Supporting Information

A gold nanoparticle-based four-color proximity immunoassay for one-step, multiplexed detection of protein biomarkers using ribonuclease H signal amplification

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Experimental

Materials and reagent

Human serum albumin (HSA), immunoglobulin G (IgG), immunoglobulin E (IgE), carcinoembryonic antigen (CEA), vascular endothelial growth factor (VEGF), prostate-specific antigen (PSA), and alpha-fetoprotein (AFP) were purchased from Sigma-Aldrich. (St. Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA). CEA antibodies (Ab1 and Ab2, No. ab4451 and ab133633), PSA antibodies (Ab3 and Ab4, No. ab68466 and ab166713), AFP antibodies (Ab5 and Ab6, No. ab3969 and ab3980), and VEGF antibodies (Ab7 and Ab8, No. ab1316 and ab46154) were supplied by the Abcam (Cambridge, MA, USA). Ribonuclease H (RNase H) were obtained from New England Biolabs (NEB, U.K.). All oligonucleotides were provided by Sangon Biotech Co. (Shanghai, China) and purified by HPLC. The sequences of the oligonucleotides involved are shown in Table S1. Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carbo-xylate (SMCC) and hydrogen tetrachloroaurate (III) ($HAuCl_4 \cdot 4H_2O$, 99.99%) were purchased from J&K (Beijing, China). All other reagents were of analytical grade and used without further purification. Ultrapure water from a Milli-Q plus 185 equip (Millipore Co., Bedford, MA, USA) was used throughout the work.

Apparatus

The fluorescence measurements were carried out using a LS-55 spectrofluorometer (Perkin-Elmer, USA) with a 300 μL cuvette. Fluorescent dyes in the sample solution were excited at 353 nm for AMCA, 494 nm for FAM, 570 nm for ROX and 645 nm for Cy5, and the fluorescence signals were measured at 445 nm for AMCA, at 520 nm for

FITC, 608 nm for ROX and 665 nm for Cy5, respectively. And slits for both excitation and emission were set at 10 nm. Transmission electron microscopy (TEM) was carried out on a JEM-100CX II electron microscope. Absorption spectra were performed using a TU-1901 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co, Ltd., China). HPLC assays were carried out using an LC-10ATVP system equipped with a RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). The assays were carried out on a C18 column (250×4.6 mm i.d., 5 μm particle sizes, Elite, China). The HPLC assay used 494 nm and 520 nm as excitation and detection wavelengths, respectively.

Synthesis of gold nanoparticles

Gold nanoparticles (AuNPs, 13 nm in size) were synthesized by the sodium citrate reduction method as previously reported.[1] Briefly, 0.01% $HAuCl_4$ solution (100 mL) was first heated to boiling with vigorous stirring, and 1% trisodium citrate solution (2.0 mL) was then added. The color of the solution turned from pale yellow to colorless and finally to burgundy. The resulting solution was kept boiling for 10 min, and then cooled to room temperature under stirring. Finally, the prepared AuNPs was filtered through a 0.45 μm Millipore membrane filter, and were stored at 4 ℃. Transmission electron microscopy (TEM) images (Figure S1) indicated the average sizes of AuNPs are 13 nm.

Preparation of the nanobeacon

Four-color gold nanobeacon was prepared by modification of AuNPs with thiolated oligonucleotides as previously reported.[2] Equimolar dye-labeled RNA-DNA hairpins (H1, H2, H3, and H4) were mixed and then added to a AuNPs solution (20 nM) to reach

the final concentration of 5.0 μM each. Then, the mixture was incubated for 12 h. Subsequently, 10% SDS solution was added to the mixture to reach the final concentration of 0.1% SDS, and 100 mM phosphate solution (pH 7.4, containing 1 M NaCl) was added to achieve final concentration of 0.1 M NaCl. The resulting solution was further incubated for 12 h. Excess reagents were then removed via centrifugation at 12, 000 rpm for 25 min at 4 ℃. The precipitate was washed three times with phosphate buffered saline (PBS) (pH 7.2), with repetitive centrifugation and dispersion, which were finally dispersed in PBS (pH 7.2). The prepared nanobeacon was stored at 4 ℃. The concentration of the nanobeacon was determined via the absorption of AuNPs.

Quantitation of each RNA-DNA hairpin loaded on the nanobeacon

The quantitation of four RNA-DNA hairpins on AuNPs was as previous reported.[3] Mercaptoethanol (ME) was added to the nanobeacon solution (1 nM) to reach the final concentration of 20 mM ME, and the mixture was incubated overnight with shaking at room temperature. The dye-labeled RNA-DNA hairpins were released, and released dye-labeled RNA-DNA hairpins were separated via centrifugation and the fluorescence of dye-labeled RNA-DNA hairpins was measured using a LS-55 spectrofluorometer. The fluorescence of each released dye-labeled RNA-DNA hairpin was converted to molar concentrations by interpolation from a standard linear calibration curve that was prepared with known concentrations of the corresponding dye-labeled RNA-DNA hairpins with identical buffer pH, ionic strength, and ME concentrations. By dividing molar concentrations of each dye-labeled RNA-DNA hairpin by the original nanobeacon concentration, the amount of dye-labeled RNA-DNA hairpins per nanobeacon was calculated.

Preparation of DNA-antibody conjugates

The DNA-conjugated antibodies were prepared according to the previously reported method.[4] Briefly, each antibody (2 mg/mL) was first activated with a 20-fold molar excess of SMCC in 55 mM PBS (150 mM NaCl, 20 mM EDTA, pH 7.4) for 2 h at 25 ℃. Excess SMCC was removed by ultrafiltration using a 10 KD mi-llipore (12,000 rpm, 15 min), and antibody-SMCC conjugate was obtained. Then, the antibody-SMCC conjugate and the thiolated DNA strand (100 μM, 3 μL) was mixed in 55 mM PBS (containing 150 mM NaCl, 5 mM EDTA, pH 7.2) and incubated overnight at 4 ℃. After that, unreacted DNA strands were removed by ultrafiltration using a 100 KD Millipore for five times (12,000 rpm, 15 min for each times), and the DNA-conjugated antibody was obtained. Finally, the obtained DNA-conjugated antibody products (T-DNA1-Ab1, T-DNA2-Ab3, T-DNA3-Ab5, T-DNA4-Ab7, A-DNA1-Ab2, A-DNA2-Ab4, A-DNA3-Ab6, or A-DNA4 -Ab8) were collected in 50 μL of 20 mM PBS (containing 20 mM NaCl and 5 mM EDTA, pH 7.2) for further experiments.

Fluorescent protein assays

In a typical assay, 95 μL of the RNase H buffer (20 mM Tris-HCl, 20 mM KCl and 10 mM $MgCl_2$, pH 7.5) containing 200 nM each DNA-conjugated antibody, 10 nM nano-beacon, and 60 U RNase H were mixed with 5 μL of different concentrations of target proteins (CEA, AFP, PSA and VEGF) or 5 μL of the diluted serum samples, and the mixture solution was incubated at 37 ℃ for 1 h. After incubation, the resulting solution was analyzed via spectrofluorometer. All assay experiments were repeated five times.

RNA cleavage assay by HPLC

HPLC was used to confirm the target binding-induced RNase H-catalyzed cleavage reaction of dye-labeled RNA-DNA hairpins on AuNPs. The experiments was carried out using PSA detection as the model system. Samples for HPLC assays were prepared as following: 50 μL of the RNase H buffer (20 mM Tris-HCl, 20 mM KCl and 10 mM $MgCl_2$, pH 7.5) containing 200 nM T-DNA2-Ab3, 200 nM A-DNA2-Ab4, 15 nM nanobeacon, and 60 U RNase H were mixed with 5 μL of 20 ng/mL PSA, and the mixture solution was incubated at 37 ℃ for 1 h. After that, the mixture solution was centrifuged at 12,000 rpm for 25 min at 4 ℃ and the supernatant was collected and analyzed by HPLC. Blank samples were prepared similarly to the procedure mentioned-above except in the absence of PSA. HPLC analysis were carried out on a C18 column at a rate of 1.0 mL/min, with a 25 min gradient from 10 to 17% acetonitrile in 0.1 M triethylammonium acetate at pH 7.0.

Results and discussion

Preparation and characterization of the four-color nanobeacon

In our design, 13 nm AuNPs were prepared for the four-color nanobeacon, because such large-sized AuNPs can efficiently quench the fluorescence of almost all fluorophores[5-7] and can be decorated with many oligonucleotide probes at one particle to provide the opportunity for design of the multicolor nanoprobe.[8-10] The prepared AuNPs and the four-color nanobeacon (AuNPs modified with four types of dye-labeled RNA-DNA hairpins) were characterized by TEM images and the results are shown in Fig. S1. As shown in Fig. S1, the sizes of AuNPs were 13±2 nm (120 particles sampled), and AuNPs

were well dispersed. After modification of four types of dye-labeled RNA-DNA hairpins, the AuNPs were still well dispersed. In addition, the prepared AuNPs and the four-color nanobeacon were characterized by UV−vis absorption spectra measurements. As shown in Fig. S2, a new peak (at 260 nm) in the UV−vis absorption spectra was observed after modification of dye-labeled RNA-DNA hairpins at the surface of AuNPs. Moreover, the maximum absorption of AuNPs was red-shifted from 519 nm to 524 nm after functionalization of the AuNPs with dye-labeled RNA-DNA hairpins. These results indicated that AuNPs were successfully decorated with dye-labeled RNA-DNA hairpins. Quantification of dye-labeled RNA-DNA hairpin surface loading by fluorescence[3] indicated that each AuNP carried about 13 AMCA-labeled H1, 13 FAM-labeled H2, 15 ROX-labeled H3, and 14 Cy5 labeled H4 (Fig. S3).

HPLC characterization

To evaluate the viability of our assay strategy, the RNase H-catalyzed cleavage of dye-labeled hairpin probes on the AuNPs initiated by the target-antibody binding was verified by using HPLC assays. A mixture solution of the three-color gold nanobeacon, four pairs of DNA-conjugated antibodies and RNase H with or without target proteins was incubated at 37 ℃ for 60 min and the resulting solution was centrifuged to collect the supernatant. The supernatant obtained was analyzed by HPLC. As shown in Fig. 3, a peak in each chromatogram was observed (traces a-d) when target proteins were present. However, when the target proteins were absent, there was no peak in each chromatogram (traces e-i). These results indicate that the target proteins can trigger the RNase H-catalyzed cleavage of the dye-labeled RNA-DNA hairpin probes linked to AuNPs, which

provides a solid foundation for the proposed multicolor fluorescent proximity immunoassay.

Optimization of assay conditions

For our design, the target binding-induced assembly of trigger DNA, assistant DNA, and the RNA-DNA hairpin probe is key to the success of the proposed strategy. Thus, it is necessary to optimize the complementary sequence pairs (T and T*, A and A*, and B and B*) to secure target binding-induced assembly of trigger DNA, assistant DNA, and the RNA-DNA hairpin probe that causes a detection signal and to obviate the target-independent assembly that leads to background. To attain the best signal to background ratio, we investigated the effect of these complementary sequences on protein assay by using PSA, T-DNA2, A-DNA2 and H2 as model system. Firstly, T2 and T2* containing 6 to 9 bases for the recognition of RNase H was tested and the experimental results are shown in Fig. S5. As the complementary sequences of T2 and T2* increased from 6 bases to 8 bases, no obvious background fluorescence increase was observed, suggesting that self-assembly of T2 and T2* can be eliminated. Further increase of the complementary sequences of T2 and T2* to 9 bases, the background fluorescence increased significantly. Meanwhile, the fluorescence for CEA detection increased with increasing the complementary sequences of T2 and T2*. The complementary sequences of T2 and T2* containing 8 bases provided the best signal to background ratio. Subsequently, T-DNA2 containing 8 bases for T2 and 6 bases for B was used to study the impact of three A-DNA2 strands containing 7 to 9 bases for A2 (A2-7, A2-8, and A2-9). As shown in Fig. S6, the fluorescence for CEA detection increased with an increase of the bases of A, However, when the A2-9 was tested, the high background fluorescence was observed, suggesting the use of A2-9 can increase the incidence of target-independent assembly of T-DNA2, A-DNA2 and H2. A2-8 provided the best signal to background ratio. Therefore, A-DNA2 with A2-8 to couple with four T-DNA1 strands containing 6 to 9 bases for B (B6, B7, B8, B9) were used to study the effect of the length of B on CEA detection and the experimental results are shown in Fig. S7. As the complementary bases increased, the fluorescence increased. B6 provided the best signal-to-background ratio.

Based on the experimental results mentioned above, we selected 8 bases, 8 bases and 6 bases as the optimal complementary sequence pairs of T2 and T2*, A2 and A2*, and B and B*, respectively, and used them for further experiments. In addition, the effect of the reaction time on CEA detection was also examined. The experimental results revealed that the fluorescence increased as the reaction time was prolonged, and kept constants at 60 min. The reaction time of 60 min provided the best signal to background ratio (Fig. S8).

References

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Supporting tables

Table S1. Oligonucleotide sequences used in this work.

Oligonucleotides	Sequences
T-DNA-1	5'-SH-TTT$(T)_{27}$TTTACCAACTATGGACA
T-DNA-2 (T2-6)	5'-SH-TTT$(T)_{27}$TTTACACCATTAGAG
T-DNA-2 (T2-7)	5'-SH-TTT$(T)_{27}$TTTACACCATTAGAGA
T-DNA-2 (T2-8, B6)	5'-SH-TTT$(T)_{27}$TTTACACCATTAGAGAC
T-DNA-2 (T2-9)	5'-SH-TTT$(T)_{27}$TTTACACCATTAGAGACC
T-DNA-2 (B7)	5'-SH-TTT$(T)_{27}$TTTGACACCATTAGAGAC
T-DNA-2 (B8)	5'-SH-TTT$(T)_{27}$TTTAGACACCATTAGAGAC
T-DNA-2 (B9)	5'-SH-TTT$(T)_{27}$TTTAAGACACCATTAGAGAC
T-DNA-3	5'-SH-TTT$(T)_{27}$TTTACACACTGATACAG
T-DNA-4	5'-SH-TTT$(T)_{27}$TTTACCCAAGATTAGCA
A-DNA-1	5'-GTTACAAGGTTGGTTTT$(T)_{27}$TTT-SH-3'
A-DNA-2 (A2-7)	5'-ATAGTAGTGGTGTCTT$(T)_{27}$TTT-SH-3'
A-DNA-2 (A2-8)	5'-CATAGTAGTGGTGTCTT$(T)_{27}$TTT-SH-3'
A-DNA-2 (A2-9)	5'-CCATAGTAGTGGTGTCTT$(T)_{27}$TTT-SH-3'
A-DNA-3	5'-GCATATAGTGTGTGTTT$(T)_{27}$TTT-SH-3'
A-DNA-4	5'-TACACTGATGGGTTTTT$(T)_{27}$TTT-SH-3'
H1	5'-ROX-ACGACGGUGUCCAUAUACUUGUAACGTCGT$(T)_6$-SH-3'
H2	5'-FAM-GAATCCGGUCUCUAAUACUACUAUGGATTC$(T)_6$-SH-3'
H3	5'-Cy5-CAGTTGGCUGUAUCAUACUAUAUGCAACTG$(T)_6$-SH-3'
H4	5'-AMCA-CAGTGTGUGCUAAUCUAUCAGUGUACACTG$(T)_6$-SH-3'

Table S2. Assay results of CEA, PSA, AFP, and VEGF in clinical serum samples using the proposed four-color immunoassay and commercial electrochemiluminescent immunoassay methods.

Biomarkers	1	2	3	4	5	
CEA	36.4	69.4	46.5	86.8	2.05	This work
(ng/mL)	38.2	64.3	49.1	89.7	2.06	Reference method
	-4.7	-7.9	5.2	3.2	4.9	Relative deviation (%)
PSA	12.2	11.3	11.6	13.4	2.66	This work
(ng/mL)	12.6	11.5	11.1	12.6	2.69	Reference method
	2.4	1.7	-4.5	-6.3	1.1	Relative deviation (%)
AFP	2.86	1.73	6.82	5.78	1.22	This work
(ng/mL)	2.70	1.65	6.95	5.71	1.28	Reference method
	-5.9	-4.8	1.1	-1.2	4.7	Relative deviation (%)
VEGF	0.28	0.41	0.64	0.45	0.18	This work
(ng/mL)	0.29	0.39	0.66	0.44	0.19	Reference method
	3.4	-5.1	3.0	-2.2	5.3	Relative deviation (%)

Supporting figures

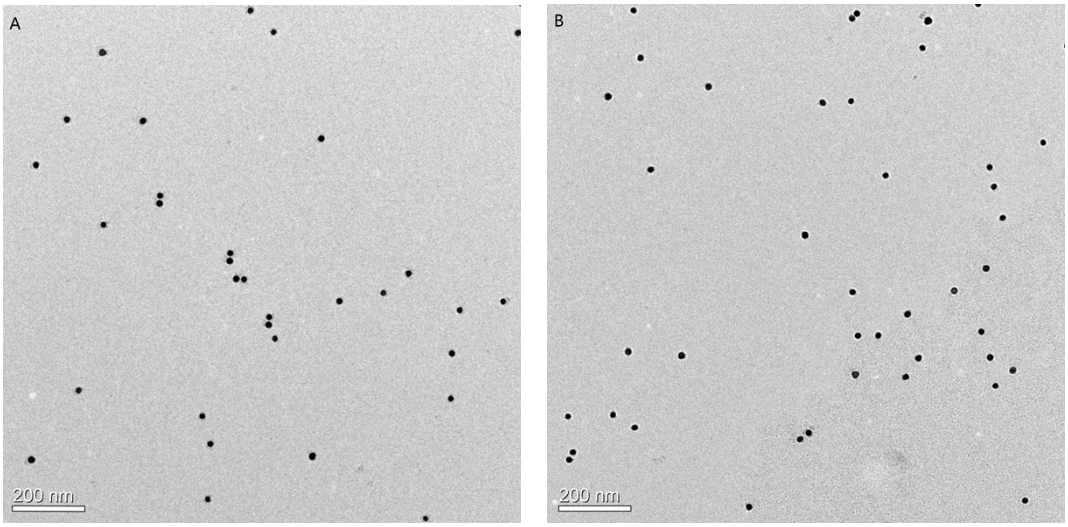


Fig. S1 TEM images of the four-color nanobeacon (a) and AuNPs (b).

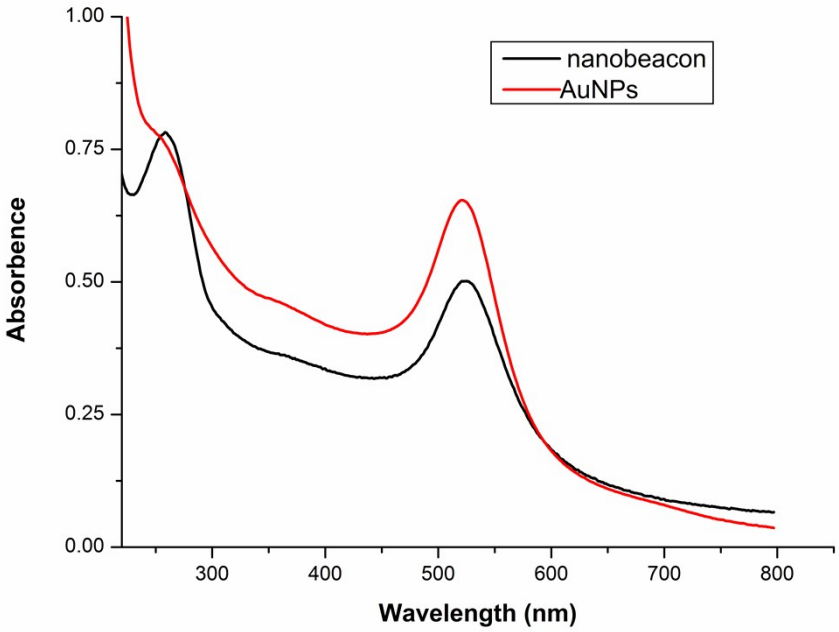
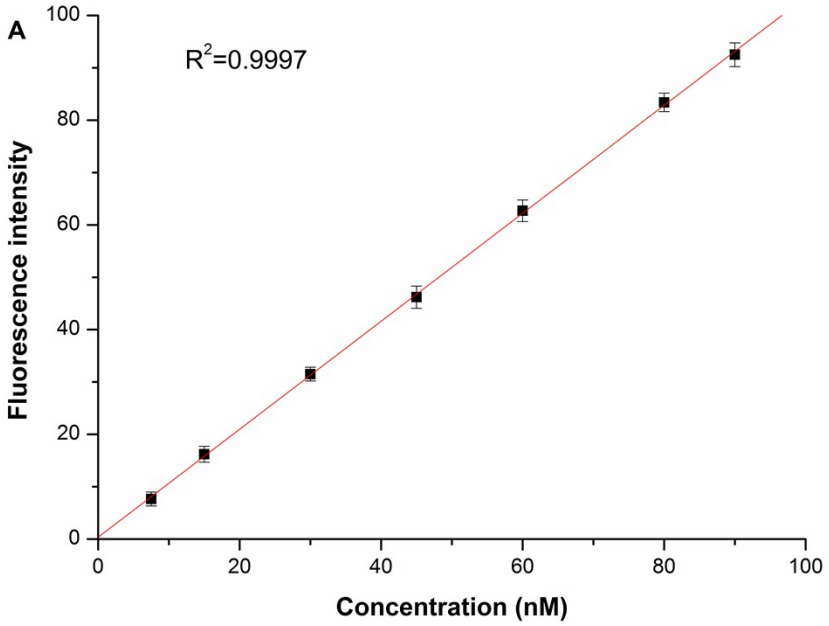


Fig. S2 UV-via spectra of AuNPs and the four-color nanobeacon.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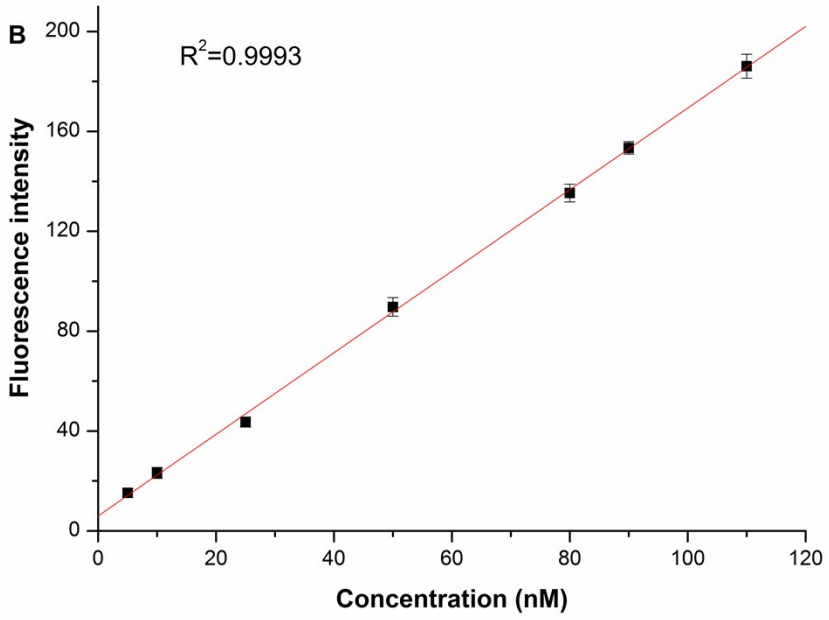
40
60
80
100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160
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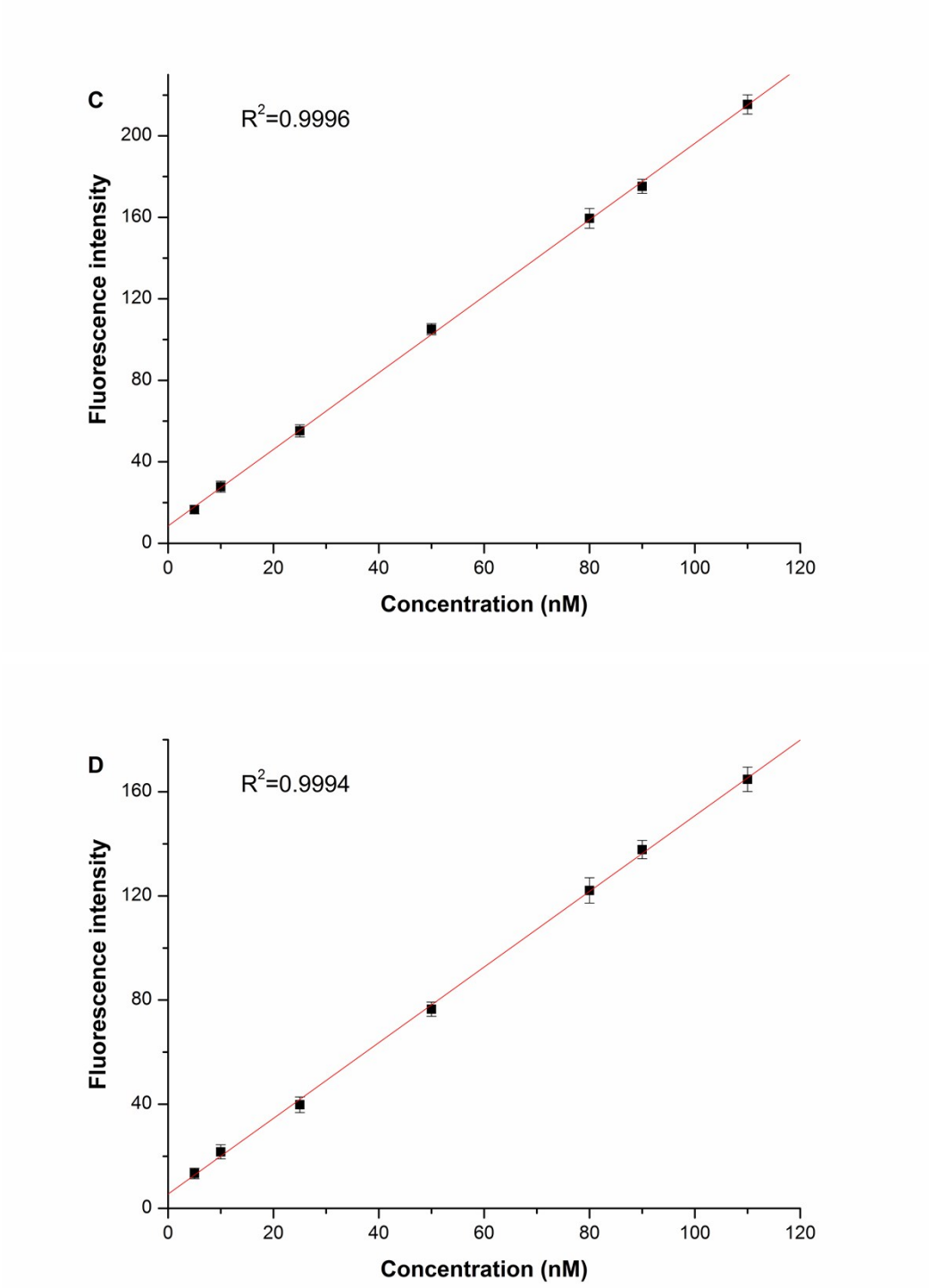


Fig. S3 Standard linear calibration curves of dye-labeled RNA-DNA hairpin probes. A) AMCA-labeled H1; B) FAM-labeled H2; C) ROX-labeled H3; and D) Cy5-labeled H4. Error bars were derived from N=5 experiments.

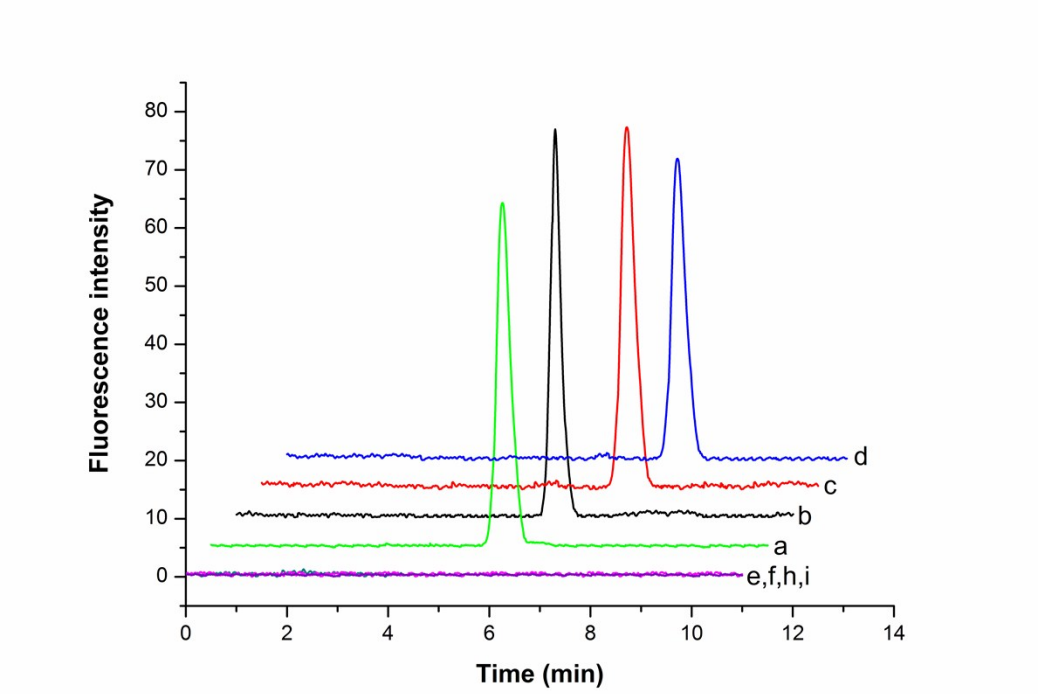


Fig. S4 HPLC chromatograms obtained from an incubation solution of the four-color nanobeacon and DNA-conjugated antibodies with target proteins (a-d) and without target proteins (e-i). Fluorescence detection wavelengths: 665 nm for traces a and e; 607 nm for traces b and f; 520 nm for traces c and h; and 442 nm for traces d and i.

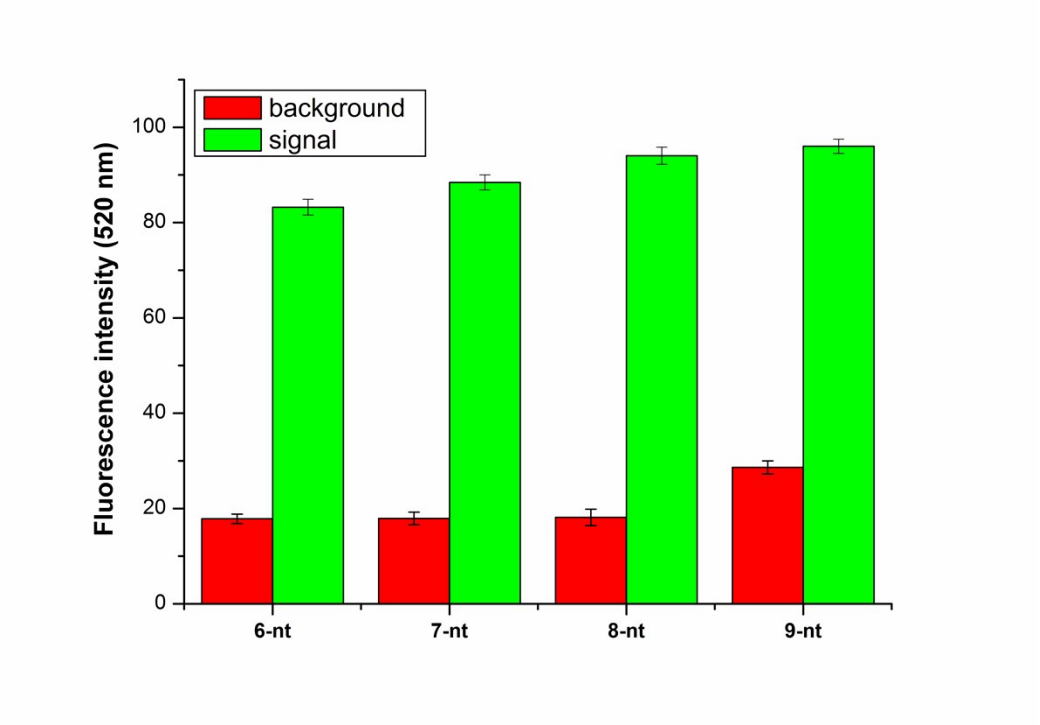


Fig. S5 Impact of the length of T2 on (with 200 pg/mL PSA) and background (without PSA). Error bars were derived from N=5 experiments.

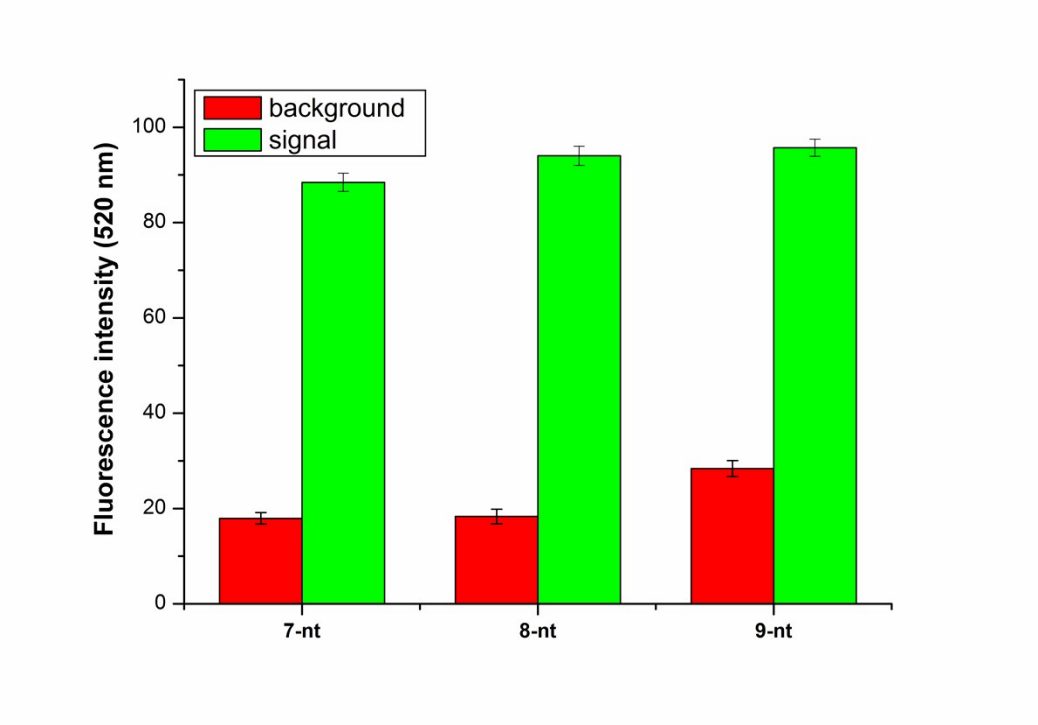


Fig. S6 Impact of the length of A2 on signal (with 200 pg/mL PSA) and background (without PSA). Error bars were derived from N=5 experiments.

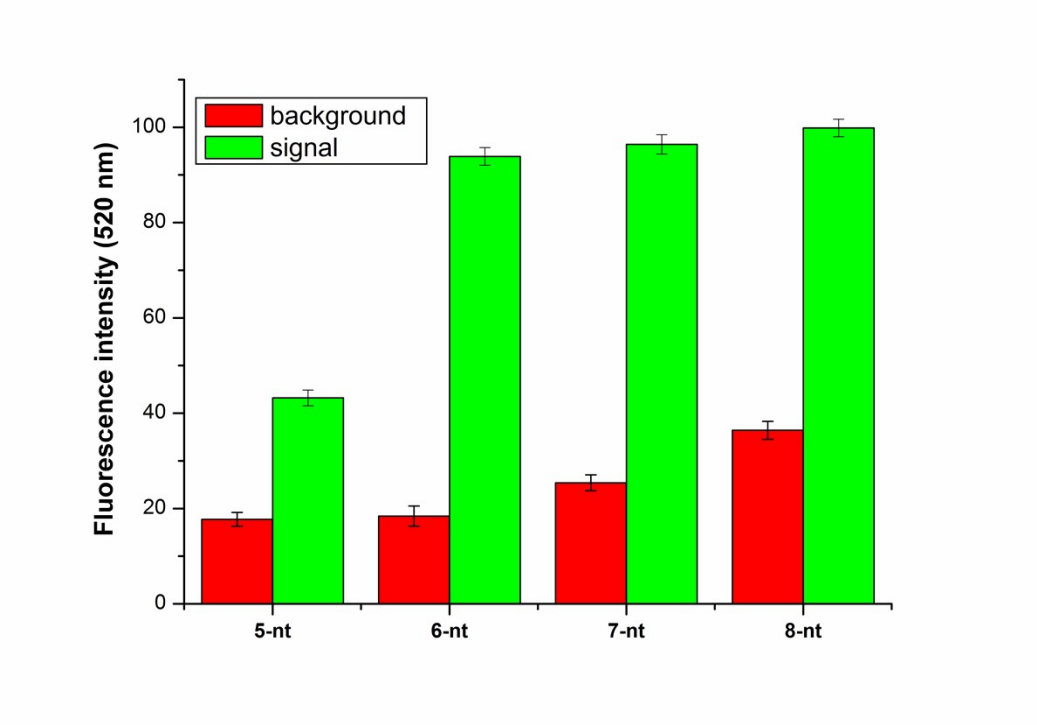


Fig. S7 Impact of the length of B on (with 200 pg/mL PSA) and background (without PSA). Error bars were derived from N=5 experiments.

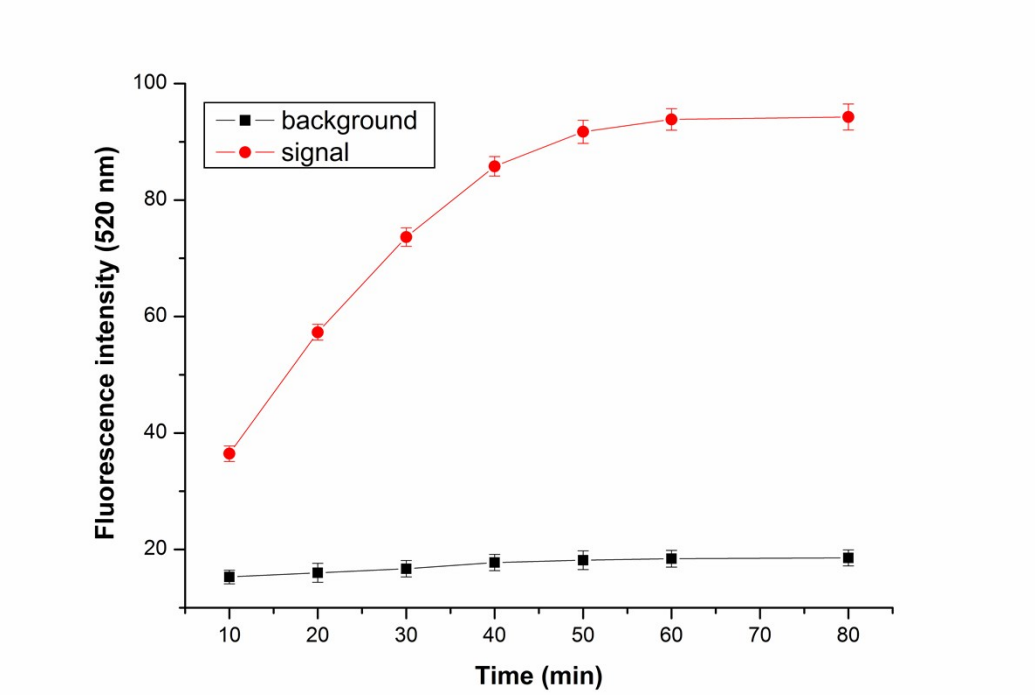
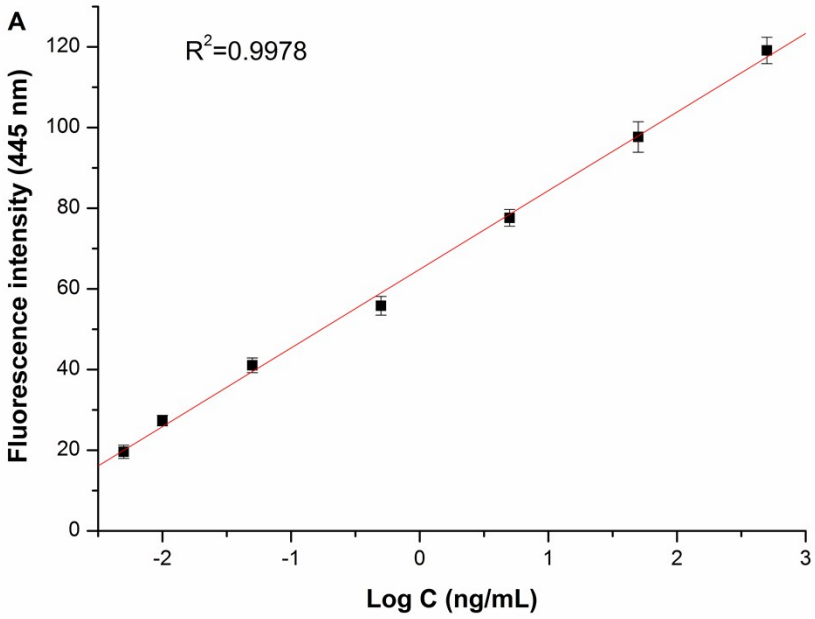


Fig. S8 The effect of reaction time on (with 200 pg/mL PSA) and background (without PSA). Error bars were derived from N=5 experiments.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100
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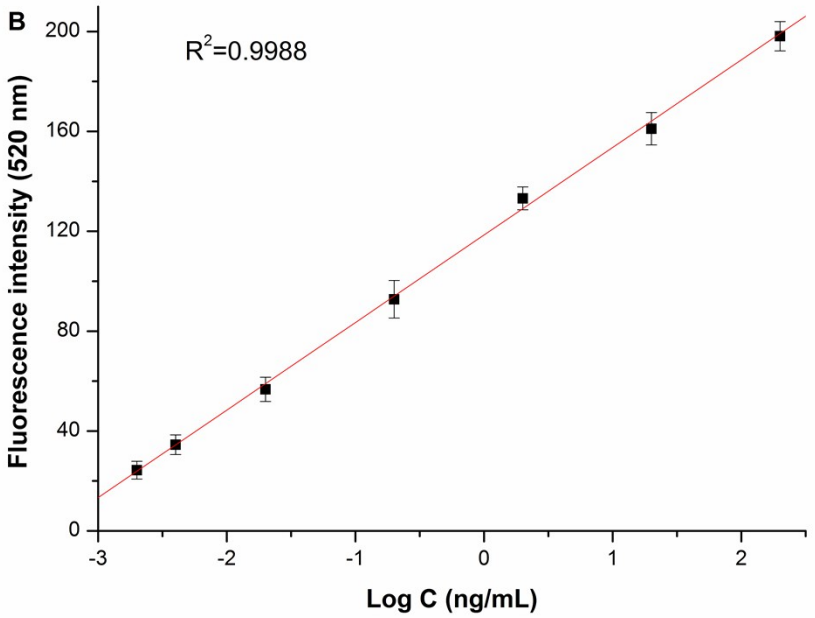
-2
-1
0
1
2
3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Fluorescence intensity (520 nm)
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80
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-1
0
1
2

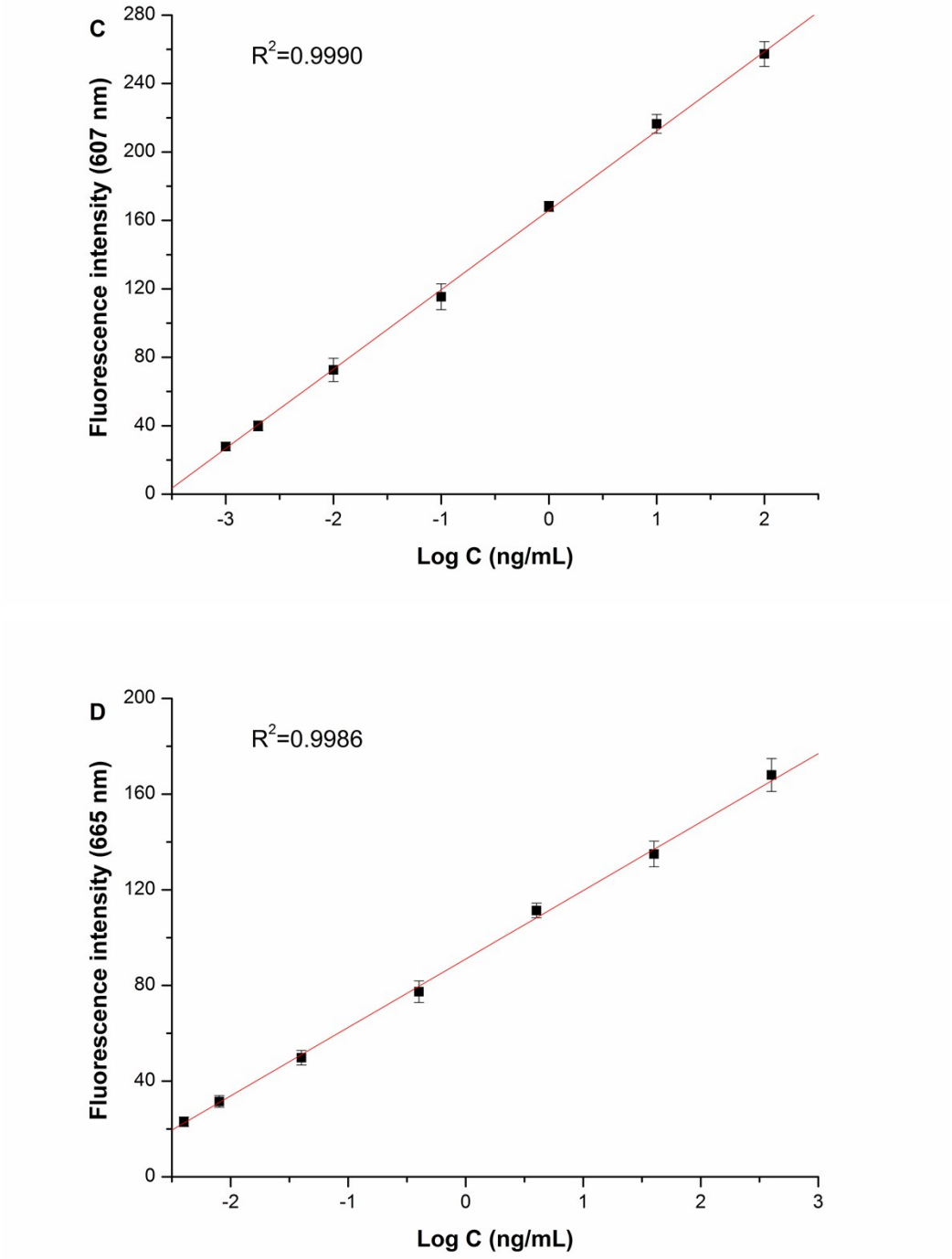
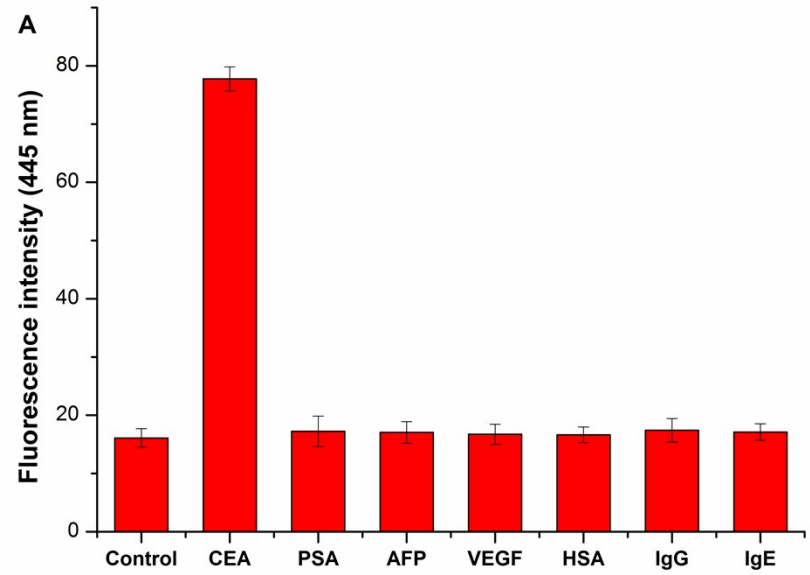


Fig. S9 Calibration curves corresponding to the proposed four-color proximity immunoassay system. A) CEA; B) PSA; C) AFP; and D) VEGF. Error bars were derived from N=5 experiments.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40
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Control
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PSA
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IgG
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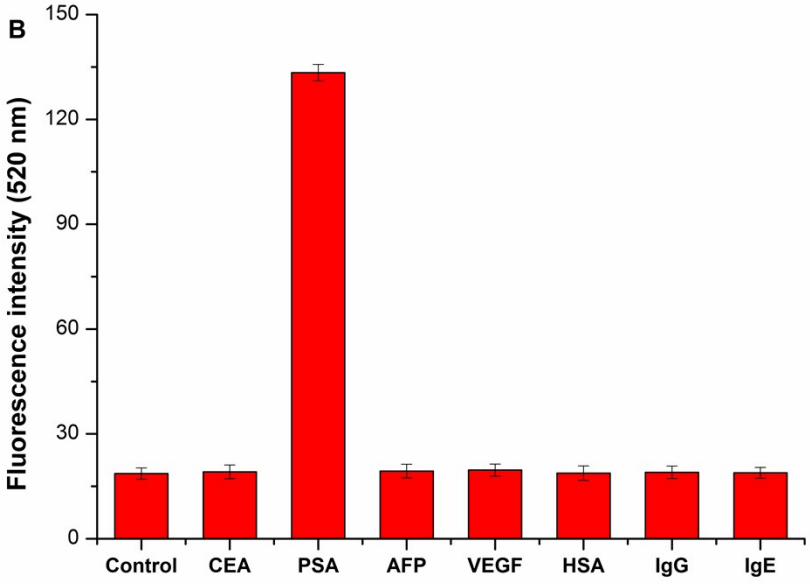
B
Fluorescence intensity (520 nm)
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30
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90
120
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Control
CEA
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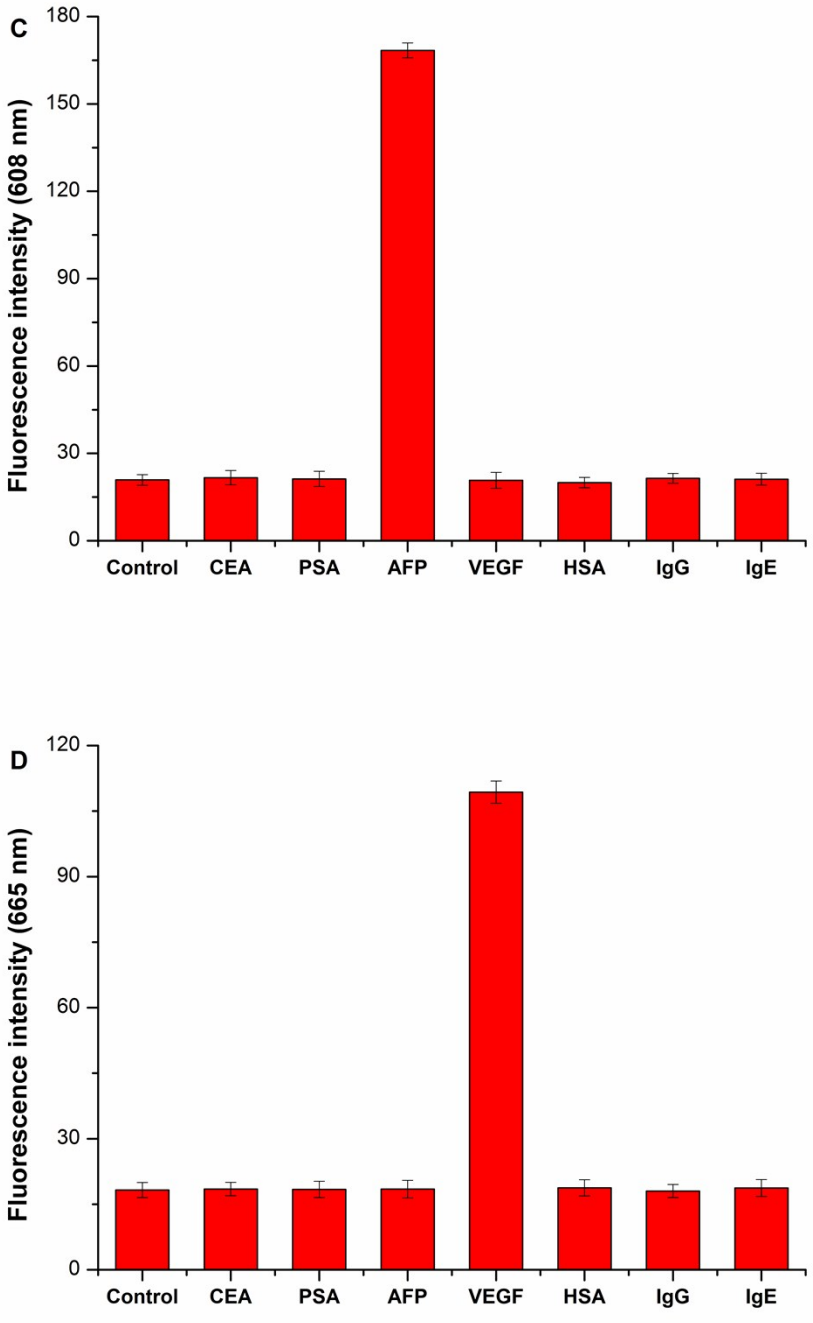

Fig. S10 Specificity of the proposed four-color proximity immunoassay. The concentration of CEA, PSA, AFP and VEGF was 5 ng/mL, 2 ng/mL, 1 ng/mL, and 4 ng/mL, respectively. Other nonspecific proteins were 1000 ng/mL. Error bars were derived from N=5 experiments.