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Supporting Information

Phage amplification-assisted SEA-CRISPR/Cas12a system for viable bacteria detection

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Material:

PAP was a gift provided by the third Military Medical University (Chongqing, China), was isolated from the sewage of Southwest Hospital (Chongqing, China) based on lambda bacteriophage isolation protocol. The bacteriophage titer was measured by the standard plaque assay.

Methods:

1. PAP genome acid extraction and purification

1.1 pure DNA extraction

The Phenol-chloroform extraction method was used to extract genomic DNA from PAP. DNA concentration was determined using a Bio Photometer, and DNA integrity was assessed using 2% agarose gel electrophoresis. Subsequently, the DNA solution was diluted with ddH_2O to a concentration of 10 ng/μL, according to the measured DNA concentration, and stored at -20℃ for further analysis.

1.2 crude DNA extraction

For rapid genomic DNA extraction, a simple thermal lysis DNA extraction protocol was selected for ease of use and rapidity. Briefly, 1 mL of the PAP sample was taken and heated at 100℃ for 10 min. Then, the samples were centrifuged at 8000×g for 10 min and 4℃. The supernatant was transferred to a new tube and stored at -20℃ for further analysis.

2.Preparation of Fe_3O_4@UiO-66-NH_2@CPBA magnetic bead

Based on our group's previous work[1], Fe_3O_4 was firstly synthesized. $FeCl_3 \cdot 6H_2O$ (2.0 g) was dissolved in ethylene glycol (20 mL) and diethylene glycol (DEG, 20 mL) to form a faint yellow clear solution, followed by addition of poly (ethylene glycol) (average MW = 800 Da, 2.0 g) and sodium acetate (3.0 g). The yellow mixture was stirred vigorously for 30 min, and then divided and sealed in a 20 mL Teflon autoclaves, which were heated at 200°C for 6 h. The black products were separated by a permanent magnet, washed three times with ethanol and re-suspended in 50 mL ethanol for further use.

And then 10 mL of as-washed Fe_3O_4 was exchanged into 45 mL N, N-dimethylformamide (DMF) through three repeated magnetic separation/re-dispersion.

After that, $ZrOCl_2 \cdot 8H_2O$ (161 mg), 2-aminoterephthalic acid (NH_2-BDC, 90 mg), and 5 mL glacial acetic acid were added successively. The mixture was mechanically stirred at 120°C for 12 h. The brown products (Fe_3O_4@UiO-66-NH_2) were separated by a permanent magnet, washed three times with DMF and ethanol successively before final lyophilization and storage at 4°C.

Finally, carboxyphenylboronic acid (CPBA) with a carboxyl group can be modified with the amino group of nanoprobes by EDC/NHS chemistry. Specifically, 4-carboxyphenylboronic acid (200 mg) was dissolved in DMSO (15 mL) by sonication. Then, EDC (40 mg), NHS (80 mg), and DMSO (5 mL) were added to the above solution and mechanically stirred at room temperature for 30 minutes. Subsequently, the mentioned Fe_3O_4@UiO-66-NH_2 nanoparticles (50 mg) were added above. The reaction was then carried out at room temperature with mechanical stirring for 24 hours. The products (Fe_3O_4@UiO-66-NH_2@CPBA) were washed sequentially with water and ethanol and finally dispersed in 10 ml of ddH_2O at a concentration of 5 mg/mL.

3. Optimization of the dosage of magnetic bead

P. aeruginosa was diluted to 10^4 CFU/mL with Tris-HCl (20 mM, pH=7.4), 1 mL of bacterial suspension was taken from each tube, and 40, 60, 80, and 100 μL of magnetic beads were added respectively. Following sufficient mixing with a vortex oscillator, the sample was incubated in a constant temperature shaker (37℃, 180 r/min) for 30 minutes. Subsequently, magnetic separation was conducted for 5 minutes, after which the supernatant was transferred to a new 1.5 mL centrifuge tube. The magnetic beads were then resuspended with 1 mL Tris-HCl buffer and diluted to 10^3 CFU/mL. A volume of 0.1 mL of the supernatant and the isolation solution were aspirated uniformly. The beads were coated on LB solid medium and placed in a constant temperature incubator (37℃) for 12 h. The number of *P. aeruginosa* colonies in the supernatant and resuspension was obtained by plate counting. The capture efficiency was calculated according to the following formula: The capture efficiency (CE) is calculated using the following formula: CE (%) = [1-M/(M+S)] × 100, where S is the number of colonies in the resuspension-coated plate. M is the number of colonies in the supernatant-coated plate.

4. Optimization of incubation time for magnetic beads and bacteria

P. aeruginosa was diluted to 10^4 CFU/mL with Tris-HCl (20 mM, pH=7.4), and 1 mL of bacterial suspension was added to each tube respectively, and 80 μL of magnetic beads were added. After sufficient mixing with a vortex oscillator, it was incubated in a constant temperature shaker (37°C, 180 r/min) for 15, 20, 25, and 30 min, followed by magnetic separation for 5 min, and plate coating and capture efficiency calculations are the same as in the previous steps.

5. Capture efficiency of magnetic beads for different concentrations of bacteria

The *P. aeruginosa* was diluted to 10^6, 10^5, 10^4, and 10^3 CFU/mL with Tris-HCl (20 mM, pH=7.4) in a gradient, and 1 mL of bacterial suspension was added to each tube respectively, and 80 μL of magnetic beads were added. After sufficient mixing with a vortex shaker, the sample was incubated in a constant temperature shaker (37℃, 180 rpm/min) for 15 min, followed by magnetic separation for 5 min. the supernatant was transferred to a new 1.5 mL centrifuge tube, and the magnetic beads were resuspended in 1 mL Tris-HCl buffer. In order to facilitate the subsequent counting, the resuspended solution was diluted to 10^3 CFU/mL, and the supernatant was diluted to 10^4 CFU/mL. Plate coating and capture efficiency calculations are the same as in the previous steps.

6. Preparation of Rhodamine-labeled Phage Probe

Rhodamine-labeled phage PAP (Rhodamine-PAP) is prepared via EDC/NHS chemistry. Firstly, 60 μL of Rhodamine solution (20 mg/mL) and adding 140 μL of PBS (pH 6.8). EDC solution (10 mg/mL) and freshly prepared NHS solution (5 mg/mL) were prepared in PBS (pH 6.8), and 200 μL of each solution was added to the Ep tube, and the reaction was carried out at 37°C and 180 rpm for 30 minutes. Then, phage PAP solution (10^{10} PFU/mL, 600 μL) was added to the Ep tube and shaken for 12 hours. Finally, purification was performed using a dialysis bag and PBS buffer (pH 7.2-7.4) to remove excess dye and collect the purified Rhodamine-PAP and keep at 4℃ for further use.

7. Fluorescence co-localization of bacteria and phage

Bacteria (*P. aeruginosa* and *A. baumannii*, 10^8 CFU/mL) were labeled with Hoechst (20 μg/mL) dye at 37°C for 20 min, followed by removal of excess dye by

centrifugal washing and capture of bacteria with magnetic beads (80 μL, 37°C, 15 min). Subsequently, 50 μL of Rhodamine-PAP was added and incubated for 15 min at 37°C. Unbound Rhodamine-PAP was removed by washing with Tris-HCl buffer and magnetic separation for 5 min, and the final slide was prepared for fluorescence observation.

8. Urine collection and culture

Mid-stream urine was collected from volunteers using a sterile container. For urine cultures, specimens were processed immediately after collection to prevent contamination and minimize the impact of bacterial die-off on results. Typically, the urine sample was gently shaken, and a 10 μL aliquot was inoculated onto an LB plate. The plate was then incubated at 37°C for 18–24 hours, after which colony formation was observed and quantified by plate counting.

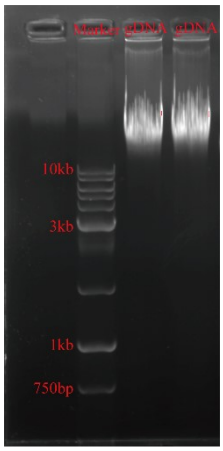

Figure S1. 2% agarose gel electrophoresis of extracted PAP DNA.

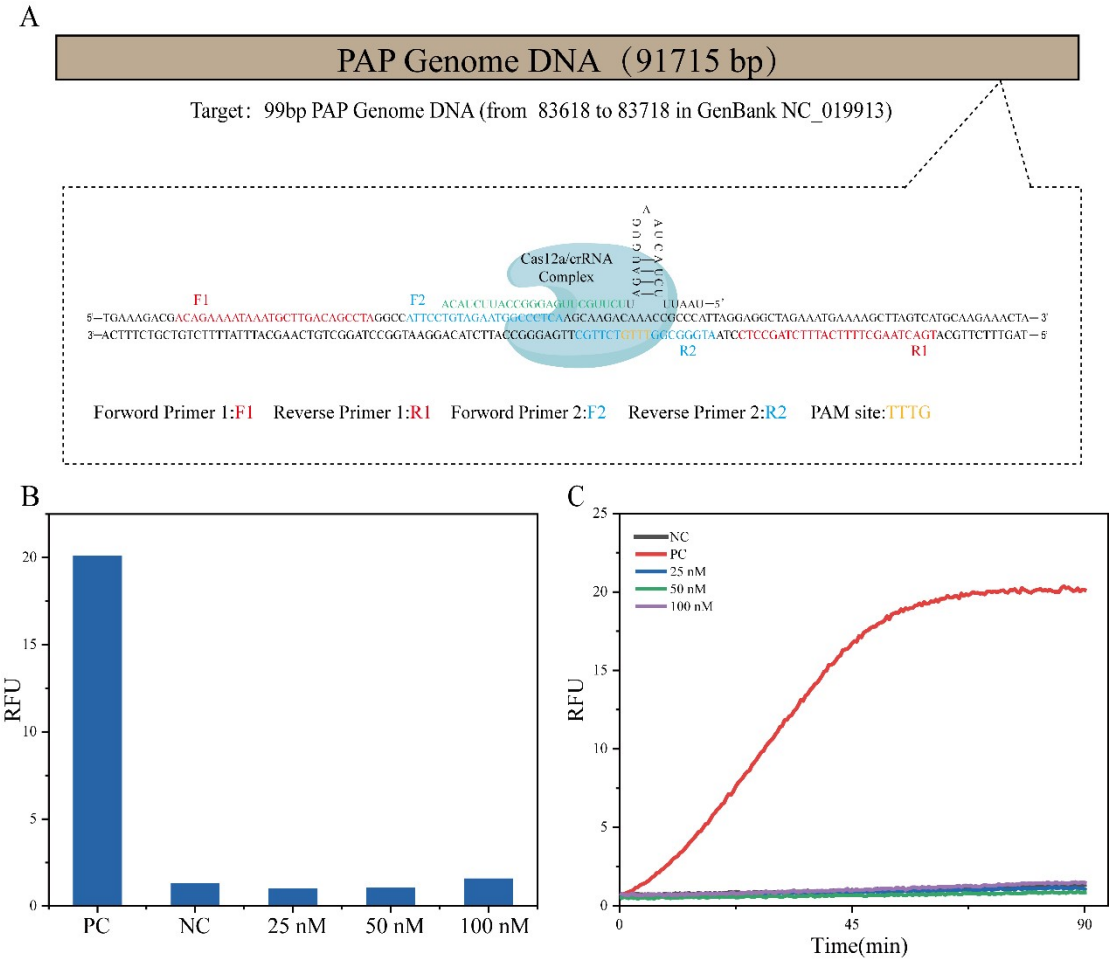

Figure S2. Target, primer, crRNA design, and primer specificity validation. (A) Illustration of Target selection, primer design, and crRNA design. (B) Endpoint fluorescence of the reaction of primer F2 (50 nM) with different concentrations of Cas12a (25 nM, 50 nM, 100 nM). (C) Kinetic results of the reaction of primer F2 (50 nM) with different concentrations of Cas12a (25 nM, 50 nM, 100 nM). Positive control (Cas12a/crRNA+dsDNA mimic target, denoted as PC), Negative control (Cas12a/crRNA-dsDNA mimic target, denoted as NC).

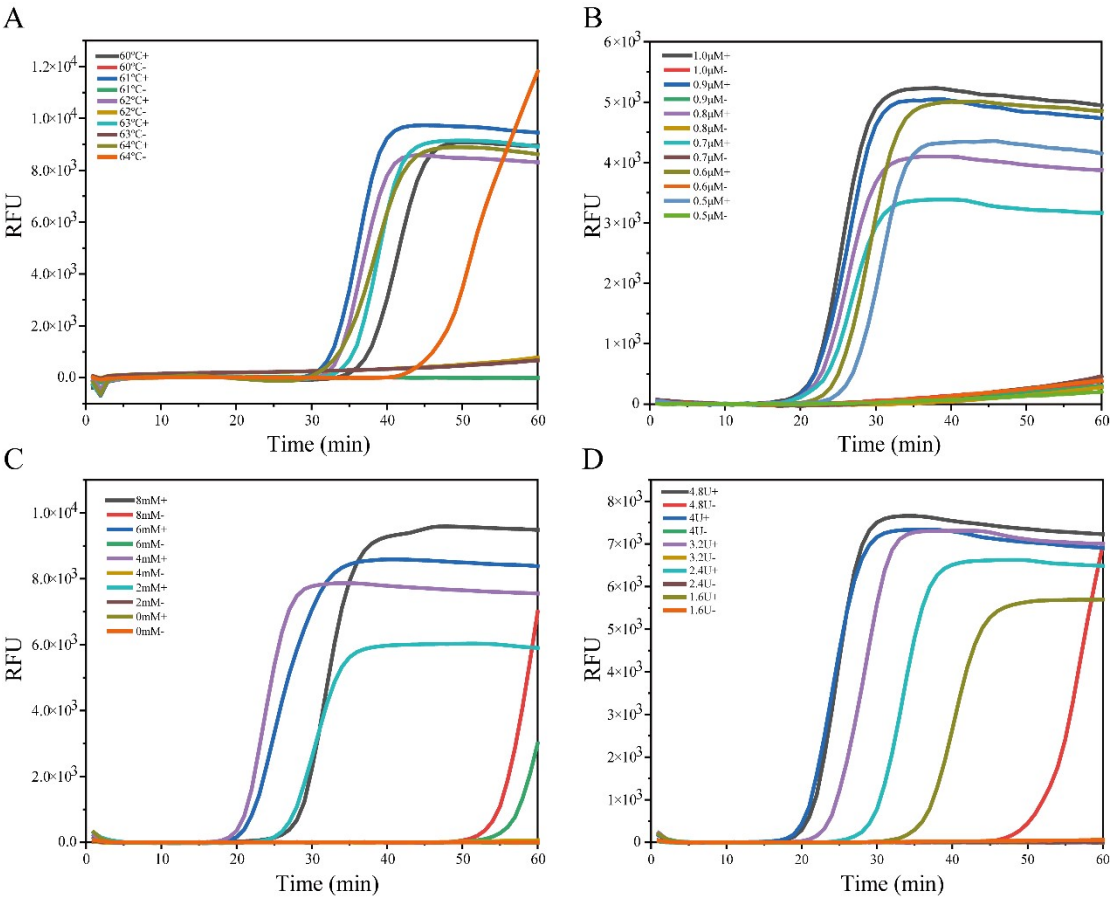

Figure S3. SEA Amplification Parameter Optimization of (A) temperature, (B) primer concentration, (C) magnesium ion concentration and (D) enzyme amount.

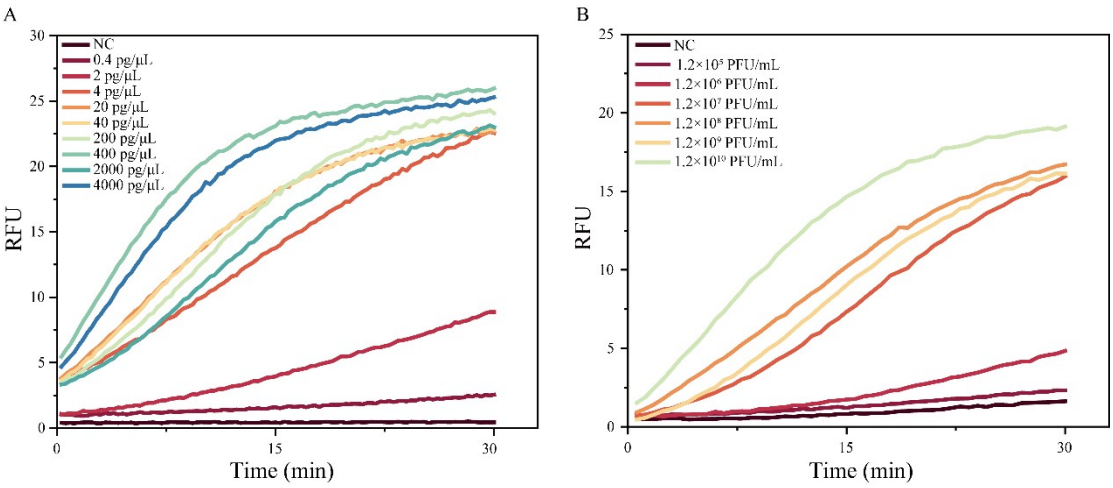

Figure S4. Reaction kinetics of the SEA-Cas12a assay for detecting different concentrations of PAP genomic DNA (A) and for detecting PAP particle (B).

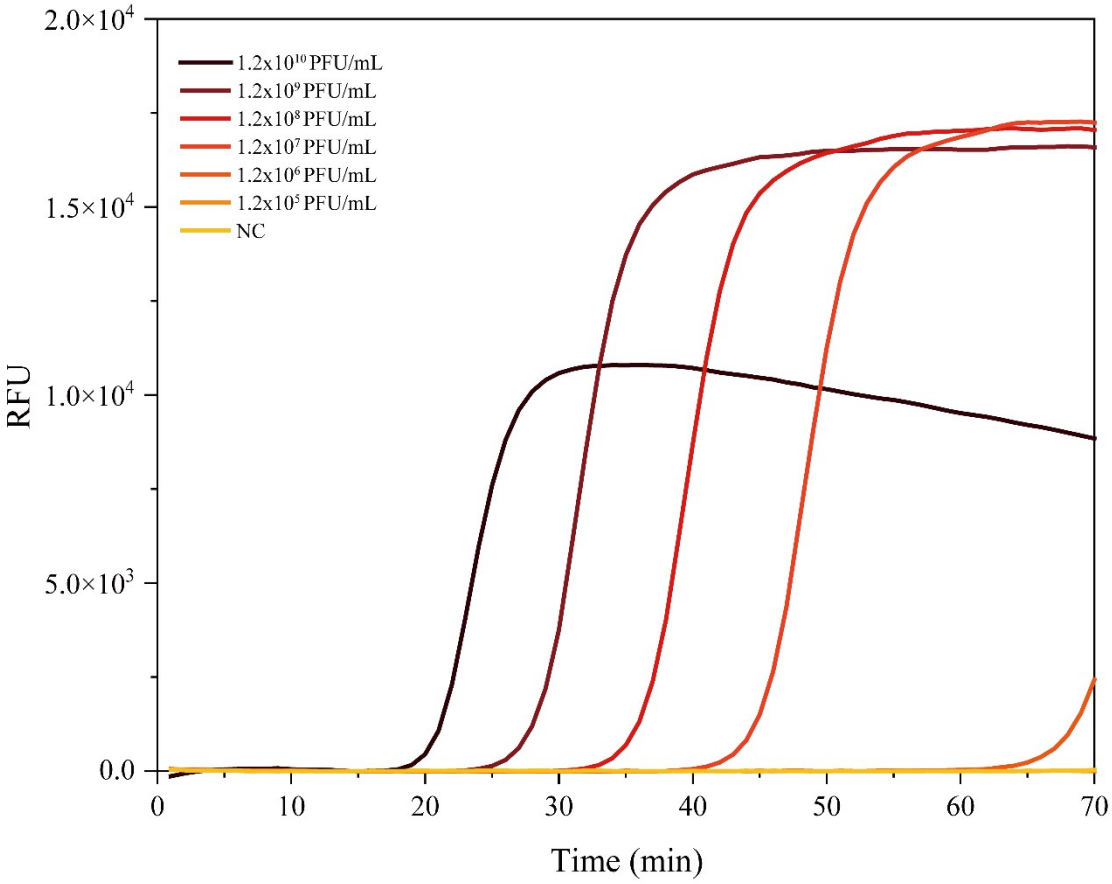

Figure S5. Sensitivity of single SEA for detecting phage particles.

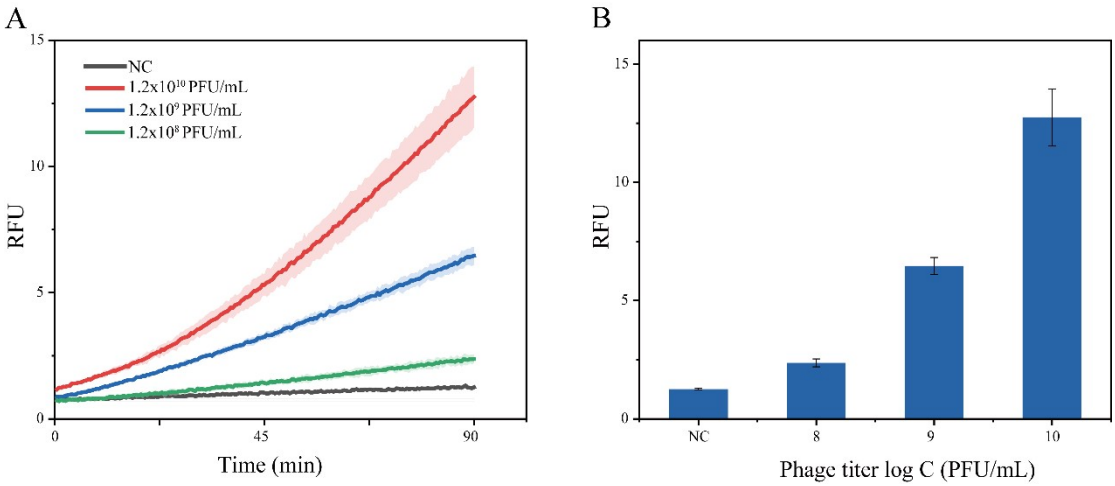

Figure S6. Sensitivity of single CRISPR/Cas12a for the detection of PAP particle. (A) Kinetic results of PAP detection at different concentrations. (B) Endpoint Fluorescence measurement for PAP detection at different concentrations.

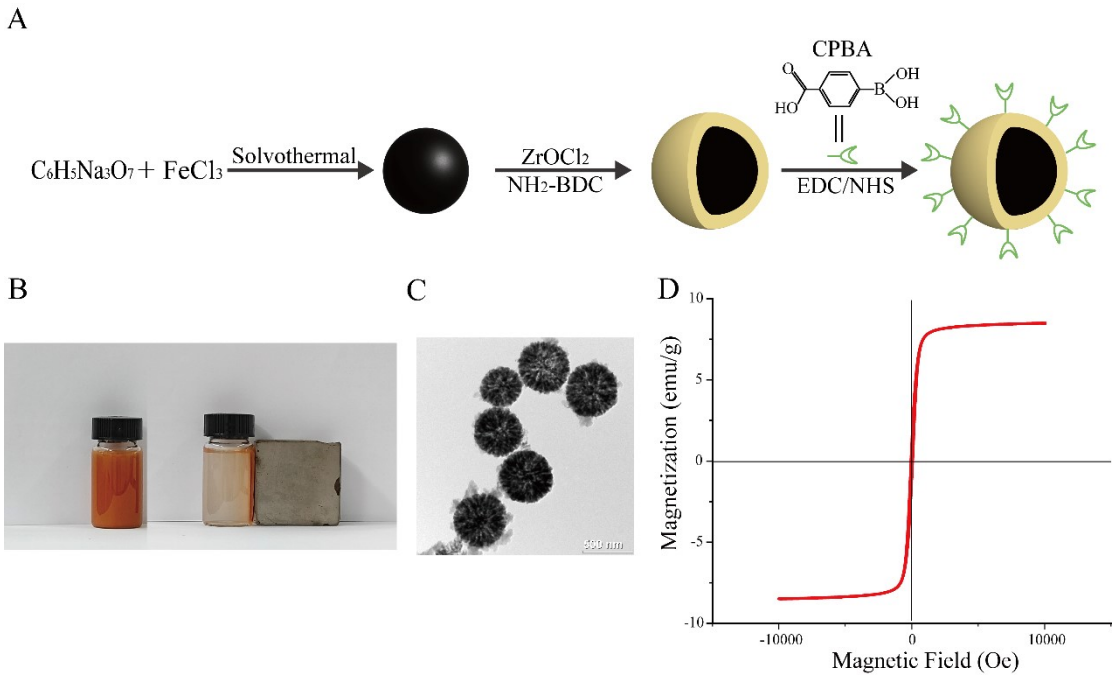

Figure S7. (A) Schematic of magnetic bead (Fe_3O_4@UiO-66-NH_2@CPBA) synthesis. (B) Photographs of the magnetic beads in the absence and presence of an applied magnetic field. (C) TEM characterization of magnetic bead. (D) The magnetic responses of magnetic beads.

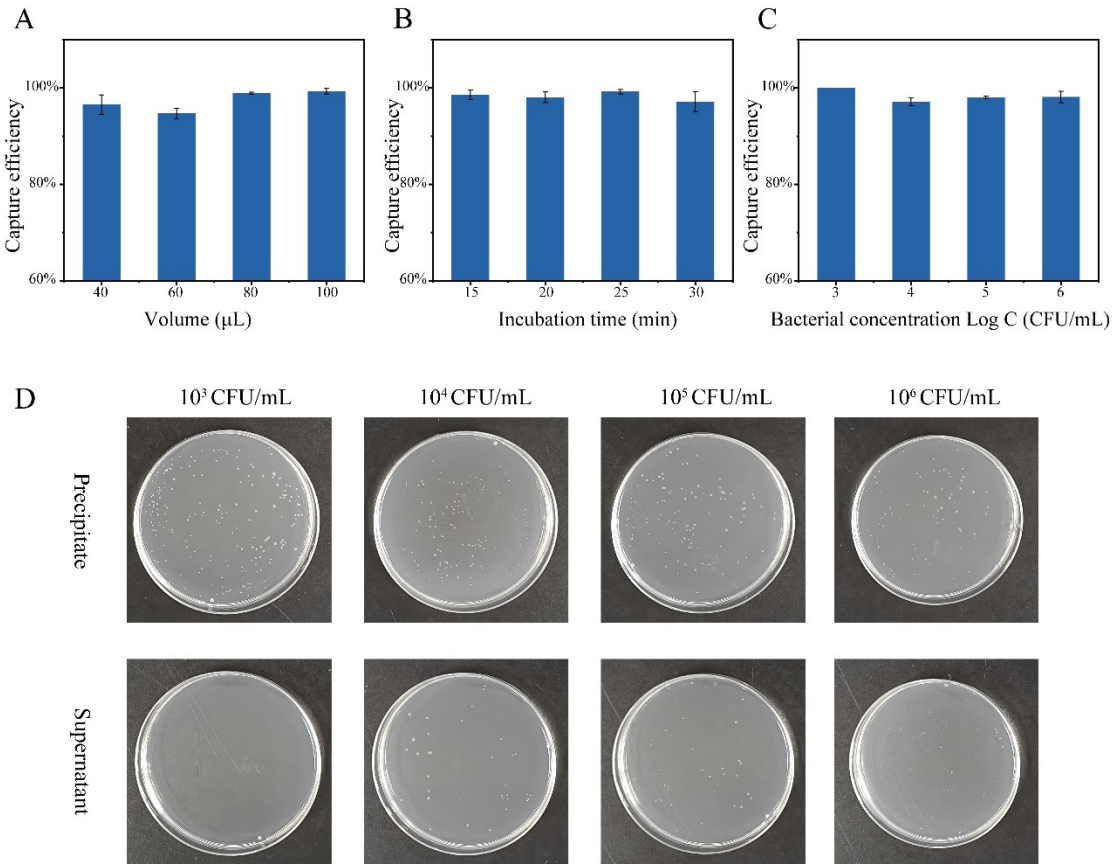

Figure S8. Validation of magnetic bead capture performance. (A) The optimum volume of Magnetic bead(5mg/mL). (B) Incubation time optimization. (C) Capture efficiency for different concentrations (10^3, 10^4, 10^5, 10^6 CFU/mL) of bacteria. (D) Plate counting results after capture and isolation of different concentrations of bacteria (10^3, 10^4, 10^5, 10^6 CFU/mL). 10^4, 10^5, and 10^6 CFU/mL bacteria were coated with the corresponding precipitates diluted to 10^3, and the supernatants were coated with the supernatants diluted to 10^4; the supernatants and resuspensions of 10^3 CFU/mL were coated with the plates directly.

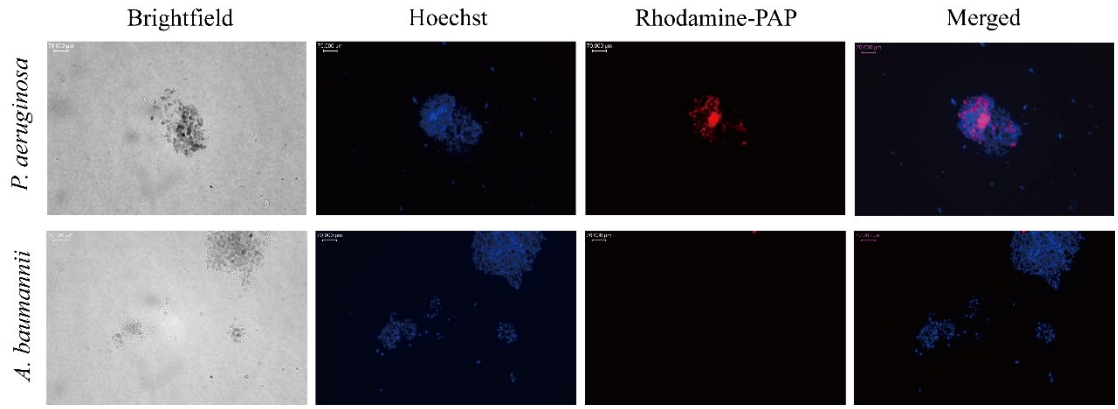

Figure S9. Phage specificity validation. Magnetic bead-based capture of target and non-target bacteria and fluorescence co-localization of phage (Rhodamine-PAP) with target (*P. aeruginosa*) and non-target bacteria (*A. baumannii*). Scale bars, 70 μm.

Table S1. Customized nucleic acids sequence.

Name	Sequence (5′-3′)
Target	ACAGAAAATAAATGCTTGACAGCCTAGGCCATTCCT GTAGAATGGCCCTCAAGCAAGACAAACCGCCCATTA GGAGGCTAGAAATGAAAAGCTTAGTCA
mimic target-F	ATTCCTGTAGAATGGCCCTCAAGCAAGACAAACCGC CCAT
mimic target-R	ATGGGCGGTTTGTCTTGCTTGAGGGCCATTCTACAGG AAT
T7-crRNA	TGTAGAATGGCCCTCAAGCAAGAATCTACACTTAGT AGAAATTACCCTATAGTGAGTCGTATTAATTTC
T7-crRNA-F	GAAATTAATACGACTCACTATAGGG
crRNA	UAAUUUCUACUAAGUGUAGAUUCUUGCUUGAGGGC CAUUCUACA
Forward primer 1(F1)	ACAGAAAATAAATGCTTGACAGCCTA
Reverse Primer 1(R1)	TGACTAAGCTTTTCATTTCTAGCCTC
Forward primer2 (F2)	ATTCCTGTAGAATGGCCCTCA
Reverse Primer2 (R2)	ATGGGCGGTTTGTCTTGC
ssDNA Reporter	FAM-TTATTCCCCC-BHQ1

Table S2 Comparison of different amplification methods combined with CRISPR/Cas system

Preamplification method	Cas type	Target	LOD	Reference
Multienzyme Isothermal Rapid Amplification (MIRA)	LbCas12a	*E. coli* O157:H7	6.5×10^4 CFU/mL	[2]
thermophilic Helicase-Dependent Amplification (tHDA)	LbCas12a	*E. coli* O157:H7	10^3 CFU/g	[3]
dUTP-LAMP	AapCas12b	*Salmonella*	2.33×10^4 CFU/mL	[4]
Competitive Annealing mediated isothermal Amplification (CAMP)	Cas14a	*S. pyogenes*	10^3 CFU/mL	[5]
Strand Invasion Based Amplification (SIBA)	LbCas12a	*Bacillus anthracis Sterne*	10^5 CFU/mL	[6]
PCR	LbCas12a	*S. aureus*	10^3 CFU/mL	[7]
SEA-Cas12a	LbCas12a	Phage	4×10^2 copy/μL	This study

Table S3 Results of plate culture count

Sample1	0.959×10^5 CFU	Sample11	6.48×10^4 CFU
Sample2	4.55×10^4 CFU	Sample12	6.15×10^4 CFU
Sample3	3.4×10^4 CFU	Sample13	500 CFU
Sample4	0.8×10^5 CFU	Sample14	833 CFU
Sample5	0.765×10^5 CFU	Sample15	466 CFU
Sample6	5.83×10^4 CFU	Sample16	433 CFU
Sample7	5.12×10^4 CFU	Sample17	566 CFU
Sample8	0.75×10^5 CFU	Sample18	600 CFU
Sample9	5.01×10^4 CFU	Sample19	700 CFU
Sample10	4.54×10^4 CFU	Sample20	300 CFU

Table S4 Comparison of Viable Bacteria Detection Methods

Method	Target bacteria	Target molecule	Sample type	Assay time (h)	LOD	References
Phage amplification assay / qPCR	*Salmonella Enteritidis*	DNA of phage	chicken	10 h	＜10 CFU/25g	[8]
Phage amplification assay / multiplex qPCR	*Salmonella enterica, Staphylococcus aureus*	DNA of phage	Milk and lettuce	＜4 h	10 CFU/mL	[9]
Phage amplification assay /LAMP	*Salmonella Enteritidis*	DNA of phage	chicken breast	≈8 h	1.1 CFU/25 g	[10]
Phage-assisted bacteria enrichment / qPCR	*Salmonella*	DNA of bacteria	milk and lettuce	≈1.5 h	30 CFU/mL	[11]
Phage-based real-time PCR	*Acinetobacter baumannii*	DNA of phage	blood	4 h	10^2 CFU/mL	[12]
Phage-assisted bacteria lysis / Argonaute	*Salmonella*	DNA and ATP of bacteria	chicken	2 h	20 CFU/mL	[13]
reporter phage-induced bioluminescence	*E. coli, Enterococcus spp., and Klebsiella spp*	Metabolic activity of bacteria	Urine	5h	≥10^3 CFU/mL	[14]
PMA combined with ddPCR	*Vibrio parahaemolyticus*	DNA of bacteria	aquatic products	2.5 h	7.32×10^1 CFU/mL	[15]
PMA combined with RAA	*S. aureus*	DNA of bacteria	milk sample	4 h	5.4×10^2 CFU/mL	[16]
PMA Combined with Flow Cytometry	*Lactobacillus*	DNA of bacteria	infant formula	2.5 h	2.7×10^4 CFU/g	[17]
Phage amplification assay /SEA-CRISPR/Cas12a	*P. aeruginosa*	DNA of phage	Urine	3.5 h	10^3 CFU/mL	This study

Reference:

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