Supporting Information

Nano-immunosorbent assay based on Cas12a/crRNA for ultra-sensitive protein detection

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Experimental procedures

1. Materials and reagents

All oligonucleotides were synthesized and HPCL-purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). CEA, anti-CEA antibodies, PSA, and anti-PSA antibodies were purchased from Linc-Bio Science Co., Ltd (Shanghai, China). PSA ELISA kit was purchased from Enzyme-Linked Biotechnology Co., Ltd. (Shanghai, China). Lab Cas12a (cpf1) and NEB 2.1 buffer were purchased from New England Biolabs Inc. (Ipswich, USA). Bovine serum albumin (BSA) was purchased from BBI Life Science. Tris[2-carboxyethyl] phosphine (TECP) and Tween-20 were obtained from Sigma-Aldrich, Inc. (St. Louis, Mo). $HAuCl_4 \cdot 3H_2O$ and other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Clinical serum samples were collected from Nanjing Drum Tower Hospital.

PBS buffer: 0.1 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4.

PBST buffer: 0.05% v/v Tween-20 in PBS buffer.

Block buffer: 1% BSA in PBS buffer.

2. Methods

2.1 Preparation of gold nanoparticles (AuNPs)

AuNPs (13 nm) were synthesized according to the standard citrate method[1]. The reaction glassware was soaked in aqua regia (HNO_3/HCl = 3:1) and washed with plenty of water for pretreatment. Briefly, 2 mL of 50 mM $HAuCl_4$ was added to 98 mL of Millipore water in a two-neck flask. The solution was heated to reflux and 10 mL of 38.8 mM sodium citrate was added quickly into the flask. The color changed from pale yellow to bright red indicating the formation of AuNPs. Allow the mixture to reflux for another 20 min, then cooled down to room temperature under stirring. The resulting solution was stored at 4°C and in dark for subsequent use. AuNPs were observed by transmission electron microscopy to determine the morphology and size. The concentration was detected by UV spectrum (Fig. S10)[2].

2.2 Characterization

AuNPs before and after modified with nucleotides were redistributed into PBS buffer for characterization to measure UV, zeta potential and TEM. The activator or AuNPs-DNA (0.1 nM) was added to extension solution, containing 1 × NEB 2.1 buffer, 20 nM Cas12a, 250 nM crRNA and 500 nM FAM-T_{36}. The solution was incubated in dark at 37°C for 60 min. For the negative group, the same amount of buffer or AuNPs was used. The cleavage activity of Cas12a was analyzed by 12% polyacrylamide gel and the final reaction product of 10 μL was loaded on the gel. Electrophoresis was carried out in 1 × TBE buffer at 200 V for 40 min in dark. After separation, the gel was imaged with the fluorescence gel imaging system. The procedure of activation of Cas12a cleavage activity by AuNPs-DNA was analyzed by PAGE, and the negative group was AuNPs.

2.3 Cleavage assay of Cas12a/crRNA recognition target DNA

The Cas12a cleavage assay was carried out in 100 μL reaction volume with 250 nM crRNA, 20 nM Cas12a, 100 nM FQ-reporter and various concentrations of target ssDNA at 37°C. Fluorescence spectrometer (F-4600, Hitachi) was used for fluorescence detection at temperature (excitation at 480 nm and emission at 520 nm), and the fluorescence signal was recorded every 120 s.

2.4 ELISA reaction

According to the protocol of the commercial kit, 50 μL of standard control group or samples were added to the plate. Subsequently, 100 μL of enzyme conjugate were added to each well and incubated for 1 h at 37°C. The microtiter plate was washed with wash solution in kit five times. Next, 100 μL of chromogenic substrate was added to each well and incubated for 15 min at 37°C in the dark. Finally,

50 μL of stop solution in kit was added to each well and the optical density (OD) at 450 nm was measured within 15 minutes immediately.

3. Supplementary figures

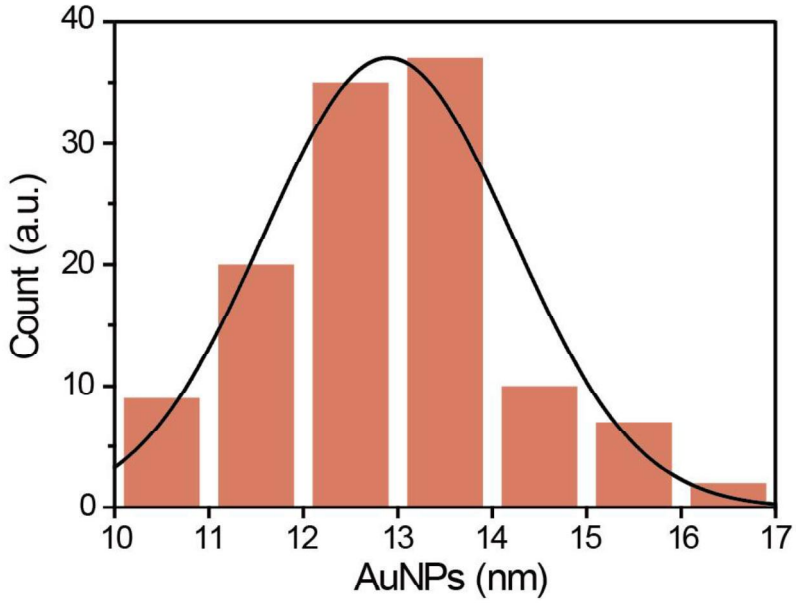

Fig. S1. Statistical size distribution of AuNPs.

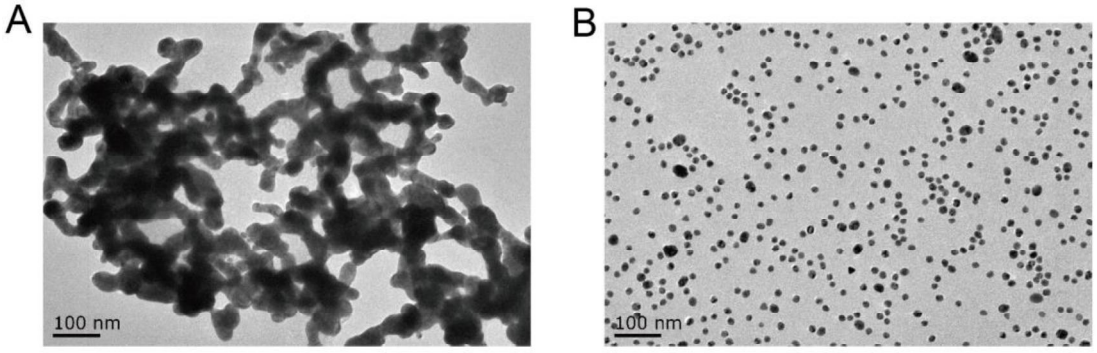

Fig. S2. TEM images of AuNPs before (A) and after (B) modification with DNA in PBS buffer.

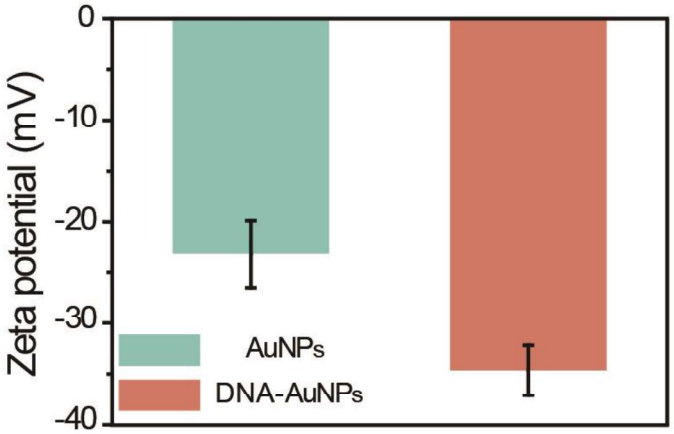

Fig. S3. Zeta potential of AuNPs and DNA-AuNPs.

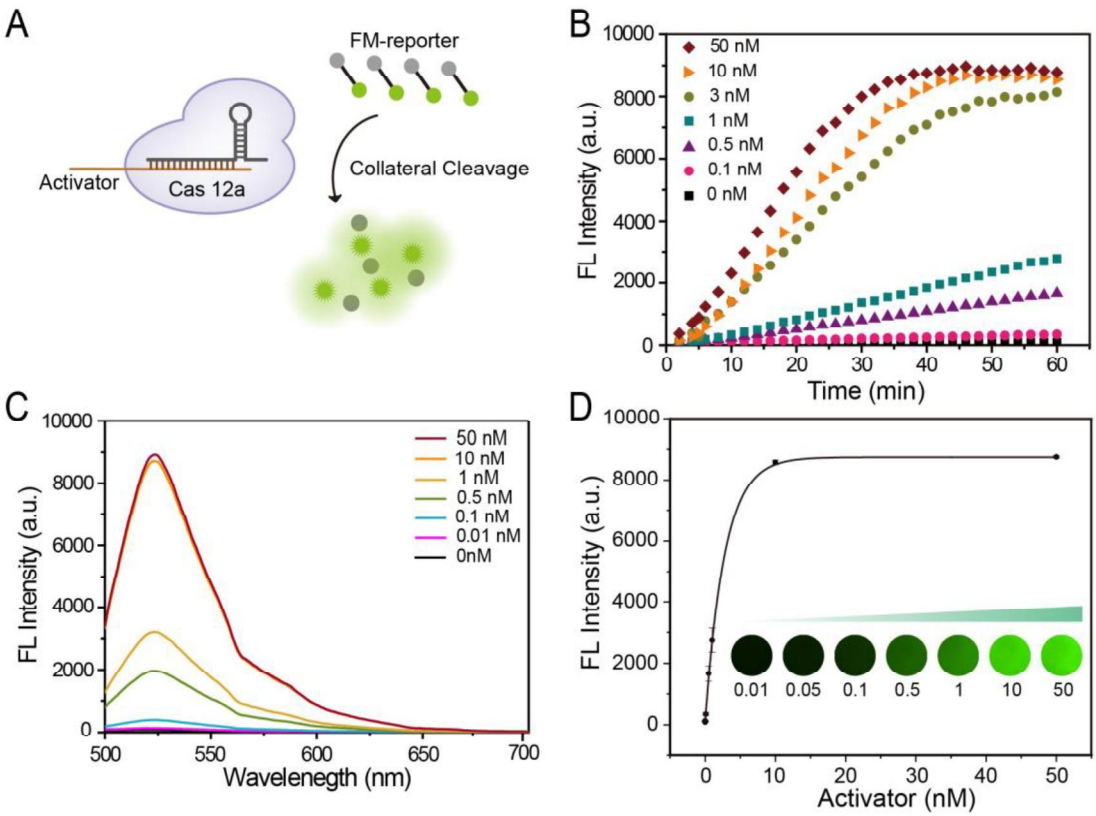

Fig. S4. Assay of Cas12a/crRNA *trans*-cleavage activity. (A) Schematic of ssDNA targets detected with the Cas12a/crRNA. (B) Real-time fluorescence assay of Cas12a/crRNA *trans*-cleavage activated by various concentrations of ssDNA targets. (C) Fluorescence spectra of FQ-reporters in the presence of ssDNA targets with different concentrations. (D) Calibration curve of ssDNA targets against the fluorescence signal at 520 nm. (inset: the corresponding fluorescence images; n = 3)

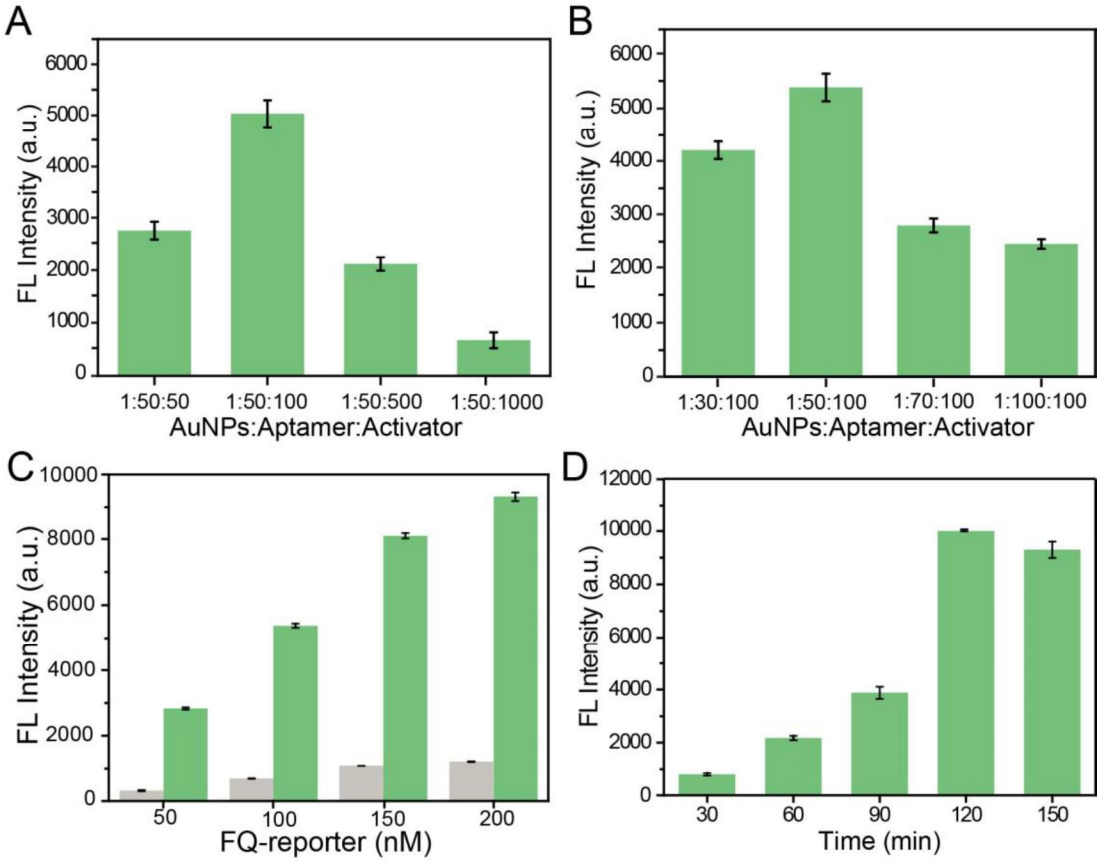

Fig. S5. Optimization of (A) (B) the molar ratio of AuNPs, aptamers and activators, (C) the concentration of FQ-reporters and (D) the reaction time. Error bars represent standard deviations obtained in three parallel experiments.

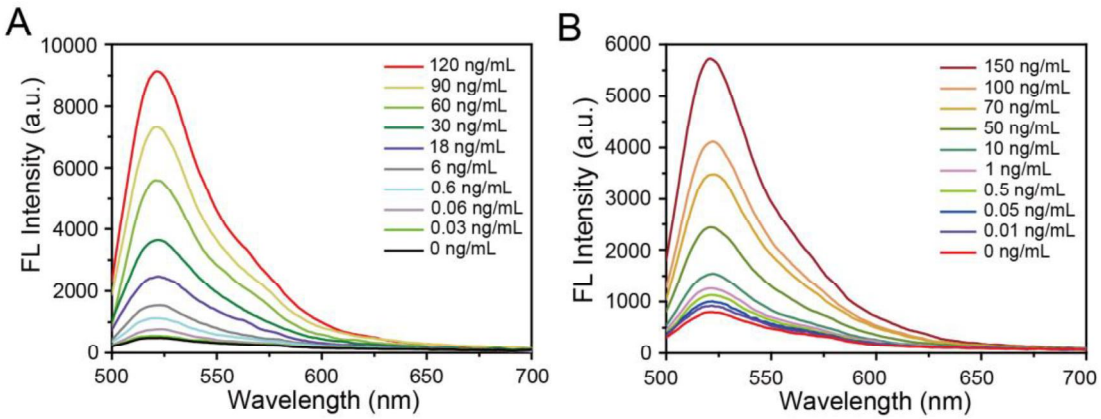

Fig. S6. (A) Fluorescence spectra at different concentrations of CEA between 0 and 120 ng/mL. (B) Fluorescence spectra at different concentrations of PSA between 0 and 150 ng/mL.

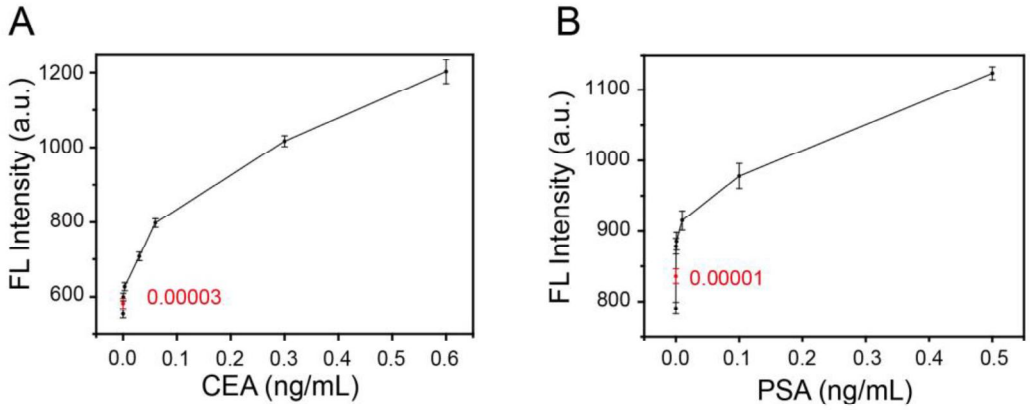

Fig. S7. (A) Detection of CEA. CEA was added to the coated plate at a series of 5-fold dilutions from 0 to 0.6 ng/mL. Data represent mean ± s.d., n = 3, three technical replicates. (B) Detection of PSA. PSA was added to the coated plate at a series of 5-fold dilutions from 0 to 0.5 ng/mL. Data represent mean ± s.d., n = 3, three technical replicates.

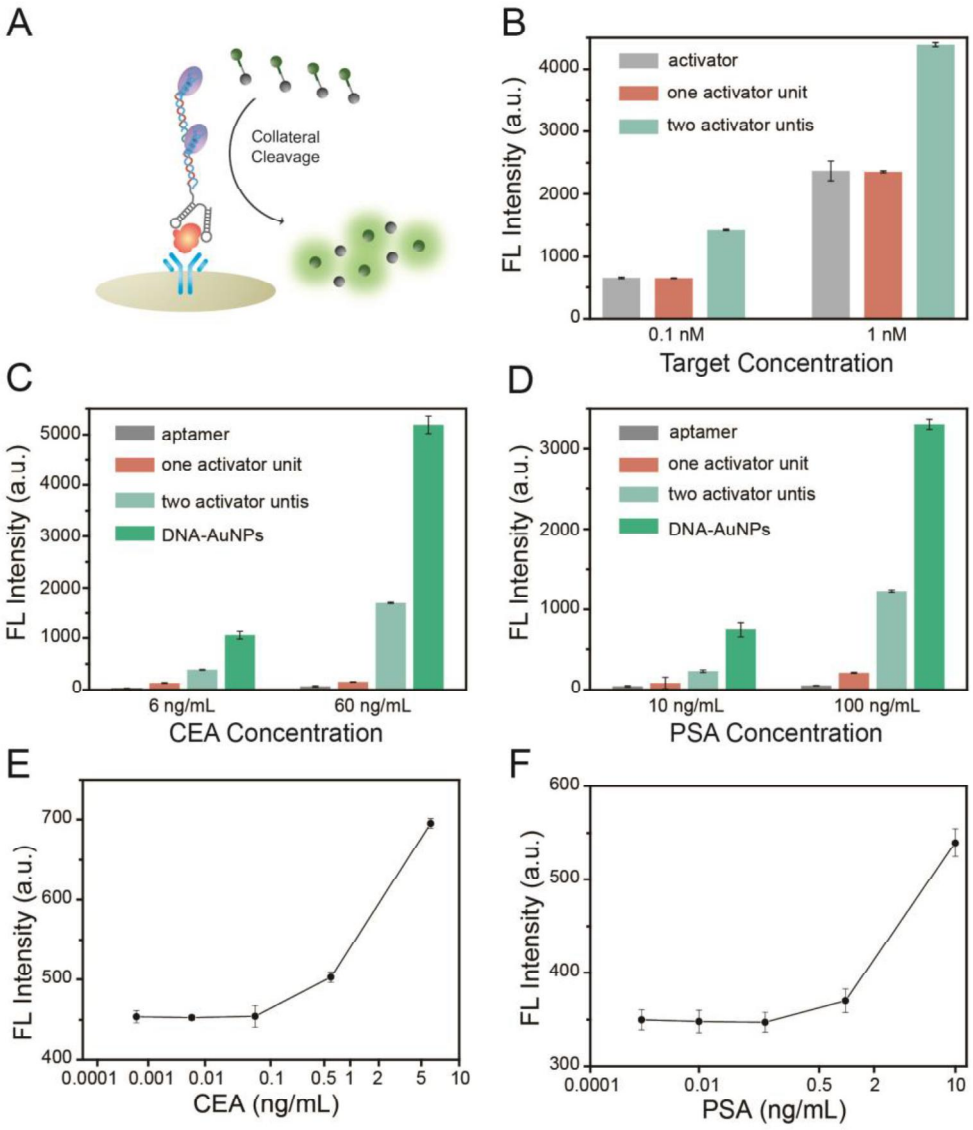

Fig. S8. (A) Schematic of using a single DNA strand as signal conversion strategy in CRISPR/Cas12a system to detect proteins. (B) Fluorescence intensity of CRISPR/Cas12a system in presence of activator and an aptamer strand including one or two repetitive activator units on a single DNA strand. Data represents mean ± s.d., n = 3, three technical replicates. Four kinds of signal transduction strategies (aptamer, an aptamer strand includes one or two repetitive activator units on a single DNA, DNA-AuNPs) were used in analyzing (C) CEA and (D) PSA. Data represents mean ± s.d., n = 3, three technical replicates. (E) CEA was added to the coated plate at a series 10-fold dilutions from 0.0006 to 6 ng/mL. Data represent mean ± s.d., n = 3, three technical replicates. (F) PSA was added to the coated plate at a series of 10-fold dilutions from 0.0001 to 10 ng/mL. Data represents mean ± s.d., n = 3, three technical replicates.

Four kinds of signal transduction strategies (aptamer, an aptamer strand includes one or two repetitive activator units on a single DNA, DNA-AuNPs) were compared in detection of CEA and PSA. An aptamer strand includes two repetitive activator units on a single DNA at most, due to the complexity and high cost of synthesizing DNA with more than 100 nt. The DNA sequence was shown in Table S1

and the principle was shown in Fig. S8A. Fig. S8B shows that the *trans*-cleavage ability of Cas12a could still be activated normally using an aptamer strand including activator units on a single DNA strand. As shown in Fig. S8C and S8D, the fluorescence signal intensity of DNA-AuNPs as signal transduction strategy was significantly higher than that of others, while the fluorescence signal intensity of aptamer system was the lowest due to the lack of activators. An aptamer strand including two repetitive activator units on a single DNA strand as a signal transduction strategy to detected CEA and PSA, with the lowest detection concentration was 0.3 and 0.7 ng/mL respectively (Fig. S8E and S8F), which was 10^4 times higher than that of Nano-CLISA method.

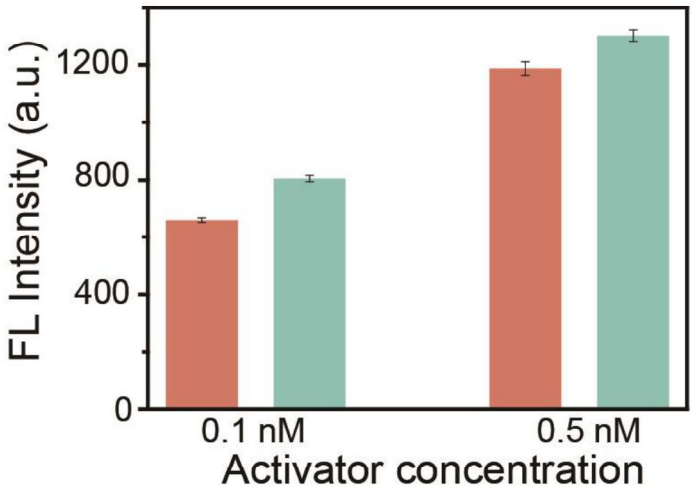

Fig. S9. Verification of the enzyme catalysis rate increase by modification on gold nanoparticles. The DNA of unmodified (red) and modified (green) -SH was respectively reacted with AuNPs and then reacted with the Cas12a system to measure the fluorescence signal. Data represents mean ± s.d., n = 3, three technical replicates.

Enzyme catalysis was affected by the approximation. Due to the local concentration of activators on gold nanoparticles in the Cas12a reaction system was higher than the free activators, and the reaction rate increased. As shown in Fig. S9, keeping other conditions consistent, it was found that the fluorescence signal intensity of the system with DNA-AuNPs was always higher than that of the system with only free DNA and AuNPs, which suggested the enzyme catalysis rate increase after modification on gold nanoparticles. The fluorescence signal difference was not very large, due to the addition of excessive DNA when connecting with AuNPs. The free DNA in the final system accounted for a large proportion.

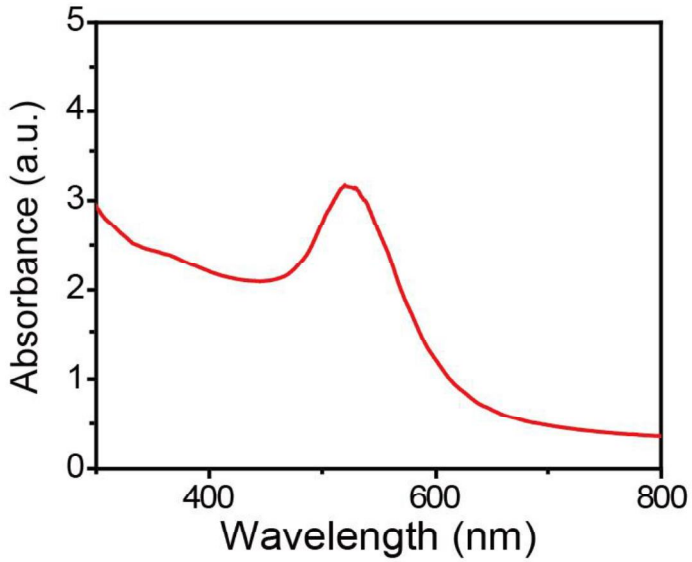

Fig. S10. The ultraviolet-visible (UV-visible) spectrum of AuNPs.

4. Supplementary tables

Table S1. Oligonucleotides used in this study.

Name	Sequence (5'-3')
PSA aptamer	SH-C_6-TTTTTTTTAATTAAAGCTCGCCATCAAATAGC
CEA aptamer	SH-C_6-TTTTTTTTTTTGCTGCTATACCAGCTTATTCAA
crRNA	UAAUUUCUACUAAGUGUAGAUAUCCAUGAGGUACUACUGGC CAA
Activator	SH-C_6- TTTTTTTTTTTGGCCAGTACCTCATGGAT
FQ-reporter	FAM-TTATT-BHQ
FAM-T_{36}	FAM-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
PSA-1-activator	TTAATTAAAGCTCGCCATCAAATAGCTTTTTTTTTTTGGCCAGTA CCTCATGGA
CEA-1-activator	GCTGCTATACCAGCTTATTCAATTTTTTTTTTTTTGGCCAGTACCT CATGGA
PSA-2-activator	TTAATTAAAGCTCGCCATCAAATAGCTTTTTTTTTTTGGCCAGTA CCTCATGGATTTTTTTTTTTTGGCCAGTACCTCATGGAT
CEA-2-activator	GCTGCTATACCAGCTTATTCAATTTTTTTTTTTTTGGCCAGTACCT CATGGATTTTTTTTTTTTGGCCAGTACCTCATGGAT

Table S2. Comparison of protein test results across published reports.

Analytical methods	Sensitivity (ng/mL)	Dynamic range (ng/mL)	Target
Nano-CLISA (this work)	0.0000139	0.6-120	CEA
	0.0000056	0.5-150	PSA
Metal-enhanced fluorescence[3]	0.1	0.1-40	CEA
	0.027	0.1-100	PSA
QDs based FRET[4]	0.56	1-100	CEA
	0.22	1-100	PSA
Chemiluminescence assay[5]	0.21	0.4-250	CEA
	0.11	0.3-50	PSA
Chemiluminescence assay[6]	0.034	0.05-20	CEA

5. Michaelis-Menten equation

$$V_0 = V_{max}\frac{[S]}{K_m + [S]}$$

where K_m is the Michaelis constant, V_{max} is the reaction rate of enzyme saturated with substrate, [S] is the substrate concentration.

References

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