Supplementary Information

On-chip filtration enrichment and ultrasensitive nanozyme-catalyzed colorimetric detection of *Streptococcus pneumoniae* in saliva samples

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S1.1. Reagents and Materials

Chloroauric acid ($HAuCl_4$) and trisodium citrate ($C_6H_5Na_3O_7$, 10019418) were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). 1-Step Ultra TMB-ELISA substrate was obtained from Thermo Fisher Scientific. Polyvinylpyrrolidone (PVP10-100G), L-ascorbic acid (L-AA, V900134-100G), hexachloroplatinic acid (H_2PtCl_6, 206083-1G), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), phosphate-buffered saline (PBS), 2-(N-morpholino)ethanesulfonic acid (MES), mouse monoclonal anti-*Streptococcus pneumoniae* antibody (Catalog # Ab8274), bovine serum albumin (BSA), fetal bovine serum (FBS), and Tween-20 were all purchased from Sigma-Aldrich (USA). Nylon, nitrocellulose, and polycarbonate membrane filters with a pore size of 0.4 μm were obtained from GE Healthcare Life Sciences (Whatman). A carbon dioxide generating agent was purchased from Mitsubishi Gas Chemical Co., Inc. ELISA kits were provided by Nanjing SenBeiJia Biological Technology Co., Ltd.

Transmission electron microscopy (TEM) and high-resolution TEM (HR-TEM) images were acquired using a Hitachi H-7800 TEM and a Tecnai G2 F20 microscope, respectively. Elemental mapping was performed via energy-dispersive X-ray spectroscopy (EDS) on a Philips Tecnai G2 F20 microscope equipped with a scanning transmission electron microscopy (STEM) unit. UV–vis absorption spectra were recorded using a UV-2600 spectrophotometer (Shimadzu, Japan). The optical density at 652 nm (OD_{652}) was measured using a BioTek microplate reader (BioTek Instruments, USA). Quantitative PCR (qPCR) Ct values were obtained using an Applied Biosystems 7500 Real-Time PCR System.

S1.2 Preparation of 25 nm Au nanoparticles

Gold nanoparticles (AuNPs) were synthesized using a standard trisodium citrate (TSC) reduction method.[1] Briefly, 1 mL of 1% (w/w) chloroauric acid ($HAuCl_4$) solution was diluted with 100 mL of deionized water in a clean flask. The solution was heated to boiling under constant magnetic stirring (300–500 rpm) to ensure uniform

temperature distribution. Upon reaching boiling, 2 mL of 1% (w/w) TSC solution was rapidly added to initiate nanoparticle formation. The reaction was maintained at 100 °C with continuous stirring for 15 minutes. After completion, the AuNP suspension was removed from heat and allowed to cool to room temperature under stirring to prevent aggregation. The resulting gold seed solution was stored in a cool, dark place until further use.

S1.3 Preparation of Au@Pt nanozymes

Au@Pt nanozymes were synthesized using a modified protocol based on previously reported methods.[2] Briefly, 400 μL of the pre-synthesized Au seed solution was centrifuged at 10,000 rpm for 5 minutes to collect the AuNPs, which were then washed twice with deionized water and redispersed in 600 μL of deionized water. To stabilize the particles, 40 μL of 10% (w/w) poly(vinylpyrrolidone) (PVP) solution was added, and the mixture was gently swirled and allowed to stand for 5 minutes to ensure uniform PVP coating on the Au seeds. Subsequently, 40 μL of L-ascorbic acid (L-AA, 100 mg/mL) and 40 μL of chloroplatinic acid hydrate (100 mM) were added dropwise to the mixture. The reaction was conducted at 60 °C with continuous shaking at 800 rpm for 20 minutes, promoting the deposition of platinum (Pt) onto the Au cores to form a porous Pt shell. The resulting Au@Pt nanozymes were collected by centrifugation at 10,000 rpm for 5 minutes, washed twice with deionized water, and finally redispersed in 1 mL of deionized water for subsequent use.

S1.4 Preparation of *S. pneumoniae* samples

The preparation of *S. pneumoniae* samples involves a series of steps to ensure accurate concentration and purity for subsequent experimental analysis. Initially, *S. pneumoniae* strains are inoculated onto 5% sheep blood agar plates and incubated at 37°C in a 5% CO_2 environment overnight (approximately 12–18 hours). Following incubation, several colonies are isolated using a sterile inoculation loop or pipette tip and transferred into 1 mL of phosphate-buffered saline (PBS, 10 mM, pH 7.4) to create a bacterial suspension with gentle mixing. The suspension is then serially diluted with sterile water, typically ranging from 1×10^5 to 1×10^8-fold (e.g., 10 μL of suspension

added to 990 μL of sterile water for a 1:100 dilution), to obtain various concentrations for quantitative analysis. Subsequently, 0.2 mL of the diluted bacterial solution is evenly spread onto blood agar plates and incubated at 37°C for 12 hours. After incubation, colony-forming units (CFUs) are counted using a colony counter or manually to calculate the original bacterial concentration (e.g., 25 CFUs in a $1:10^6$ dilution indicates an original concentration of 2.5×10^7 cfu/mL). Based on experimental requirements, the bacterial solution is further diluted to desired concentrations (e.g., 10^7 to 10 cfu/mL) and aliquoted into sterile centrifuge tubes, stored at 4°C or -20°C for future use. This protocol ensures reliable bacterial sample preparation with controlled concentration and purity.

S1.5 Preparation of a Microfluidic Immunosensor Based on HRP Assay

First, 2 μL of freshly prepared 10 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) solution in ethanol was added to 2 mL of an aqueous gold nanoparticle (AuNP) suspension and gently agitated for at least 4 hours to allow surface modification, resulting in carboxyl-terminated AuNPs. These carboxyl groups enabled covalent conjugation of anti-*S. pneumoniae* antibodies and horseradish peroxidase (HRP) via EDC/sulfo-NHS chemistry. To activate the AuNP surface, 2 mL of the modified AuNP suspension was centrifuged and redispersed in 500 μL of MES buffer (2 mM, pH 5.5), followed by the addition of 10 μL each of 10 mM EDC and 10 mM sulfo-NHS. The reaction was allowed to proceed for 15 minutes. Excess crosslinking reagents were removed by centrifugation, and the pellet was resuspended in 200 μL of phosphate-buffered saline (PBS, 2 mM, pH 7.4). Next, 10 μg of anti-*S. pneumoniae* antibody and 100 μg of HRP were added to the activated AuNPs, and the mixture was incubated for 2 hours at room temperature. To block any remaining active sites, 90 μL of 10% (w/w) bovine serum albumin (BSA) was added, followed by incubation for an additional 60 minutes. The resulting immuno-Au nanotags were purified by centrifugation, washed twice with 2 mM PBS, and finally resuspended in 300 μL of storage buffer (2 mM PBS containing 1 mg/mL BSA, 0.5% w/v sucrose, and 0.02% w/v sodium azide). Aliquots (15 μL each) were dispensed into 1.5 mL microcentrifuge tubes and lyophilized under vacuum for future use.

S1.6 PCR gel electrophoresis

To verify that all collected saliva samples were negative, the following procedure was conducted. First, 10 μL of each saliva sample was collected and thoroughly mixed. DNA was extracted from the pooled saliva samples using the TIANamp DNA extraction kit according to the manufacturer's instructions. Subsequently, PCR amplification was performed in a 30 μL reaction mixture, comprising 15 μL of the extracted saliva DNA, 7.5 μL of 4× Probe dPCR Promix, 4.7 μL of RNase-free deionized water (Tiangen), and 2.4 μL each of forward and reverse primers (100 μM). The amplified products were then analyzed via agarose gel electrophoresis. Briefly, 0.45 g of agarose was dissolved in 30 mL of 1× TAE buffer by heating in a microwave for 1–2 minutes until fully dissolved. After cooling slightly, 10000× Gelred nucleic acid dye was added and mixed. The agarose solution was poured into a gel tray with a comb and allowed to solidify. The gel was placed in an electrophoresis tank containing 1× TAE buffer. Each PCR product was mixed with 6× RNA/DNA loading buffer to a final concentration of 1×, and 3 μL of each sample, along with 3 μL of DL5000 plus marker, was loaded into the wells. Electrophoresis was conducted at 200 V and 120 mA for 30 minutes. Post-electrophoresis, the gel was imaged using a gel imaging system.

S1.7 Optimization the size of Au@Pt nanozymes

Initially, varying volumes of AuNPs solution (200, 300, 400, 500, 600, and 700 μL) were each mixed with complementary volumes of deionized water (800, 700, 600, 500, 400, and 300 μL, respectively) to maintain a consistent total volume. Subsequently, 40 μL of a 10% (w/w) PVP solution was added to each mixture. The solutions were gently swirled and allowed to incubate for 5 minutes to ensure uniform coating and stabilization of the AuNP seeds by the polymer. Following this, 40 μL of L-ascorbic acid solution (100 mg/mL) and 40 μL of chloroplatinic acid hydrate (100 mM) were added dropwise to each preparation. The mixtures were then subjected to constant shaking at 800 rpm for 30 minutes at 65 °C. During this step, platinum was progressively deposited onto the surface of the gold seeds, resulting in the formation of a Pt shell. The synthesized Au@Pt nanozymes were subsequently harvested by centrifugation, washed twice with deionized water, and finally redispersed in 200, 300,

400, 500, 600, and 700 μL of deionized water, respectively, for storage and further use.

In the experiments for detecting *S. pneumoniae* using the microfluidic immunosensor, the size of Au@Pt nanozymes plays a critical role in their performance during membrane-based immunoassays. Larger Au@Pt nanozymes offers several advantages, including enhanced catalytic activity due to their increased surface area, resulting in stronger signal amplification during TMB oxidation. Their superior visibility and increased retention on the filter membrane further enhance the sensitivity and stability of bacterial detection. However, their larger size can lead to nonspecific binding during the immune reaction, low diffusion efficiency, and potential membrane clogging, which may compromise uniform signal generation and elevate background signals. In contrast, smaller Au@Pt nanozymes exhibit lower catalytic activity, resulting in weaker colorimetric signals. Nevertheless, their small size minimizes the risk of membrane clogging, ensures more uniform distribution, and helps reduce background signals, thereby improving detection specificity and reproducibility. Therefore, an optimal balance between nanozyme size and catalytic performance must be considered to achieve high analytical sensitivity and reliability. As shown in the TEM images (i) and particle size distributions (ii) in Figure S10a-f, Au@Pt nanoparticles with diameters of 107 ± 1.51, 102.27 ± 0.88, 96.52±1.51, 93.19 ± 1.05, 89.55 ± 1.33, 82.23 ± 1.85nm were synthesized using different volumes of Au particles (200, 300, 400, 500, 600, and 700 μL, at a concentration of 2.55 μg/μL). As the diameter of the Au@Pt nanozyme particles decreased, the intensity of the colorimetric signal generated by the oxidation of TMB gradually decreased (Figure S10g,h). To identify the optimal particle size of Au@Pt nanozymes, nanozymes of different diameters were individually introduced into the microfluidic immunosensor for the detection of *S. pneumoniae*. As shown in Figure S10g,f, the Au@Pt nanozymes with an average diameter of approximately 93 nm generated the highest SNR upon catalyzing the oxidation of TMB.

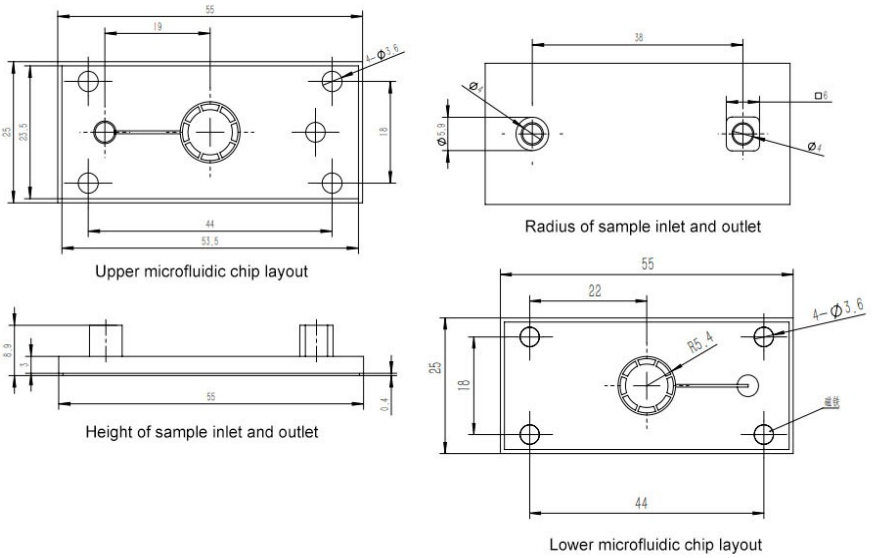

Figure. S1 Planar schematic parameters of the microfluidic chip.

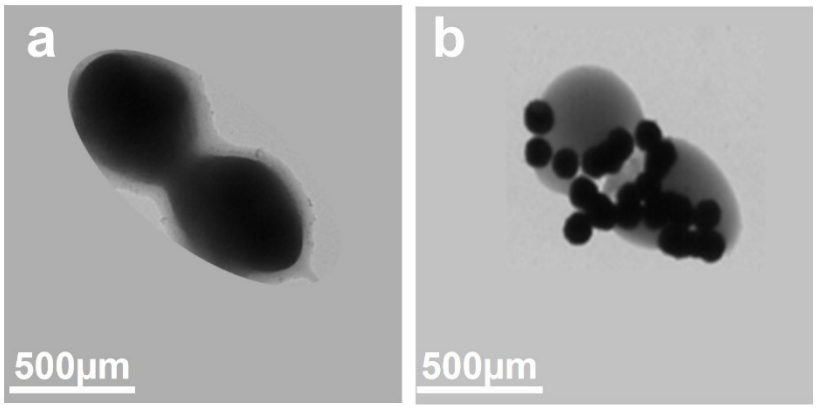

Figure. S2 TEM images of (a) *S. pneumoniae*, and (b) immunocomplex of Au@Pt- *S. pneumoniae*.

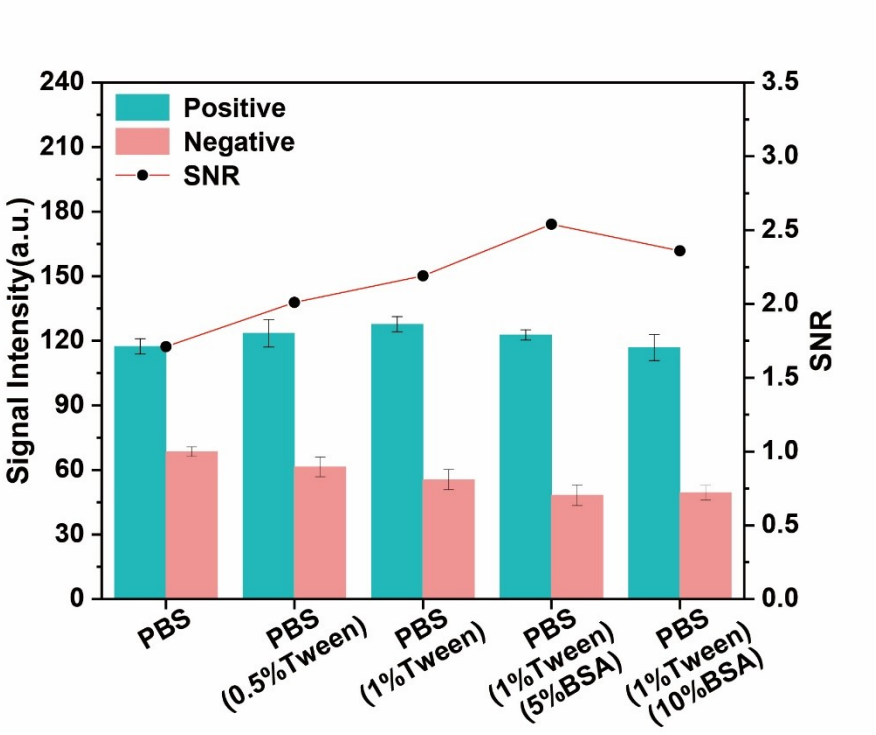

Figure. S3 Optimization of the composition and ratio of the running buffer solution.

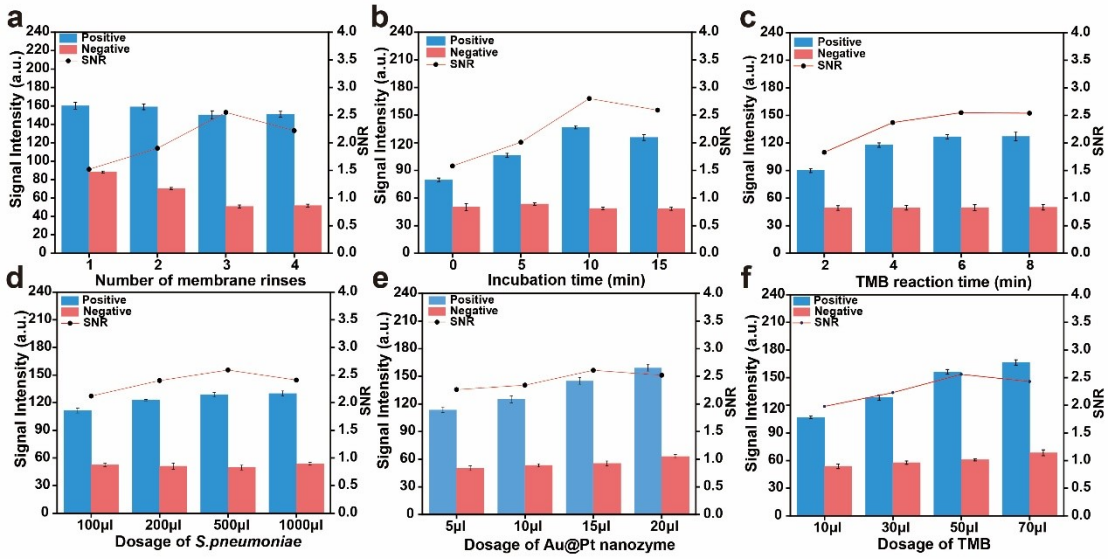

Figure. S4 Optimization of the number of filter membrane washes with PBST solution (a), incubation time (b), TMB reaction time (c), dosage of TMB (d), dosage of Au@Pt nanozyme with concentration of 2.55μg/μL (e), dosage of *S. pneumoniae* with concentration of 10^4 cfu/mL (f) for microfluidic immunosensor.

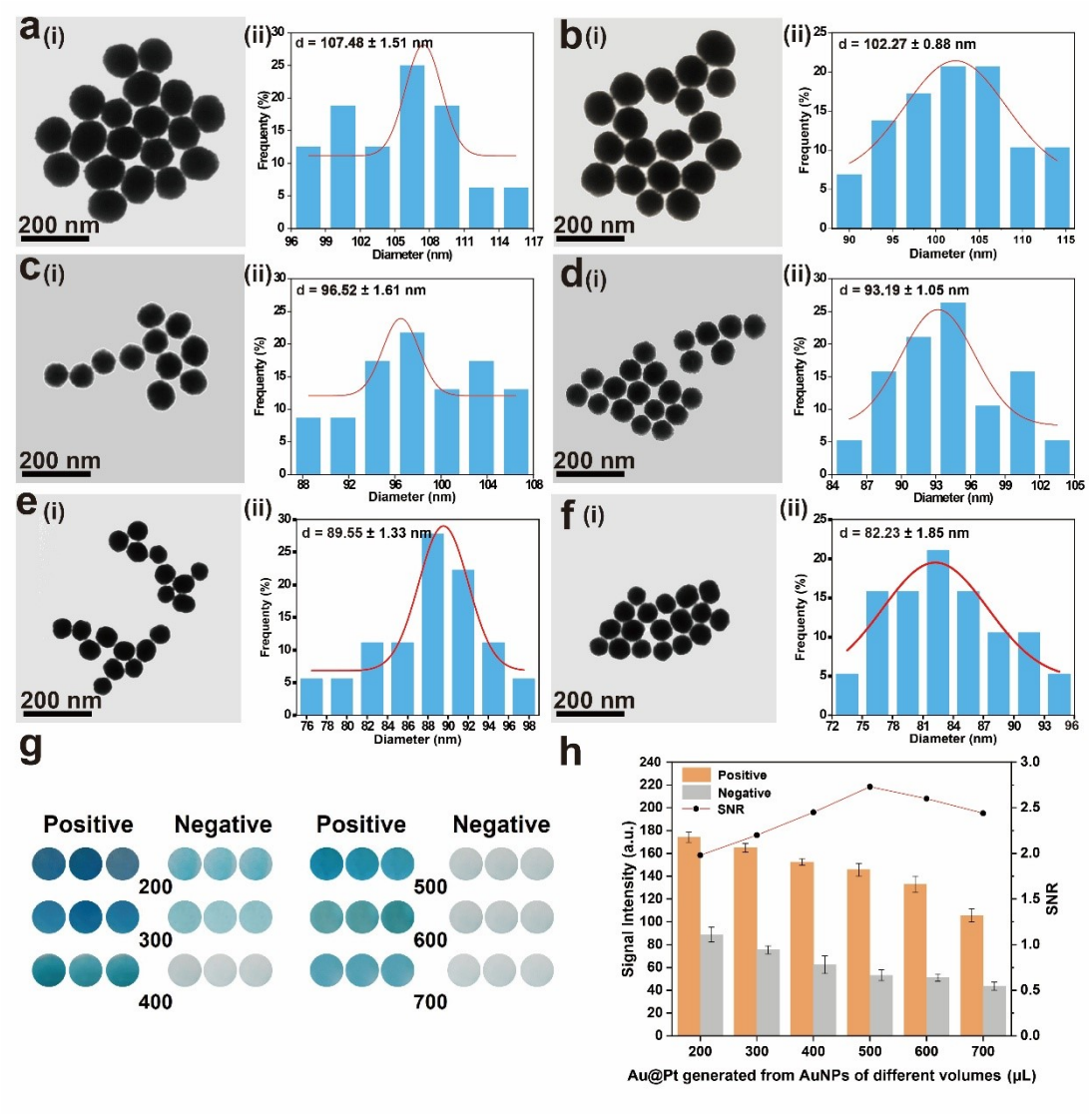

Figure. S5 (i) TEM images and (ii) particle size distribution histograms of Au@Pt nanozymes with different amount of Au seeds added:(a) 200,(b) 300,(c) 400,(d) 500,(e) 600,(f) 700μL. (g) Photographs and (h) detailed signal intensity of microfluidic immunosensor with different Au@Pt nanozyme with different amount of Au nanoparticles added (200, 300, 400, 500, 600, and 700 μL) for detection *S. pneumoniae* at the concentration of 10^6 and 0 cfu/mL.

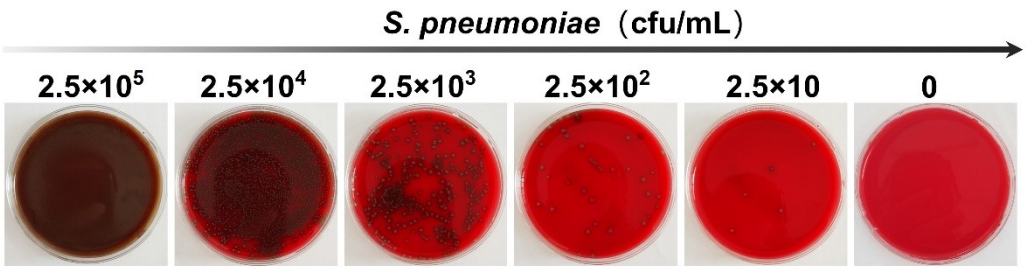

Figure. S6 Standard plate counting method for quantitative determination of *S. pneumoniae*

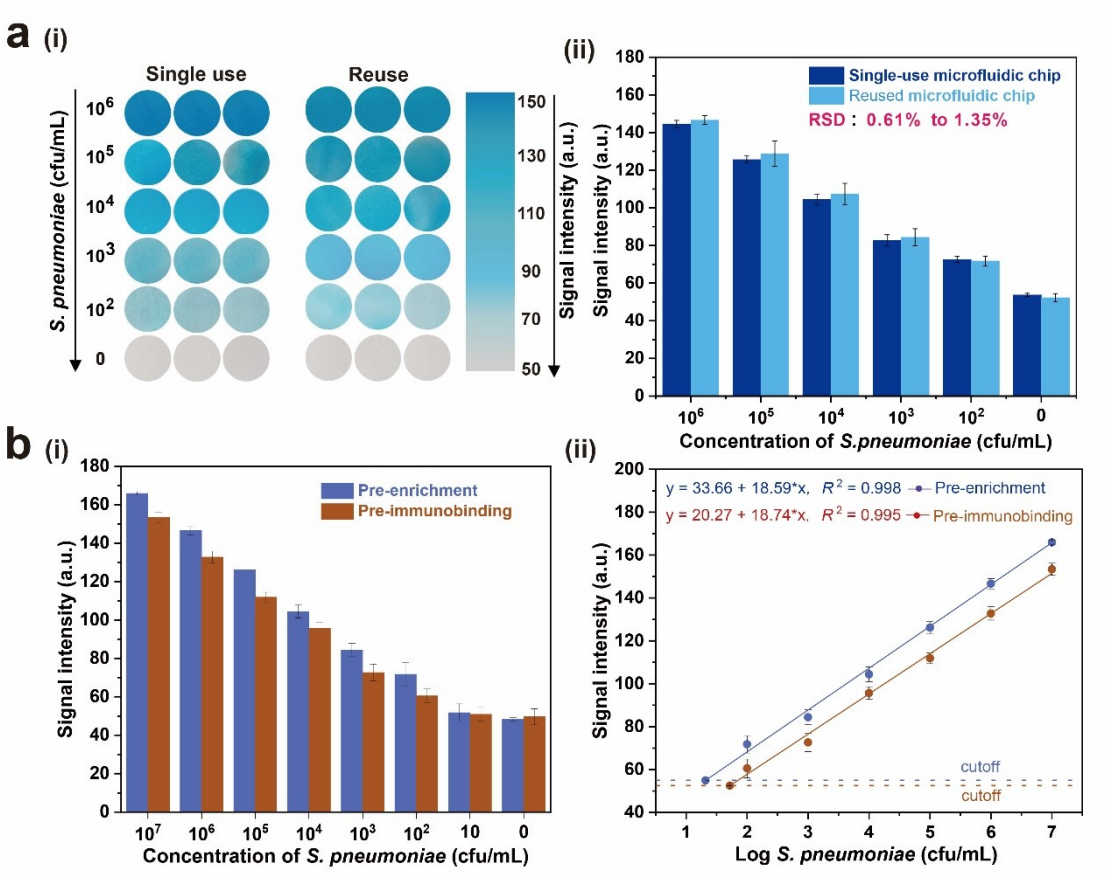

Figure S7. (a) Photographs of single-use microfluidic chip and reused microfluidic chip for detection of *S. pneumoniae* (i), and colorimetric signal intensity of single-use microfluidic chip and reused microfluidic chip for detection of *S. pneumoniae* (ii). (b) Signal intensity of *S. pneumoniae* enriched by the membrane before immune binding compared to that enriched after immune binding (i), corresponding calibration curves of *S. pneumoniae* enriched by the membrane before immune binding compared to that enriched after immune binding (ii).

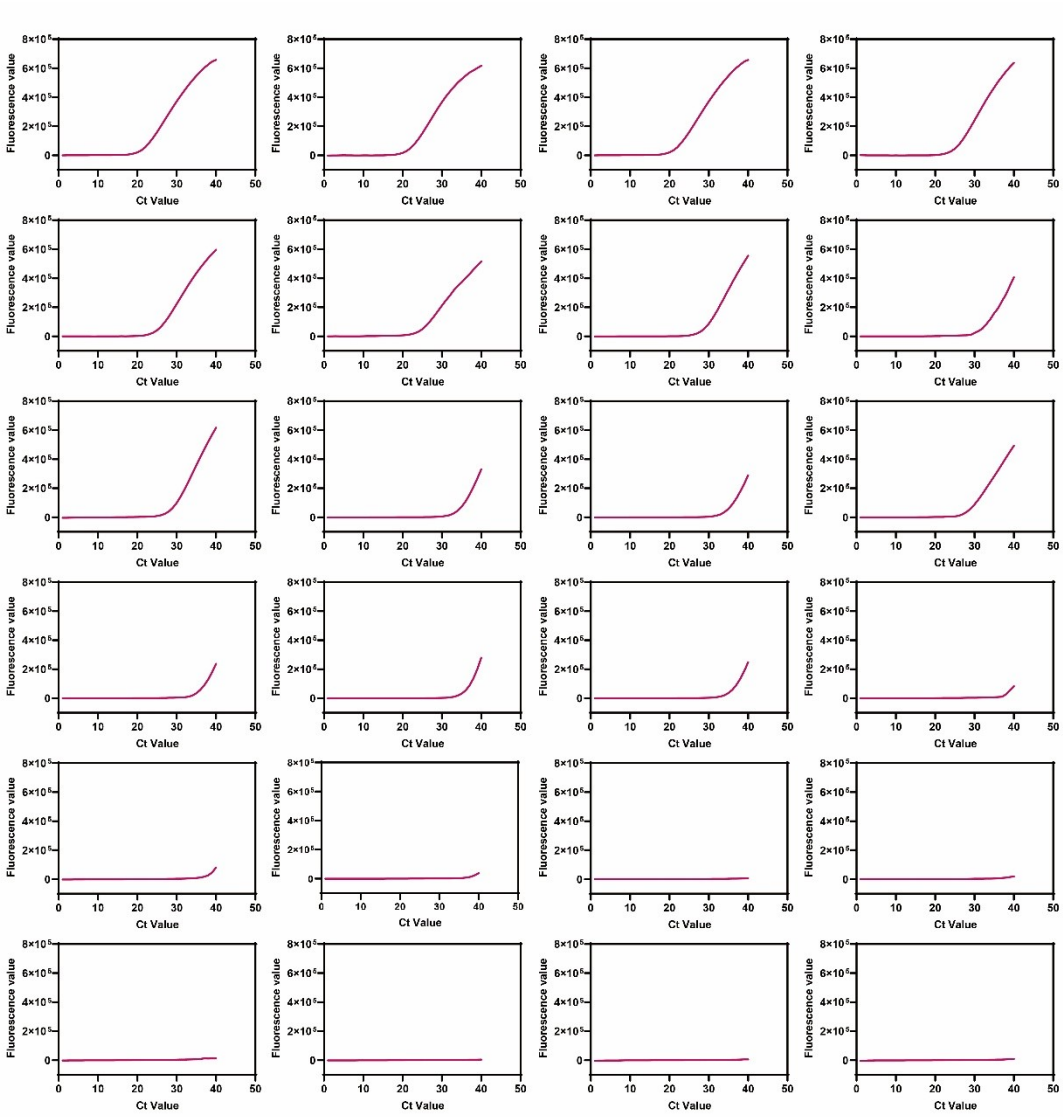

Figure. S8 The real-time fluorescence curves of positive and negative saliva samples tested by qPCR.

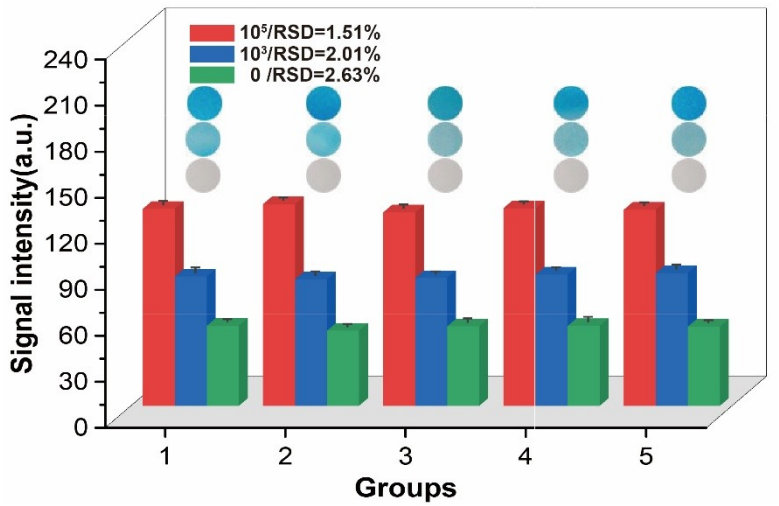

Figure. S9 Reproducibility of microfluidic immunosensor. Colorimetric images (inset) and corresponding signal intensities for *S. pneumoniae* detection at the concentrations of 10^5 , 10^3 and 0 cfu/mL.

Table S1. Analytical performances of the microfluidic immunosensor and other approaches for bacteria detection.

Approaches	Pathogens	Assay time	LoDs	References
Electrochemical	*S.pneumoniae*	~2 minutes	962 cfu/mL	[3]
Electrochemical	*E.coli*	35 minutes	50 cfu/mL	[4]
Microfluidic biosensor	*S.pneumoniae*	~35 minutes	1.2×10^3 cfu/mL	[5]
Microfluidic biosensor	*S.pneumoniae*	~2 hours	10^2 cfu/mL	[6]
Fluorescent-ICA	*S.aureus*	10 minutes	660 cfu/mL	[7]
Immunochromatographic	*S.pneumoniae*	~15 minutes	5×10^4 cfu/mL	[8]
PCR of genomic DNA	*E.coli*	50 minutes	10 cfu/mL	[9]
Conventional ELISA	*S.pneumoniae*	30 minutes	10^6 cfu/mL	[10]
Real-Time PCR	*S.pneumoniae*	~1-2 hours	10-100 cfu/mL	[11]
Microfluidic immunosensor	*S.pneumoniae*	~20 minutes	21 cfu/mL	This work

Table S2. Recovery efficiency of *S. pneumoniae* in spiked samples.

Sample	Add (cfu/mL)	Detected (cfu/mL)	Recovey (%)	RSD (%)
Saliva	1×10^6	1.047×10^6	104.7	2.44
	1×10^5	0.892×10^5	89.2	5.71
	1×10^4	0.933×10^4	93.3	3.63
	1×10^3	1.125×10^3	112.5	5.88

Table S3. Primers sequences used in this study.

Bacteria	Target gene	Primer sequence (5'-3')	Length	Reference
S.pneumoniae	lytA	Forward primer:		[12]
		ACGCAATCTAGCAGATGA	22bp	
		Reverse primer:		
		TCGTGCGTTTTAATTCCAG	22bp	
		TaqMan probe:		
		TGCCGAAAACGCTTGATACAGG	23bp	

Table S4. qPCR amplification procedure.

Procedure	Temperature	Time	Cycle number
Predegeneration	95℃	30 s	1
Denaturation	94℃	10 s	
Anneal	53℃	30 s	40
Extension	58℃	30 s	

Reaction system: 4×ProMix 7.5 μL, forward primer (10 μM) 2.4 μL, reverse primer(10 μM) 2.4 μL, TaqMan probe(10 μM) 0.75 μL, ultrapure water 1.95 μL, and DNA template 15 μL.

References

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