Supporting Information

GOx signaling triggered by aptamer-based ATP detection

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General Experimental Procedure. MagnaBind™ Streptavidin coated beads in phosphate-buffered saline (PBS) pH 7.5 containing 0.1% BSA, EDTA and sodium azide, were supplied by Thermo Scientific. Beads were saved at 4°C and opened just prior to use. The binding capacity of beads is 8 nmol of biotin/mL of beads. The 5' biotinylated, native and mutated anti-ATP aptamers and complementary sequences of these aptamers in desalted form were supplied by IDT and were used as received. Adenosine–triphosphate disodium salt hydrate (ATP), Cytidine 5'–triphosphate disodium salt (CTP), Guanosine 5' triphosphate sodium salt hydrate (GTP), Glucose oxidase from *Aspergillus niger* Type VI-A, Flavin adenine dinucleotide disodium salt hydrate (FAD), 4-Aminoantipyrine, 3, 5–dichloro-2-hydroxybenzesulfonate, sodium phosphate monobasic, and glucose were purchased from Sigma Aldrich. Sodium phosphate dibasic (EMD), magnesium chloride (Acros Organic) and Ultrapure glycerol (Invitrogen) were used as received. Sodium chloride, magnesium chloride, ammonium sulfate and Tris hydrochloride were purchased from Fischer Scientific. The anti-ATP aptamer (ATP-A) binding buffer composition was adapted from a previously reported procedure (Tris 20 mM, NaCl 300 mM , $MgCl_2$ 5 mM, pH 7.54)(1).

Water from Milli-Q-Ultra pure system (resistivity of 18.0 megaohm-cm) was autoclaved prior to use in buffers and in the preparation of other reagents. Glucose assay was carried out by measuring the initial rate of the enzyme (reactivated or native) by using the kinetic mode on a BeckMan Coulter Du-800 Spectrophotometer at 510 nm. ATPA absorbance was measured in Synergy NEO-HTS Multimode Microplate Reader. Disposable semi-micro cuvettes (1.5 mL) of an optical path length 10 mm were used for absorbance measurements. MicroCal Auto-ITC_{200} from GE Healthcare was used for binding studies. Nunc 96 deep well -1.3 mL microplates were purchased from Thermo Scientific.

Purification of apo-Gox

Dissociation of FAD from glucose oxidase (GOx) was adapted from a previously reported method (2). Concentration of purified apoGOx was calculated by comparing absorbance of GOx (2mg/mL) and apo-GOx measured at 278 nm using the UV-Vis. The activity of purified apo-GOx was determined by incubating apo-GOx (2mg/mL) with concentrations of FAD. Aliquots of apo-GOx were diluted to 2mg/mL prior to carrying out the glucose assay.

Glucose assay

Glucose (550 mM) was prepared in water and left to mutarotate for 3 h. 4-Aminoantipyrine (9.98mM) and 3, 5 –dichloro-2-hydroxybenzesulfonate (11.36mM) stocks were prepared in water and saved at 4°C. Stock 4-Aminoantipyrine 3.5 mL and 3,5-dichloro-2-hydroxybenzenesulfonate 3.5 mL were mixed together and diluted with 3 mL phosphate buffer (0.1M, pH 6.2) just prior to the assay. Horseradish peroxidase (POD) was diluted to 60U/mL.

A typical glucose assay was carried out by mixing the following reagents to a total volume of 1 mL in a 1.5 mL disposable cuvette. Diluted and pre-mixed solutions of 4-aminoantipyrine and 3, 5-dichloro-2-hydroxybenzensulfonate in 0.1M phosphate buffer pH 6.2 (500 µL) was transferred to a cuvette. Solutions of glucose (550 mM, 50 µL) and POD (60U/mL, 50 µL) were added to the cuvette followed by the addition of water (390 µL). Reactivated apo-Gox (10 µL) was added to cuvette and intial rate of change in absorbance was measured every 15seconds for 2 mins.

FAD displacement experiment

The experimental details of dose dependent FAD displacement by ATP are described herein. MagnaBind beads (250 µL, 2 nmol biotin) were transferred to six different tubes respectively. The beads were washed with 0.1M phosphate buffer pH 7.4 (200 µL × 2) followed by 200 µL binding buffer (Tris 20 mM, NaCl 300 mM , $MgCl_2$ 5 mM, pH 7.5). After the washing step, 5'-Biotinylated ATP-A (50 µM, 200 µL) was added to the beads contained within the six reaction tubes respectively. The control tubes contained only 200 µL of binding buffer. The reaction tubes were incubated at 50 °C for 30 min followed by 2 h at room temperature. Excess 5'-biotinylated ATP-A was separated from the beads by using a magnet to separate the bead and solution slurry. The amount of ATP-A present on the beads was calculated by subtracting ATP-A concentration before and after incubation with the beads by measuring absorbance at 260 nm. On average, 5.7 nmol ATP-A was calculated to be bound on the beads under the conditions mentioned in these experments. Beads containing bound ATP-A in the reaction tubes were washed with binding buffer (100 µL, 2X). The remaining trace buffer was completely removed from the beads using the magnet. Solutions of FAD (50 µL, 1.2×10^{-6}M) were added to each tube containing ATP-A conjugated to MagnaBind. The mixture in the tubes were incubated at 4 °C overnight. The excess unbound FAD supernatant was removed from the beads using the magnet and saved in reaction tubes labeled as No ATPA- control, F-1, F-2, F-3, F-4 and F-5 (see main text Figure 2B). The beads were washed with binding buffer (50 µL). Solutions of ATP (50 µL) at concentrations in the range of 10 to 500 µM were added to the tubes containing the ATP-A/FAD (and controls) bead complex. The control tubes only received the 25µM ATP (50 µL) solution. The tubes were gently vortexed at 4 °C for 8h. The respective reaction supernatants were removed from the beads and collected in separate tubes labeled appropriately for each of the concentrations. Solutions (20 µL) of the supernatant containing displaced FAD were transferred to 0.5 mL reaction tubes respectively. Aliquots of apo-GOx (20 µL, 2 mg/mL) were added to each individual tube containing the displaced (and control) FAD solutions. All the tubes were incubated at room temperature and gently vortexed for 3 h. Simultaneously, solutions of FAD 1.2×10^{-6} M in binding buffer (positive control) and an aliquot of only binding buffer (negative control) were also incubated with apo-GOx (2mg/mL) and vortexed. The previously described Glucose assay was carried out with 10 µL of the reactivated apo-GOx using freshly prepared reagents.

For the selectivity experiments, GTP and CTP were used to displace FAD in addition to ATP. The remaining procedures were similar to those described in the previous section.

ATP-A was mutated (G to A) at positions G22, G9 and both G9/22 to determine the putative binding site for FAD. In this particular experiment, mutated 5'-biotinylated ATP-A sequences were conjugated with MagnaBind by adapting experimental details used in the FAD displacement experiments. In addition to ATP-A, G22 mutated ATP-A (G22M), G9 mutated ATP-A (G9M) and G9/22 mutated ATPA (G9/22M) were also conjugated to different aliquots of MagnaBind beads respectively. The complementary

sequence of each of the respective aptamers (60 µM, 50 µL) was added to the reaction tubes. FAD displaced by complementary sequences was used for reactivation of apo-GOx as previously described.

Total amount of FAD displaced by ATP

The experimental procedures for ATP-A bead binding and FAD complexation were described in the previous section. After removal of the excess FAD supernatant, the ATP-A/FAD complexed beads were washed with 100µl binding buffer. ATP (250 and 500 µM) was added to different tubes containing the complex under similar conditions. The released FAD was used to reactivate apo-GOx and determine percent release. With concentrations of 250µM ATP, ~96% of the ATP-A bound FAD is released (Total ATP-A bound FAD is determined from the subtraction of FS-1 and FS-2 from FS-Ctrl respectively, Figure S1). Addition of 500 µM ATP showed a further 2% increase in FAD release, and signifies the upper limit of this assay.

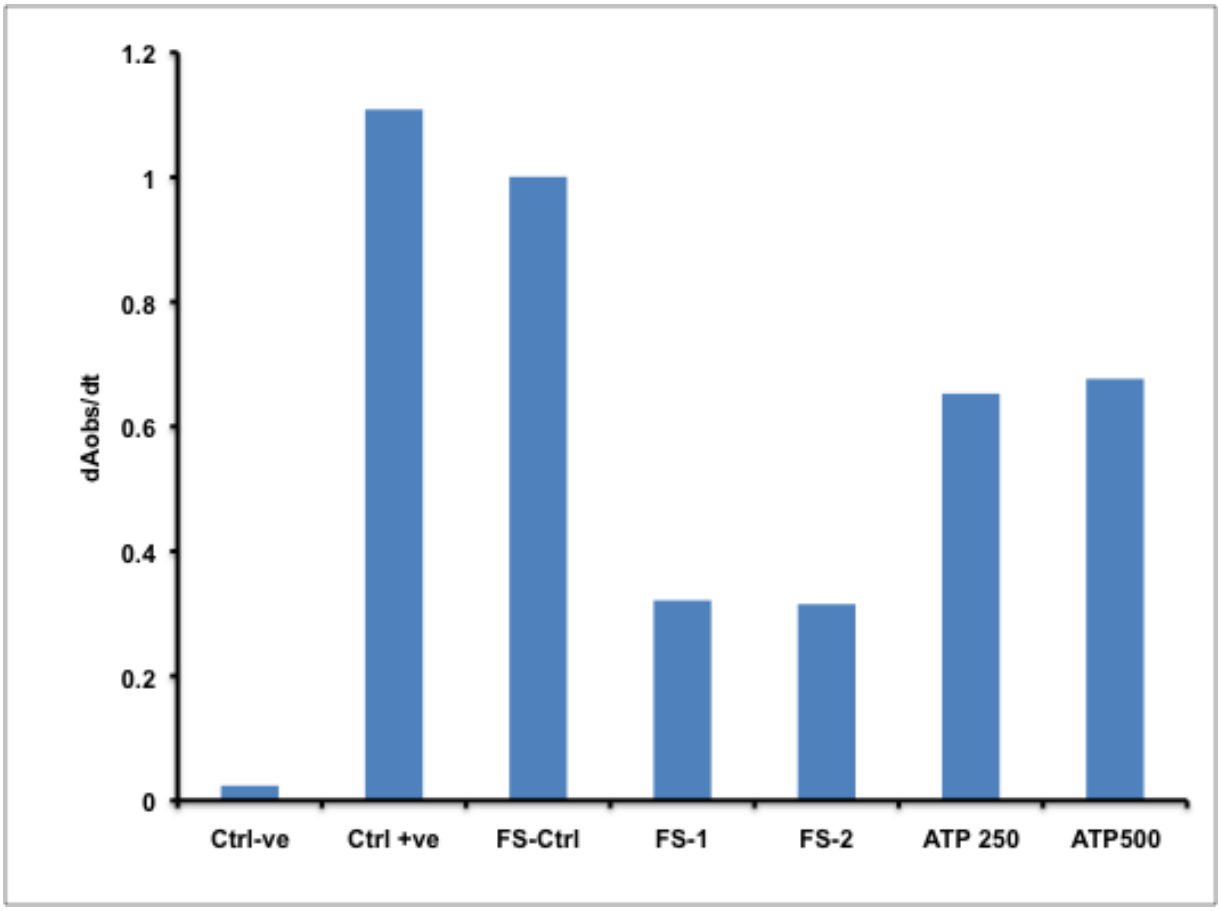

Figure S1: The data shows the leftover FAD (FS-1 and FS-2) from the initial ATP-A/FAD complex. This data can be subtracted from the FS-control peak in order to gauge the maximum bound FAD. The ATP 250 and ATP 500 data show the initial velocity measured from the FAD that is released from the ATP-A/FAD complex in the presence of 250µM and 500µM ATP respectively. The data set also includes the positive and negative controls showing the maximum FAD related signal (if 100% of the FAD added was

bound to ATP-A) and the residual signals from apo-GOx.

Stability of ATPA-FAD complex

Magna beads (250 µL) were transferred in 5 tubes. All the steps were similar as in previous experiments as mentioned above. After removal of supernatant from FAD -ATPA magnabeads, the beads were washed with 100 µL binding buffer. Then, binding buffer (50 µL) was added in each tubes and vortexed gently for 1 to 4 days at 4 °C. The glucose assay was carried out for supernatant of tube 1 after day 1 whereas tube 2, 3, 4 were measured after 2, 3, 4 days respectively. Figure S2 shown below is the initial rate versus buffer supernatants of tubes from the different days. The fourth day sample shows less than 15 % FAD dissociated from FAD-ATPA complex in buffer when compared to total amount of FAD bound to the ATP-A. There is minimal difference between the FAD that is released from day 1 to day 4 of the study.

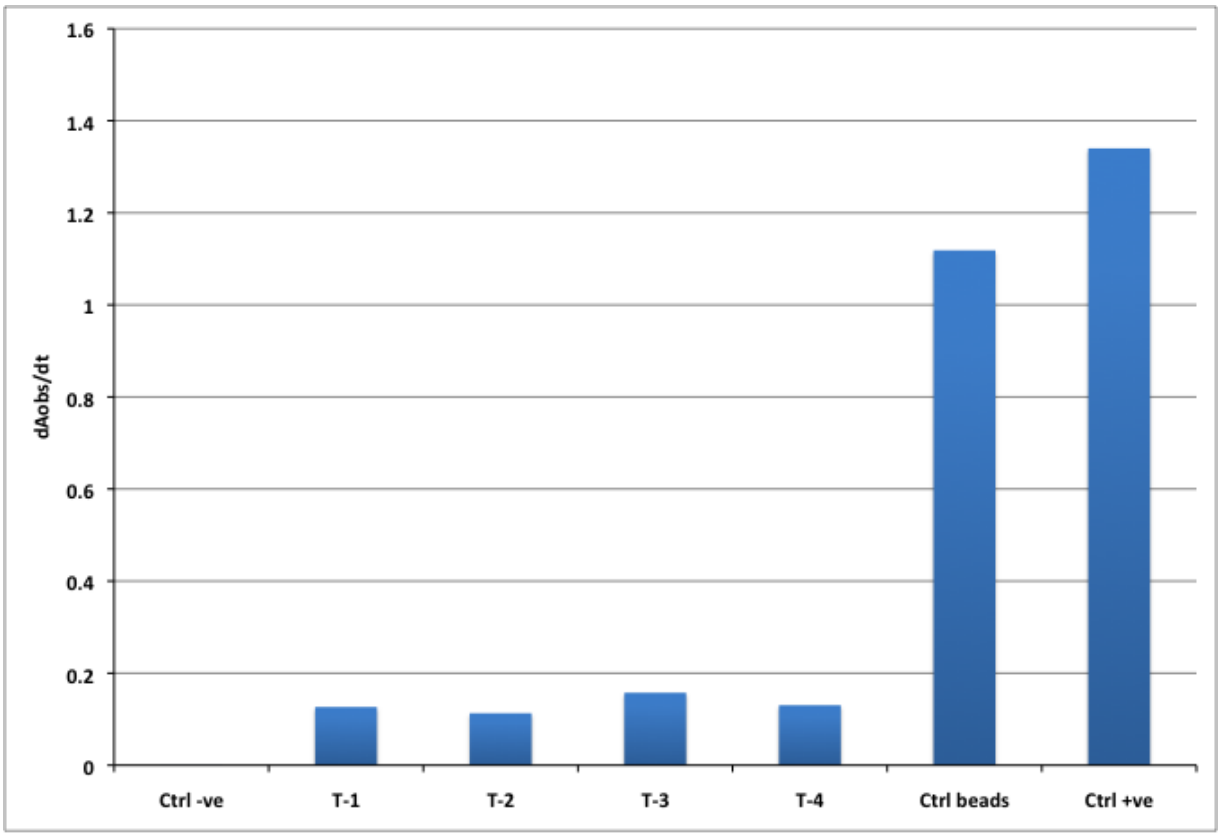

Figure S2: Tubes T-1 to T-4 contained ATP-A bound to FAD. This data (T-1 to T-4) shows the initial velocity measured from the FAD that is released from the ATP-A/FAD complex in the absence of ATP after excess FAD is removed. T-1 was measured after day 1, T-2 after day 2, T-3 after day 3 and T-4 after day 4. The control bead represents the maximum amount of reactivation from FAD in the absence of aptamer. The positive control aliquot does not contain aptamer or beads and thus represents the maximum reactivation of enzyme with the total FAD that was initially added to the ATP-A.

Mutated and Native ATP-A and their respective complementary sequences used in FAD displacement assay are listed below

(ATPA) 5'Biosg/ACC TGG GGG AGT ATT GCG GAG GAA GGT3'
CS-ATPA-5'ACCTTCCTCCGCAATACTCCCCCAGGT3'

(G9/22MATPA) 5'Biosg/ACC TGG GGA AGT ATT GCG GAG AAA GGT3'
CS-G9/22MATPA-5'ACCTTTCTCCGCAATACTTCCCCAGGT3'

(G9MATPA) 5'Biosg/ACC TGG GGA AGT ATT GCG GAG GAA GGT3'
CS-G9M-5'ACCTTCCTCCGC AAT ACT TCC CCA GGT3'

(G22MATPA) 5'Biosg/ACC TGG GGG AGT ATT GCG GAG AAA GGT'
CS-G22M-5'ACCTTTCTCCGCAATACTCCCCCAGGT3'

Isothermal Titration Calorimetry (ITC)

Anti-ATP aptamer solutions (ATP-A) (400 µL, 50 µM) were prepared in binding buffer (Tris 20 mM, NaCl 300 mM, $MgCl_2$ 5 mM, pH 7.4). ATP 200 µL (700 µM) and FAD 200 µL (900 µM) solutions were also prepared in binding buffer. All the samples and buffers for the washing syringe were sonicated for 15 mins at RTP. A solution of ATPA (or mutated aptamers) (400 µL) was loaded in a deep well plate assigned as the target while ATP or FAD solutions were loaded in a deep well plate assigned as the ligand, for syringe injection. The first injection was set at 0.1 µL and the remaining 18 injections were set at 2 µL for total injections of 19. The ATP or FAD was injected to the cell containing the ATP-A (or mutated aptamer) solution at 25 °C. Upon completion of the injections, the data was processed using MicroCal $Auto_{itc}$ Origin Software. The corresponding thermodynamics parameters are shown in Table S1.

Table S1 Thermodynamics analysis of target (mutated and native ATP-A) binding to the ligand (FAD or ATP) at 25 °C. N is stoichiometry binding site. K_d is binding constant and calculated from association constant K from ITC.

Target (50µM)	Ligands	K_d (µM)	ΔH Cal/mol	T (°K)	ΔS Cal/mol/deg	ΔG cal/mol	N
G22/9mutated ATPA	FAD	NA	NA	298	NA	NA	NA
G9mutated ATPA	FAD	19.5	$-1.541E^4$	298	-30.1	-6440.2	0.81
G22mutatedATPA	FAD	377.3	$-1.36E^7$	298	$-4.58E^4$	48400	NA
ATPA	FAD	12.9	-8421	298	-5.88	-6668.7	1.2
ATPA	ATP	14.5	-6986	298	-1.3	-6598.6	1.8

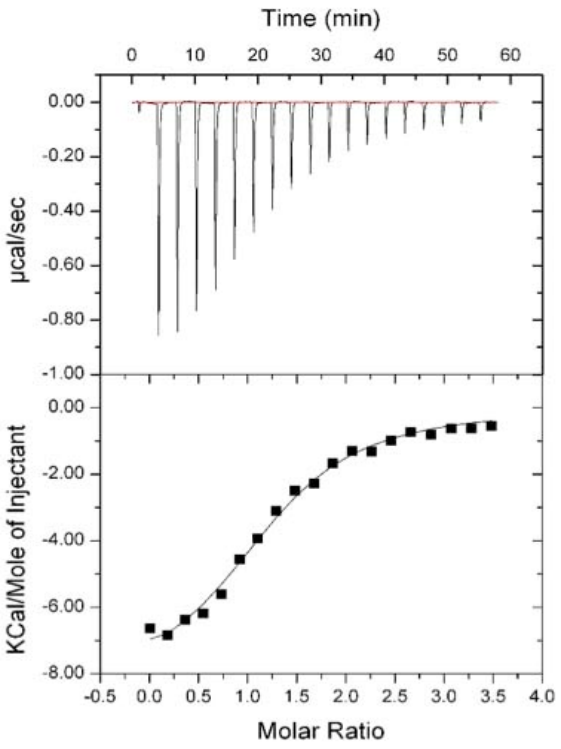

Figure S3: Isothermal Titration Calorimetry profiles for the titration of FAD into a 50µM of ATP-A in binding buffer (Tris 20mM, NaCl 300mM, 5mM $MgCl_2$), pH 7.54 at 25°C. (upper panel) Each heat burst curve is the result of a 2µL injection from 900µM solution of FAD into ATP-A. Lower panel represents corresponding heat signals versus molar ratio of FAD to ATP-A. The data points reflect experimental injection heat while the solid line represents the calculated fit of the data.

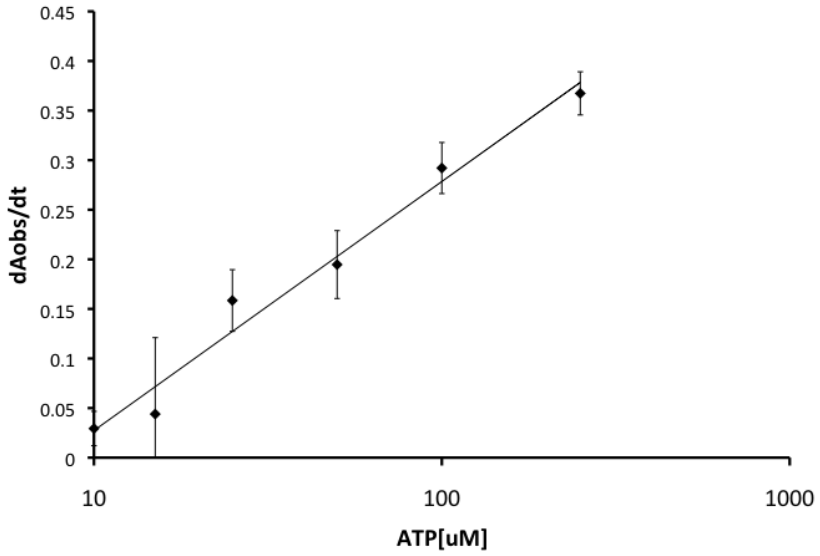

Figure S4: Dose dependent curve of initial velocity versus ATP concentration. Error bars were obtained from three or more trials.

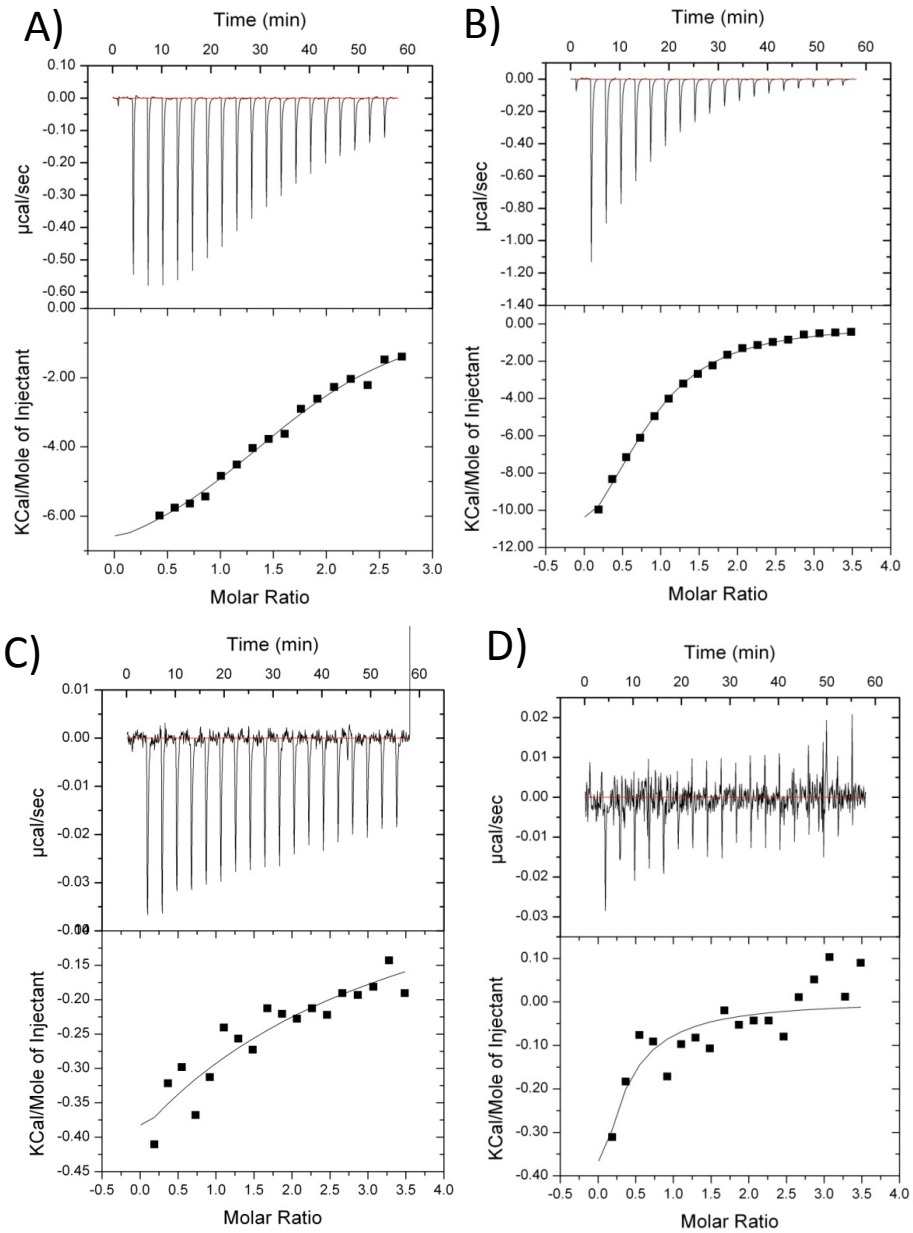

Figure S5: Isothermal Titration Calorimetry. Upper panel exothermic heat evolved upon addition of ligands (ATP or FAD) to the target (Native or mutated ATP aptamer). A) ATP 700µM to native ATPA 50µM; B) FAD 900 µM to G9 mutated ATPA 50µM; C) FAD 900µM to G22mutated ATPA 50 µM; D) FAD 900µM to G9/22 mutated ATPA 50 µM ; Lower panel Normalized ITC data for titration plotted vs the molar ratio of ATP and FAD to ATP-A and Mutated ATP-A respectively.

References

1) David E. Huizenga and Jack W. Szostak, *Biochemistry 1995, 34, 656-665*
2) B.E.P. Swoboda *Biochim. Biophys. Acta, 1969, 175, 365-379.*