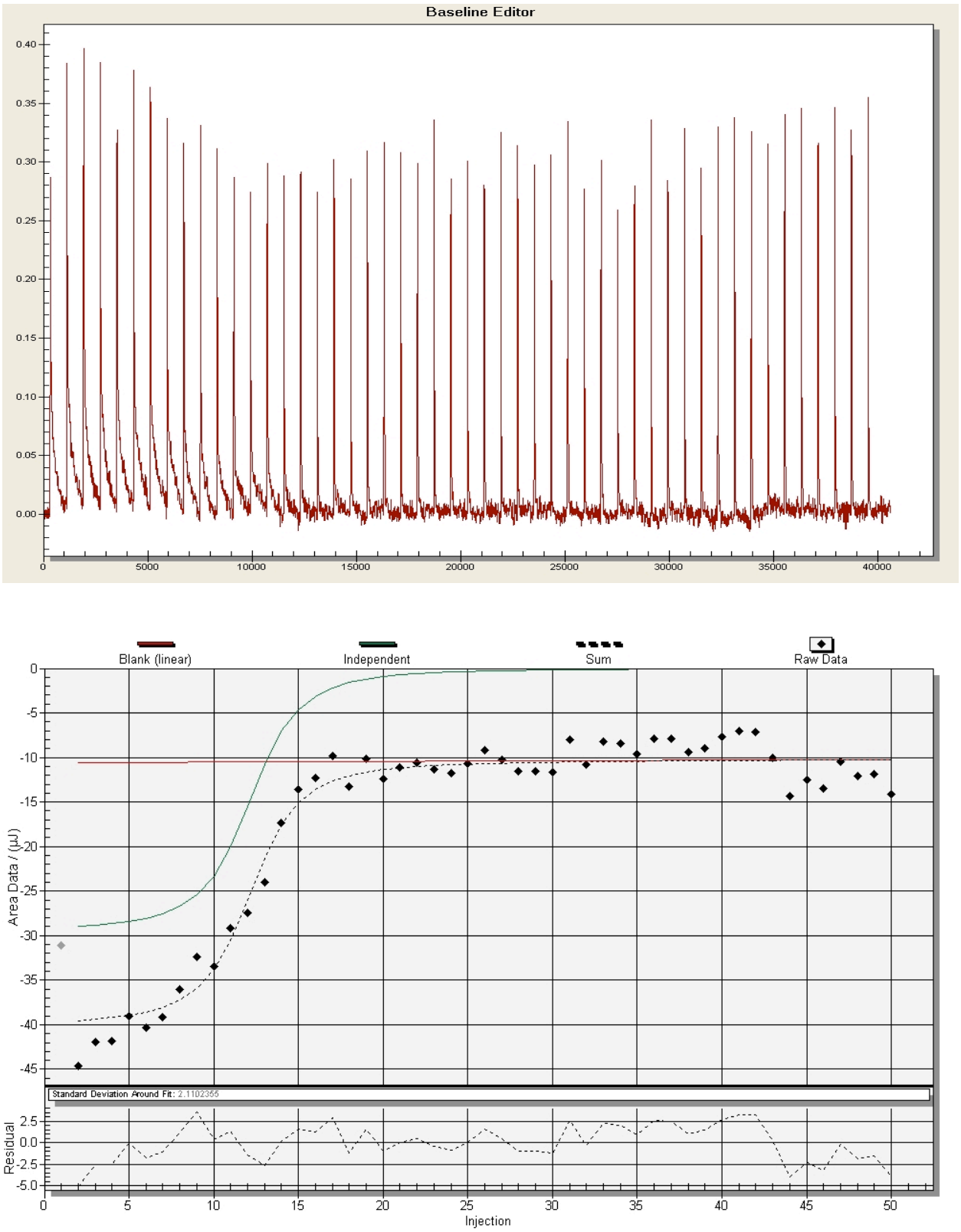


Figure S35. ITC data for binding of **PNA5 (E)** to **HRP4 (U-A)** pH 6.25.

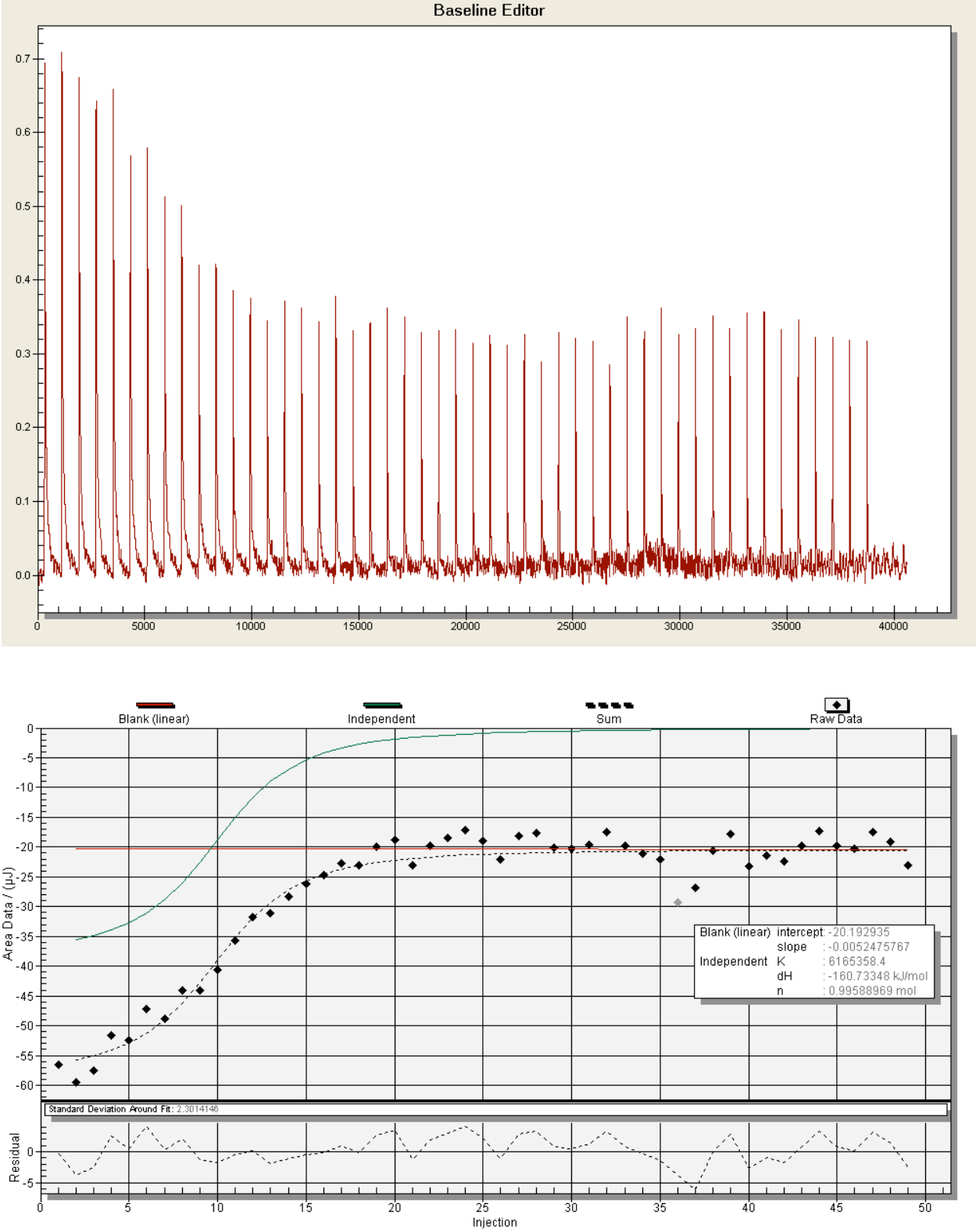


Figure S36. ITC data for binding of **PNA6** to **HRP5** (bacterial A-site) at pH 6.25.

Table S1. Experimental results of ITC titrations – analyzed data.

Sequence	K_a	ΔH	ΔS	ΔG	order
		HRP1			
PNA1 (5.5)	**8.90E+08**	**-24.8**	**-42**	**-12.2**	**1.0**
PNA2 (5.5)	**7.20E+07**	**-35.6**	**-83**	**-10.7**	**1.1**
PNA3 (5.5)	**9.49E+06**	**-25.8**	**-55**	**-9.5**	**1.2**
PNA4 (5.5)	**4.71E+07**	**-46.1**	**-120**	**-10.5**	**1.2**
PNA5 (5.5)	**2.70E+08**	**-33.9**	**-75**	**-11.5**	**1.0**
PNA1 (6.25)	6.80E+06	-22.5	-44	-9.3	1.3
	9.40E+06	-32.5	-77	-9.5	1.2
average	**8.10E+06**	**-27.5**	**-61**	**-9.4**	**1.3**
standard dev	1.84E+06	7.1	23	0.1	0.1
PNA2 (6.25)	**7.50E+06**	**-25.3**	**-53**	**-9.4**	**1.0**
PNA4 (6.25)	**<10E+4**	**No binding detected**			
PNA5 (6.25)	2.90E+06	-27.2	-62	-8.8	0.7
	3.20E+06	-18.6	-33	-8.9	1.2
average	**3.05E+06**	**-22.9**	**-47**	**-8.8**	**1.0**
standard dev	2.12E+05	6.1	21	0.0	0.4
		HRP2			
PNA1 (5.5)	**9.60E+07**	**-23.4**	**-42**	**-10.9**	**2.0**
PNA2 (5.5)	**8.20E+08**	**-39.6**	**-92**	**-12.2**	**1.4**
PNA3 (5.5)	**2.81E+06**	**-62.3**	**-180**	**-8.8**	**0.3**
PNA4 (5.5)	1.53E+08	-43.0	-107	-11.2	1.2
	1.91E+08	-40.8	-99	-11.3	1.2
average	**1.72E+08**	**-41.9**	**-103**	**-11.2**	**1.2**
standard dev	2.69E+07	1.5	5	0.1	0.0
PNA5 (5.5)	**2.10E+08**	**-40.6**	**-98**	**-11.3**	**1.1**
PNA1 (6.25)	**<10E+4**	**No binding detected**			
PNA2 (6.25)	3.70E+07	-23.2	-43	-10.3	0.9
	1.20E+07	-33.4	-80	-9.7	1.3
	1.10E+07	-31.3	-73	-9.6	1.1
average	**2.00E+07**	**-29.3**	**-65**	**-9.9**	**1.1**
standard dev	**1.47E+07**	**5.4**	**19**	**0.4**	**0.2**

PNA4 (6.25)	**<10E+4**	**No binding detected**			
PNA5 (6.25)	**4.70E+05**	**-43.2**	**-119**	**-7.7**	**0.9**
		HRP3			
PNA1 (5.5)	**7.40E+07**	**-47.1**	**-122**	**-10.7**	**0.9**
PNA2 (5.5)	**6.20E+07**	**-40.4**	**-100**	**-10.6**	**1.2**
PNA3 (5.5)	5.40E+07	-35.3	-83	-10.5	1.1
	3.88E+07	-39.6	-98	-10.3	0.8
average	**4.64E+07**	**-37.5**	**-91**	**-10.4**	**1.0**
standard dev	1.07E+07	3.0	11	0.1	0.2
PNA4 (5.5)	**2.93E+08**	**-47.5**	**-121**	**-11.5**	**1.0**
PNA1 (6.25)	**<10E+4**	**No binding detected**			
PNA2 (6.25)	**<10E+4**	**No binding detected**			
PNA4 (6.25)	4.10E+06	-24.8	-53	-9.0	0.9
	4.78E+06	-19.8	-36	-9.1	0.9
average	**4.44E+06**	**-22.3**	**-45**	**-9.1**	**0.9**
standard dev	4.81E+05	3.5	12	0.1	0.0
PNA5 (6.25)	**<10E+4**	**No binding detected**			
		HRP4			
PNA1 (5.5)	**3.20E+06**	**-29.6**	**-70**	**-8.9**	**1.7**
PNA2 (5.5)	**8.00E+06**	**-42.3**	**-110**	**-9.4**	**1.0**
PNA3 (5.5)	**3.16E+06**	**-38.2**	**-98**	**-8.9**	**0.5**
PNA4 (5.5)	**5.61E+06**	**-41.6**	**-109**	**-9.2**	**0.9**
PNA5 (5.5)	**3.80E+07**	**-46.1**	**-120**	**-10.3**	**0.9**
PNA1 (6.25)	**<10E+4**	**No binding detected**			
PNA2 (6.25)	**8.90E+05**	**-24.4**	**-55**	**-8.1**	**1.0**
PNA4 (6.25)	**<10E+4**	**No binding detected**			
PNA5 (6.25)	2.00E+07	-29.4	-65	-10.0	1.2
	3.60E+07	-39.2	-97	-10.3	0.8
average	**2.80E+07**	**-34.3**	**-81.0**	**-10.1**	**1.0**
standard dev	1.13E+07	6.9	22.4	0.2	0.3

HRP5

PNA6 (6.25)	4.22E+06	-44.2	-118	-9.0	1.1
	6.16E+06	-38.5	-98	-9.3	1.0
average	**5.19E+06**	**-41.3**	**-108**	**-9.1**	**1.1**
standard dev	1.37E+06	4.1	14	0.2	0.1

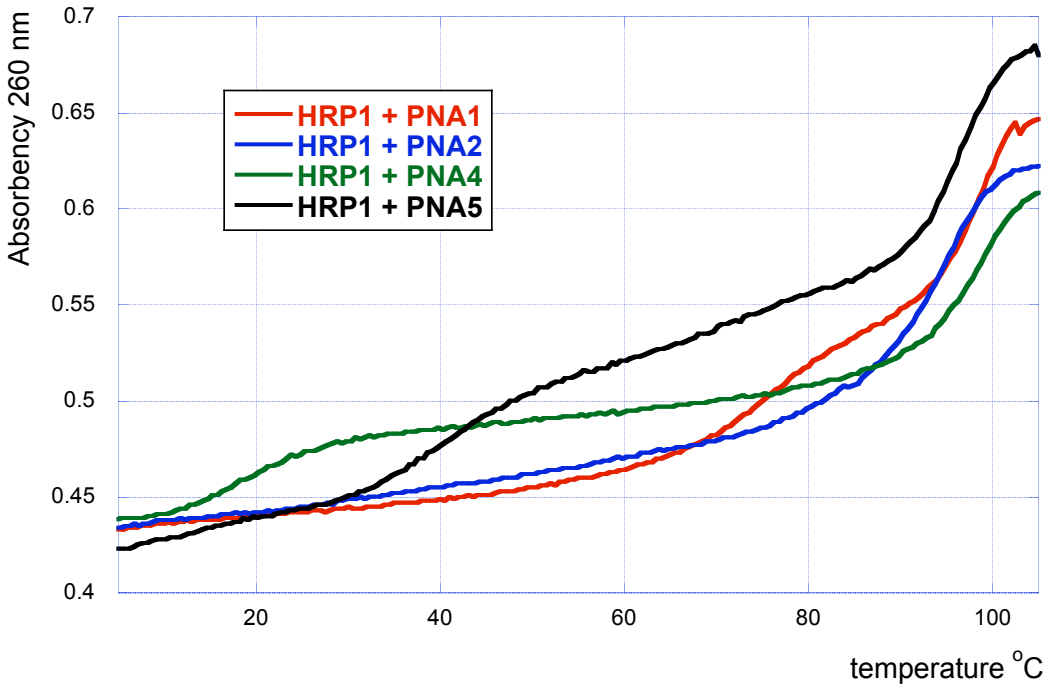


Figure S37. UV thermal melting of **HRP1** triplexes at pH 6.25.

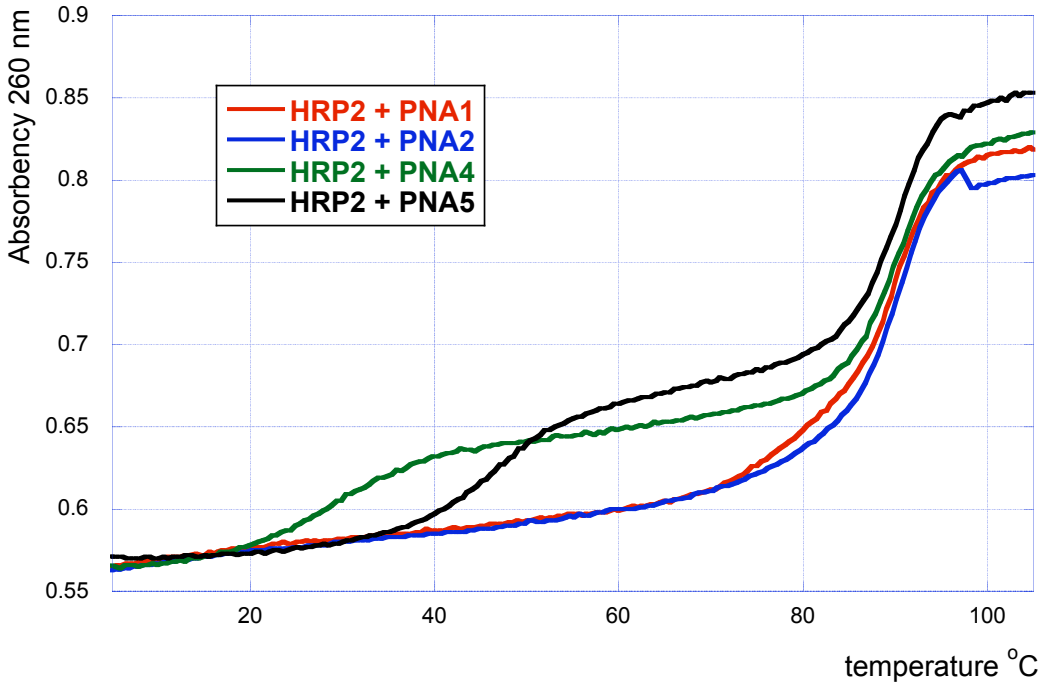


Figure S38. UV thermal melting of **HRP2** triplexes at pH 6.25.

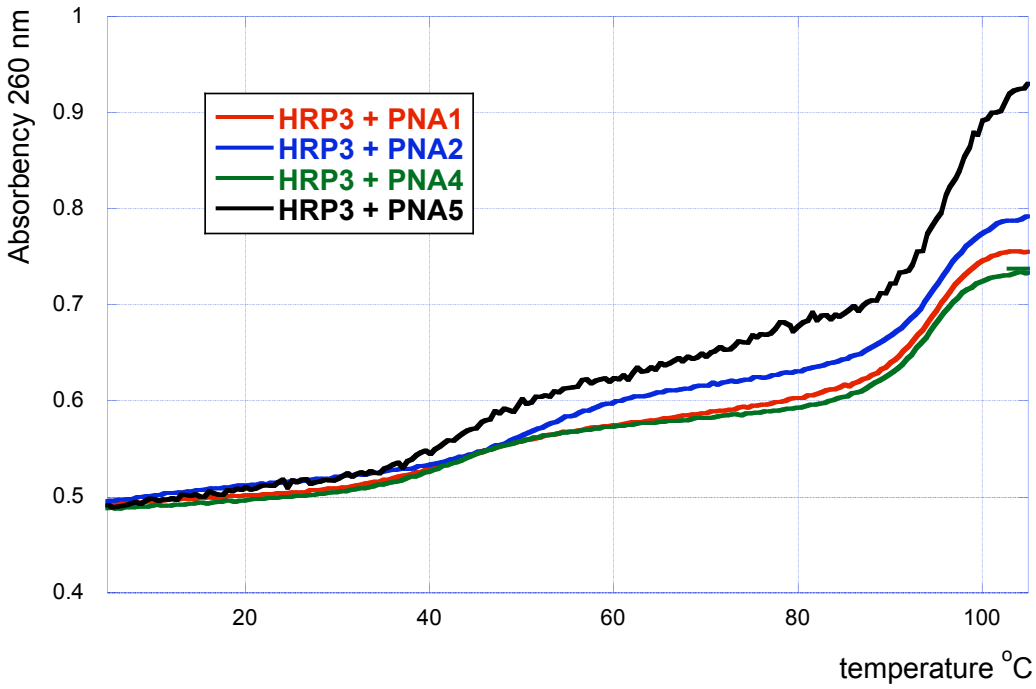


Figure S39. UV thermal melting of **HRP3** triplexes at pH 6.25.

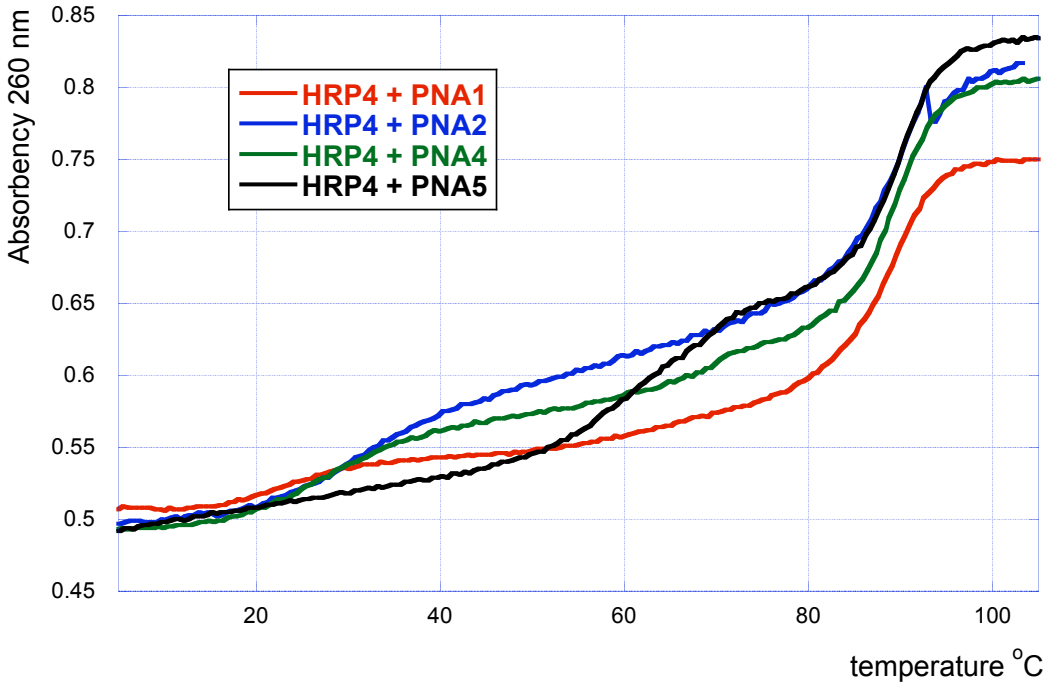
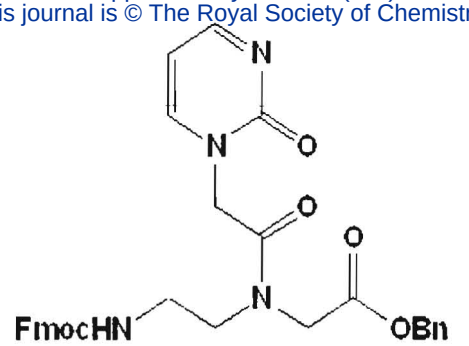


Figure S40. UV thermal melting of **HRP4** triplexes at pH 6.25.

Table S2. UV thermal melting of PNA-RNA complexes at pH 6.25 [a]

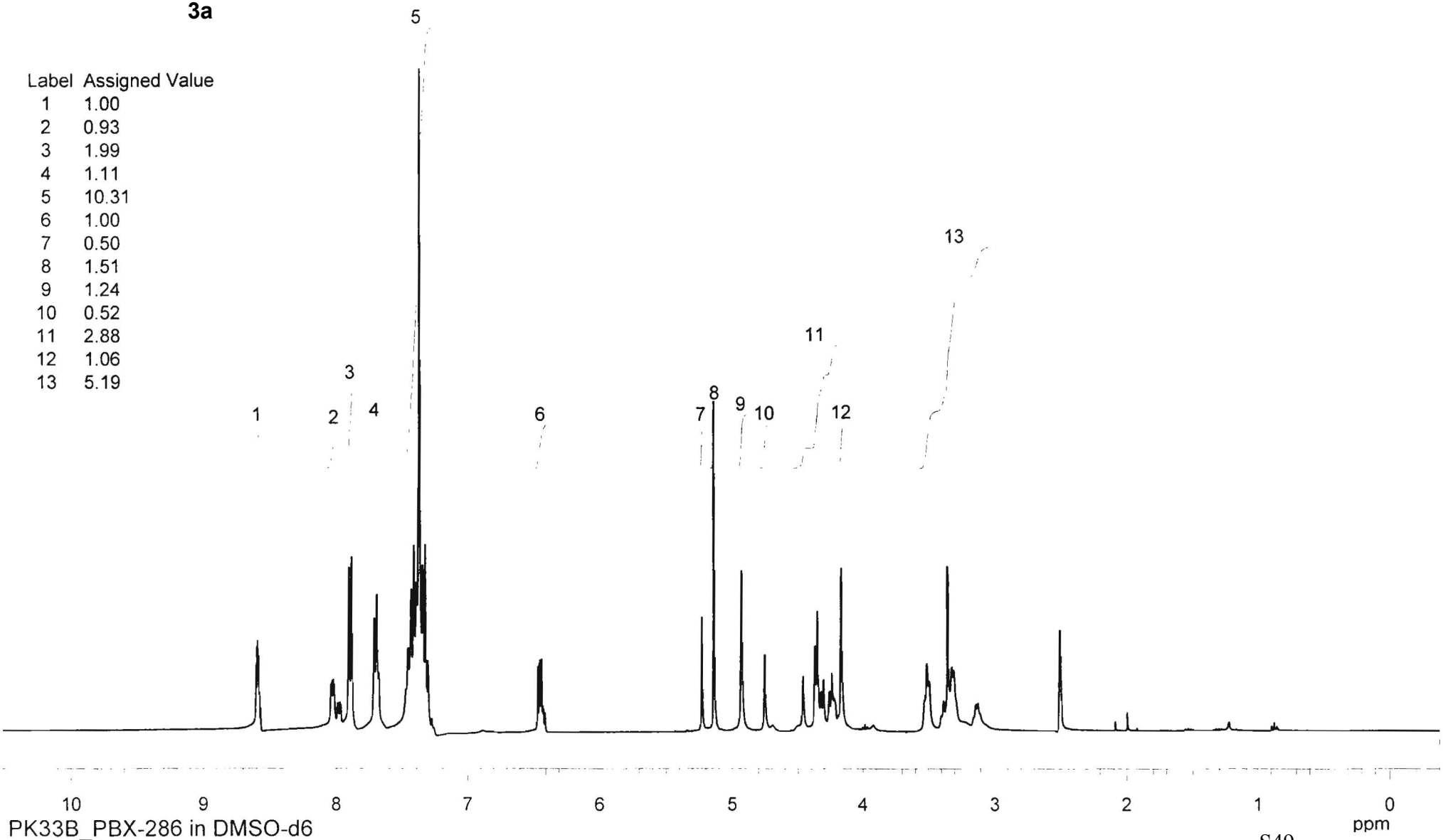
Entry	PNA (variable base)	HRP1 (**G**-C)	HRP2 (**A**-U)	HRP3 (**C**-G)	HRP4 (**U**-A)
1	**PNA1** (C)	**>70** [b]	NT [c]	40	23
2	**PNA2** (T)	NT [c]	**NT** [c]	51	31
3	**PNA4** (P_{ex})	19	29	**43**	27
4	**PNA5**(E)	39	45	44	**>60** [b]

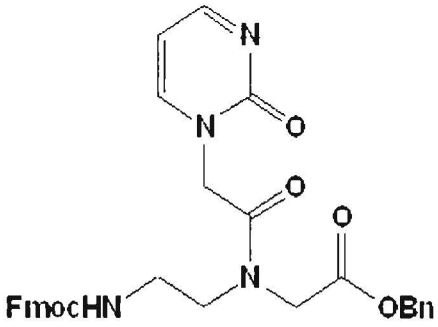
[a] Melting temperatures (°C) in sodium acetate buffer, pH 6.25; [b] Lower estimate; overlap with the duplex melting hinders precise measurement; [c] In some cases we observed only the high temperature transition, which we interpreted as either one step triplex to single strands melting for the highly stable **HRP2-PNA1** and **HRP2-PNA2** complexes, or as no triple helix formation for **HRP2-PNA1**, which had low stability according to ITC results.



3a

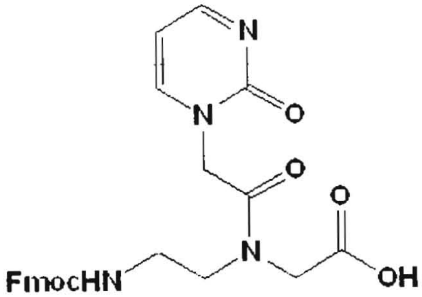
Label	Assigned Value
1	1.00
2	0.93
3	1.99
4	1.11
5	10.31
6	1.00
7	0.50
8	1.51
9	1.24
10	0.52
11	2.88
12	1.06
13	5.19





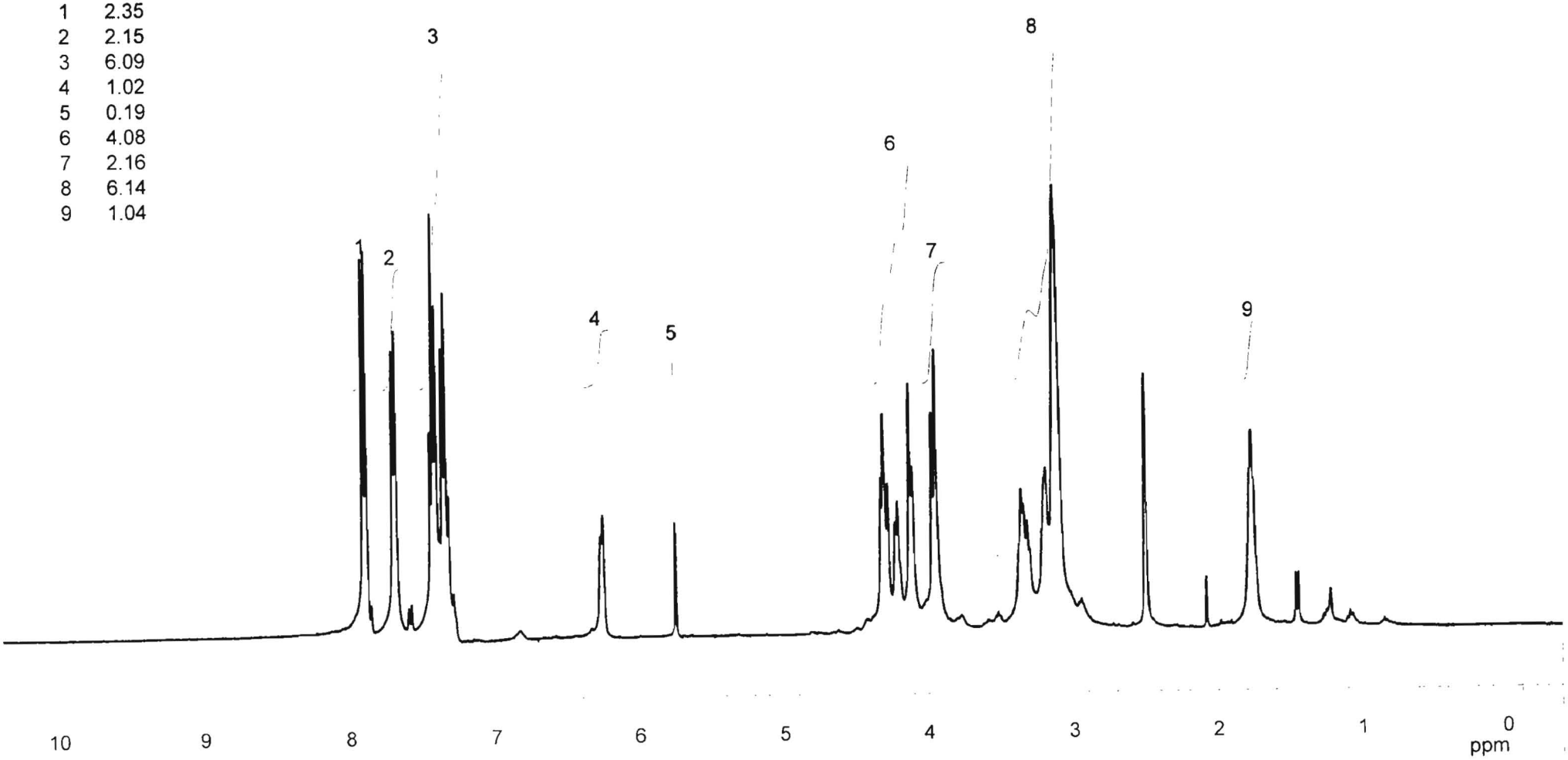
3a

PK33B_PBX-286 in DMSO-d6

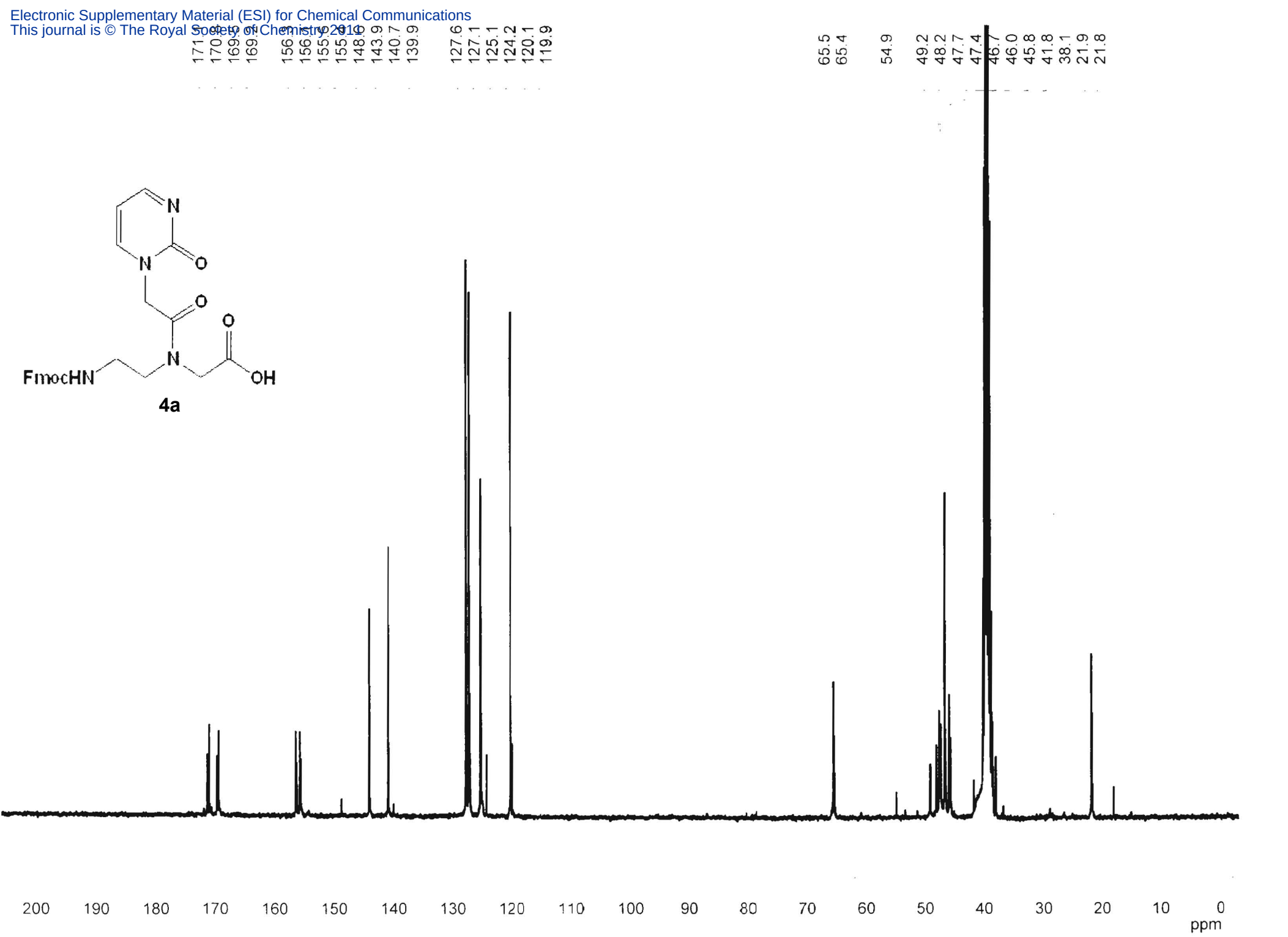


4a

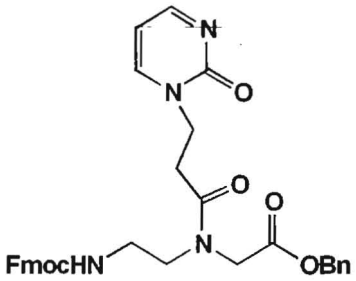
Label	Assigned Value
1	2.35
2	2.15
3	6.09
4	1.02
5	0.19
6	4.08
7	2.16
8	6.14
9	1.04



PK34_PBXM-287 in DMSO-d6

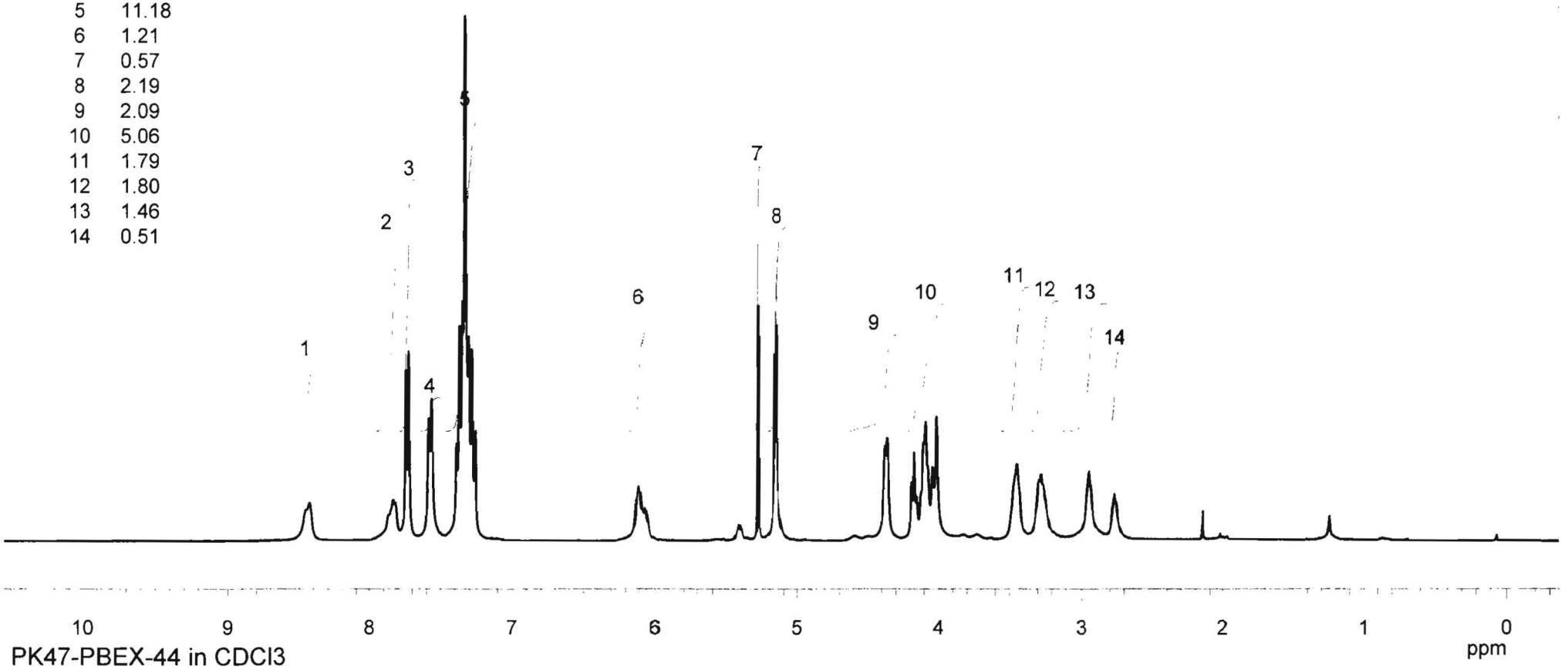


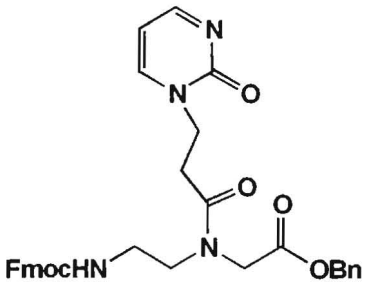
PK34_PBXM_in DMSO-d6



3b

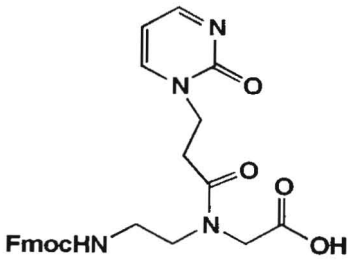
Label	Assigned Value
1	1.00
2	0.85
3	2.04
4	2.19
5	11.18
6	1.21
7	0.57
8	2.19
9	2.09
10	5.06
11	1.79
12	1.80
13	1.46
14	0.51





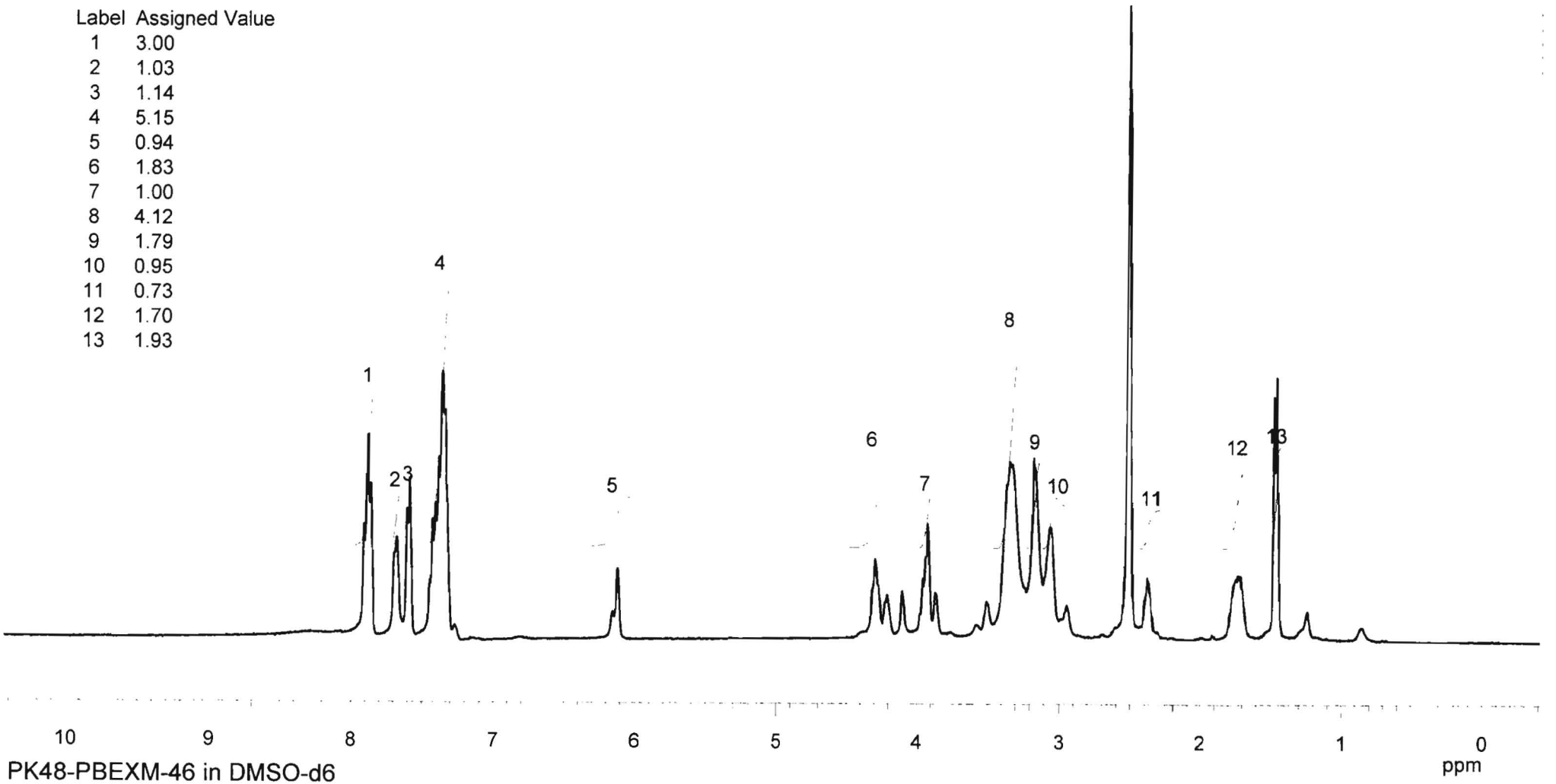
3b

PK47-PBEX-44 in CDCl3

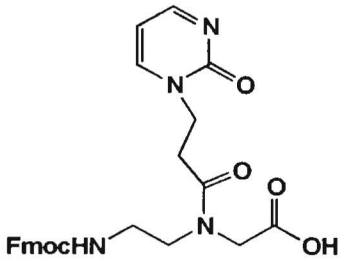


4b

Label	Assigned Value
1	3.00
2	1.03
3	1.14
4	5.15
5	0.94
6	1.83
7	1.00
8	4.12
9	1.79
10	0.95
11	0.73
12	1.70
13	1.93



PK48-PBEXM-46 in DMSO-d6



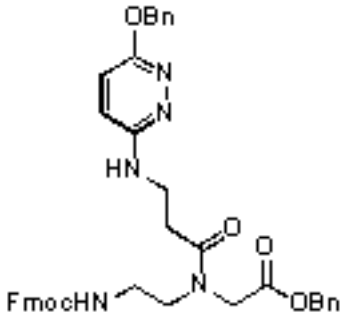
4b

PK48-PBEXM-46 in DMSO-d6

H-1 in DMSO-d6

ppm

Label	Assigned Value
1	2.00
2	1.54
3	13.52
4	1.66
5	0.84
6	1.62
7	1.63
8	1.85
9	0.62
10	0.88
11	0.60
12	2.31
13	1.61
14	0.95
15	1.01
16	0.14
17	0.89
18	0.20

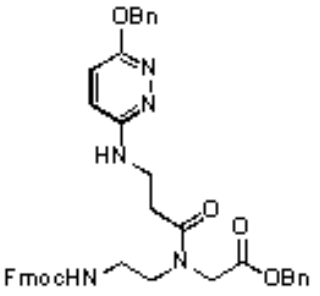


6

C-13 NMR Spectrum in DMSO-d6

ppm

171.90 171.65 169.79 169.37 158.50 156.04 143.90 143.84 140.73 137.30 135.90 128.43 128.30 128.13 128.03 127.94 127.82 127.71 127.58 127.03 125.11 125.03 120.55 120.08 119.20 67.31 65.83 65.43 65.33 47.65 47.52 46.73 37.35 37.27 33.34 31.45 24.45



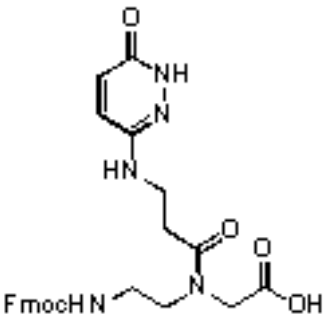
6

230.0 220.0 210.0 200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0 0.0 -10.0

F-1 in DMSO-d6

ppm

Label	Assigned Value
1	1.00
2	2.72
3	2.65
4	2.87
5	3.04
6	1.29
7	1.22
8	0.95
9	0.11
10	2.86
11	2.04
12	1.51
13	1.30
14	1.06
15	0.16
16	1.08
17	0.95

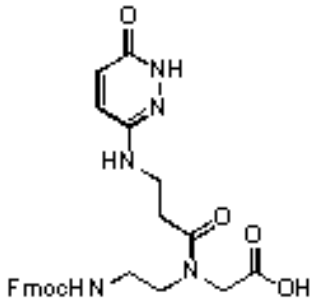


7

C-13 NMR Spectrum in DMSO-d6

ppm

174.76
171.70
171.17
170.97
158.66
156.12
147.29
143.90
143.84
140.71
130.75
130.70
128.10
127.60
127.05
125.17
125.05
120.09
65.43
65.40
54.90
47.47
46.72
46.55
38.26
37.32
37.24
31.53
31.12



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