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DNA-Crowded Enzyme Nanoparticles with Enhanced Activities and Stabilities

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Methods and Materials

Materials: Sodium phosphate dibasic, sodium chloride, Tris base, magnesium chloride, magnesium acetate, 10 × TBS (Tris-buffered saline), 1 M triethylammonium acetate (TEAA), glycine, sodium citrate and DNA grade water were all purchased from Fisher Scientific (Waltham, MA). Horseradish peroxidase (HRP), β-Nicotinamide adenine dinucleotide (NAD^+), glucose-6-phosphate (G6p), glucose, and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were all purchased from Sigma Aldrich (St. Louis, MO). Glucose-6-phosphate dehydrogenase (G6PDH) was purchased from Worthington Biochemical Company (Lakewood, NJ). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Initiator strand (I): 5'- AGT CTA GGA TTC GGC GTG GGT TAA AAA AAA AAA A-amine -3'; hairpin 1 (HP-1): 5'- TTA ACC CAC GCC GAA TCC TAG ACT CAA AGT AGT CTA GGA TTC GGC GTG -3'; hairpin 2 (HP-2): 5'- AGT CTA GGA TTC GGC GTG GGT TAA CAC GCC GAA TCC TAG ACT ACT TTG -3'.

SPSC Buffer: It was prepared as previously reported in the literature.[1] A 1×SPSC (sodium phosphate sodium chloride) contains 50 mM sodium phosphate and 500 mM sodium chloride. The pH of the buffer solution was adjusted to 7 using 1 M hydrochloric acid (HCl).

Universal Buffer: A 5× buffer solution was prepared by dissolving glycine, sodium citrate, magnesium acetate, and tris base in distilled water to a concentration of 100 mM for each component. This solution was then used to prepare multiple buffers (20 mM, 1×) from pH 4-10 by adding HCl or NaOH.[2]

Purification of oligonucleotides: Oligonucleotides purchased from Integrated DNA Technologies (IDT) were purified by HPLC using 3 μm Oligo-RP column (50 x 4.6 mm, Phenomenex, Torrance, CA) in 100 mM TEAA and a methanol gradient from 25 % to 35 %.

Bio-conjugation: Thiol-modified DNA initiator ordered from IDT was first reduced by adding 20 × access T-CEP in 1 × TBS buffer, and was incubated for one hour. After the reduction, extra T-CEP and free thiols were removed by filtration with 10 kDa Amicon MW filter, three times with distilled water at 4 °C. The reduced thiol-DNA was stored at – 20 °C. Enzyme was conjugated to an initiator strand using a previously published SPDP chemistry.[3,4] Briefly, SPDP first reacted with lysine residues on protein surface in pH 8.5, 50 mM HEPES buffer. SPDP-modified enzyme was then reacted with an 8-fold excess

thiol-modified DNA for one hour. All reactions were incubated on a rocker (Armalab, Arma/rock 100) (50 rpm) at room temperature in the dark. The unconjugated DNA was removed from the solution mixture by first filtering it with a 10 kD Amicon MWCO filter using 50 mM HEPES buffer (pH 7.5) with 1.5 M NaCl for one time, followed by two more washes with 50 mM HEPES buffer (pH 7.5).

Purification of DNA-conjugated enzyme: DNA-conjugated enzyme was further purified by anion-exchange liquid chromatography (Mono Q^{TM} 4.6/100 PE, GE Healthcare) to separate enzymes labelled with the exact number of oligonucleotides. As shown in Figure S1 and S2, using an NaCl gradient increased from 50% (vol/vol) solvent B (~500 mM NaCl) to 70% (vol/vol) solvent B (~700 mM NaCl), a DNA-conjugated G6PDH was separated into four component peaks, identified as the unmodified G6PDH and G6PDH with 1, 2 and 3 DNA labels. The concentrations of the distinct peaks are determined using their *A*260 and *A*280 absorbance values, as shown in **Table S1**. Similarly, a DNA-conjugated HRP was separated into four component peaks of unmodified HRP, HRP labelled with 1, 2, 3 DNA molecules.

Assembly of DNA crowded, Enzyme-HCR Complexes: Enzyme-HCR complexes were prepared by first snap cooling DNA hairpins of HP-1 and HP-2 separately in 1×SPSC (pH 7.0), by heating to 95 °C for 5 minutes, followed by cooling at 23 °C for 30 minutes. 300 nM of initiator-conjugated enzyme solution (enzyme-I) was prepared in 1×SPSC (pH 7.0), followed by the addition of HP-1 and HP-2 strands to a final enzyme concentration of 100 nM. The mixture solution was incubated in the dark for 3 hours on a rocker at 50 rpm. Control enzyme solutions without the addition of hairpins were incubated similarly in 1×SPSC.

Atomic Force Microscope: 2 μL of 100 nM sample solution was first deposited onto a freshly cleaved mica surface (Ted Pella, Redding, CA) and left to adsorb for 2 minutes. 80 μL of 1 x TAE-Mg^{2+} buffer was added to the sample and 2 μL of 100 mM Ni^{2+} was added to enhance DNA adsorption on mica. Then, the extra buffer solution was removed and the surface was rinsed using distilled water for three times. After the mica surface was dried under air, the samples were scanned with SCANASYST-Air probe (Bruker, Billerica, MA) using "Scanasyst in air mode" of Multimode 8 AFM (Bruker, Billerica, MA).

Electrophoresis: Native PAGE characterization of DNA structures: Native PAGE gels (3.5 %) were prepared at room temperature and run for 2.5 hours at a constant voltage of 200 V and subsequently stained with SYBR Green I and visualized with a ChemiDoc-It^2 Imager (UVP).

Activity Measurement of DNA-Crowded Enzymes: The enzyme-HCR solution was first diluted to 2 nM with 1×TBS-Mg (pH ~7.4; 1 mM $MgCl_2$) and was incubated for 20 minutes at room temperature in the dark. Then, the enzyme solution was mixed with substrate solution to a final concentration of 1 nM, and the activity was measured using a Cytation 3 plate reader (Biotek, Winooski, VT). The activity of enzyme

solution was determined by fitting the initial velocity of the reaction curves where the slope of the curve is a straight line prior to the reaction reaching equilibrium. For G6PDH, the activity was evaluated by measuring the increased absorbance at 340 nm due to the reduction of NAD^+ to NADH. For HRP, the activity was evaluated by measuring the increased absorbance at 420 nm due to the oxidation of ABTS. All reactions were carried out in a 96 well half-area plate with at least three replicates. V_{max} and K_m values were determined by fitting the curves of velocity *vs.* substrate concentration (Figure S6-S7) with the Michaelis-Menten equation, using GraphPad Prism 7. A calibration curve was used to convert V_{max} to k_{cat} (Figure S5).

Long Term Stability Assay: To measure the long term stability of DNA-crowded enzymes, 100 nM Enzyme-HCR solution was stored at room temperature (~ 25 °C) for a period of 10 weeks. For each week, an aliquot of the samples was taken, and then was assayed for activity (Figure S8-S11).

Freeze-Thaw Stability of DNA-Crowded Enzymes: An aliquot of 100 nM enzyme solution was prepared for freeze-thaw study. Prior to frozen, the activity of enzyme solution was measured. Then, the enzyme solution was frozen quickly at -80 °C for 30 mins. The frozen solution was then placed at room temperature for 20 minutes to allow the solution to thaw. Then, the enzyme activity was measured again. This process was repeated seven times (Figure S12-S16).

Thermal Stability of DNA-Crowded Enzymes: 10 nM enzyme-HCR solution was prepared in 1×TBS-Mg (pH ~7.4). Solution was then incubated in a thermocycler at a set temperature for 1 hour. A control solution was incubated at 25 °C. After the incubation was completed, the activity of the solution was assayed at 25 °C.

Enzyme absorption to centrifuge tube: To test whether enzyme was absorbing onto the walls of centrifuge tubes during the course of the experiments, which would reduce the solution enzyme concentration, we labelled HRP with Cy3 to measure the solution fluorescence intensity over long-term incubation and repeated freeze-thaw cycles (Figure S12 and Figure S17).

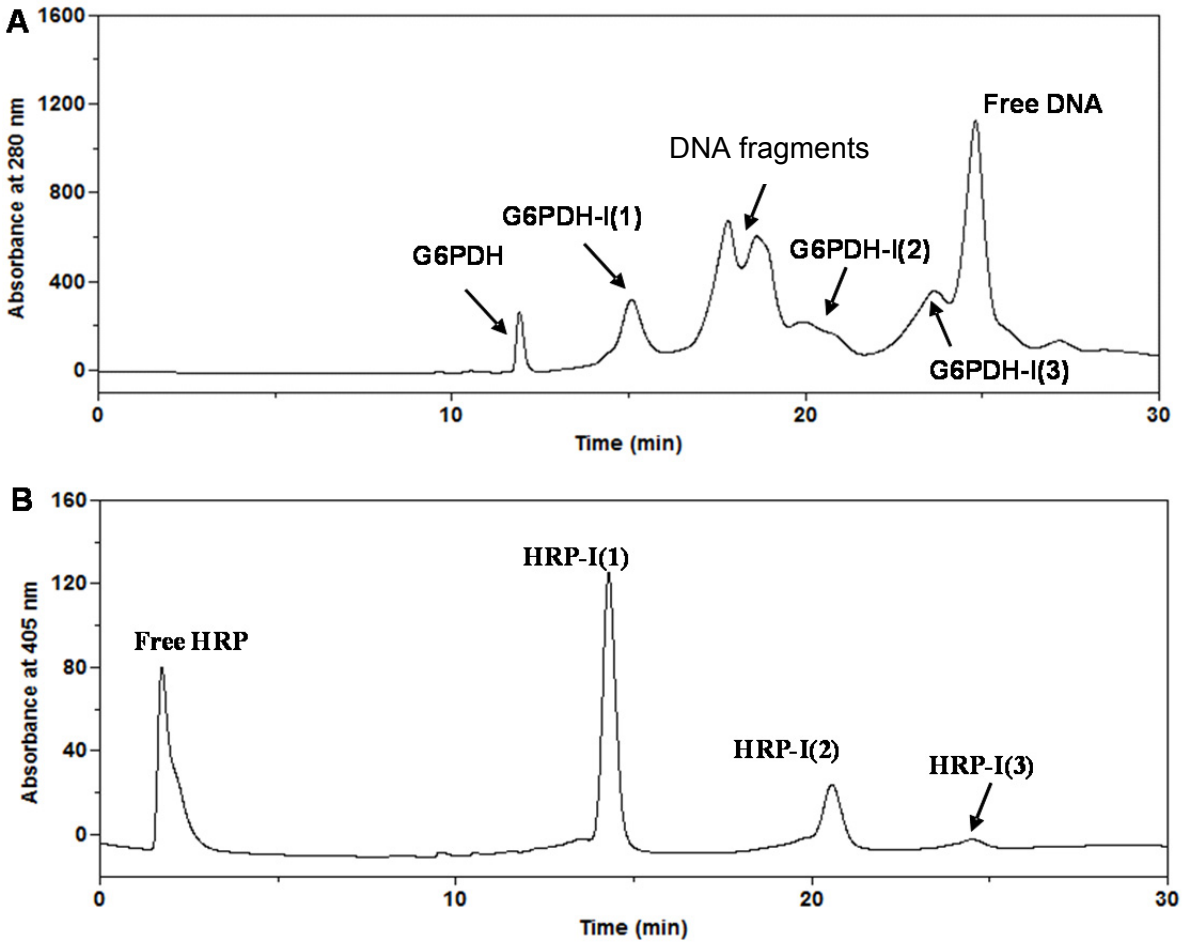

Figure S1. Anion-exchange FPLC to purify DNA conjugated enzymes. (A) G6PDH-initiator (I) conjugates, and (B) HRP-initiator (I) conjugates. The enzymes with different number of labelled DNA were separated into distinct peaks that were collected in fractions. Condition: buffer A, 50 mM sodium HEPES (pH 7.5); buffer B, 50 mM sodium HEPES, 1 M NaCl (pH 7.5). The identities of the distinct peaks were assigned using the A260 and A280 absorbance for G6PDH or A405 nm for HRP.

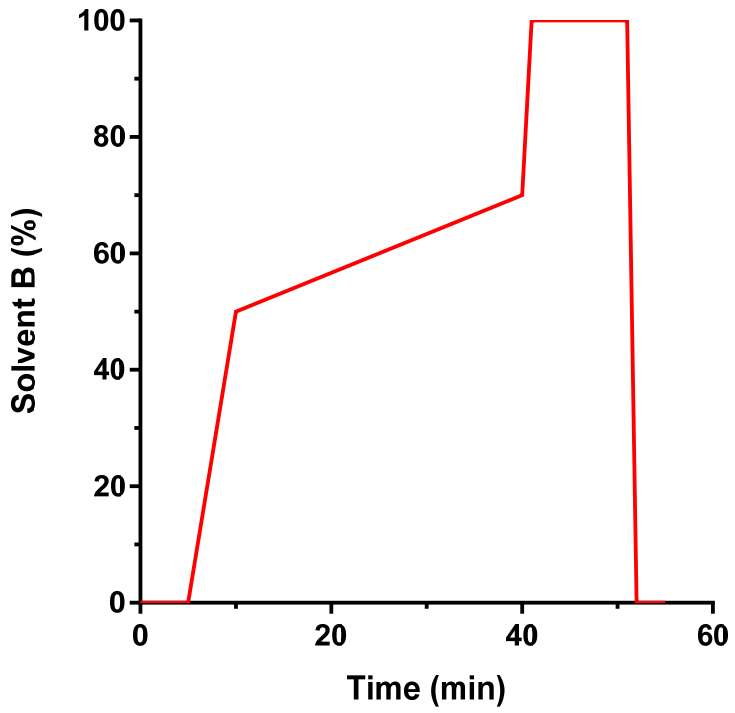

Figure S2. Solvent gradient for HPLC purification of initiator-conjugated enzymes (enzyme-I). Major enzyme-I peaks are eluted with B from 50% to 70%. Solvent A is 50 mM HEPES (pH 7.5). Solvent B is 50 mM HEPES (pH 7.5) + 1 M NaCl.

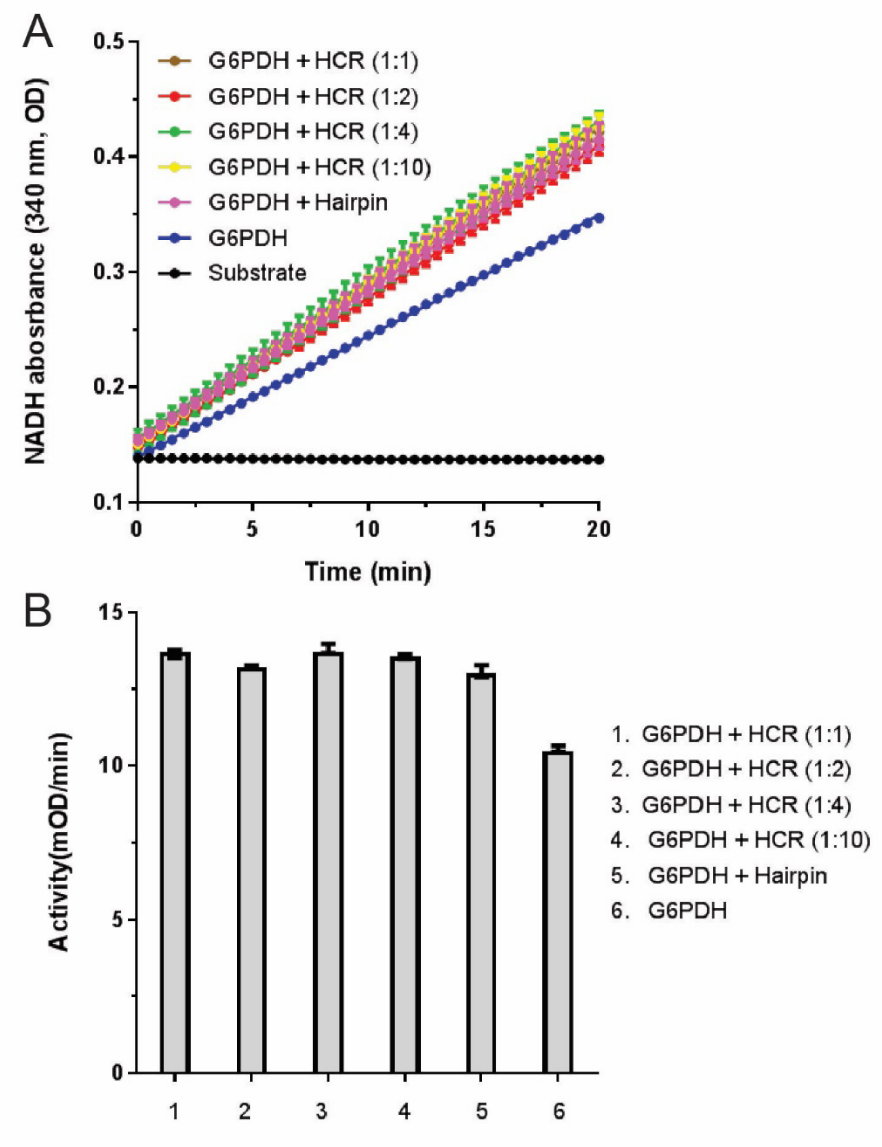

Figure S3. Evaluation of the effect of free HCR duplexes on enzyme activities. (A) Raw activity traces for G6PDH enzyme (blue), G6PDH with free hairpins (128 × access, pink); G6PDH with free hairpins and initiators with initiator –to-hairpin ratio of 1: 10 (yellow), 1: 4 (green), 1: 2 (red) and 1: 1(brown). (B) Activities for G6PDH solution and the enzyme solution with the addition of free HCR duplexes at various initiator-to-hairpin ratios. Assay condition: 1 nM enzyme.

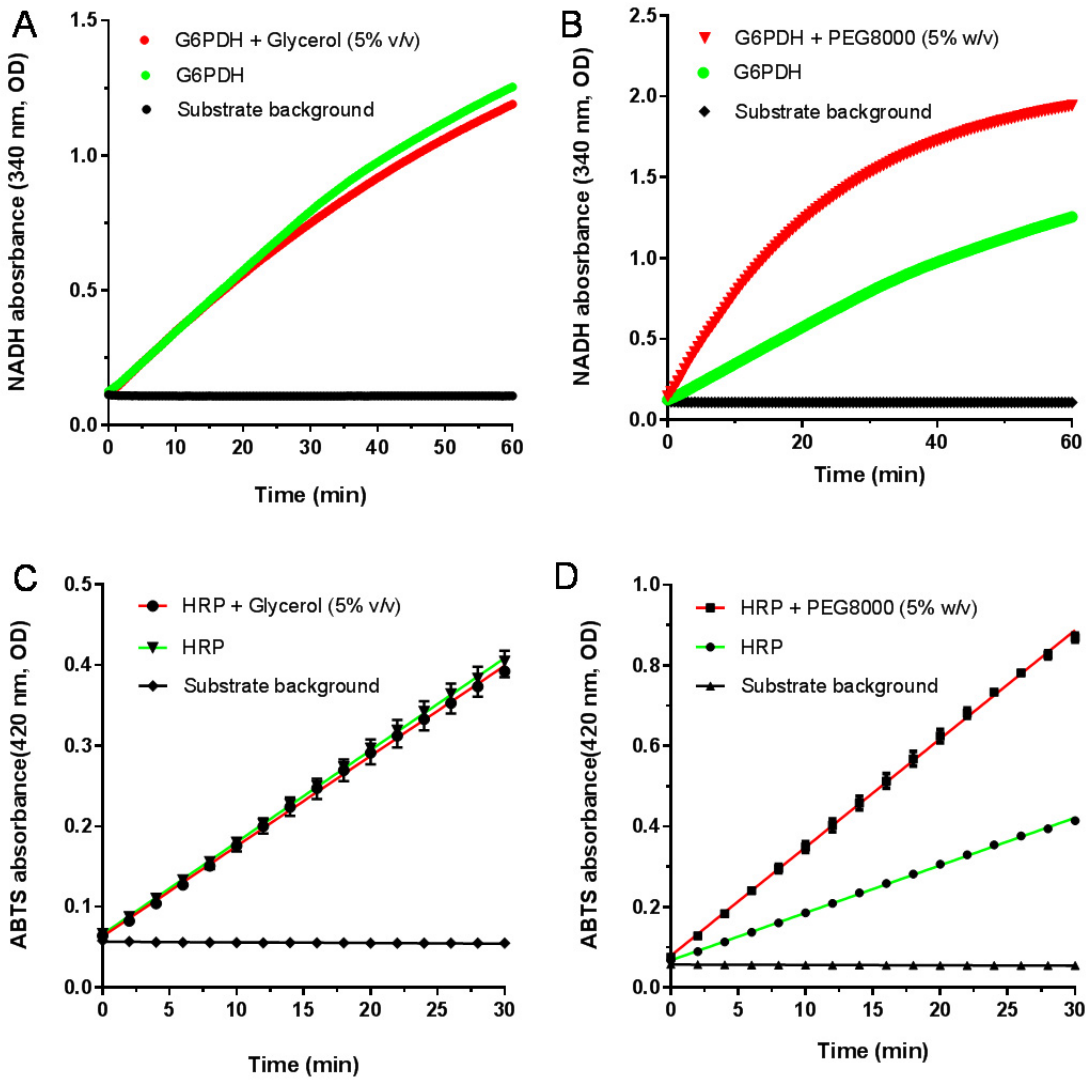

Figure S4. Evaluation of crowding agents for affecting enzyme activity. (A) The addition of 5% glycerol (v/v) into G6PDH and (B) the addition of PEG8000 (5% w/v) into G6PDH. (C) The addition of 5% glycerol (v/v) into HRP and (B) the addition of PEG8000 (5% w/v) into HRP. Small-molecule crowding agent of glycerol affects little of enzyme activity. Large-molecule crowding agent of PEG8000 can boost enzyme activity when enzyme is incubated with 5% PEG (w/v). However, such a high concentration of PEG8000 also increases the overall viscosity of the solution.

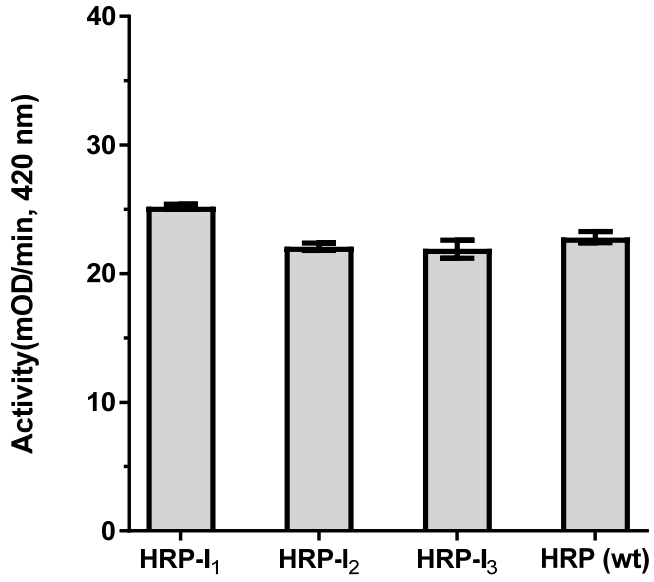

Figure S5. Activity comparison of HRP-I conjugates that are labelled with 1, 2 and 3 initiator strands, and none-labelled HRP. Conditions: 1 nM enzyme is assayed with 500 μM H_2O_2 and 2 mM ABTS. Error bar: range of data for three replicates.

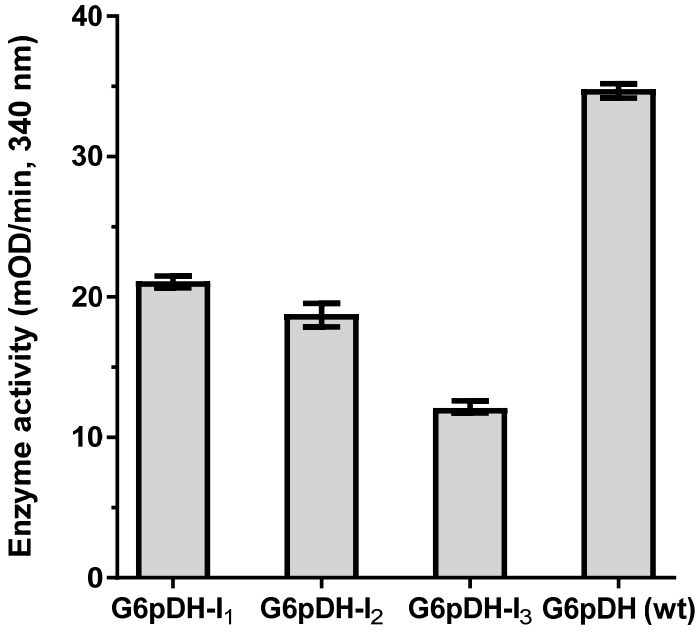

Figure S6. Activity comparison of G6PDH-I conjugates that are labelled with 1, 2 and 3 initiator strands, and none-labelled G6PDH. Conditions: 1 nM enzyme is assayed with 1 mM G6P and 1 mM NAD^+. Error bar: range of data for three replicates.

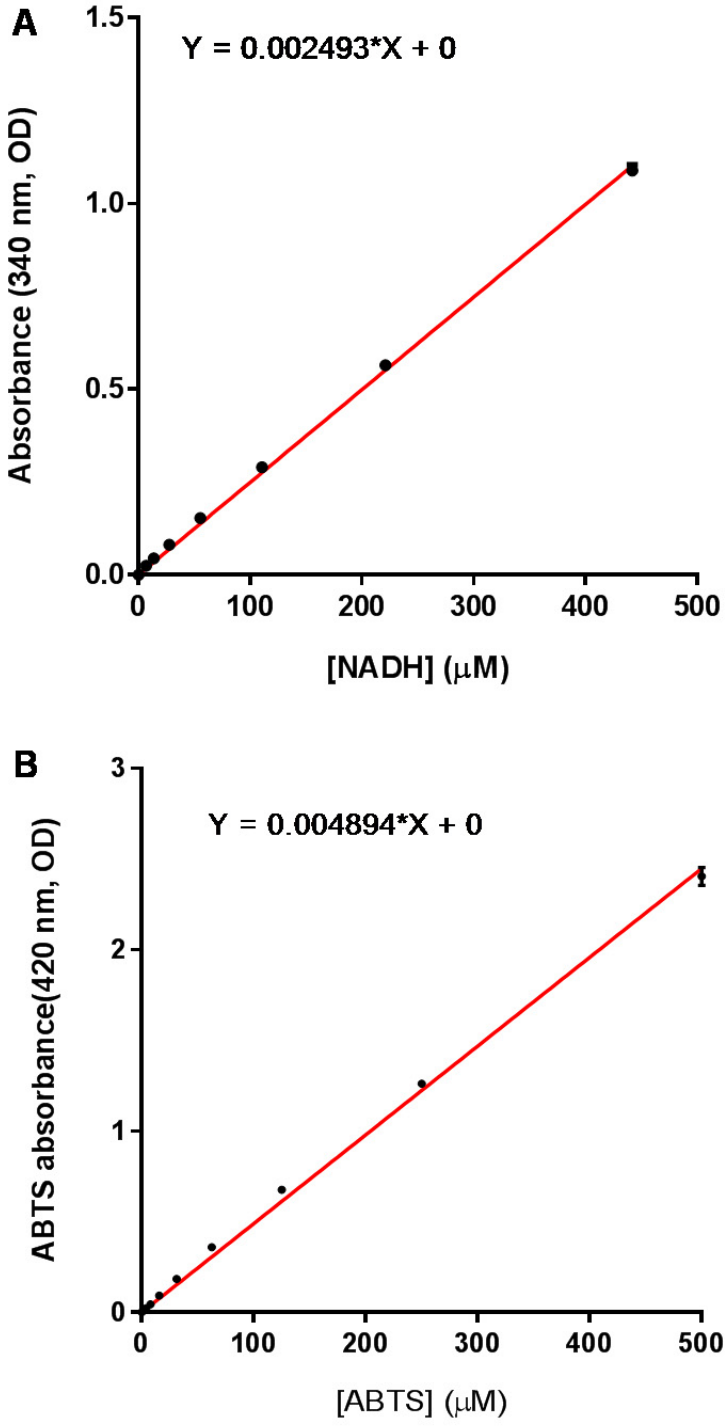

Figure S7. Standard calibration curves for (A) NADH at 340 nm and (B) ABTS at 420 nm in in pH7.5, 1 ×TBS with 1 mM $MgCl_2$.

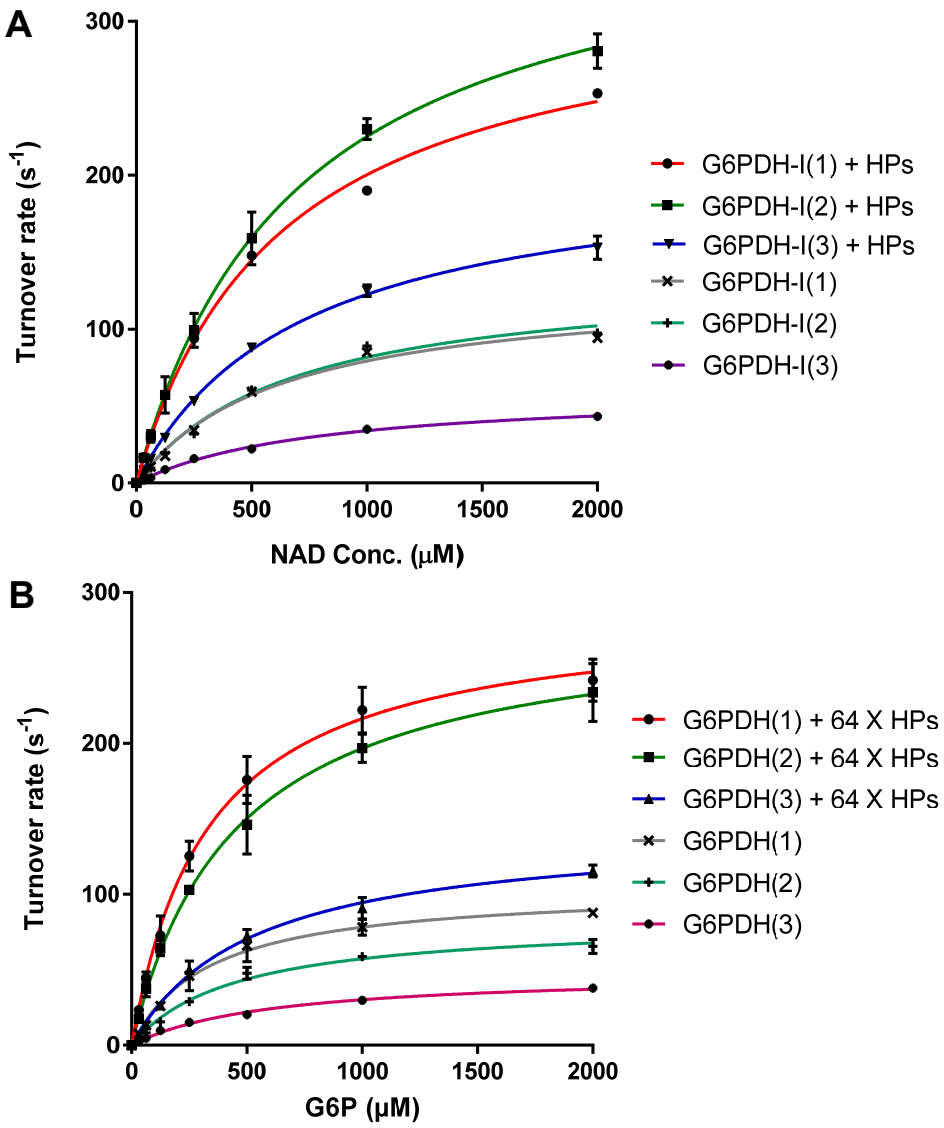

Figure S8. Michaelis-menten curves for DNA-crowded G6PDH-I/HCR complexes and G6PDH-I control with the titration of (A) NAD concentration and (B) G6P concentration. Error bar: range of data for three replicates.

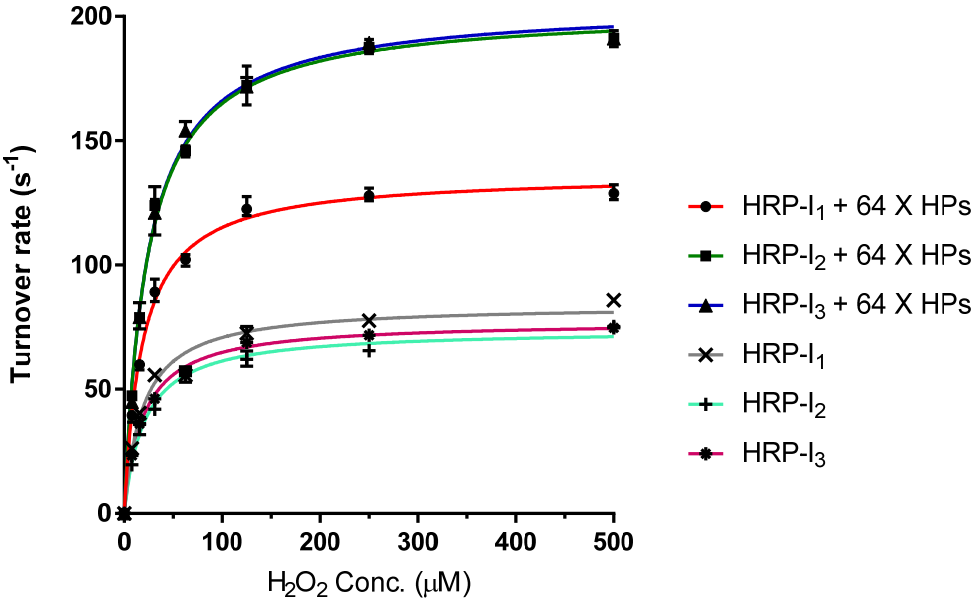

Figure S9. Michaelis-menten curves for DNA-crowded HRP-I/HCR complexes and HRP-I control with the titration of H_2O_2 concentration and 2 mM ABTS. Error bar: range of data for three replicates.

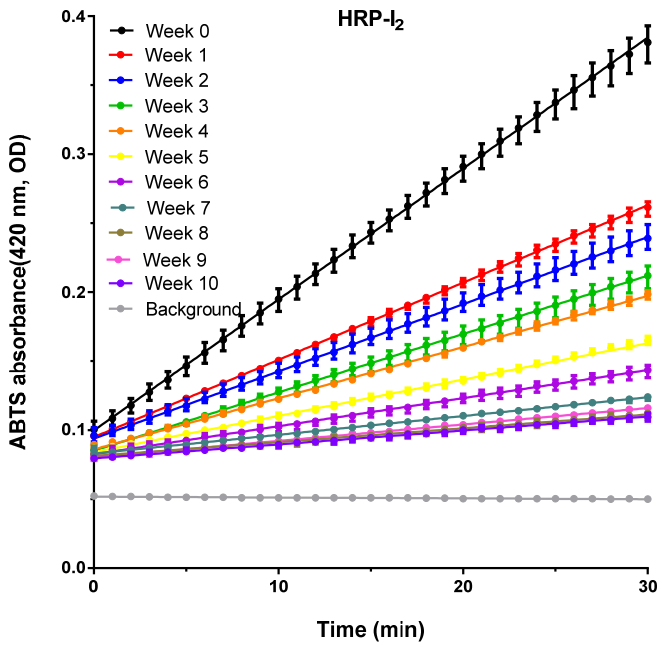

Figure S10. Long-term stability assay for HRP-I_2 complex with the incubation from week 0 – week 10 at 25 °C in the dark. Error bar: range of data for three replicates.

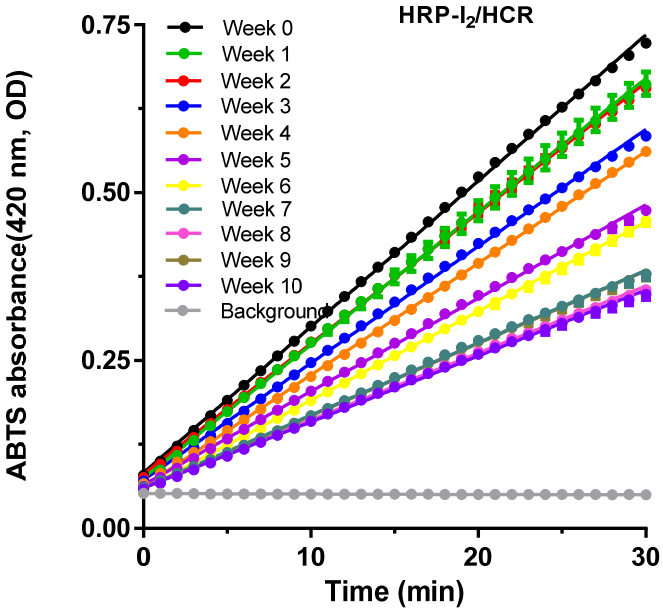

Figure S11. Long-term stability assay for HRP-I_2/HCR complex with the incubation from week 0 – week 10 at 25 °C in the dark. Error bar: range of data for three replicates.

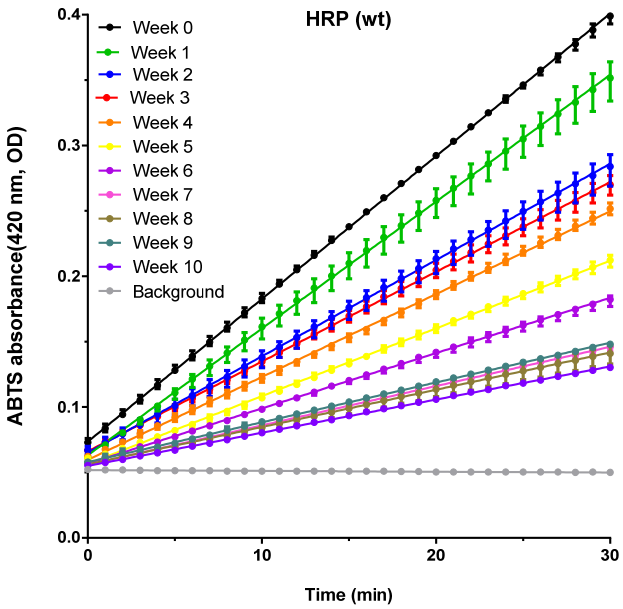

Figure S12. Long-term stability assay for wild type HRP with the incubation from week 0 – week 10 at 25 °C in the dark. Error bar: range of data for three replicates.

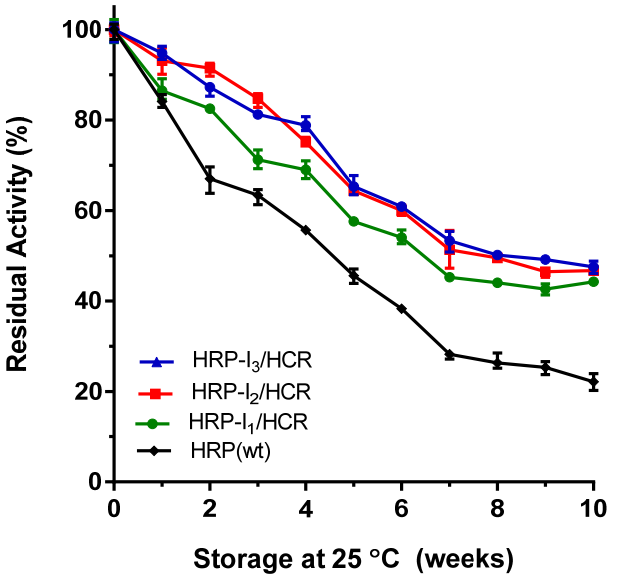

Figure S13. Long-term stability assay for HRP-I_1/HCR, HRP-I_2/HCR, HRP-I_3/HCR and HRP (wt) with the incubation from week 0 – week 10 at 25 °C in the dark. Error bar: range of data for three replicates.

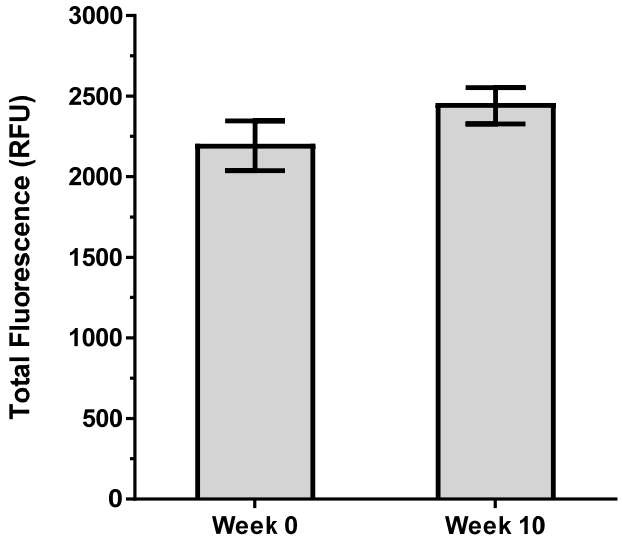

Figure S14. Solution fluorescence intensity of Cy3-labeled HRP during the long-term incubation. The stable fluorescence intensity indicated that HRP solution concentration did not vary significantly over the incubation period.

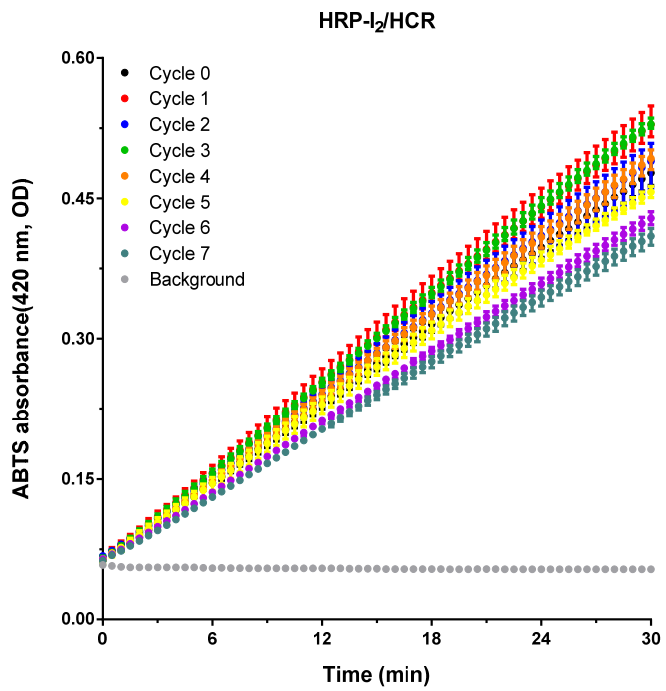

Figure S15. Raw activity curves of the freeze-thaw stability test for HRP-I_2/HCR. Error bar: range of data for three replicates.

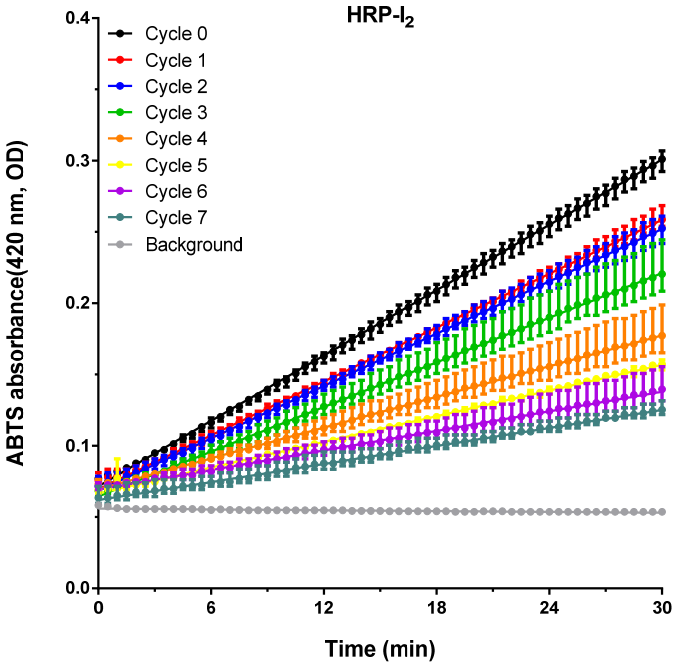

Figure S16. Raw activity curves of the freeze-thaw stability test for HRP-I_2. Error bar: range of data for three replicates.

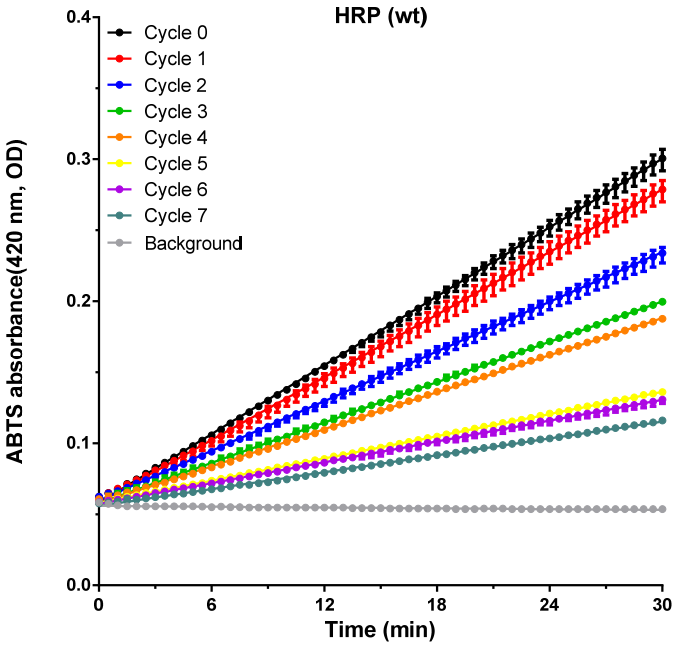

Figure S17. Raw activity curves of the freeze-thaw stability test for wildtype HRP. Error bar: range of data for three replicates.

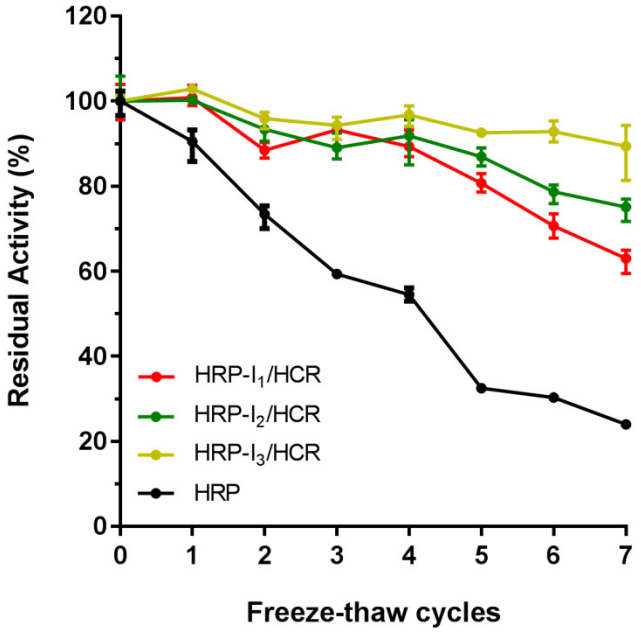

Figure S18. Freeze-thaw stability assay for HRP-I_1/HCR, HRP-I_2/HCR, HRP-I_3/HCR and HRP (wt). Error bar: range of data for three replicates.

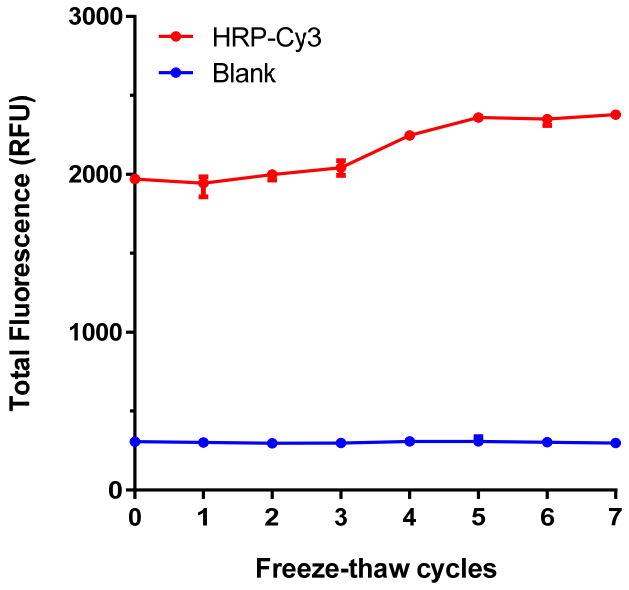

Figure S19. Fluorescence intensity of HRP-Cy3 solution over the freeze-thaw cycles. The relatively stable fluorescence signal indicates that HRP solution concentration does not fluctuate significantly during the freeze-thaw cycles.

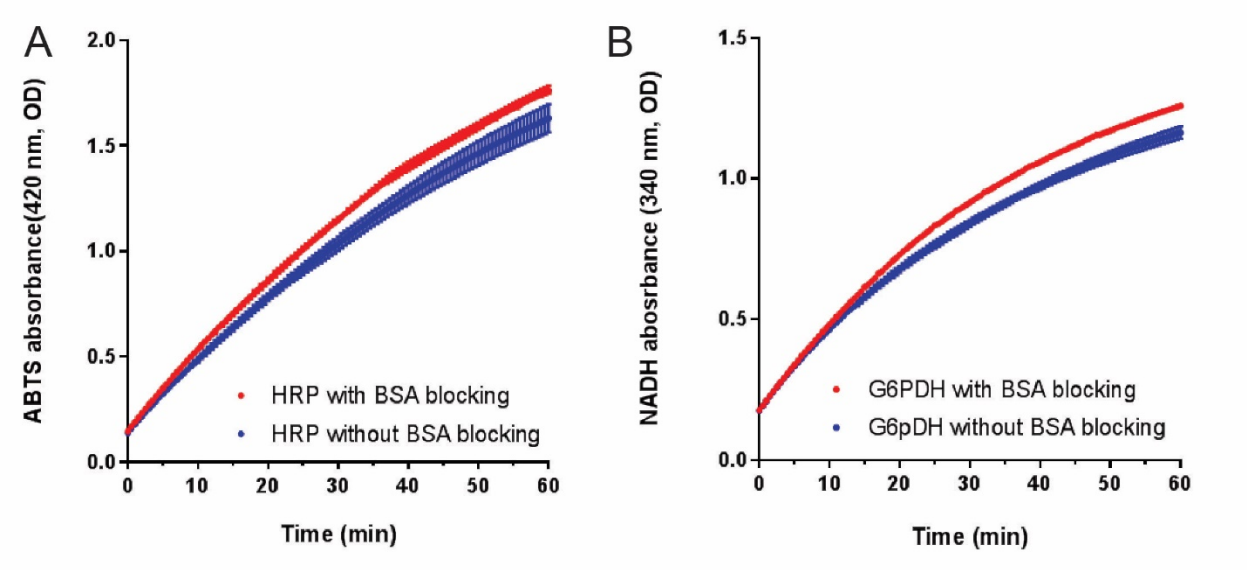

Figure S20. The test of enzyme activities with and without BSA pre-blocking for (A) HRP and (B) G6PDH. The activity is assayed at 1 nM enzyme concentration in 96-well plate. For BSA pre-blocking, the 96-well plate is pre-blocked with 200 μL 1 mg/ml BSA for two hours.

DNA	A260/ A280	ε_{260} (M^{-1} cm^{-1})	ε_{280} (M^{-1} cm^{-1})	Enzyme	A260/ A280	ε_{260} (M^{-1} cm^{-1})	ε_{280} (M^{-1} cm^{-1})	Collection peaks	A260/A280	A260	A280	DNA - to-Protein Ratio	Protein Conc. (μM)
I	2.15	359600	167220.3	G6pDH	0.52	61594	118450	G6pDH-I (1)	1.44	9.065	6.296	0.92	23.17
I	2.15	359600	167220.3	G6pDH	0.52	61594	118450	G6pDH-I (2)	1.69	6.602	3.900	1.82	9.24
I	2.15	359600	167220.3	G6pDH	0.52	61594	118450	G6pDH-I (3)	1.84	5.549	3.023	2.96	4.93
DNA	A260/ A280	ε_{260} (M^{-1} cm^{-1})	ε_{280} (M^{-1} cm^{-1})	Enzyme	A260/ A405	ε_{260} (M^{-1} cm^{-1})	ε_{405} (M^{-1} cm^{-1})	Collection peaks	A260/A405	A260	A405	DNA - to-Protein Ratio	Protein Conc. (μM)
I	2.15	359600	167220.3	HRP	0.38	38000	100000	HRP-I (1)	3.80	11.60	3.06	0.95	30.57
I	2.15	359600	167220.3	HRP	0.38	38000	100000	HRP-I (2)	8.23	9.22	1.12	2.18	11.20
I	2.15	359600	167220.3	HRP	0.38	38000	100000	HRP-I (3)	10.88	10.04	0.92	2.92	9.23

Table S1. Quantification of the concentration and DNA labeling ratio of the purified G6PDH-I and HRP-I conjugates by measuring the absorbance at 260 and 280 nm for G6PDH and 405 nm for HRP, and using the following equations:

$$A_{260}(DNA-protein) = \varepsilon_{260}(protein) * Conc.(protein) + \varepsilon_{260}\ (DNA) * Conc.(DNA)$$

$$A_{280}(DNA-protein) = \varepsilon_{280}(protein) * Conc.(protein) + \varepsilon_{280}\ (DNA) * Conc.(DNA)$$

$$Ratio\left(\frac{DNA}{protein}\right) = \frac{Conc.(DNA)}{Conc.(protein)}$$

Reference

(1) Dirks, R. M.; Pierce, N. A. Triggered amplification by hybridization chain reaction. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15275-15278.

(2) Brooke, D.; Movahed, N.; Bothner, B.: *Universal buffers for use in biochemistry and biophysical experiments*, 2015; Vol. 2.

(3) Fu, J.; Yang, Y. R.; Dhakal, S.; Zhao, Z.; Liu, M.; Zhang, T.; Walter, N. G.; Yan, H. Assembly of multienzyme complexes on DNA nanostructures. *Nat. Protoc.* **2016**, *11*, 2243-2273.

(4) Fu, J.; Yang, Y. R.; Johnson-Buck, A.; Liu, M.; Liu, Y.; Walter, N. G.; Woodbury, N. W.; Yan, H. Multi-enzyme complexes on DNA scaffolds capable of substrate channelling with an artificial swinging arm. *Nat. Nanotechnol.* **2014**, *9*, 531-536.