Electronic Supplementary Information

Exonuclease-Assisted Cascaded Recycling Amplification for Label-Free Detection of DNA

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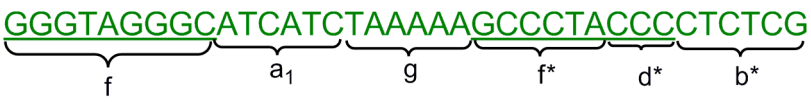
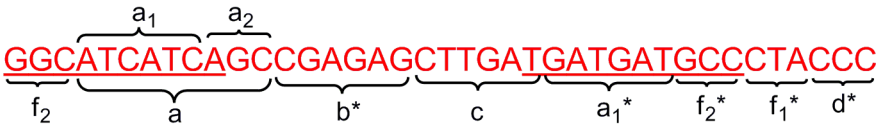
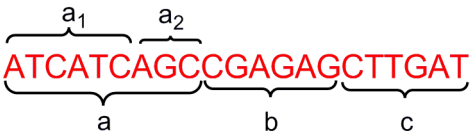
Experimental Section

Materials. Oligonucleotides were custom-synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (sequences see Table S1).

Table S1. Oligonucleotide Sequences Used in This Study

Name	Sequences (5′ to 3′)[a]
MB_1	
MB_2	
MB_3	
perfectly matched DNA target	
single-base mismatched DNA target	ATCATCA*T*CCGAGAGCTTGAT
three-base mismatched DNA target	ATCATCA*T*CC*T*A*T*AGCTTGAT
completely mismatched DNA target	*AGAGGTAGTAGGTTGCATAGT*

[a]Underlines indicate complementary sequences of stem-loop DNA molecular beacons. Italic letters indicate mismatched bases. Domain x* is the complement of domain x.

The stock solution of DNA (0.1 mM) were prepared in the TE buffer (10 mM Tris-HCl, 1 mM EDTA, 12.5 mM $MgCl_2$, pH 8.0) and diluted to desired concentration using the same TE buffer. Exo-III was purchased from Fermentas (Canada). Hemin, tris(hydroxymethyl)aminomethane hydrochloride (Tris), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) disodium salt

(ABTS^{2-}), 4-(2-hydroxyethyl)piperazine-1 ethanesulfonic acid sodium salt (HEPES) were ordered from Aladdin Chemistry Co. Ltd (China), and used as supplied. A hemin stock solution (5 mM) was prepared by dissolving 0.0326 g of hemin in 10 mL of DMSO and stored in dark at -20 ^{o}C. The human serum sample was supplied by Qingdao Center Hospital. All chemicals were used as received without further purification. Water was used in all of the experiments.

Exo-CRA Reaction for DNA Assay. Previously, the DNA solutions of MB_1 (1.0×10^{-5} M), MB_2 (1.0×10^{-5} M) and MB_3 (1.0×10^{-5} M) were heated at 90 ^{o}C for 10 min, followed by gradually cooling down to room temperature. The Exo-CRA reaction was performed by mixing 2.0×10^{-6} M MB_1, 2.0×10^{-6} M MB_2, 2.0×10^{-6} M MB_3, 4.5 μL of 10 × reaction buffer, 50 units of exonulease III and varying concentrations of DNA target to a final volume of 45 μL, followed by incubating at 25 ^{o}C for 30 min.

Colorimetric Measurement. The experiment was performed by mixing the above products, 800 μL of hemin (2.5×10^{-6} M), and 400 μL of ABTS^{2-} (10 mM) in a buffer solution consisting of 25 mM HEPES, 20 mM KCl, 200 mM NaCl, Triton X-100 (0.05%, w/v), and DMSO (1%, v/v), pH 7.4. The reaction started upon the quick addition of 755 μL of H_2O_2 (5.3 mM). The absorption spectra were recorded with a Cary50 UV-vis spectrophotometer (Varian, USA).

Nondenaturing Polyacrylamide Gel Electrophoresis. The assembly and disassembly of the fabricated DNA nanoball structure were characterized by 12.5% native polyacrylamide gel electrophoresis. The samples were by 6×loading buffer. 1×Tris-acetate-EDTA (TAE) was used as the separation buffer. Electrophoresis were carried out at 120 V for 1 h at room temperature. The gels were stained with ethidium bromide (EB, 0.5 μg/mL) for 30 min. The visualization and photography were performed using a digital camera under UV illumination.

Fluorescence Imaging. Three samples were prepared as follows. Sample 1: the mixture of annealed MB_1 (1.0×10^{-6} M), MB_2 (1.0×10^{-6} M) and MB_3 (1.0×10^{-6} M). Sample 2: the mixture of annealed MB_1 (1.0×10^{-6} M), MB_2 (1.0×10^{-6} M), MB_3 (1.0×10^{-6} M) and target DNA (1.0×10^{-7} M). Sample 3: the mixture of annealed MB_1 (1.0×10^{-6} M), MB_2 (1.0×10^{-6} M), MB_3 (1.0×10^{-6} M), target DNA (1.0×10^{-7} M) and Exo-III (50 units). All the samples were incubated at 25 ^{o}C for 30 min, followed by reacting with 5 μL of 10 × SYBR Green I dye (Invitrogen) to final volume of 50 μL. The fluorescence imaging was carried out on a Leica DMI600B CS inverted microscope. The samples were excited with Leica FL6000 fluorescence light source through I3 filter (450~490 nm), and observed with Leica HCX PLAN APO 100x/1.46 objective. The images were recorded with an Andor iXon X3 EMCCD camera.

Optimization of the Exo-CRA Machine.

Effect of Reaction Temperature. The activity of Exo-III is highly sensitive to temperature that is also closely related to the conformation of the constructed MBs. Thus, as an essential factor of this assay, the reaction temperature was first investigated by detecting 5.0×10^{-12} M target DNA at 4, 25, 37, 45 ^{o}C, respectively. The blank sample at each temperature was treated in the same way for Exo-CRA without target DNA. As shown in Fig. S1, the UV-vis absorption for not only target DNA but also blank was increased with an increase of reaction temperature. It is reasonable that as the temperature increased, the activity of Exo-III increased, which facilitate the Exo-CRA reaction. However, on the other hand, the conformation of the MBs could be destroyed as the increase of temperature, which increases the background signal. Additionally, the exonuclease also degrades some of the closed molecular beacon under relatively high temperature.[S1] Fig. S1B shows the

maximum interval of the UV-vis absorption between target and the blank. Therefore, 25 °C was considered to be the optimum reaction temperature used in the Exo-CRA reaction.

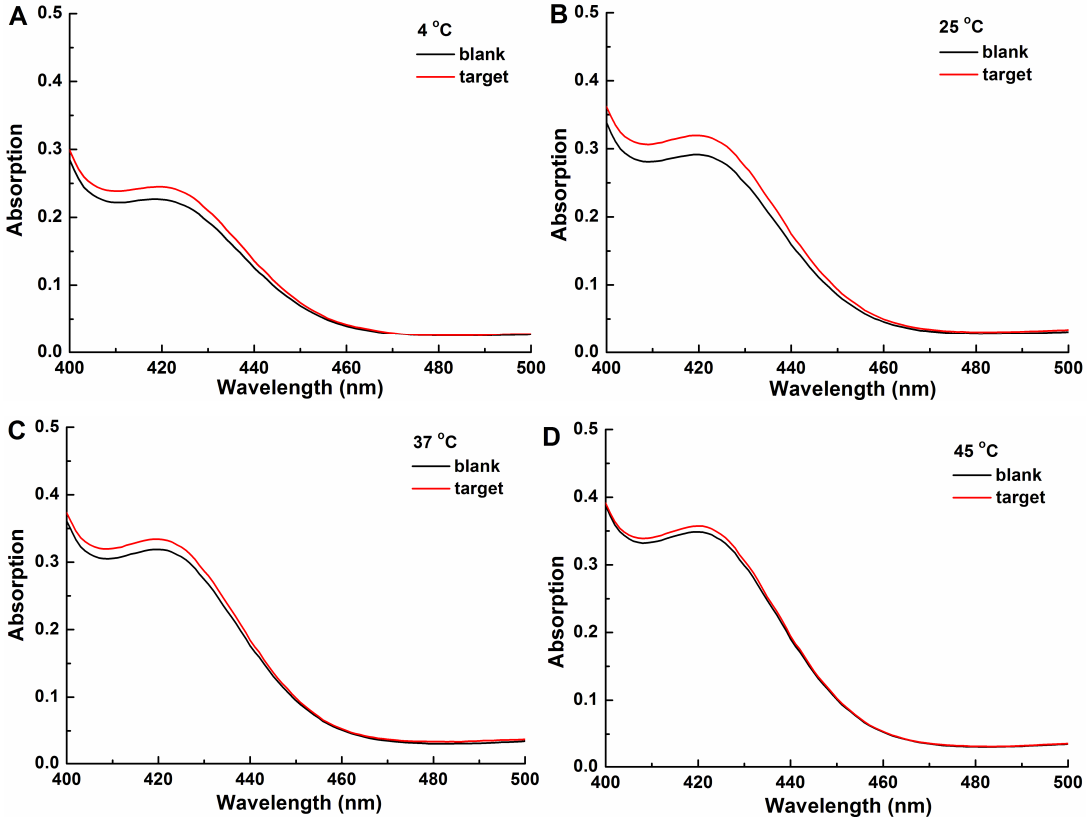


Fig. S1 The influence of reaction temperature. The UV-vis curves were obtained by 5.0×10^{-12} M target DNA (red) and the blank (black) with Exo-CRA machine produced DNAzymes. The Exo-CRA reaction and UV-vis measurement were carried out according to the procedures mentioned in the Experimental Section except the temperature used for Exo-CRA as (A) 4 °C, (B) 25 °C, (C) 37 °C, and (D) 45 °C.

Effect of the Amount of Exo-III. To investigate the influence of the amount of Exo-III used in Exo-CRA machine on DNA detection, the UV-vis curves produced by 5.0×10^{-12} M target DNA was measured by using 0, 25 units, 50 units, and 100 units Exo-III, respectively. The blank sample was treated in the same way for Exo-CRA without target DNA. Fig. S2 depicts the influence of

the amount of Exo-III used in the proposed Exp-CRA machine on the UV-vis intensities of target DNA and the blank. The maximum interval of the UV-vis absorption between target and the blank was obtained at 50 units Exo-III (Fig. S2C). Therefore, 50 units of Exo-III was selected for the Exo-CRA machine.

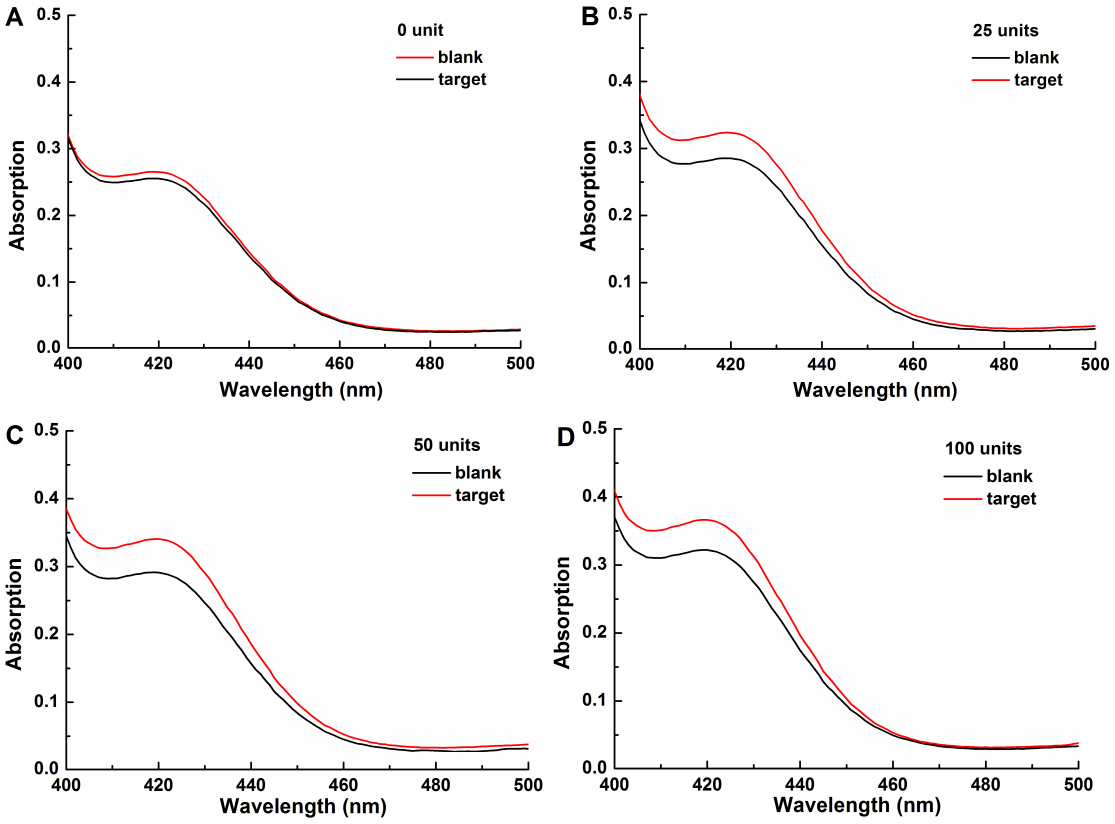


Fig. S2 The influence of the amount of Exo-III. The UV-vis curves were obtained by 5.0×10^{-12} M target DNA (red) and the blank (black) with Exo-CRA machine produced DNAzymes. The Exo-CRA reaction and UV-vis measurement were carried out according to the procedures mentioned in the Experimental Section except the amount of Exo-III used for Exo-CRA as (A) 0, (B) 25 units, (C) 50 units, and (D) 100 units.

Effect of Reaction Time. Subsequently, we investigated the influence of the reaction time for the performance of the Exo-CRA machine upon analyzing target DNA at a concentration corresponding to 5.0×10^{-12} M. As shown in Fig. S3, the UV-vis absorption intensities were greatly increased with increasing the time at the early stage, and reached the maximum after ~30 min, while no obvious UV-vis absorption change was observed for the blank. Therefore, the reaction time of 30 min was adopted for the proposed Exo-CRA machine used in DNA assay.

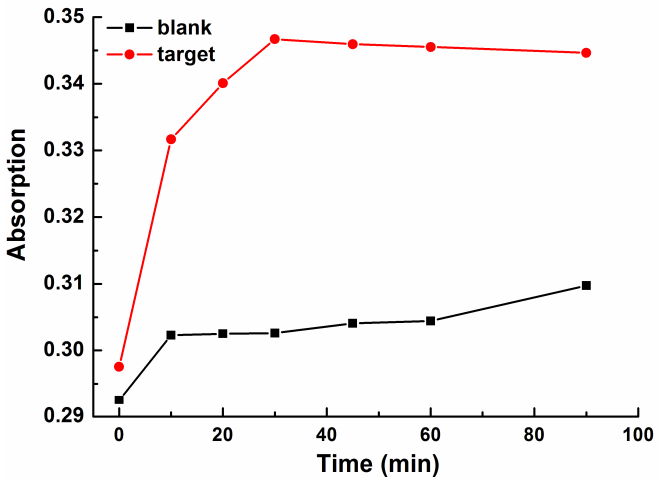
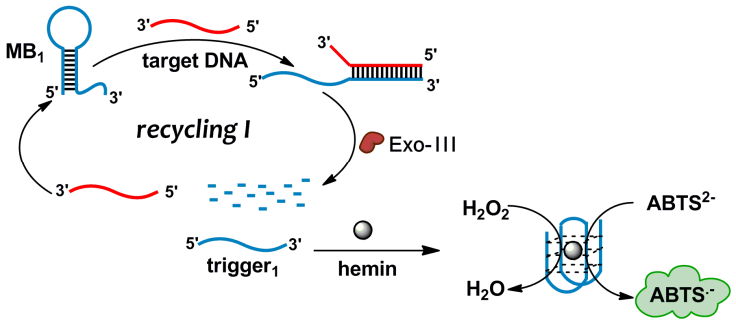


Fig. S3 The influence of the reaction time. The UV-vis absorption was obtained by 5.0×10^{-12} M target DNA (red) and the blank (black) with Exo-CRA machine produced DNAzymes. The Exo-CRA reaction and UV-vis measurement were carried out according to the procedures mentioned in the Experimental Section except the reaction time used for Exo-CRA as 0, 10, 20, 30, 45, 60, and 90 min, respectively.

Control Experiments.

The first control experiment of exonulease-assisted target recycling that only employed MB_1 was carried out as Scheme S1. Upon the addition of target DNA, the stem-loop structure of MB_1 is opened and forms a blunt 3′ terminus, which facilitates the 3′- to -5′ exonucleolytic activity of

Exo-III, liberating trigger$_1$ and ultimately releasing the target. The released target can then hybridize with a new MB$_1$ and catalyze a new cycle of MB$_1$ transformation, achieving the recycling I and amplification of the signal. From the results of Fig. S4, the UV-vis absorption that is obtained by the produced DNAzymes increases with increasing concentrations of target DNA ranging from 10.0 pM to 1.0 μM with a linear correlation equation as $\Delta A = 0.0050 \lg C + 0.0595$ (ΔA is the relative UV-vis absorption intensity; C is the concentration of target DNA; n= 6, R = 0.9982) and a limit of detection (LOD) of 10.0 pM.



Scheme S1 Illustration of ssDNA target triggered exonulease-assisted amplification that only employs one kind of MB, MB$_1$. The signal can be easily readout by binding the generated trigger$_1$ with hemin to yield DNAzymes to catalyze the oxidation of $ABTS^{2-}$ by H_2O_2 to form a green colored product, $ABTS^{\cdot-}$.

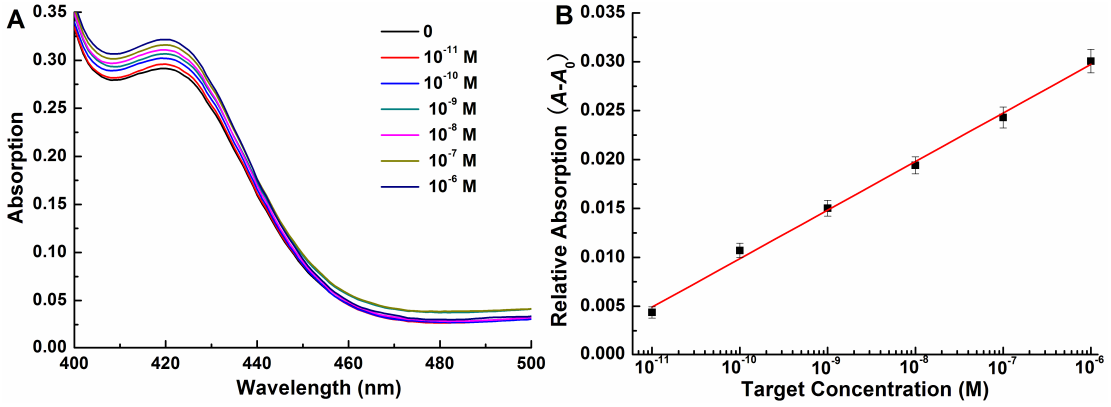
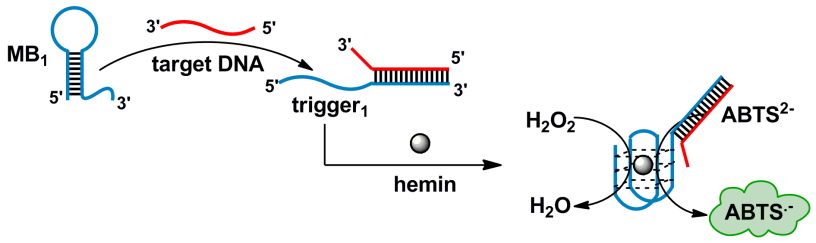


Fig. S4 (A) UV-vis absorption spectra for analyzing the peroxidation product ($ABTS^{\cdot-}$) of the $ABTS^{2-}$-H_2O_2 system catalyzed by the HRP-mimicking DNAzyme that is yielded in Scheme S1

strategy corresponding to different concentrations of target DNA from 0 to 1.0×10^{-6} M. (B) The corresponding calibration curve of relative UV-vis absorbance (at 419 nm) versus the concentration of target DNA. Relative UV-vis absorbance ($A - A_0$) is calculated by $A - A_0$, where A_0 and A are the UV-vis absorbance without and with target DNA, respectively. The concentration of each kind MB is 2.0×10^{-6} M. Error bars are standard deviation of three repetitive measurements.

The other control experiment that only employs MB_1 without exonuclease amplification was illustrated in Scheme S2. Upon the addition of target DNA, the stem-loop structure of MB_1 is opened and forms a blunt 3′ terminus. Because no Exo-III exists in this system, no recycling occurs. The signal is obtained by binding the newly exposed sticky end of MB_1 ($trigger_1$) with hemin to generate a DNAzyme. This 1:1 hybridizaiton ratio seriously limits the sensitivity. From the results of Fig. S5, the UV-vis absorption that is obtained by the produced DNAzymes only increases with increasing concentrations of target DNA ranging from 1.0 nM to 1.0 μM with a linear correlation equation as $\Delta A = 0.0127 \lg C + 0.1247$ (ΔA is the relative UV-vis absorption intensity; C is the concentration of target DNA; n= 4, R = 0.9981) and a limit of detection (LOD) of 1.0 nM.



Scheme S2 Illustration of ssDNA target detection that only employs MB_1 without exonuclease amplification. The signal can be easily readout by binding the generated $trigger_1$ with hemin to

yield DNAzymes to catalyze the oxidation of $ABTS^{2-}$ by H_2O_2 to form a green colored product, $ABTS^{\cdot-}$.

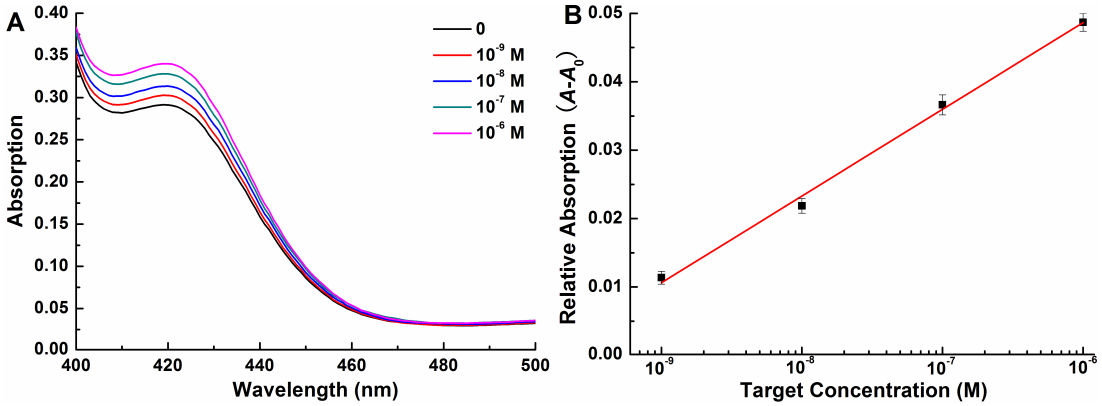


Fig. S5 (A) UV-vis absorption spectra for analyzing the peroxidation product ($ABTS^{\cdot-}$) of the $ABTS^{2-}$-H_2O_2 system catalyzed by the HRP-mimicking DNAzyme that is yielded in Scheme S2 strategy corresponding to different concentrations of target DNA from 0 to 1.0×10^{-6} M. (B) The corresponding calibration curve of relative UV-vis absorbance (at 419 nm) versus the concentration of target DNA. Relative UV-vis absorbance ($A - A_0$) is calculated by $A - A_0$, where A_0 and A are the UV-vis absorbance without and with target DNA, respectively. The concentration of each kind MB is 2.0×10^{-6} M. Error bars are standard deviation of three repetitive measurements.

Selectivity of the Assay.

To evaluate the specificity of the assay, the proposed Exo-CRA strategy was challenged to perfectly matched, single-base-mismatched, three-base-mismatched, and completely mismatched target DNA sequences, respectively. From the results of Fig. S6, no matter the targets are at low concentration (1.0×10^{-11} M) or at high concentration (1.0×10^{-7} M), the UV-vis signal produced by the completely complementary DNA could be well separated from those produced by other mismatched targets. The completely mismatched sequence showed no response that was equal to the blank. Thus, the presented DNA assay with Exo-CRA could readily discriminate between mismatched targets, even single nucleotide polymorphisms. The high specificity could be attributed to the specific hybridization of DNA and digestion property of Exo-III on the duplex from the blunt 3′-OH terminus.

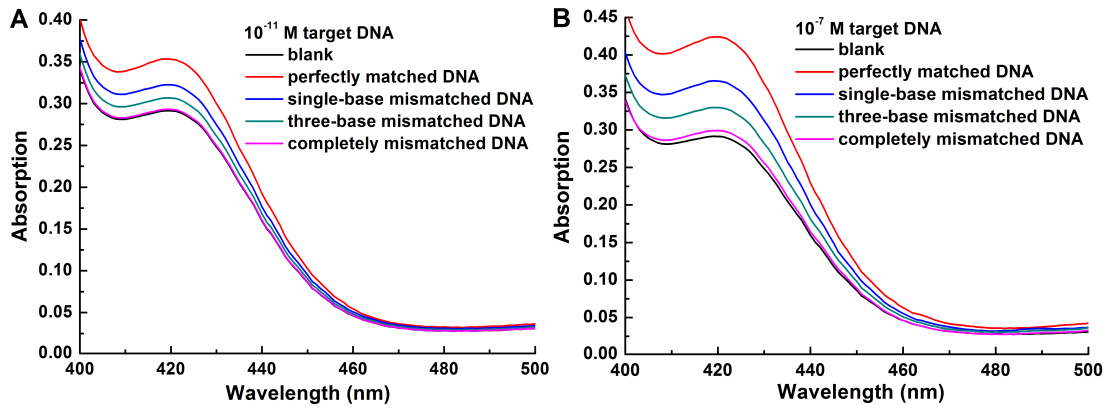


Fig. S6 UV-vis absorption spectra for analyzing the peroxidation product ($ABTS^{\cdot-}$) of the $ABTS^{2-}$-H_2O_2 system catalyzed by the HRP-mimicking DNAzyme that is yielded in the proposed Exo-CRA machine corresponding to perfectly matched and mismatched DNA targets at 1.0×10^{-11} M (A) and 1.0×10^{-7} M (B) target concentrations.

Real Sample Assay.

To investigate the applicability of the proposed Exo-CRA machine in a real human serum sample, we performed spike and recovery experiments. The serum sample was respectively spiked with 1.0×10^{-12}, 1.0×10^{-10}, and 1.0×10^{-8} M target DNA to test the performances of the assay in complex matrixes. From Fig. S7, the UV-vis absorbance obtained from serum sample decreases slightly compared to that in buffer solution. By using calibration method to determine the concentration of target DNA in serum sample, the recovery was calculated to be 99 ± 5%, indicating the potentiality of the proposed assay for DNA detection in real biological samples.

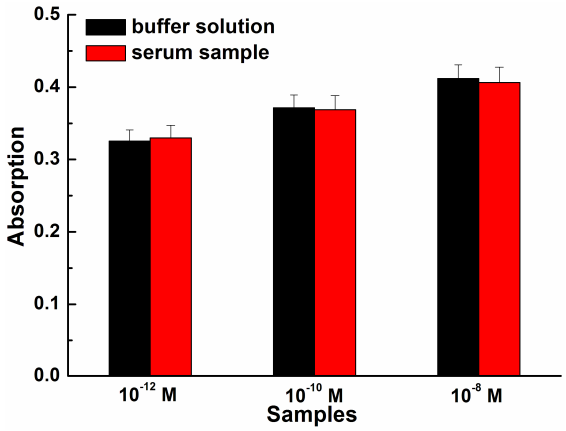


Fig. S7 UV-vis absorption spectra for analyzing the peroxidation product ($ABTS^{\cdot-}$) of the $ABTS^{2-}$-H_2O_2 system catalyzed by the HRP-mimicking DNAzyme that is yielded in the proposed Exo-CRA machine corresponding to different concentrations of target DNA (1.0×10^{-12}, 1.0×10^{-10}, and 1.0×10^{-8} M) in a buffer solution and a serum sample, respectively. Error bars are standard deviation of three repetitive measurements.

References.

S1 X. Zuo, F. Xia, Y. Xiao and K. W. Plaxco, *J. Am. Chem. Soc.*, 2010, **132**, 1816-1818.