# Electronic Supplementary Information

## Ultrasensitive electrochemical biosensor for *Pseudomonas aeruginosa* assay based on rolling circle amplification-assisted multipedal DNA walker

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## Material and methods

### Reagents and apparatus

All oligos adopted in the research were obtained from Sangon Biotech (Shanghai, China) and listed in Table S4. 40% acrylamide/bis solution (ABS, 19:1), Luria-Bertani (LB) broth, and DEPC-treated RNase-free water were obtained from Sangon Biotech (Shanghai, China). TIANamp Bacterial DNA Kit was obtained from TIANGEN (Beijing, China). Loading dye, SYBR Gold dye, DNA marker, T4 DNA ligase, Exo I, Exo III, phi29 DNA polymerase, and their related reaction buffers were purchased from New England Biolabs Inc. (Beijing, China). MagZol™ Reagent was supplied by Magen (Guangzhou, China). N,N,N',N'-tetramethylethylenediamine (TEMED), tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), 6-mercapto-hexanol (MCH), NaCl, dNTP Mix (10 mM each), human serum, ethylenediaminetetraacetic acid (EDTA), KCl, chloroform, tris base, $MgCl_2$, ammonium persulphate (APS), isopropanol, ethanol, and urea were supplied by Sigma-Aldrich Inc. (Shanghai, China). All solutions were prepared by using ultrapure water (≥18 MΩ cm, Milli-Q, Millipore). An autoclave instrument (Shenan, Shanghai, China), a Gel Imaging System (Baijing, Beijing, China), a constant-temperature incubator (Jiecheng), and an electrochemical workstation (CHI660E) were adopted in the study. A conventional three-electrode system was used including a platinum wire as the counter electrode, a planar Au electrode as the working electrode, and a saturated calomel reference electrode (SCE).

### Preparation of the MCH/SP-CP/Au electrode

The Au electrodes were carefully burnished for 3 min by using alumina oxide slurries (0.5 and 0.05 μm, respectively), and then sonicated successively the electrodes for 3 min in ultrapure water and ethanol. Then immersed the resulted electrodes for 10 min in a fresh-prepared piranha solution ($H_2SO_4$:$H_2O_2$ = 3:1, v/v) and thoroughly washed by using ultrapure water. Subsequently, the above electrodes were immersed in 0.5 M $H_2SO_4$ solution and scanned at the potential ranging from − 0.3 to 1.5 V until obtaining stable cyclic voltammograms. Finally, the electrodes were

rinsed by ultrapure water and dried at 25 °C. Prior to the fabrication of SP and CP onto the surface of Au electrode, they were immersed in 20 mM TCEP solution for 0.5 h in the dark. After that, 10 μL of 1 μM SP and 0.05 μM CP were added on the Au electrode surface and kept for 2 h at 37 °C to obtain SP-CP/Au electrode. Eventually, the resulted electrodes were immersed for 0.5 h in 3 mM MCH solution to obtain the MCH/SP-CP/Au electrode.

**Bacteria strain culture and total RNA extraction**

*Staphylococcus aureus* (*S. aureus*), *Enterobacter sakazakii* (*E. sakazakii*), *Salmonella typhimurium* (*S. typhimurium*), *Listeria monocytogenes* (*L. monocytogenes*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (*Caution: Please certainly be careful with these pathogenic bacteria*). All bacterial strains were cultured overnight in the sterile LB broth with 150 rpm/min shaking at 37 °C and collected the bacteria when the $OD_{600}$ value of broth was 1. First, picked up 3 mL bacterial medium and centrifuged for 1 min at 12 000 rpm/min at 4 °C to obtain bacteria cells. The bacterial cells were incubated in 100 uL TE/lysozyme for 10 min. 1 mL MagZol™ Reagent was added into the above solution, vortexed for 1 min, and kept at 25 °C for 3 min. Centrifuged the mixture for 10 min at 12 000 rpm/min at 4 °C, and then transferred the supernatant to a new tube. Subsequently, added 200 μL chloroform to the supernatant. After shaking for 15 s, the mixture was kept for 3 min at 25 °C and centrifuged at 12 000 rpm/min at 4 °C for 15 min, then transferred the supernatant to a new tube carefully. An equivoluminal isopropanol was added to the tube, vortexed and kept for 15 min at 25 °C. Centrifuged the mixture for 15 min at 12 000 rpm/min at 4 °C, discarded the supernatant and added 1 mL of 75% ethanol into the tube. Then the solution was centrifuged at 7500 rpm/min for 5 min at 4 °C. Discarded the supernatant and dried the pellets for 10~15 min at 25 °C to obtain total RNA. Resuspended the total RNA by adding RNase-free water and stored at – 80 °C for future use.

**Extraction of *P. aeruginosa* 16S rRNA from simulated sputum samples**

Added 6 mL of 4% sodium hydroxide solution and 3 mL simulated sputum to a centrifuge tube. After sealing with the spiral cover, shaked on the above tube on a vortexer for 1 min, and then kept it at 25 °C for 15 min to ensure the sputum is fully and evenly distributed. Subsequently, added 9 mL sterile phosphoric acid buffer (67 mM, pH 6.8) to the resulted tube and thoroughly mixed. Finally, the mixture was centrifuged for 15 min at 4000 rpm/min. Discarded the supernatant and washed the precipitate using 15 mL sterile PBS buffer and M7H9 medium. The prepared sample was used for the extraction of 16S rRNA via the above-mentioned method.

**Polyacrylamide gel electrophoresis analysis**

First, 10% native polyacrylamide gel electrophoresis (PAGE) was made by adding 0.1% TEMED and 0.04% APS into 40% ABS (19:1) in 1× TBE buffer. Subsequently, the gel was pre-run at 200 V for 30 min. After that, the gel was run for 2.5 h at 180 V with the addition of samples in 1× loading dye, then stained it in SYBR Gold dye solution for 15 min. Finally, the gel picture was captured by the gel imaging system.

**Electrochemical analysis of 16S rRNA fragment**

10 μL of 16S rRNA fragment with different concentrations was dropped onto the surface of MCH/SP-CP/Au electrode and incubated for 1 h at 37 °C. After being washed, 10 μL of 1 μM AP was added onto the surface of 16S rRNA/MCH/SP-CP/Au electrode and incubated at 37 °C for 1 h to obtain AP/16S rRNA/MCH/SP-CP/Au electrode. Next, the above electrode was washed completely and kept in 20 μL reaction solution including 10 μL CPP (1 μM) analysed by PAGE (Fig. S5), 2 μL phi29 DNA polymerase (10 U/μL), 2 μL dNTPs (10 mM), 2 μL of 10× phi29 DNA polymerase reaction buffer, 4 μL RNase-free water, and kept for 1.5 h at 30 °C to perform the RCA reaction. Next, added 10 μL of 10 μM WP and 100 μM $Pb^{2+}$ solution onto the AP/16S rRNA/MCH/SP-CP/Au electrode surface and kept for 1.5 h at 37 °C. Eventually, after washing step, the electrochemical measurement of the biosensing electrodes were performed with differential pulse voltammetry (DPV) in 10 mM dioxygen-removed PBS buffer (50 mM NaCl, 5 mM $MgCl_2$, pH 7.4).

**The preparation of CPP and PAGE analysis**

20 μL ligation reaction solution containing 1 μM linear padlock probe (LPP), 3 μM primer probe, 10 U/μL T4 DNA ligase, and 1 mM ATP was incubated at 37 °C for 1 h to obtain CPP. Subsequently, heated at 65 °C for 10 min to inactivate T4 ligase. After that, added 20 U/μL Exo I and 100 U/μL Exo III to the above solution and kept at 37 °C for 1.5 h. After that, the obtained mixture was heated at 70 °C for 20 min to stop the enzymes cleavage process.

After T4 ligation and enzymes degradation, 10% PAGE was operated in 1× TBE buffer at 250 V for 2 h and followed by staining in SYBR Gold dye solution. As shown in Fig. S5, lane 1 displayed the band of LPP. Lane 2 displayed a clear band of CPP after T4 ligation and enzymes degradation, which ran a little slower than that of LPP in lane 1 maybe owing to its circular structure, which could not be degraded by Exo I and Exo III. However, compared to lane 2, lane 3 displayed many different side-bands after T4 ligation without enzymes degradation. Therefore, these results indicated that the clean CPP was obtained in lane 2 after T4 ligation and enzymes degradation.

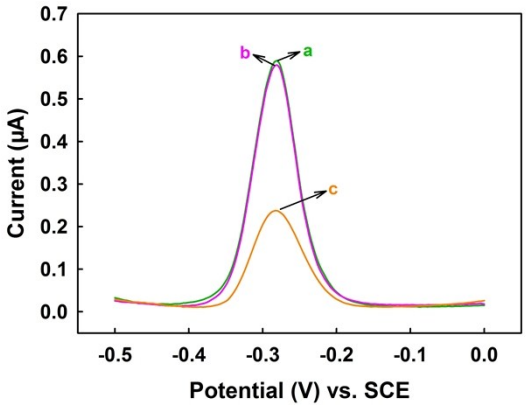
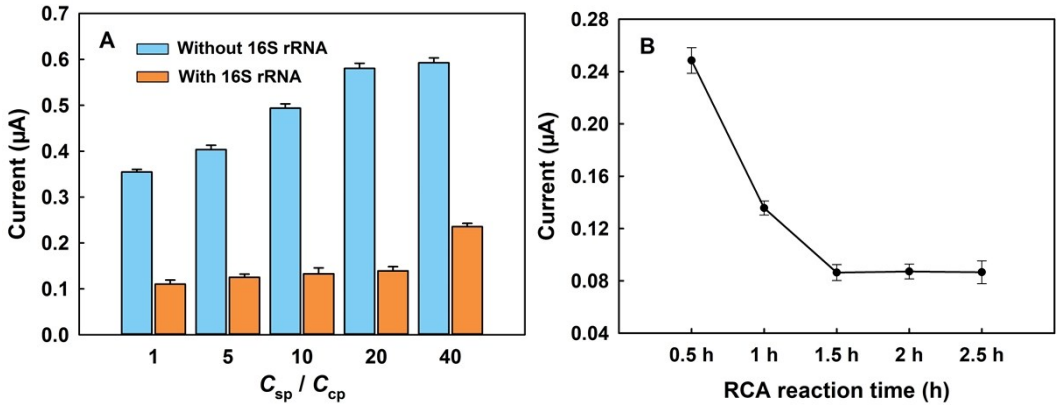



**Fig. S1** Feasibility of the electrochemical biosensor. DPV curves of (a) MCH/SP-CP/Au electrode without the subsequent amplification reactions, (b) MCH/SP-CP/Au electrode without 16S rRNA, (c) MCH/SP-CP/Au electrode with 10 pM 16S rRNA.

After getting the optimal reaction conditions including the ratio of SP/CP and RCA reaction time (Fig. S2A and B), the complementary base number, $Pb^{2+}$ concentration, cleavage temperature, and cleavage time were also evaluated, respectively (Fig. S2C, D, E, and F). As we know, some parameters are also very vital to the multipedal DNA walking process that may affect the hybridization kinetics and the DNAzyme cleavage efficiency directly, such as complementary base number, $Pb^{2+}$ concentration, cleavage temperature, and cleavage time. In this study, the 3'-end of the DNAzyme tail is immobilized through 5 bases, and the length of 5'-end is in the range from 5 to 9 bases. Ideally, the nonspecific cleavage can be reduced to some extent by a shorter DNAzyme tail; whereas, it is hard to achieve a specific signal intensity for a too short DNAzyme tail. Meanwhile, the DNA duplex formed by an overlength of DNAzyme tail can generate a strong false positive signal. As shown in Fig. S2C, the peak current of MB decreased gradually in the absence of 16S rRNA due to the nonspecific cleavage with the enhancement of complementary base number on the 5'-end of the DNAzyme tail, and the signal-to-noise ratio reached a maximum value when the base number was six. Therefore, the DNAzyme tail with 6 bases was used in the work. As shown in Fig. S2D, with the increase of $Pb^{2+}$ concentration, the peak current of MB decreased and reached a platform at the concentration of 100 μM, hence, the $Pb^{2+}$ concentration at 100 μM was chosen in the whole experiment. Subsequently, the cleavage temperature was evaluated. The peak current of MB achieved minimum at 37 °C, as shown in Fig. S2E, demonstrating an optimized cleavage efficiency. Thus, 37 °C was used in the DNAzyme cleavage system. Finally, we evaluated the effect of cleavage time. As displayed in Fig. S2F, the peak current of MB decreased with the enhancement of cleavage time and achieved a platform at the time of 1.5 h, so the optimized cleavage time was 1.5 h.



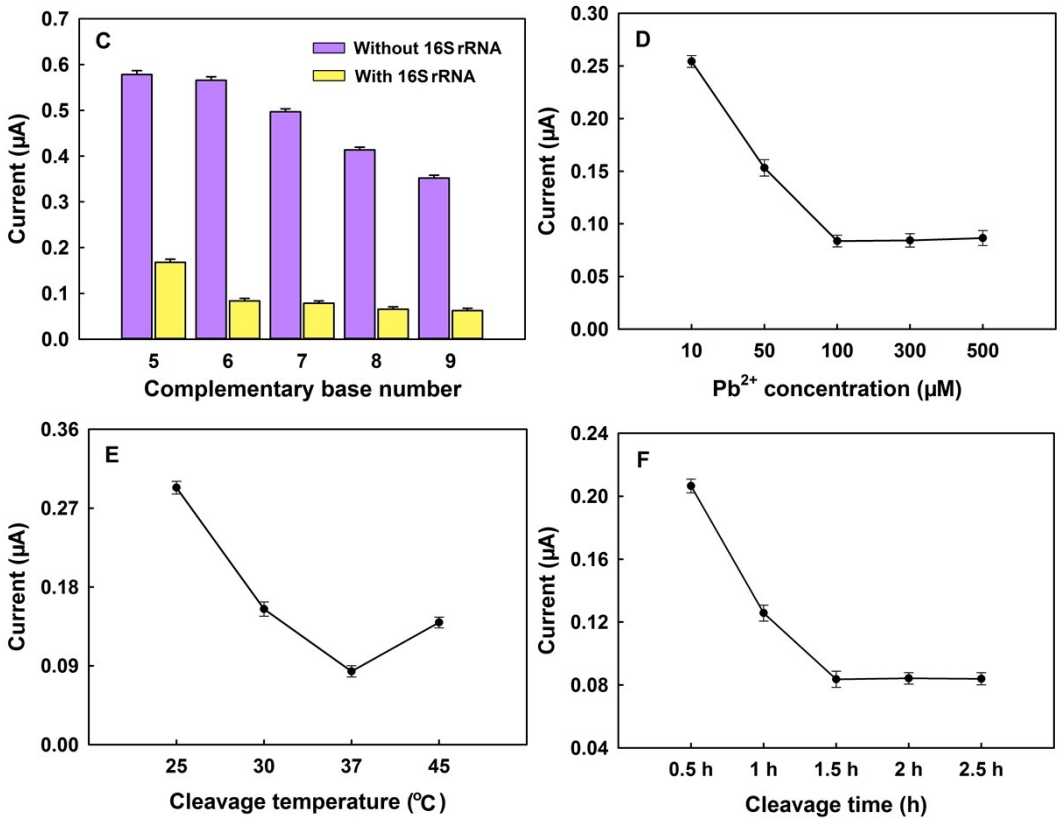



**Fig. S2** Optimization of experimental conditions for the proposed electrochemical biosensor in the presence of 16S rRNA. (A) The concentration ratio of SP and CP, (B) RCA reaction time, (C) Complementary base number between SP and WP, (D) $Pb^{2+}$ concentration at 37 ℃ for 1.5 h, (E) Cleavage temperature in the presence of 100 μM $Pb^{2+}$ for 1.5 h, (F) Cleavage time at 37 ℃ in the presence of 100 μM $Pb^{2+}$. Error bars represent the standard deviation of three individual assays.

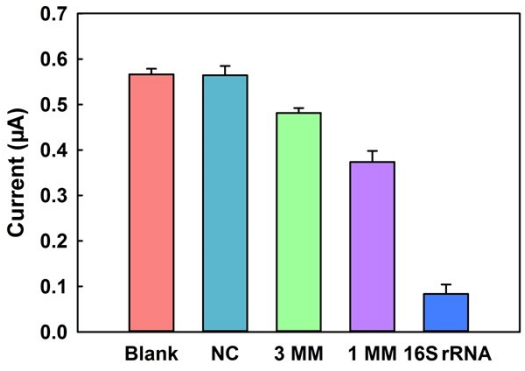



**Fig. S3** Specificity of the electrochemical biosensor for 0.5 nM 16S rRNA against 5 nM NC, 1MM, and 3MM. Error bars represent the standard deviation of three individual assays.

**Recovery test**

The recovery experiment was performed to assess the analytical reliability and application potential of the newly proposed electrochemical biosensor for the 16S rRNA assay. Briefly, the real biological sample (human serum) was diluted 10-fold with 10 mM PBS, followed by adding different concentrations of 16S rRNA (0.1 pM, 1 pM, and 10 pM). As shown in Table S1, the recovery varied from 98.6% to 103.0%, demonstrating that detection of 16S rRNA in real biological samples was promising.

**Table S1** Recovery test of 16S rRNA detection in 10-fold diluted human serum samples.

| Samples (Nos.) | Added | Found | Recovery |
|---|---|---|---|
| 1 | 0.1 pM | 0.103 ± 0.06 pM | 103.0% |
| 2 | 10 pM | 9.86 ± 0.97 pM | 98.6% |
| 3 | 100 pM | 99.68 ± 1.83 pM | 99.7% |

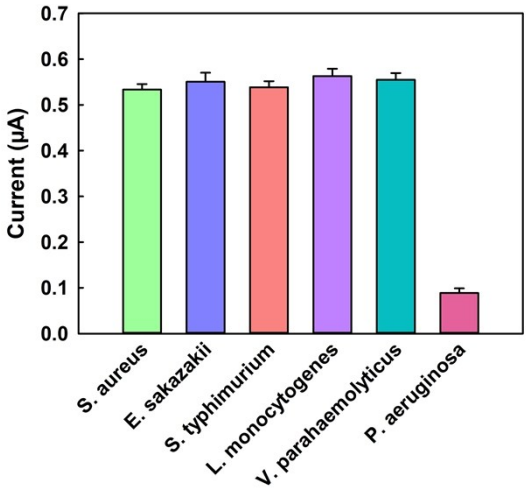



**Fig. S4** Specific responses of the electrochemical biosensor to 16S rRNA from *P. aeruginosa* and other interfering bacterial strains including *S. aureus*, *E. sakazakii*, *S. typhimurium*, *L. monocytogenes*, and *V. parahaemolyticus*. The concentration for all bacteria was $1 \times 10^8$ CFU/mL. Error bars represent the standard deviation of three individual assays.

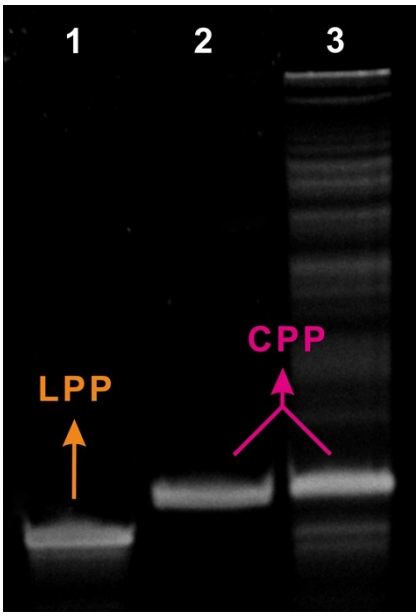



**Fig. S5** PAGE analysis after T4 ligation and enzymes degradation. Lane 1: LPP; lane 2: CPP after T4 ligation and enzymes degradation; lane 3: CPP after T4 ligation without enzymes degradation.

**Table S2** Comparison of various analytical methods for *P. aeruginosa* assay.[a]

| Analytical methods | Detection limit (CFU/mL) | Linear range (CFU/mL) | Time (h) | Reference |
|---|---|---|---|---|
| PCR | $2.7 \times 10^2$ | $2.7 \times 10^2 \sim 2.7 \times 10^6$ | ~1.5 | 1 |
| LSPR | 10 | $10 \sim 10^3$ | ~3 | 2 |
| Fluorescence | 100 | $1.28 \times 10^3 \sim 2 \times 10^7$ | 2 | 3 |
| Fluorescence | 5 | $5.64 \sim 100$ | 3 | 4 |
| Fluorescence | 46 | $4.0 \times 10^3 \sim 1.8 \times 10^4$ | ~1.5 | 5 |
| Impedimetric method | $10^2$ | $10^2 \sim 10^6$ | 2.5 | 6 |
| MSPQC method | 9 | $81 \sim 8.1 \times 10^5$ | 2 | 7 |
| Electrochemiluminescence | 56 | $1.4 \times 10^2 \sim 1.4 \times 10^6$ | ~2 | 8 |
| Chemiluminescence | 10 | $10 \sim 10^3$ | ~6 | 9 |
| Magnetic relaxation switch | 50 | $10^2 \sim 10^6$ | ~4.7 | 10 |
| Electrochemistry | 10 | $10 \sim 10^8$ | 5 | this work |

[a]Polymerase Chain Reaction (PCR), Localized Surface Plasmon Resonance (LSPR), Multichannel Series Piezoelectric Quartz Crystal (MSPQC).

As displayed in Table S3, among the 14 positive samples, only one sample was not monitored via the developed electrochemical biosensor maybe due to the unsuccessful 16S rRNA extraction. Among the 31 negative samples, two samples measured by the developed electrochemical biosensor were positive, which may be owing to the low bacteria concentrations (less than $10^2$ CFU/mL) in the samples or slow bacterial growth. Based on the value of the McNemar test, there was no distinct change between these two methods. Compared with the conventional bacteria culture method, the proposed biosensing platform exhibited a sensitivity of 92.86% (13/14) and a specificity of 93.55% (29/31), respectively. Additionally, the bacteria culture method takes several weeks to obtain the result for identification of the bacteria, while the proposed electrochemical biosensor only takes a few hours. Hence, the proposed electrochemical biosensor provides a novel, rapid, and precise platform for *P. aeruginosa* assay.

**Table S3** Comparison of the proposed electrochemical biosensor for 45 simulated samples evaluated with the culture method.

| **Proposed biosensor** | **Culture method** | | **Total** |
|---|---|---|---|
| | **Positive** | **Negative** | |
| **Positive** | 13 | 2 | 15 |
| **Negative** | 1 | 29 | 30 |
| **Total** | 14 | 31 | 45 |

**Table S4** Sequences of oligonucleotides used in this work.

| Oligonucleotides | Sequences (from 5' to 3') |
|---|---|
| Linear padlock probe | Phosphate- TCTGACGG ACT TGA GAC ACT TATATA ACT TGA GAC ACT GCACTTCA |
| Primer probe | AGT CCGTCAGA TGAAGTGC AGT |
| Capture probe | TTACTGCC CTTCCTCC TTTTT-$(CH_2)_6$-SH |
| Auxiliary probe | CCGTCAGA TGAAGTGC ACAGCAAG GTATTAAC |
| Signal probe | MB-ACTATrAGGAAGAGATGTTTTT-$(CH_2)_6$-SH |
| Walker probe-9 bases | CATCTCTTCTCCGAGCCGGTCGAAATAGT $T_{25}$ ACT TGA GAC ACT |
| Walker probe-8 bases | ATCTCTTCTCCGAGCCGGTCGAAATAGT $T_{25}$ ACT TGA GAC ACT |
| Walker probe-7 bases | TCTCTTCTCCGAGCCGGTCGAAATAGT $T_{25}$ ACT TGA GAC ACT |
| Walker probe-6 bases | CTCTTCTCCGAGCCGGTCGAAATAGT $T_{25}$ ACT TGA GAC ACT |
| Walker probe-5 bases | TCTTCTCCGAGCCGGTCGAAATAGT $T_{25}$ ACT TGA GAC ACT |
| 16S rRNA | GGAGGAAG GGCAGUAA GUUAAUAC CUUGCUGU |
| NC | CACUAGCA UUACACGU GGAUUCUG CCUCAUCA |
| 3 MM | GGA*U*GAAG *U*GCA*U*UAA GUUAAUAC CUUGCUGU |
| 1 MM | GGAGGA*U*G GGCAGUAA GUUAAUAC CUUGCUGU |

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