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Natural Phage Nanoparticles-Mediated Real-time Immuno-PCR for Ultrasensitive Detection of Protein Marker

Heng Zhang,[a §] Ye Xu,[a] Qiuying Huang,[a] Changqing Yi,[b] Tan Xiao[b] and Qingge Li[a]*

[a] Engineering Research Centre of Molecular Diagnostics, Ministry of Education, Department of Biomedical Sciences, School of Life Sciences, Xiamen University, Xiamen 361005, China. Fax 86-592-2187363; e-mail qgli@xmu.edu.cn

[b] School of Engineering, Sun Yat-sen University, Guangzhou 510275, China

[§] Present address: Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen, Guangdong 518045, China

Materials and Methods

MATERIALS

T7 bacteriophage and BLT5615 host strain were kindly provided by Professor Jun Cheng of Peking Ditan Hospital, China. PEG 8000, CsCl and DNase I for purification of T7 bacteriophage were purchased from Shanghai Sangon Ltd. Fluorescein isothiocynate (FITC) was obtained from Sigma Chemical Co. (St. Louis, MO). Reagent Sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC), 2-iminothiolane (Traut's reagent) were obtained from Pierce Biochemicals Inc. (Rockford, IL, USA). Nanosep™ ultracentrifuge filters (10 kDa, 100 kDa, 300 kDa Cutoff) for separation and purification of the T7-antibody complex were purchased from Pall Corporation (East Hills, NY, USA). Monoclonal capture antibody B20 and monoclonal detection antibodies S04 were kindly

provided by InTec Products, Inc. (Xiamen, China). The standard solutions of HBsAg were prepared by diluting HBsAg (InTec Products, Inc.) with 0.05 M Tris-HCl buffer containing 5% bovine serum albumin (BSA), 0.9% NaCl, and 0.1% NaN_3 (pH 7.8). Regular polypropylene PCR tubes were purchased from Axygen Scientific, Inc. (Union City, CA, USA). Taq DNA polymerase and dNTP (dATP, dCTP, dGTP and dTTP) were purchased from Takara (Dalian, China). Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification. Ultrapure water (18.0 MΩ) was used throughout the experiments.

PREPARATION OF T7 BACTERIOPHAGE

The protocols of amplification and purification of T7 bacteriophage referred to T7 Select® System Manual (Novagen, USA). In brief, BLT5615 was inoculated into 200 mL of sterile M9 LB cultures, which contained 100 μg/mL of ampicillin, and incubated with shaking at 250 rpm at 37 °C until the OD_{600} reached 0.4, and then added 1 mM of IPTG and continue shaking for another 30 min. After being infected with 50 μL of high titer T7 phage (10^7 pfu/mL), the culture was gently shaken until lysis. Upon lysis, 40 μL of DNase (1 U/μL) and 5 g of solid NaCl were added, swirling to dissolve, and pouring the lysate into a centrifuge bottle. Then, centrifuge at 10,000 rpm for 12 min at 4 °C. After transferring the supernatant, 20 g of PEG 8000 was added slowly with Continue stirring at room temperature until the PEG was dissolved. The lysate-PEG mixture was stored overnight at 4 °C. The lysate-PEG mixture was centrifuged at 10,000 rpm for 10 min at 4°C, and the PEG pellets were resuspended in 1.5 ml of 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. And then T7 bacteriophage was further purified followed by banding in a CsCl step gradient. And T7 bacteriophage purified was

stored at 0.1 M PBS, pH 7.4 for the following use.

DETERMINATION OF PRIMARY NH_2 GROUP ON THE T7 BACTERIOPHAGE SURFACE

Firstly, 100 μL of T7 bacteriophage (OD_{260}=4.32) was dialyzed against carbonate buffer (0.01 M, pH 9.0) at 4 °C followed by the addition of 0.1 mg FITC. After gentle stirring, the reaction was performed for 12 hr at 4 °C. The solution was then dialyzed against the PBS buffer at 4 °C overnight to remove residual FITC. And then the solution was irradiated under a Dark Reader™ Device (Clare Chemical Research, Inc., Dolores, CO, USA). The determination of the amino group on the T7 bacteriophage surface was performed by trinitrobenzene sulfonic acid (TNBS) method. Reagent Glycin was used as the standard of the amino group measurement. Prior to the analysis, 1.5 mL of 0.1 M Na_2SO_3 was mixed with 98.5 mL of 0.1 M NaH_2PO_4 (reagent A). To 50 μL of the T7 bacteriophage solution was added 50 μL of 0.1 M $Na_2B_4O_7$/0.1 M NaOH, and 10 μL of TNBS solutions (1%). After incubation for 5 min at room temperature, 200 μL of reagent A was added to the mixture. Absorbance at 420 nm of the reaction solution was measured with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA).

PREPARATION OF T7 BACTERIOPHAGE-LABELED ANTIBODY

A mixture of 75 μL T7 bacteriophage (0.1 M PBS, pH 7.4, OD_{260}=14.3) and 0.1 mg of Sulfo-SMCC was reacted with periodic mixing for 30-60 min at room temperature. And then the maleimide-activated T7 bacteriophage was purified immediately by applying the reaction mixture to a desalting Nanosep™ ultracentrifuge filter (100 kDa), and washed with PBS buffer three times to remove unreacted materials. In the meanwhile, modification of detection

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antibody with Traut's Reagent was performed. Briefly, 0.2 mg of detection antibody S04 was dialyzed against 0.16 M sodium borate (pH 8.0) for 6 hr to remove residual small molecules. And the Traut's reagent was dissolved in water at a concentration of 2 mg/mL, which should be used immediately. 1 μL of Traut's reagent was added into the above antibody solution. After stirring for 1 hr at room temperature, the thiolated antibody was centrifuged and washed three times with PBS buffer using a Nanosep™ ultracentrifuge filter (10 kDa) to remove unreacted Traut's reagent. A mixture of the maleimide-activated T7 bacteriophage and the thiolated detection antibody were reacted with stirring to form the T7-antibody complex. After the mixture was further incubated for 24 hr at 4 °C, the solution was centrifuged using an ultracentrifuge filter (300 kDa), and washed several times with PBS buffer to thoroughly remove residual antibody molecular. Then, the T7-antibody complex was stored at 4 °C in 0.01 M PBS buffer, pH 7.4, containing 1% BSA, 0.09% NaN3, 1 mM EDTA before use.

DESIGN OF PRIMERS AND PROBES

All primers and probes (Table S1) were designed using Primer v 5.0 and Tm utility v 1.3. The positive and negative strands of displacing probes and all primers were synthesized and PAGE-purified by Shanghai Sangon Ltd (Shanghai, China). The primers were dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (TE buffer) at a concentration of 50 μM. Displacing probes were prepared by mixing 5.0 nmol positive strand and 5.0 nmol negative strand in 50 μL of TE buffer.

IMMUNO-PCR OF HBsAg USING T7-ANTIBODY COMPLEX

A schematic representation of sandwich immuno-PCR based upon the T7-antibody complex is shown in Figure S1. Briefly, after 25 μL of 1% glutaraldehyde was added into

regular polypropylene PCR tubes and incubated for 1-2 hr at 37 °C, these tubes were rinsed several times with water. Then, Anti-HBsAg monoclonal capture antibody B20 (diluted to 5 μg/mL in 0.05 M carbonate buffer, pH 9.5) was coated inside the PCR tubes (25 μL/tube) by conjugating with glutaraldehyde, incubating overnight at 37 °C. After washing with washing buffer A (10 mM PBS containing 0.05% Tween 20, pH 7.4), 120 μL of the blocking buffer (0.01 M Tris-HCl containing 0.05% Tween 20, 1% BSA, 2% sucrose, 0.5% glycine, 0.1% NaN_3, pH 7.8) was added to each tube, and incubated overnight at 4 °C. After washing with washing buffer A, 25 μL of serial dilutions of human HBsAg standard solution (or sera samples) was added, and incubated for 1 hr at 37 °C. After washing with washing buffer A, 25 μL of the T7-antibody complex (diluted 10000-fold with the dilution buffer of 0.01 M Tris-HCl containing 0.9% NaCl, 0.2% BSA and 0.1% NaN3, pH 7.8) was added to each tube, and these tubes were incubated at 37 °C for 1 hr. the tubes were washed six times with washing buffer B (10 mM PBS containing 2 mM EDTA, 0.05% Tween 20, 0.1% SDS, pH 7.4) and washed three times with water to remove unbound phages, then, subjected to be added the PCR mixture containing 2.5 μL of 10×PCR buffer (160 mM $[NH_4]_2SO_4$, 670 mM Tris-HCl pH 8.8, and 0.1 % wt/vol Tween 20), 3.0 μL of 25 mM $MgCl_2$, 0.2 μL of a mixture of dNTPs (2.5 mM each of dATP, dCTP, dGTP and dTTP), 0.2 μL each of sense primers and anti-sense primers (50 μM final concentration), 0.2 μL each of probes (50 μM final concentration) and 1.0 U Taq DNA polymerase and distilled water in a total of 25 μL. Real-time PCR amplification was performed in Rotor-Gene 3000 (Corbett Research, Sydney, Australia), and the amplification reaction conditions included denaturation at 95 °C for 6 min, followed by 45 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 20 s. And fluorescence signal from

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FAM channels was recorded at the annealing step of 58 °C in the last 45 cycles. In addition, negative control containing no phages was included in each batch of PCR tests. Subsequent data analysis was accomplished with Rotor-Gene 3000 software and Origin v 6.1.

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Table S1. Sequence of primer and probe.

	Name	Sequence	GC (%)	TM (°C)
primer	T7G9-F2	5′>CCAGCGAGAGTACGAGGAGA<3′	60	60.9
	T7G9-R2	5′>GTCACGATTGGTCAACGCATTAT<3′	43.5	59.9
Probe	T7-Ps	5′>FAM-AAGCTCTGGTGGAGCAGTACGT<3′	59.1	63.8
	T7-Ns	3′>DABCYL-TTCGAGACCACCTCGTCAT<5′	52.6	58.5

“

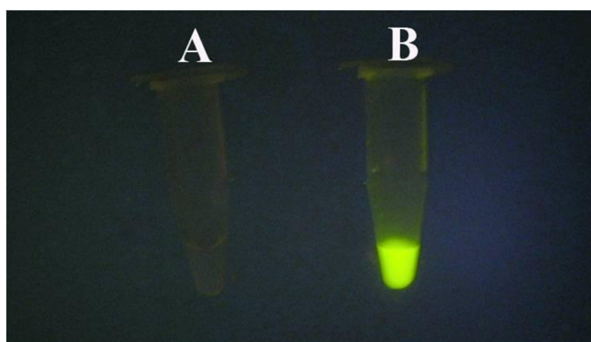

Figure S1. Photograph of T7-FITC on the Dark Reader™ device. (A). Control tube. No FITC added. (B). T7 phage-FITC.

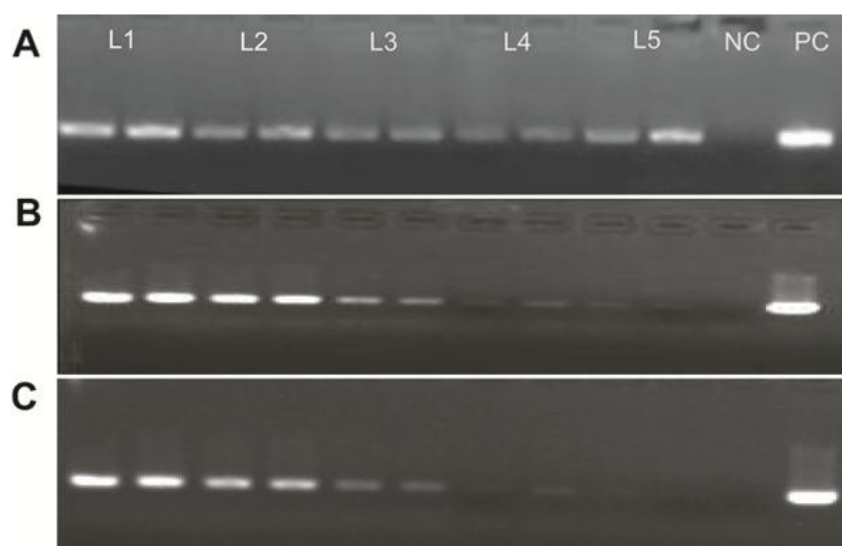

Fig S2. The results of immuno-PCR for detection of HBsAg under different conditions.

(A) The blocking buffer (without 0.05% Tween 20) and the washing buffer A. (B) The blocking buffer (containing 0.05% Tween 20) and washing buffer A. (C) The blocking buffer (without 0.05% Tween 20) and washing buffer B (containing 0.1% SDS). L1~L4 represent in duplicate serial 10-fold dilutions of HBsAg from 800 to 0.8 ng/ml, and L5 stands for 0 ng/ml on a 2% agarose gel. NC: negative control, PC: positive control.

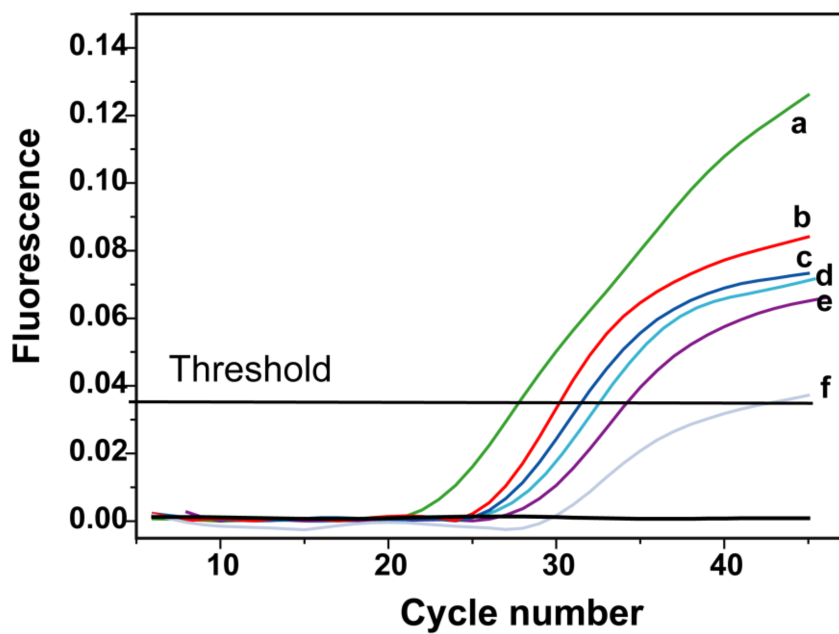

Fig S3. Detection limits of sandwich Immuno-PCR. Curves a-e represent serial 10-fold dilutions of HBsAg from 1.0 ng/ml to 0.1 pg/ml. Curve f stands for the negative control without HBsAg.