Electronic Supplementary Information (ESI)

for

Microfluidic chip based multivalent DNA walker amplification biosensor for simultaneous detection of multiple food-borne pathogens

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1. Experiments

1.1 Materials and reagents.

Staphylococcus aureus (ATCC 25923) and *Salmonella typhimurium* (ATCC 14028) were purchased from Sole bold Co., Ltd. (Beijing, China), and *Vibrio parahaemolyticus* (ATCC 17802) was obtained from Luwei Technology Co., Ltd. (Shanghai, China), Tryptone Soy Broth Medium (TSB) and LB Medium were acquired from Shanghai Chemical Reagent Co., Ltd., gold stirring rods were gained from Shanghai Test Company (Shanghai, China), 4-ethynylphenylboronic acid (PBA), dibenzo Cyclooctyne-N-hydroxy succinimide ester (DBCO-NHS) were got from Sigma-Aldrich (Milan, Italy), *Salmonella* antibody, and *Vibrio parahaemolyticus* antibody were bought from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China) , copper sulfate pent hydrate and ascorbic acid were obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). Phosphate buffer saline (PBS, pH=7.4) solution was obtained from Sigma-Aldrich Co., Ltd (Milan, Italy). Zinc chloride ($ZnCl_2$) and trisodium citrate solution were purchased from Aladdin Co., Ltd (Shanghai, China). SYBR Gold and Tris-EDTA (TE) buffer solution (10 mM Tris-HCl, 1mM EDTA-2Na, pH = 8.2) were provided by Invitrogen Co., Ltd (Shanghai, China). DNA-500 buffer and DNA-500 marker were obtained from Shimadzu Co., Ltd (Kyoto, Japan). TMB one-component chromogenic solution, bovine serum albumin, and Rabbit Anti-Chicken HRP antibody were received from Sole bold Co, Ltd. (China, Beijing). Ultrafiltration centrifuge tube (30KD) was bought from Millipore (USA). All oligonucleotide sequences used in this work were purchased from Shanghai Sheng gong Biological Engineering Co., Ltd., and the specific sequences were shown in Table S1 and S2.

1.2 Apparatus

The centrifuge was gained from Sigma (Germany). The UV-vis spectra were recorded by a UV-1800 spectrophotometer Shimadzu Co, Ltd. (Tokyo, Japan). The

morphology of biological materials was photographed by scanning electron microscope (SEM) from Hitachi Company (Japan). The DNA fragments were separated and detected by the MCE-202 MultiNA microchip electrophoresis from Shimadzu Co., Ltd (Tokyo, Japan) coupled with laser induced fluorescence detector with the excitation wavelength at 488 nm and emission wavelength at 523 nm. The micro-channel of microfluidic chip was 23 mm in length, 104 μm in width and 48 μm in depth. The sample injection volume was 150 pL. The separation buffer containing a commercial DNA-500 kit (25-500 bp DNA fragments) with 10μM SYBR Gold as a fluorescent dye was injected by a syringe pump into the channel of the microfluidic chip. Subsequently sample addition and separation detection were achieved by adding different voltages in the microchannel. The voltage power of the microfluidic chip was ranged from 0 to 5000 V for sample loading and separation, and the specific voltage parameters were shown in Table S3. Briefly, samples were introduced into the microchannel under different voltages, namely S = 280 V, SW = 510 V, B = 320 V and BW = 0 V for 1 min (S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir). And then the samples were separated and detected within 3 min at S = 250 V, SW = 250 V, B = 0 V, and BW = 1000 V. After analysis, the micro-channels were washed for three times with ultrapure water and cleaning solution-RA (Lot 5071) provided by the Shimadzu Co., Ltd (Tokyo, Japan). The picture of the chip was shown in Fig. S8A.

1.3 Preparation and purification of yolk antibody

1.3.1 Preparation

S.A was used to immunize SPF (specified pathogen-free animals) laying hens to obtain IgY. The steps were as follows: 1.0 mL of S.A with 10^9 CFU·mL^{-1} was taken to centrifuge at 5000 rpm for 5 minutes, and discarded the supernatant. Next, PBS solution was added to the pellet to wash 3 times, discarded the supernatant also. Then 1.0 mL of 0.5% formaldehyde solution was added and incubated at 37°C for 24 h to inactivate the bacteria. Mixing 1:1 (v/v) with Freund's adjuvant and inactivated bacteria solution and injected it into SPF chickens for immunity. Two months after immunization, the yolk was obtained.

1.3.2 Purification

The purification of the yolk antibody was obtained as following. 1.0 mL egg yolk and 9.0 mL 0.1 M HAc-NaAc solution (pH 5.0) were mixed, and stirred at 4°C stayed overnight. It was taken from refrigerator to centrifuge at 10,000 r/min the next day and the supernatant was given up. Na_2SO_4 was added follow up to a final concentration of 18% (wt %), and stirred to dissolve, stood for 1h at room temperature. Then the above solution was centrifuged again (10,000 r/min), discarded the supernatant to obtain a precipitate. After that, PBS (pH 7.4) solution was added to make the precipitate dissolved, Na_2SO_4 was put into it to obtain a final concentration of 14% (wt %). After 1 hour, it was conducted centrifuge, deserted the supernatant to acquire the precipitate, and dissolved the precipitate in 1 mL of PBS solution. Later, the above solution was in an ultrafiltration centrifuge tube (MW=10 KD, 5000 r/min) to centrifuge for 1 h, thus, the IgY concentrate can be obtained. It was diluted with PBS to 10 μM, and stored at 4°C for subsequent experiments.

1.4 Cultivation of pathogenic bacteria

Staphylococcus aureus (ATCC 25923) *and Salmonella typhimurium* (ATCC 14028) were all cultured by LB medium. *Vibrio parahaemolyticus* (ATCC 17802) was cultured in TSB liquid medium containing 3% NaCl, all of them were incubated at 120 r/min for 6 h in a shaker at 37°C. The finished bacteria were added in 2 mL centrifuge tubes respectively and centrifuged at 5000 r/min for 3 minutes. After that, the supernatant medium was abandoned, and PBS solution was added to wash the pellet with 3 times, discarded the supernatant after every time. Next, the obtained bacterial solution was resuspended in 1mL PBS solution, and took 200μL bacterial fluid to measure its absorbance at 600 nm by ultraviolet-visible spectrophotometer, and the bacterial concentration-absorbance standard curve will be achieved to determine its concentration. The processed bacteria stored in a refrigerator at 4 °C for subsequent experiments.

1.5 Detection of V. P, S.T and S.A in the real samples

The 400 mL water samples were collected from tap water (aquatic water 1 and 2), local shrimp aquaculture water (aquatic water 3), yongjiang river water (aquatic

water 4), and local restaurant dishwater (aquatic water 5) to verify the suitability of the sensor. The water sample was filtered by 30-50 μm Whatman filter papers firstly to remove solid particles and other insoluble substances such as the stones and dirt. Then, 1.5 mL supernatant of the sample was spiked with different concentrations of three targets bacteria, respectively. The water sample was centrifuged at 5,000 r/min for 5 min to precipitate the targets bacteria, then the supernatant was discarded. The obtained precipitate was resuspended with 10 mM pH 7.4 PBS (1.0 mL) and further centrifuged. After it was washed for 3 times, the precipitate obtained was suspended with 10 mM pH 7.4 PBS, thus to obtain the sample suspension for measurement. Afterwards, the functionalized gold stirring rod was taken into the above suspension, and it had been stirred at 120 rpm for 40 minutes to achieve the capture and enrichment of pathogenic bacteria. The above stirring rod, capture probes and signal probe were then mixed simultaneously (molar ratio of capture probes to signal probe 1:10, pH = 8.0), and incubated at room temperature for 30 min. Later, the reacted stirring rod was taken out and immersed in the solution containing Zn^{2+} (100 μM), and incubated for 30 minutes at room temperature. Next, the solution obtained after the reaction was detected by MC, and the separation and detection of DNA were obtained by controlling the voltage change, and then the detection of the target pathogenic bacteria was realized.

1.6 Animal ethical statement

The animal study was reviewed and approved by the Ethics Committee for Animal Experiments of Animal Laboratory Center of Ningbo University (approval number NBU-A-2021-0026).

2. Supporting Tables

Table S1. The targets sequence oligonucleotides used in this work.

Name	Sequence (5'—3')
E 17	**Azide (N3)- TTT TTT ACC CTT AT/rA/ GGA AGA GAT CAC CTT /rA/GG ACC TAT TAA CTG T/rA/G ACA TGG AGT TTT TTT TTT TTT**
P-VP	**Azide (N3)-TTT TTT TTT TTT TTT TGA TCT CTT CTC CGA GCC GGT CGA AAT AAG GGT**
P-SA	**Azide (N3)-TTT TTT TTT TTT TTT TTT TAA TAG CTC TCC GAG CCG GTC GAA AAG GTG AT**
P-ST	**Azide (N3)-TTT TTT TTT TTT TTT TCT CCA TGT TCC GAG CCG GTC GAA ACA GTT AAT**

Note: Green represents the active region of Zn^{2+} cleavage that forms 8-17 DNAzyme. /rA/ stands for adenine ribonucleotide, which is 8-17 DNAzyme cutting site.

Table S2. The others sequence oligonucleotides used in this work.

Name	Sequence (5'-3')
1	**TTTTTTTTTTTTTTTTGATCTCTTCTCCGAGCCGGTCGAAATAAGGGT**
1-2	**TTTTTTTTTTTTTTTAGATCTCTTCTCCGAGCCGGTCGAAATAAGGGT**
1-3	**TTTTTTTTTTTTTTTGATCTCTTCTCCGAGCCGGTCGAAATAAGGGA**
1-4	**TTTTTTTTTTTTTTGTGATCTCTTCTCCGAGCCGGTCGAAATAAGGGT**
1-5	**TTTTTTTTTTTTTTTGATCTCTTCTCCGAGCCGGTCGAAATAAGGGTA**
2	**TTTTTTTTTTTTTTTTTAATAGGTCTCCGAGCCGGTCGAAAAGGTGAT**
2-2	**TTTTTTTTTTTTTTTATAATAGGTCTCCGAGCCGGTCGAAAAGGTGAT**
2-3	**TTTTTTTTTTTTTTTTTAATAGGTCTCCGAGCCGGTCGAAAAGGTGAA**
2-4	**TTTTTTTTTTTTTTTTAATAGGTCTCCGAGCCGGTCGAAAAGGTGATC**
2-5	**TTTTTTTTTTTTTGTTGATCTCTTCTCCGAGCCGGTCGAAATAAGGGT**
3	**TTTTTTTTTTTTTTTCTCCATGTTCCGAGCCGGTCGAAACAGTTAAT**
3-2	**TTTTTTTTTTTTTTTACTCCATGTTCCGAGCCGGTCGAAACAGTTAAT**
3-3	**TTTTTTTTTTTTTTTCTCCATGTTCCGAGCCGGTCGAAACAGTTAATA**
3-4	**TTTTTTTTTTTTTTTTCTCCATGTTCCGAGCCGGTCGAAACAGTTAA**
3-5	**TTTTTTTTTTTTTTTTTCCATGTTCCGAGCCGGTCGAAACAGTTAAT**

Table S3. Voltage program applied for sample injection and separation modes.

Scheme	Time (s)	Voltage applied into the reservoirs (V)			
		S	B	SW	BW
Loading	**30**	**280**	**320**	**510**	**0**
separation	**180**	**250**	**0**	**250**	**1000**

Note: S: sample reservoir; B: buffer reservoir;

SW: sample waste reservoir; BW: buffer waste reservoir.

Table S4. Comparison of the analytical performance of other works for the simutaneous determination of pathogenic bacteria.

Method	Bacteria	Linear range ($CFU \cdot mL^{-1}$)	LOD ($CFU \cdot mL^{-1}$)	Time	Ref.
Fluorescence immunoassay	E.coli O157:H7 S.T	1.5×10^{1} - 1.5×10^{6} 4.0×10^{1} - 4.0×10^{6}	40	2h	1
Fluorescent	S.A S.T V.P	50 - 10^{6}	25 15 10	>2h	2
Microfluidic chip	S.T P. aeruginosa	5×10^{1}-5×10^{6} 1×10^{1}-5×10^{4}	15 5	>2.5h	3
SERS	S.T V.P	27 - 2.7×10^{5} 18 - 1.8×10^{5}	27 18	>2h	4
ECA	E.coli O157:H7 Vibrio cholerae O1	50 - 1×10^{6}	39 32	>0.5h	5
Thermal sensor	S.T	300-1000	300	1.5h	6
Microfluidic chip	V.P S.T S.A	1×10^{2} - 1×10^{8} 1×10^{1} - 1×10^{8} 1×10^{1} - 1×10^{8}	28 10 9	<1.0 h	This Work

Table S5. Recoveries for the determination of V.P, S.T and S.A in different aquatic samples (n=5).

Samples	Spiked (CFU•mL^{-1})			Measured (CFU•mL^{-1})			Recovery (%)			RSD (%)		
	V.P	S.T	S.A	V.P	S.T	S.A	V.P	S.T	S.A	V.P	S.T	S.A
Aquatic 1	0	0	0	ND	(102±10)	(133±16)					9.4	8.8
	10^3	10^3	10^3	(0.97±0.04)×10^3	(1.01±0.05)×10^3	(1.05±0.06)×10^3	92.6	101.2	104.3	4.6	5.6	7.8
	10^6	10^6	10^6	(0.99±0.06)×10^6	(1.03±0.04)×10^6	(1.02±0.05)×10^6	98.9	99.6	101.4	5.2	6.1	6.2
Aquatic 2	0	0	0	ND	(116±15)	(158±23)					7.9	8.5
	10^3	10^3	10^3	(0.92±0.06)×10^3	(1.01±0.05)×10^3	(1.02±0.04)×10^3	95.3	98.5	103.1	5.9	6.2	7.4
	10^6	10^6	10^6	0.96±0.05)×10^6	(1.02±0.06)×10^6	(1.05±0.06)×10^6	99.8	102.4	103.5	4.8	5.9	7.1
Aquatic 3	0	0	0	(93±14)	ND	(127±25)				6.9		7.6
	10^3	10^3	10^3	(1.02±0.03)×10^3	(1.01±0.05)×10^3	(1.04±0.05)×10^3	97.6	99.2	105.7	5.4	7.3	6.2
	10^6	10^6	10^6	(1.00±0.04)×10^6	(1.02±0.04)×10^6	(1.03±0.04)×10^6	99.3	102.5	102.8	4.3	6.6	7.0
Aquatic 4	0	0	0	(97±20)	(112±10)	ND				6.5		8.3
	10^3	10^3	10^3	(0.94±0.06)×10^3	(1.04±0.06)×10^3	(1.03±0.054)×10^3	99.6	105.6	103.5	5.5	6.4	8.2
	10^6	10^6	10^6	0.97±0.05)×10^6	(1.03±0.04)×10^6	(1.04±0.064)×10^6	100.3	104.2	105.9	5.1	6.8	7.5
Aquatic 5	0	0	0	ND	(106±18)	(123±14)					9.2	9.5
	10^3	10^3	10^3	(0.95±0.04)×10^3	(1.02±0.05)×10^3	(1.06±0.03)×10^3	93.2	103.8	104.7	4.9	5.8	6.6
	10^6	10^6	10^6	0.93±0.03)×10^6	(1.04±0.02)×10^6	(1.05±0.03)×10^6	97.4	107.3	105.2	6.2	7.9	8.7

3. Supporting Figures

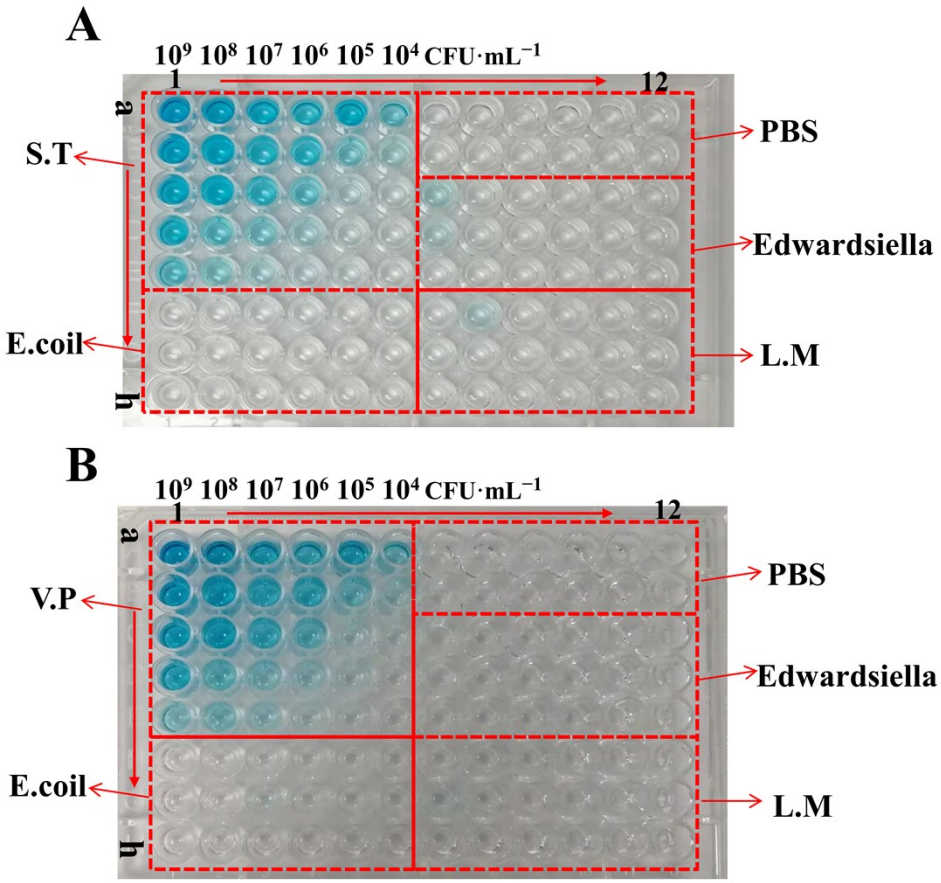

Fig. S1 Verification of binding of (A) P-ST-Ab to S.T and (B) P-VP-Ab to V.P by ELISA. (All bacteria were cultured for 6 h, and incubation time of P-ST-Ab and P-VP-Ab with the bacteria were 1 h).

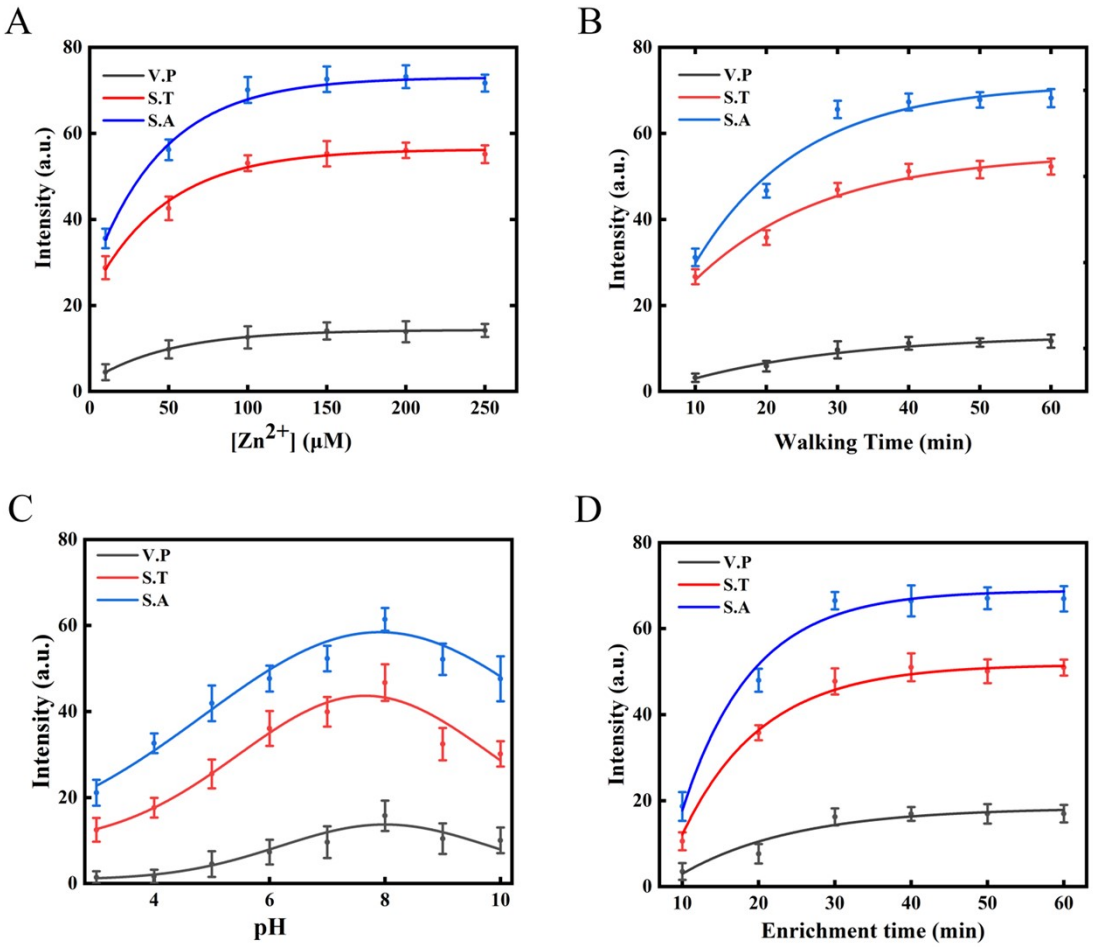

Fig. S2 Microchip electrophoresis optimized reaction conditions. (A) the concentration of Zn^{2+}, (B) cleavage time, (C) pH and (D) enrichment time of the gold stirring rod. (The concentration of V.P and S.T and S.A were 1×10^6 CFU·mL^{-1}, the above phenomena were all obtained in DNA 500 buffer).

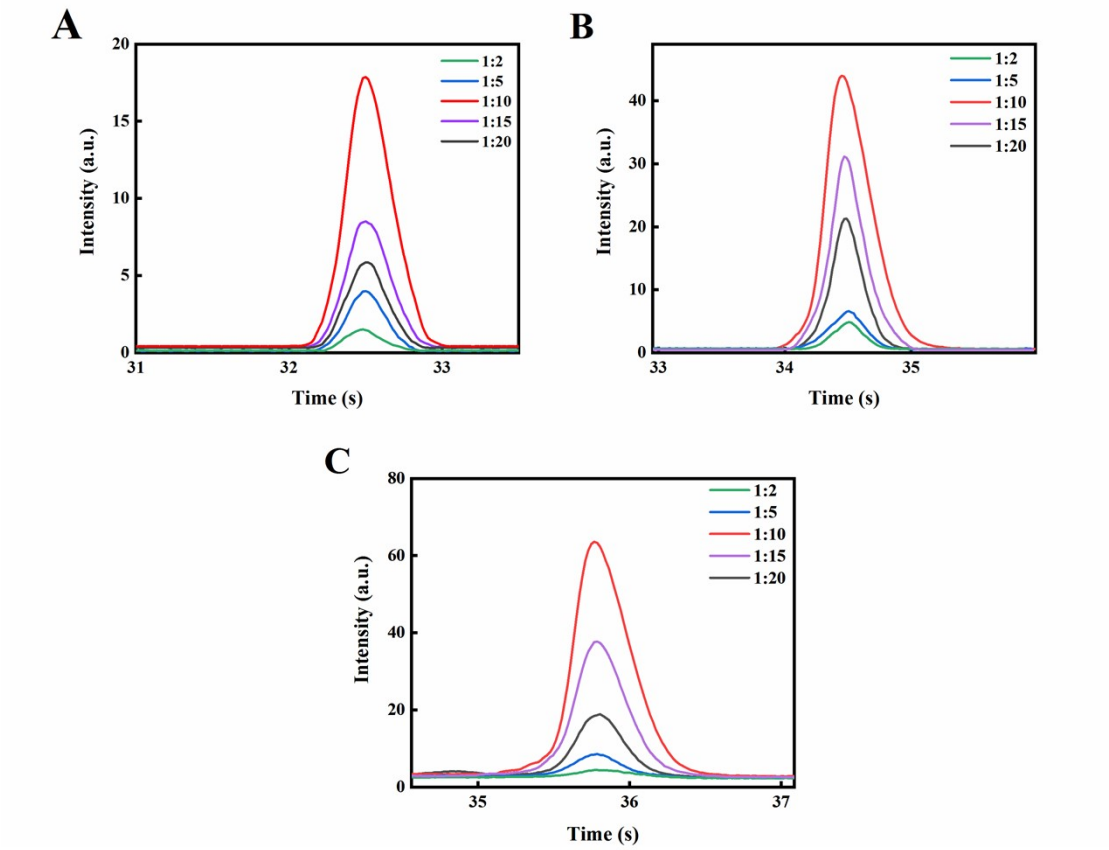

Fig. S3 Microchip electrophoresis optimized the molar ratio of three capture probes and signal probes. (A) V.P, (B) S.T (C) S.A. (The reaction buffer was PBS buffer and time was 0.5 h, pH were 8.0, the concentration of V.P and S.T and S.A were 1×10^{6} CFU·mL^{-1}).

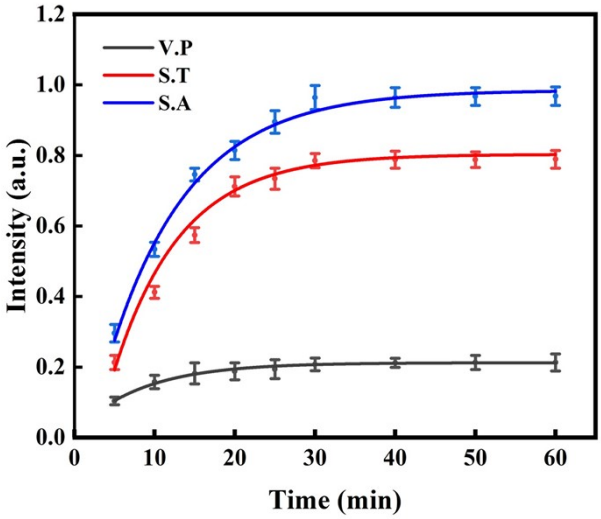

Fig. S4 Determination of the changes of probes' load number on three target pathogens over time by UV spectrophotometry. (The concentration of probes was 5.0 μM and targets bacteria was 1×10^6 CFU·mL^{-1}, pH was 8.0, the reaction buffer was PBS buffer).

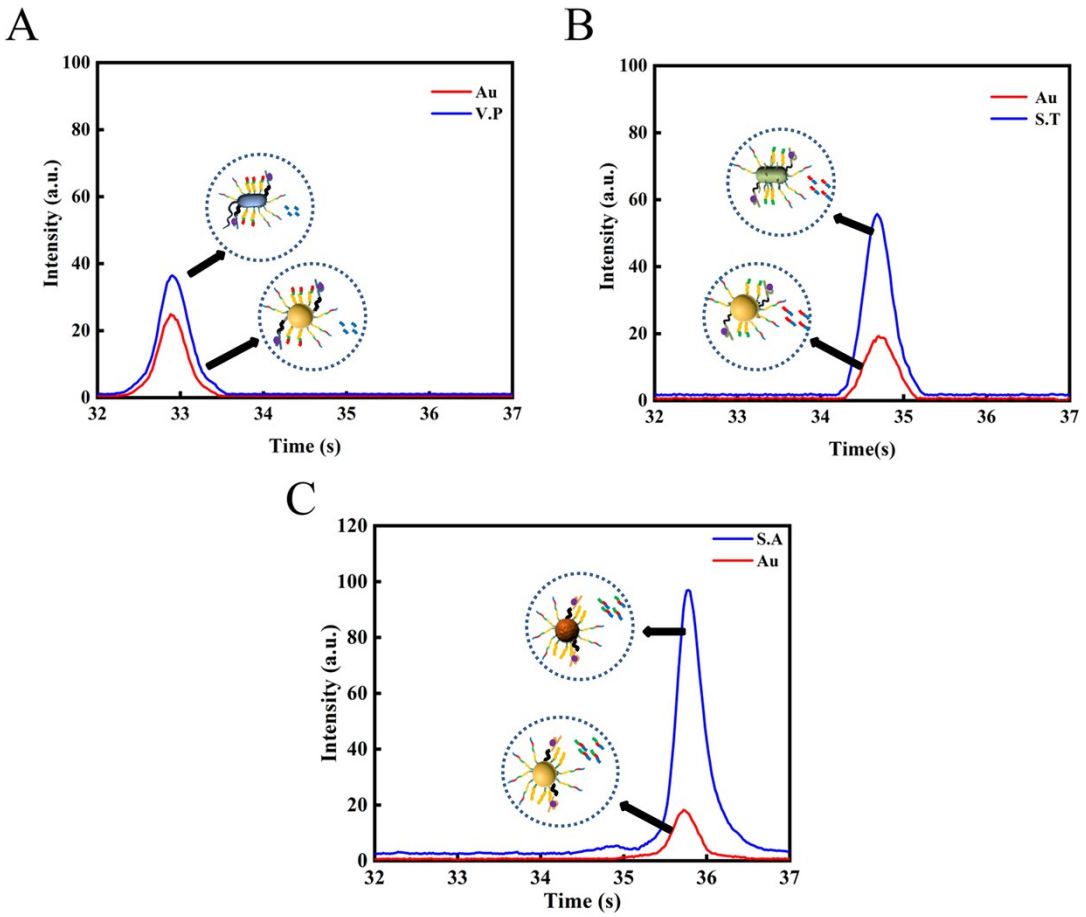

Fig. S5 Microchip electrophoresis to compare the amplification effect of targets pathogenic bacteria and gold nanoparticles (Au) as DNA walker carrier, respectively. (A) V.P, (B) S.T, (C) S.A. (The reaction buffer was PBS buffer, the concentration of V.P, S.T and S.A were 1×10^{7} CFU·mL^{-1}, the size of Au was 13nm, the reaction was carried out under optimized conditions).

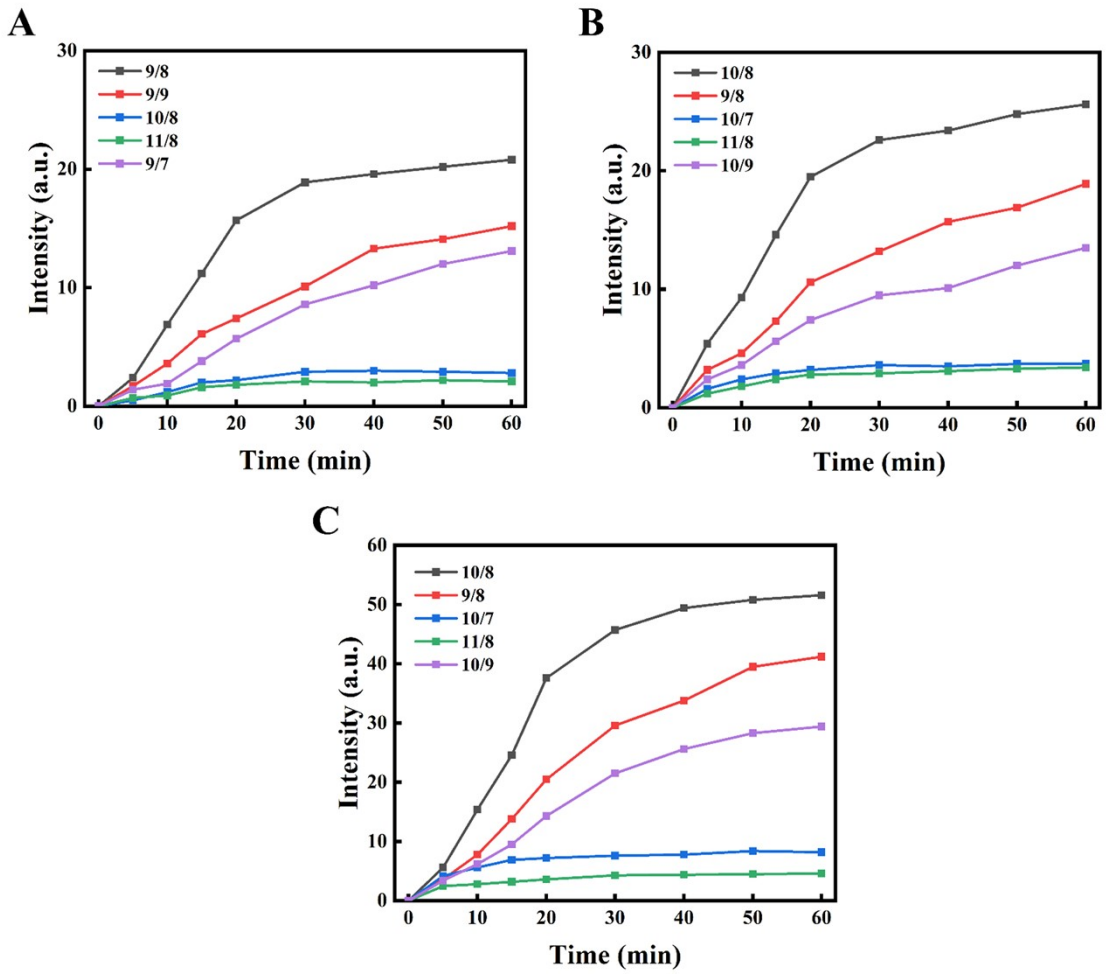

Fig. S6 The signal intensity of the number of different base pairing sequences (capture probes/signal probe) on three targets pathogenic bacteria changed with time. (A) V.P, (B) S.T (C) S.A. (The reaction buffer was PBS buffer, the concentration of V.P, S.T and S.A were 1×10^5 CFU·mL^{-1}, the reaction was carried out under optimized condition.

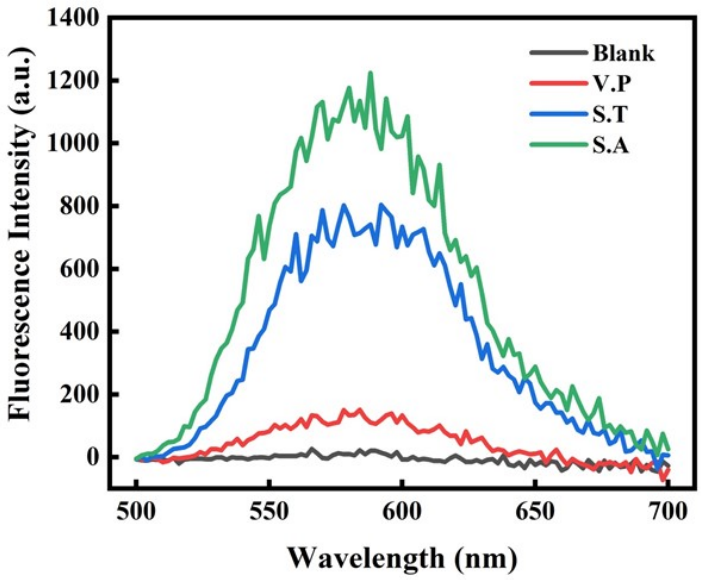

Fig. S7 Bioluminescence method to confirm the life and death of three targets pathogenic bacteria. (The reaction buffer was PBS buffer, the concentration of V.P, S.T and S.A were 1×10^5 CFU·mL^{-1}, the reaction was carried out under optimized conditions, the fluorescence intensity only existed in live bacteria)

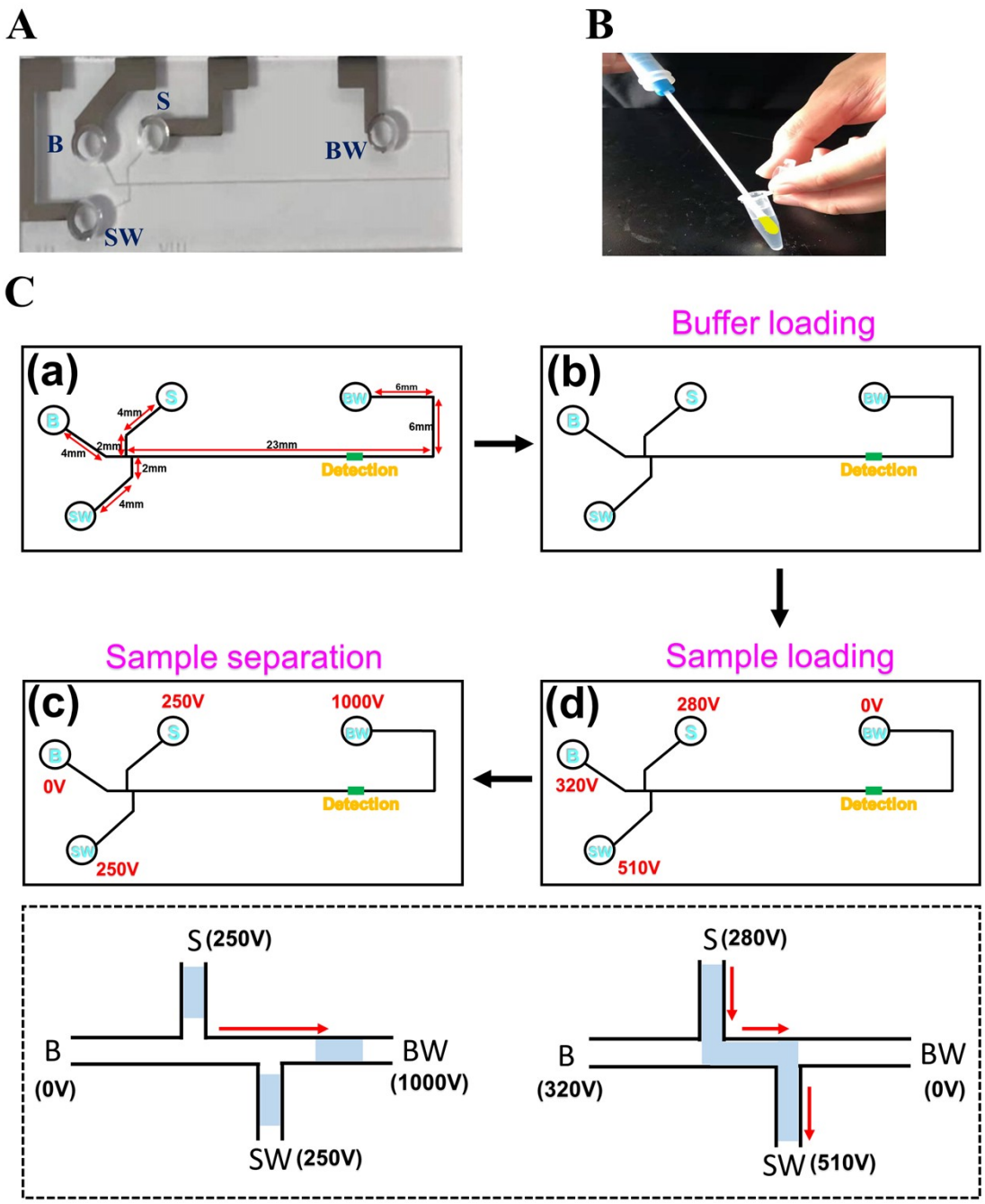

Fig. S8 (A) Physical map of microfluidic chip. (S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir). (B) Schematic diagram of gold stirring rod enrichment. (The gold stirring rod was wrapped around the bottom of the cotton swab). (C) Scheme illustration of sample analysis based on MC with (a) the surface design of microfluidic chip; (b) buffer loading; (c) sample loading; (d) sample separation and detection.

4. References

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