Supporting Information for

# Pyrroloquinoline quinone maintains redox activity when bound to a DNA aptamer

Ismaila Emahi, Isabel M. Mulvihill, and Dana A. Baum*

Department of Chemistry, Saint Louis University,
St. Louis, Missouri 63103, USA

* To whom correspondence should be addressed. D.A.B. – Tel: +1 314 977 2842; Fax: +1 314 977 2521; Email: dbaum1@slu.edu.

## Supplemental Materials and Methods

*Reagents and materials*. DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and purified by denaturing polyacrylamide gel electrophoresis (PAGE) using 1X TBE running buffer (89 mM each of Tris and boric acid, 2 mM EDTA, pH 8.3). Samples were extracted from the gel by the crush-and-soak method in TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 300 mM NaCl), recovered by ethanol precipitation, and quantified by UV absorbance.[1] The single-stranded DNA (ssDNA) random pool for in vitro selection was 5'-GAACTAGATCGCAGC-$N_{70}$-GGATCGAGGTAATCC-3'. The $N_{70}$ designation indicates 70 nucleotides with equimolar incorporation of the four DNA bases A, C, G and T at each position. Two primers, primer 1: 5'-CAACAACAACAA-X-GGATTACCTCGATCC-3' and primer 2: 5'–GAACTAGATCGCAGC-3', anneal to the 3' and 5'-ends of the random pool, respectively, during PCR amplification. The non-amplifiable spacer 18 linker in primer 1 (designated as X) allows for separation of the inactive complement from the active pool sequence via PAGE following PCR. The sequence of the unstructured DNA oligo used in the control experiments was 5'-GAACTAGATCGCAGC-$(CAA)_{23}$-CGGATCGAGGTAATCC-3'. PQQ was purchased from Berry & Associates, Inc (Dexter, MI). 2,6-dichlorophenolindophenol (DCPIP) was purchased from Fisher Science Education (Nazareth, PA). Sodium ascorbate was obtained from Sigma-Aldrich Co. (St. Louis, MO).

*In vitro selection*. DNA aptamers for PQQ were identified through in vitro selection (Figure S1) using the random pool described above. PQQ was coupled to MagnaBind amine-derivatized beads (Thermo Fisher) using 50 mM 4-(4, 6-Dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium (DMT-MM) in 100 mM MOPS pH 7.0 and 1 M NaCl. The amount of PQQ coupled to the beads was determined spectroscopically by measuring the $A_{330}$ of the coupling solution before and after the coupling reaction. Prior to each incubation with beads, the DNA pool was annealed by heating to 95 °C for 3 minutes, followed by incubation on ice for 5 min in 1X HEPES annealing buffer (5 mM HEPES pH 7.5, 10 mM NaCl, and 0.1 mM EDTA). Selections were initiated by first incubating 200 pmol of the random pool with amine-derivatized beads that had not been coupled to PQQ. This pre-selection column was intended to remove sequences that bind to the beads or the tether. The unbound sequences in the supernatant were

then incubated with 0.1 mM immobilized PQQ under the buffer conditions listed in Table 1. Separation of bound and unbound sequences was achieved by using a magnetic separator to hold the PQQ-functionalized magnetic beads. Bound aptamers remained associated with the beads while unbound aptamers in solution were removed by pipetting. Three 20 µL-washes with binding buffer were done to remove any further unbound DNA from the aptamer-bead complexes. The bound aptamers were then eluted from the beads by washing 3 times with 20 µL elution buffer (binding buffer + 200 µM PQQ). Tightly bound sequences were eluted by washing 3 times with 20 µL of an EDTA elution buffer containing 100 mM HEPES pH 7.5, 200 mM KCl, 0.2 mM PQQ, and 100 mM EDTA. EDTA was included to help disrupt divalent metal ion-dependent binding structures and to help elute aptamers that may be binding very tightly to PQQ. The eluted binding sequences were then amplified by PCR using the primers listed above and α-$^{32}$P-dCTP. The progress of the selection was monitored by determining the percent of radiolabelled DNA that was eluted from the beads. The amount of binding sequences present in the washes, elution fractions, and remaining with the beads was determined by Cerenkov counting using a Tri-Carb 3110 TR (Perkin Elmer). The percentage of eluted aptamers was estimated from radioactivity in the combined elution washes based on the following equation: % eluted = [cpm in elution washes / sum of cpm in all washes (binding, elution, and reconstituted beads)] x 100.

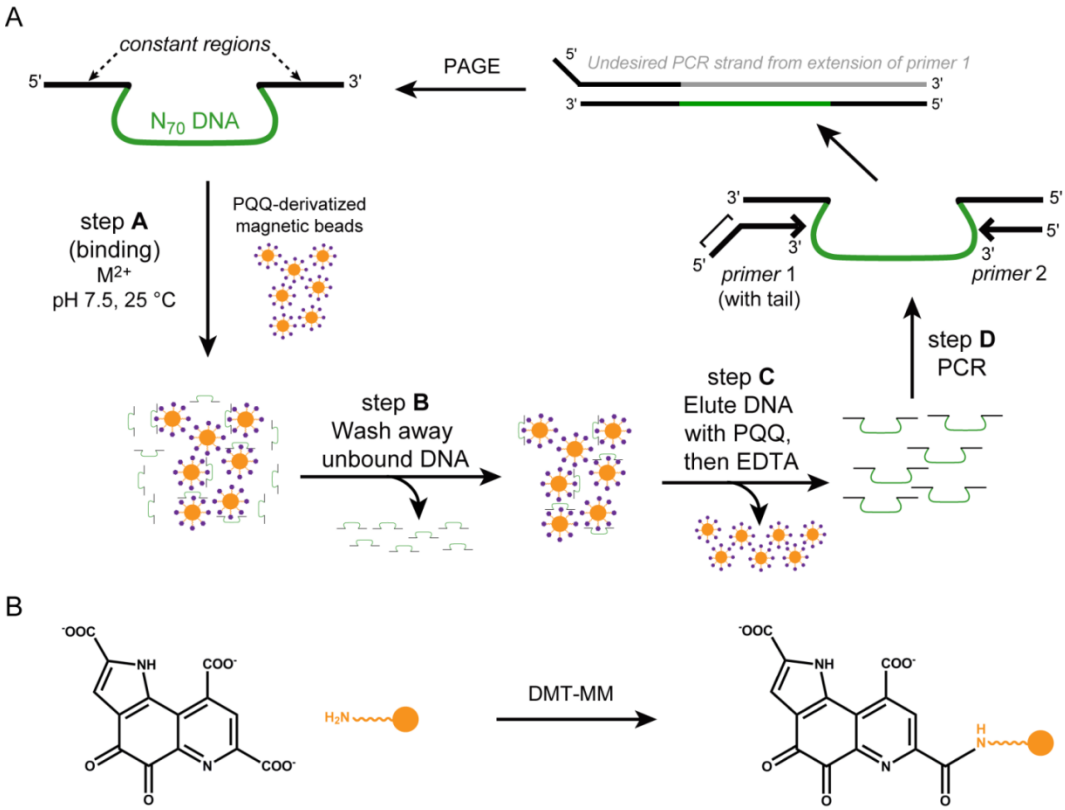


Figure S1. In vitro selection of PQQ aptamers. (A) General selection scheme. Step A involves binding of the oligonucleotide pool to PQQ immobilized on magnetic beads. In Step B, the unbound oligonucleotides are separated from the bound sequences, which are subsequently eluted (Step C) and amplified through PCR (Step D). (B) PQQ was covalently linked to amine-modified magnetic beads using DMT-MM as the coupling agent. PQQ has 3 carboxylic acid groups, so alternative attachments are possible.

*Cloning and sequencing*. The ssDNA from round 15 of the selection was PCR-amplified with two new primers to generate a double-stranded product: 5'-TAATTAATTAATTA-GGATTACCTCGATCC-3' and 5'-TAATTAATTAATTAGAACTAGATCGCAGC-3'. The underlined regions of the primers introduce stop codons to reduce false negatives in blue-white screening.[2] The resulting PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Resulting colonies were cultured and the plasmids containing the aptamer sequences were isolated via miniprep (IBI Scientific, Peosta, IA). Plasmids were then sequenced by ACGT, Inc. Unique DNA sequences were made by solid phase synthesis by IDT for testing and are listed in Table S1. We also aligned the unique DNA sequences in MEGA6[3] using MUSCLE.[4] As seen in Figure S2, consensus or conserved regions were not readily identified.

Table S1. Sequences of DNA aptamers and oligonucleotides used for testing

| Selection pool | GAACTAGATCGCAGC-$N_{70}$-GGATCGAGGTAATCC |
|---|---|
| 15ACa9 | CACCGGGGGAGCACAAGCATTTGACACAAGACGACCCGCGCGAGATCTGGTACATGCCTTACCCGCTGTG[a] |
| 15ACb5 | CACGGGGGGTGATAACAGTGTATGACAACGCTGTGTGTCGTATCGCGTCCATTGTTGTTGTTGCGTGTTG |
| 15ACb12 | CCGGGGGGTGGAATATCTGGTTCGATAACCACTGCTCCAAAATTATAGCAGCTCTCGTGTGTGTTGGTG |
| 15ADa9 | CCACCGGCGGACAGCATAGGACGACTGGCTCGAGCGCTCTACTACTGCGGCATTTCTACCCTGATTTGTA |
| 15ADb4 | CACGCACGGGGCACAGAAAAGAGAGCCACTGAGGCCTCAGTGCCAAACATCGGACCACAAAGCGCCTTCTA |
| 15ADa10 | CTAATGCCCGTACATAGCCACGCATACTGTTTTTCTAATAAAGCTCCTTGAGGTGAACCGTCGCTGGGTG |
| 15ADb11 | CCCACAGTCGAGAAGCAGTAAGACGACATGGGAACAAACATGGGGCCAAGAGATCTAGGGCACGCTGTGT |
| 15AFa2 | CCACAAGCGAGGACCAATTAATGGTGCAAGTGGCTCAGGGATTGATGGGCCTTCAATGCTGCTCGGTGTA |
| 15AFa4 | CACAGGCGGGAATCTACCGTCTTACTATTGTAAACGCAGCAGCAATGCAGAATCAGCCATTCCGTGGGTG |
| 15AFa11 | CACACGGCTGGAGAACGGGAAGGTAGAACGTAATCCCTGCGCCGGGACGATCAAGTTGTGTATCGCCGTA |
| 15AFb3 | CCAACACGGGGAGATGTGCTCTATAGACCTATTGAAGAACGCATGTGTTCGCGGGCTGTTATCGGACATG |
| 15AFb4 | CCACGGGGTAGTTACAGGAGAGTAGTTTGCAGGAAATCCCTTACGTATTGCGGACGGGGCGTGTCGTGGG |
| Unstructured DNA | CAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAAC |

[a] Sequences of the $N_{70}$ region for each aptamers and control (unstructured) DNA are shown. Tested aptamers included the 2 primer binding regions flanking the $N_{70}$ region, resulting in oligo lengths of 100 nt.

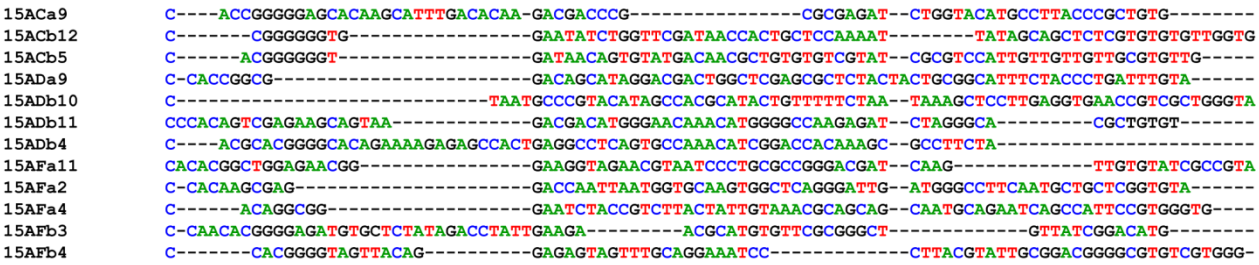


Figure S2. Alignment of unique PQQ aptamer sequences. Sequences were aligned using the MUSCLE[4] program in MEGA6.[3]

*Binding assays*. Each of the twelve sequences isolated was tested for its ability to bind to PQQ immobilized on the amine-derivatized magnetic beads. 10 pmol of unlabelled aptamer and a tracer amount of [$^{32}$P] 5'-end-labeled aptamer were annealed as described above. The annealed DNA aptamer was then incubated with 100 µM immobilized PQQ in 20 µl of the appropriate binding buffer (Table 1) with mild shaking at room temperature for 1 h. Aptamers were eluted from the beads using the same washing procedures used in selection and the fraction bound was determined as described above.

*UV-Vis absorbance spectroscopy*. Changes in either the wavelength or maximum absorption of PQQ upon addition of the DNA aptamer were measured using a NanoDrop2000c spectrophotometer (Thermo Scientific). The DNA aptamer was annealed in 1X HEPES annealing buffer as described above. Next the aptamer was brought up in 500 µL of appropriate binding buffer (Table 1) with 25, 50, or 100 µM PQQ. Final concentration of the aptamer was 1 µM. The aptamer and PQQ were allowed to bind for 1 h at room temperature. The absorbance of the complex was then measured at 330 nm. Control experiments involving PQQ alone in binding buffer, and 1 µM DNA alone in binding buffer were also performed for comparison. In addition to the isolated aptamers, the starting DNA pool and two G-quadruplex forming sequences (PS5.M and PS2.M aptamers)[5] were also tested.

*Fluorescence anisotropy studies*. Changes in the fluorescence anisotropy of PQQ upon addition of the aptamer were measured on a Fluorolog 3 fluorometer (Horiba Scientific, Edison, NJ). 10 µM PQQ was prepared in 130 µL of 1X PBS buffer (pH 7.5) containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ and initial anisotropy measurements were taken. Next, the aptamer, pre-annealed in 1X HEPES annealing buffer as described above, was titrated from a 60 µM stock into the cuvette containing PQQ. After each addition, the sample was allowed to equilibrate at RT for about 20 min in order to form a stable complex between the aptamer and PQQ before taking anisotropy measurements. The excitation and emission wavelengths used for PQQ were 365 nm and 485 nm, respectively. Excitation and emission bandpasses were set to 2 and 14, respectively, and signals were taken at 1.4 x $10^6$ counts per second (cps). For each titration, anisotropy measurements were recorded three times at 10 s integration times, and the resulting anisotropy values were averaged. The binding constant, $K_d$, was determined by fitting the anisotropy data to the equation $A = A_0 + (A_{max}*B^n/K_d^n+B^n)$ where A is anisotropy, $A_0$ is minimum anisotropy, $A_{max}$ is maximum anisotropy, B is the concentration of DNA, and n is the Hill coefficient.

*Spectroscopic assay of redox activity using DCPIP*. As a preliminary assessment of the redox ability of the PQQ-aptamer complexes, a simple spectroscopic assay involving the redox-active

dye 2,6-dichlorophenolindophenol (DCPIP) was conducted. Initially, 25 μM of the dye was reduced with 150 μM sodium ascorbate. After monitoring the solution for at least 10 min to ensure that the dye remained reduced, 100 μM PQQ complexed with 1 μM aptamer in 1X AD binding buffer (Table 1) was added, resulting in a final volume of 150 μL. Absorbance measurements were continued for up to 2 h. 100 μM PQQ alone, as well as 100 μM PQQ incubated with 1 μM unstructured DNA, were monitored in separate experiments as controls. In an equimolar control experiment, 40 μM of each aptamer was incubated with 40 μM PQQ and added to 10 μM DCPIP that had been reduced with 60 μM sodium ascorbate. The oxidation process was then monitored continuously for 1 h.

## Supplemental Results

*Selection progress*. The progress of the selections is shown in Figure S3 below. Apart from selection AE, all selections showed a consistent gradual increase in the amount of DNA eluted in the presence of PQQ each round. To eliminate sequences that were binding just to the bead or the linker without binding PQQ, we included a negative selection step at rounds 10 and 14. The DNA pool sequences were incubated with the magnetic beads that had not been functionalized with PQQ. The non-binding sequences were then carried forward into the selection step with PQQ-functionalized beads. Binding sequences from round 15 were cloned as described above.

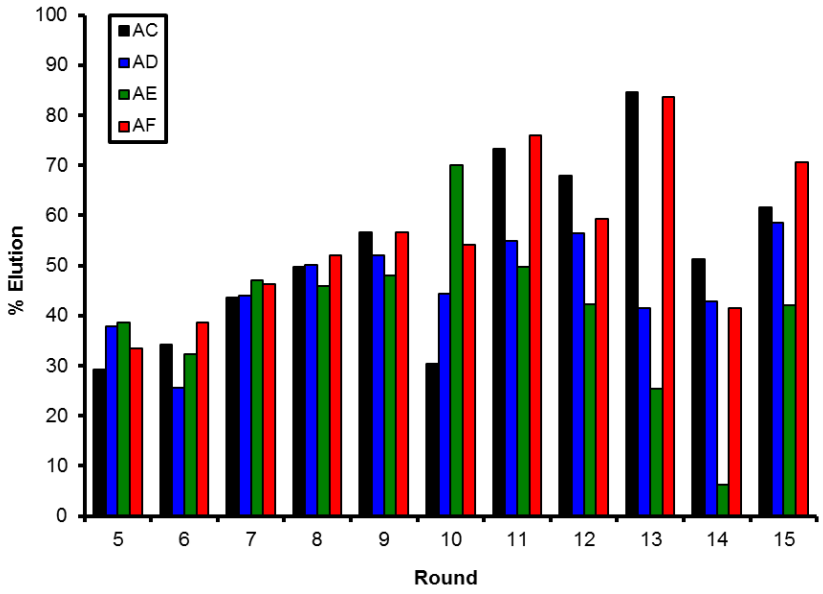


Figure S3. Progress of in vitro selection experiments for PQQ aptamers. Percentage of radiolabeled oligonucleotides that eluted from beads in the presence of PQQ was determined by Cerenkov counting. Negative selection as applied in rounds 10 and 14 to eliminate sequences binding solely to the beads or the amine linker. Binding sequences from round 15 were cloned and sequenced.

*Binding assays*. The 12 unique aptamer sequences isolated during selection were tested for their ability to bind immobilized PQQ under the same condition used during selection. As seen in Figure S4, each isolated aptamer appears to bind to PQQ, albeit with different affinities. These initial binding assays utilized immobilized PQQ, so it is possible that the aptamer binds to PQQ

and the tether used for immobilization. To ensure the aptamers are able to bind free PQQ, additional studies were undertaken.

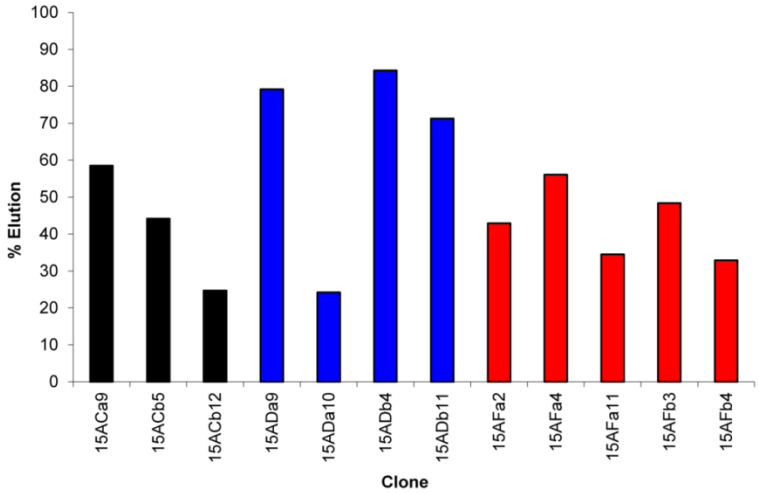


Figure S4. Binding studies of isolated aptamers to immobilized PQQ. Percentage of radiolabeled aptamer oligonucleotides that eluted from beads in the presence of PQQ was determined by Cerenkov counting. Clone names designate individual aptamer sequences.

*UV-Vis absorbance spectroscopy*. Binding of aptamers to free PQQ in solution was initially assessed by measuring changes in PQQ absorbance. PQQ did not show any shift in absorbance wavelength upon aptamer addition (data not shown). However, changes in the absorbance at 330 nm were observed (Figure S5). In each case, the absorbance at 330 nm was monitored for 30 min and remained stable over that time. The DNA oligonucleotides have no absorbance at 330 nm, while PQQ strongly absorbed at this wavelength. The DNA pool showed no change in PQQ absorbance with or without the DNA present with PQQ (Figure S5A). The pool contains a mixture of random oligonucleotide sequences, most of which are not expected to bind to PQQ. When identified aptamers were added, changes in absorbance were observed, specifically a drop in the absorbance. Aptamers 15ACb5 (Figure S5B) and 15AFb3 (Figure S5D) do not appear to bind at 25 µM PQQ, but do bind 50 µM PQQ and above. 15ADa9 (Figure S5C) appears to have greater affinity for PQQ, indicated by a drop in absorbance with just 25 µM PQQ. The change in absorbance upon DNA addition happens rapidly, as seen in Figure S5G. As additional controls, we tested two sequences known to bind hemin (PS5.M and PS2.M)[5] to determine if PQQ could bind G-quadruplexes. As was observed with the DNA pool, there is very little change in absorbance with or without the DNA (Figures S5E-F). Although aptamer 15ADa9 was selected in the presence of $Ca^{2+}$, UV-Vis absorbance results suggest that $Ca^{2+}$ is not a requirement for binding (Figure S5H).

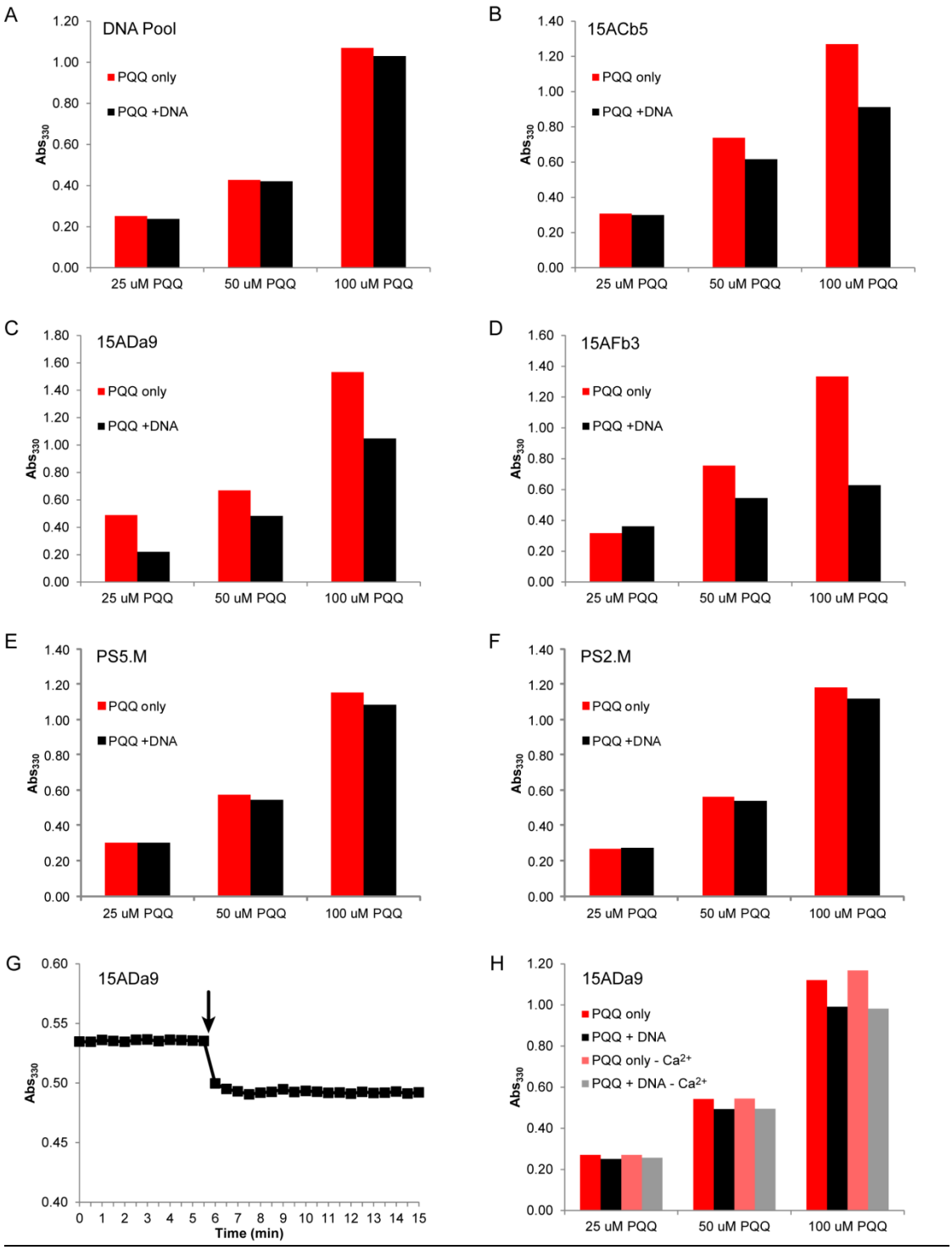


Figure S5. Binding studies of isolated aptamers and control sequences to free PQQ using UV-Vis absorbance spectroscopy. Absorbance of each solution was monitored at 330 nm for 30 min and the average absorbances are reported (A-F). PQQ absorbance drops quickly upon aptamer addition to a 50 μM solution of PQQ (indicated by arrow) and remains stable (G). Despite being selected in the presence of $Ca^{2+}$, aptamer 15ADa9 does not require $Ca^{2+}$ for PQQ binding (H).

*Fluorescence anisotropy studies*. For a more detailed binding analysis and to obtain binding constants for our aptamers, we utilized fluorescence anisotropy (Figure S6). In these studies, increasing amounts of the DNA aptamer were titrated into a 10 μM PQQ solution and changes in the fluorescence anisotropy of PQQ was monitored. Control DNA sequences were also analyzed and did not show anisotropy changes that would indicate PQQ binding, even at higher DNA concentrations (Figure S6K-M).

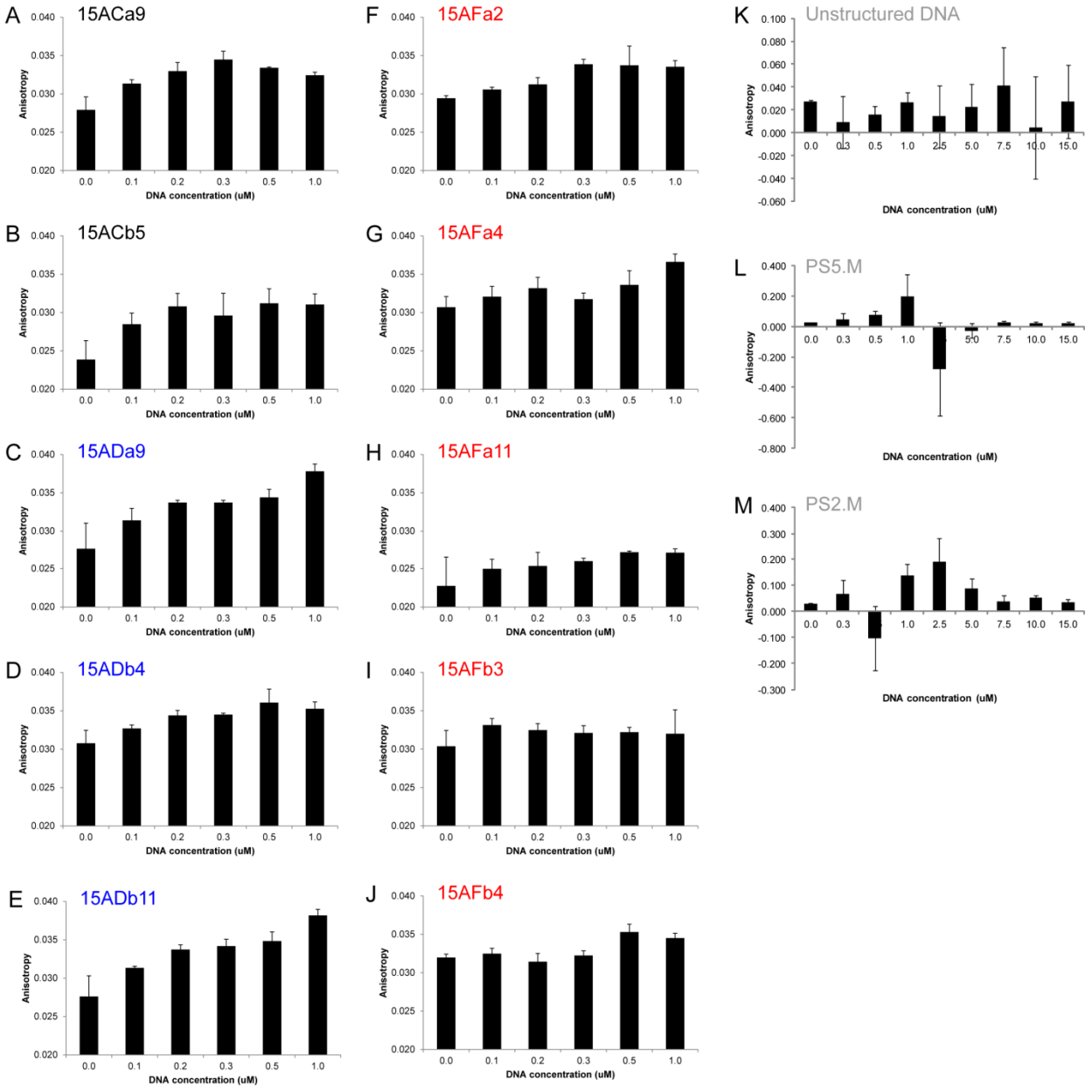


Figure S6. Binding studies of isolated aptamers and control DNA sequences to free PQQ using fluorescence anisotropy. The excitation and emission wavelengths were 365 nm and 485 nm, respectively. For each titration of DNA, anisotropy measurements were recorded three times at 10 s integration times and the resulting anisotropy values were averaged.

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