Supporting Information for

A dual-readout chemiluminescent gold lateral flow test for multiplex and ultrasensitive detection of disease biomarkers in real samples

Yiping Chen [a]※, Jiashu Sun [a]※, Yunlei Xianyu [a]※, Binfeng Yin[a], Yajing Niu [a], Songbai Wang [a,c], Fengjing Cao [a], Xiaoqing Zhang [a], Yu Wang [b], and Xingyu Jiang [a] *

[a] Beijing Engineering Research Center for BioNanotechnology and CAS Key Lab for Biological Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for NanoScience and Technology, Beijing 100190, China.

[b] Beijing Institute for Tropical Medicine; Beijing Friendship Hospital, Capital Medical University, 95,Yongan Road, Xicheng District, Beijing, 100050, China.

[c] Department of Chemistry and Chemical Engineering, Research Center of Environmental Science and Engineering, Shanxi University, Taiyuan 030006, China.

MATERIALS AND METHODS

Reagents and Materials

Alpha fetal protein (AFP), carcino embryonic antigen (CEA), anti-AFP capture monoclonal antibody (6.4 mg/mL), anti-AFP detection monoclonal antibody (4.9 mg/mL), anti-CEA capture monoclonal antibody (3.9 mg/mL) and anti-CEA detection monoclonal antibody (2.8 mg/mL) were purchased from Beijing Hotgen BioTech. (Beijing, China). Anti-PCT capture monoclonal antibody (9.8 mg/mL) and anti-PCT detection monoclonal antibody (10 mg/mL) were provided by ABZYMO Biosciences (Beijing, China). These antibodies were all from mouse. The chemiluminescence substrate, Millipore HF135 nitrocellulose (NC) membrane, the absorbent pad, the sample pad, the polyvinyl chloride pad were purchased from Millipore (Billerica, MA, USA). Phosphate buffer saline (PBS, pH 7.4, 0.01 M) was purchased from Armesco (USA). NaCl (150 mM), sucrose (0.5%, M/V), sodium caseinate (0.5%, M/V), bovine serum albumin (BSA) and horseradish peroxidase (HRP) (10 mg/mL) were purchased from Sigma-Aldrich (USA). 0.01 M Tris buffer (pH=8.0, 1% sucrose , 1% BSA and 0.02% NaN$_3$) was used as the developing solvent in the lateral flow system. Other reagents and solvents were of analytical grade and purchased from Beijing Chemical Reagents Co. (Beijing, China). The water used in the experiments was deionized and ultrafiltered using a Milli-Q apparatus.

Apparatus

A portable custom-made chemiluminescence analyzer was employed for collecting the chemiluminescent signals. Biological electron microscope (Ht-7700, Hitachi Limited, Japanese) was employed to characterize the Ab-Au-HRP conjugate. XYZ Dispense Workstations (XYZ3050, Bio dot, USA) and CM4000 cutting system (Bio dot, USA) were employed for the preparation of the chemiluminescent gold lateral flow test (C-mode GLFT). German high-speed refrigerated centrifuge (3K30, Sigma, USA) and MS3 vortex oscillator (IKA Inc, Germany) were employed to prepare the Ab-Au-HRP conjugate.

EXPERIMENTAL SECTION

Preparation of gold nanoparticles (AuNPs)

Citrate-stabilized AuNPs (~30 nm in diameter) were prepared by the citrate reduction of $HAuCl_4$. The glassware was soaked overnight in cleaning solution, rinsed with ultrapure water, and dried prior to use. 200 mL distilled water was added into a beaker, followed by 2 mL of 1 % sodium citrate. The solution was heated and 2 mL of 1% chloroauric acid solution were added and boiled for 30 min. The prepared solution of AuNPs was allowed to cool down to room temperature and stored at 4 °C.

Preparation of Ab-Au-HRP conjugate

AuNPs were modified with antibodies according to a previously optimized procedure. The pH of 10 mL 0.01% AuNPs was adjusted to 8.5 by adding 0.01M K_2CO_3 solution, and different amounts of antibody and HRP molecules were added into the solution. The resulting mixture was stirred at 650 rpm for 1 h. 0.5 mL of 10

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mg/mL BSA solution was added and stirred for another 0.5 h. The mixture was centrifuged twice (14000 rpm, 4 °C) for 20 min. Finally, the supernatant was removed and the Ab-Au-HRP conjugate was re-suspended with 0.2 mL developing solvent.

Characterization of the Ab-Au-HRP conjugate

The appropriate volume of Ab-Au-HRP conjugate and Ab-Au conjugate was added drop by drop to the surface of copper grid. After drying at the room temperature, these samples are characterized by the biological electron microscope.

Based on the relationship between the concentration of AuNPs and UV-Vis spectra,[26] we quantify the number of HRP molecules on the surface of per Au NP as follows: (1) we can quantify the number of Ab-Au-HRP conjugate based on the relationship between the absorbance of the solution of Ab-Au-HRP and its concentration.

$$N = \frac{A_{450} \times 10^{14}}{d^2[-0.295 + 1.36\exp(-(\frac{d-96.8}{78.2})^2)]}$$

"A_{450}" is the absorbance at λ=450 nm, and "d" represents the size of Au NPs in our study, d=30 nm.

(2) by preparing a series of concentration of HRP, we can build the linear relationship between the concentration of HRP and the absorbance of TMB solution (the substrate of HRP) at 450nm. This result is shown in the Figure S4, and we can obtain the amount of HRP by this result. (3) according to the step (2), we can obtain the total amount of HRP molecules on the surface of Ab-Au-HRP conjugate, and this number of HRP divide the number of AuNPs, then we can quantify the number of HRP

molecules on the surface of per AuNP.

Preparation of the C-mode GLFT device

The C-mode GLFT device is shown in **scheme 1**. The test strips consisted of four components: a sample pad, a conjugation pad, an NC membrane and an absorbent pad. All of these components were attached onto a PVC backing card. The test device was prepared by separately spraying the goat anti-mouse IgG (2.0 mg /mL) and capture antibody (1.0 mg/mL) onto an NC membrane to form a control (C) region and a test (T) region, respectively. The sample pad was treated with 1% BSA-containing PBS for 30 min, and dried at 37 °C for 2 h. The conjugate pad was prepared by dipping it into the previously prepared Ab-Au-HRP conjugate and drying under vacuum for 1 h. The different pads were sequentially laminated 2 mm from each other and pasted onto the adhesive backing card in the following order: detection, conjugation, sample and absorbent pads. Finally, the strips were cut 7 mm wide and stored at room temperature for use.

Procedures of C-mode GLFT

200 μL of sample diluted in developing solvent was dispensed onto the sample pad and kept for 15 min (**Scheme 1**). We obtained the qualitative result by observing the brightness of color of AuNPs on the surface of test line in V-mode GLFT using naked eyes. On the other hand, 200 μL of phosphate buffer saline with 0.05% tween 20 (PBST) was used to wash away the excess Ab-AuNPs-HRP conjugate twice. After drying the lateral flow strips at room temperature, 100 μL of the CL substrate was added onto the surface of strip, the HRP molecules can catalyze the H_2O_2 to

decompose into H_2O and oxygen free radical, which oxidizes the luminol to generate luminescence. After addition of CL substrate, the CL signals were generated, and we employed the portable CL analyzer to collect the CL signal from the whole area of the test line in the strip, and the exposure time was 10 s.

Detection of clinical serum samples

Serum samples (positive samples and negative samples) were collected from Tiantan hospital (Beijing) and Beijing Friendship Hospital. Before analysis, these samples were 10-fold diluted by PBS solution to eliminate the matrix interference. Each sample was detected three times (n=3).

Supplementary results
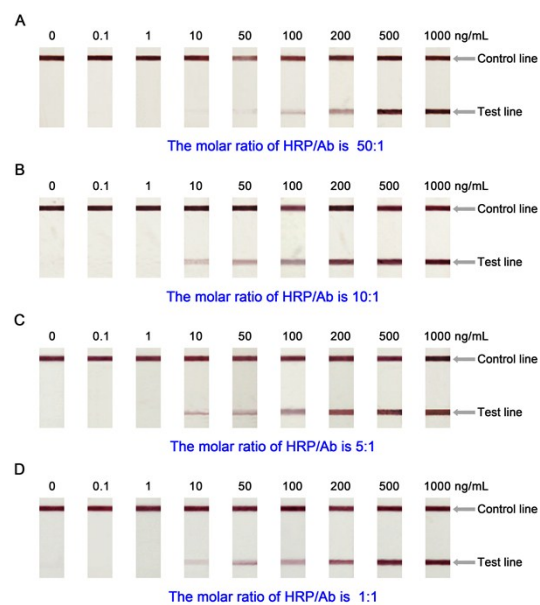
Figure S1. The effect of molar ratio of HRP/Ab for detection of CEA. The concentration of CEA from left to right is 0, 0.1, 1, 10, 50, 100, 200, 500 and 1000 ng/mL.
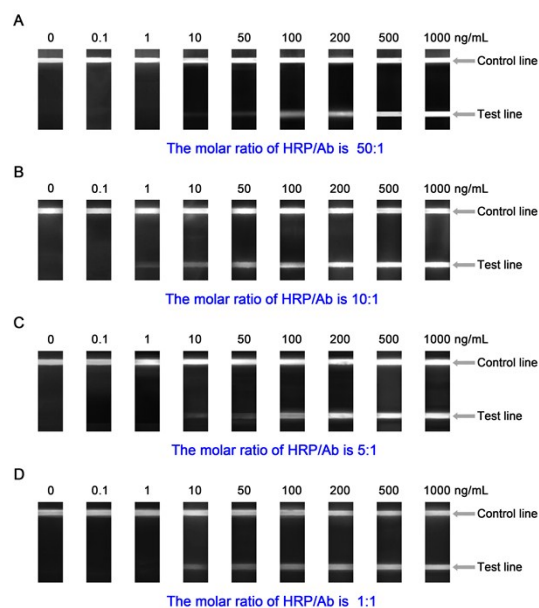
Figure S2. The effect of molar ratio of HRP/Ab for detection of CEA using CL signal.

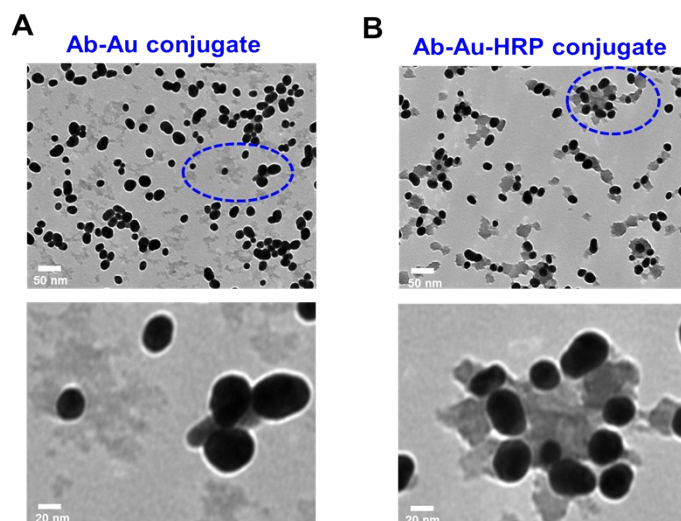The concentration of CEA is 0, 0.1, 1, 10, 50, 100, 200, 500 and 1000 ng/mL.

Figure S3. Characterizations of Au-Ab conjugate and Ab-Au-HRP conjugate using biological electron microscope. The molar ratio of HRP/Ab is 10:1.
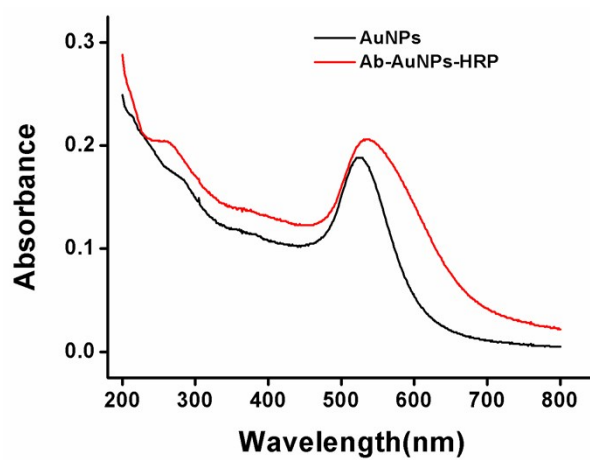
Figure S4. The UV/Vis spectra of the AuNPs solution and the "Ab-AuNPs-HRP" conjugate solution.
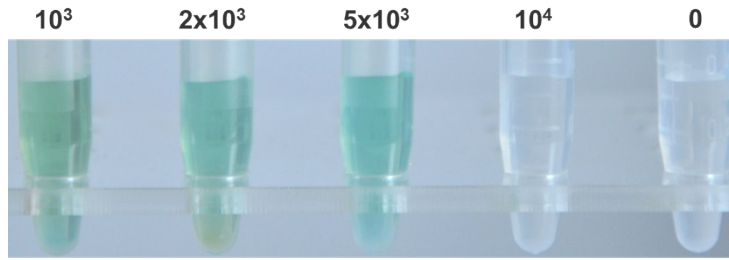
Figure S5. The result of the reaction between Ab-Au-HRP conjugate and the

substrate of HRP (tetramethylbenzidine, TMB). The diluted fold of Ab-Au-HRP

conjugate is 10^3, 2×10^3, 5×10^3 and 10^4. 0 represents the control group without Ab-Au-

HRP conjugate. The original color of TMB is colorless.
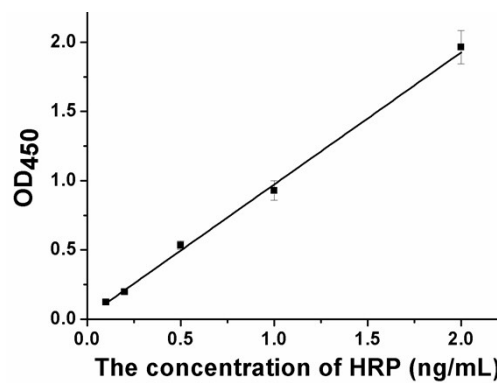
Figure S6. The relationship between the concentration of HRP and the absorbance at
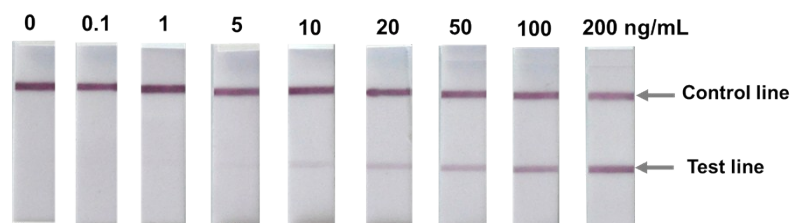
450 nm of its substrate (TMB). The concentration of HRP is 0.1, 0.2, 0.5, 1 and 2

ng/mL.

Figure S7. The result of conventional GLFT for detection of CEA (30% fetal calf

serum). The concentration of CEA from left to right is 0, 0.1, 1, 5, 10, 20, 50, 100 and
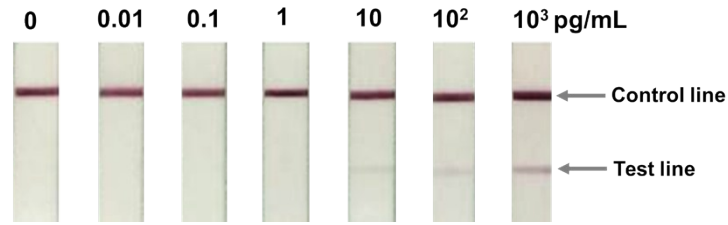
200 ng/mL.

Figure S8. The result of conventional GLFT for detection of PCT (30% fetal calf serum). The concentration of PCT from left to right is 0, 0.01, 0.1, 1, 10, 10^2 and 10^3 pg/mL.
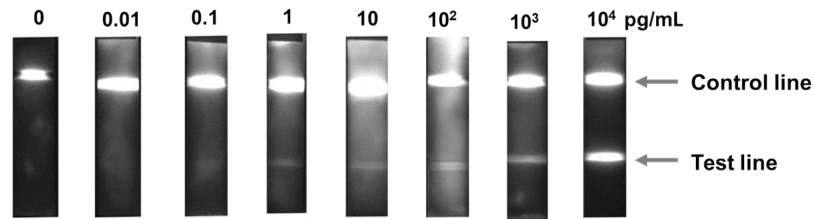

Figure S9. The result of chemiluminescent lateral flow test without AuNPs for detection of PCT based on HRP-Ab catalyzed chemiluminescent reaction of luminol.
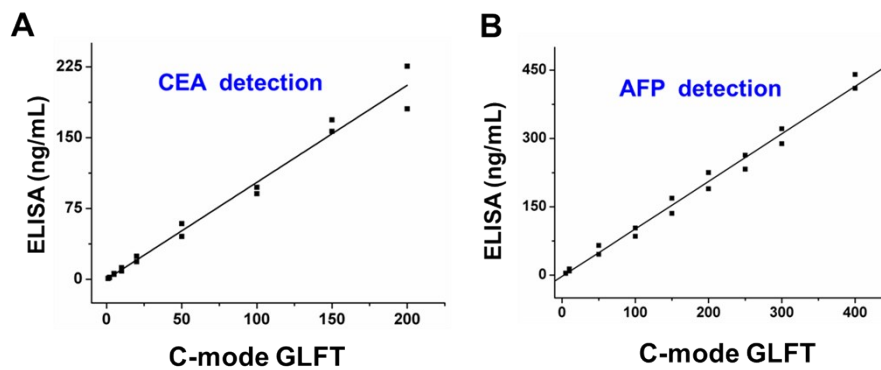

Figure S10. The results of C-mode GLFT and ELSA for detection of CEA and AFP in spiked serum samples. (A) The concentration of CEA is 0, 1, 2, 5, 10, 20, 50, 100,

150, and 200 ng/mL; (B) The concentration of AFP is 0, 5, 50, 100, 150, 200, 250, 300 and 400 ng/mL.
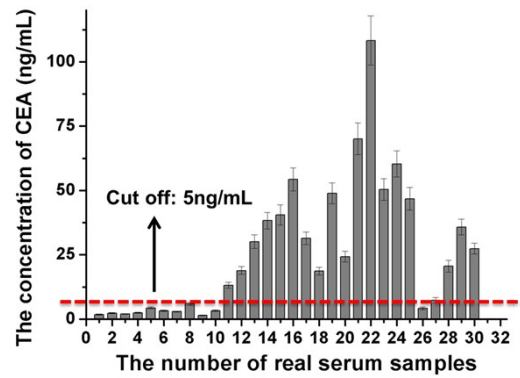

Figure S11. The results of ELISA for detection of CEA in real serum samples. Sample 1 to sample 10 are negative samples, and sample 11 to sample 30 are positive samples.
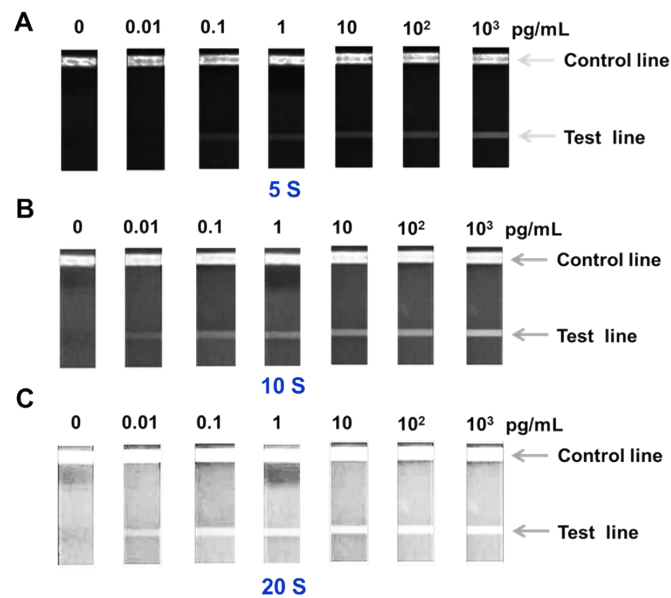

Figure S12. The results of C-mode GLFT for detection of PCT using different exposure time.

Table S1 Results from the analysis of CEA in spiked serum samples by C-mode GLFT and conventional ELISA (n =3).

	C-mode GLFT		ELISA	
Spiked (ng/mL)	Intra-RSD (%)	Inter- RSD (%)	Intra-RSD (%)	Inter- RSD (%)
50	4.8	6.9	5.5	7.4
10	5.7	8.6	6.3	8.0
5	6.7	9.3	7.2	8.9
1	7.8	11.2	8.5	11.8