# A Study of the Detection of SARS-CoV-2 by the Use of Electrochemiluminescent Biosensor Based on Asymmetric Polymerase Chain Reaction Amplification Strategy

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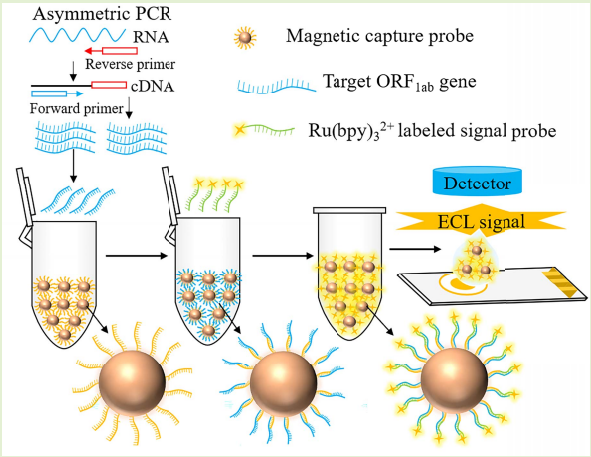
***Abstract*—A new and reliable method has been constructed for detecting severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) open reading frames 1ab (ORF1ab) gene via highly sensitive electrochemiluminescence (ECL) biosensor technology based on highly efficient asymmetric polymerase chain reaction (asymmetric PCR) amplification strategy. This method uses magnetic particles coupled with biotin-labeled one complementary nucleic acid sequence of the SARS-CoV-2 ORF1ab gene as the magnetic capture probes, and Ru(bpy)$_3^{2+}$-labeled amino-modified another complementary nucleic acid sequence as the luminescent probes, and then a detection model of magnetic capture probes–asymmetric PCR amplification nucleic acid products-Ru(bpy)$_3^{2+}$-labeled luminescent probes is formed, which combines the advantages of highly efficient asymmetric PCR amplification strategy and highly sensitive ECL biosensor technology, enhancing the method sensitivity of detecting the SARS-CoV-2 ORF1ab gene. The method enables the rapid and sensitive detection of the ORF1ab gene and has a linear range of 1–10$^6$ copies/$\mu$L, a regression equation of $Y = 534.942X + 2919.301$ ($R = 0.9983$, $N = 7$), and a limit of detection (LOD) of 1 copy/$\mu$L. In summary, it can meet the analytical requirements for simulated saliva and urine samples and has the benefits of easy operation, reasonable reproducibility, high sensitivity, and anti-interference abilities, which can provide a reference for developing efficient field detection methods for SARS-CoV-2.**




***Index Terms*—Asymmetric polymerase chain reaction (asymmetric PCR) amplification strategy, electrochemiluminescence (ECL) biosensor, open reading frames 1ab (ORF1ab) gene, severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2).**

## I. Introduction

SEVERE acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) was found in 2019 as a highly pathogenic virus strain spreading worldwide. After two years of human efforts, its rapid infection and high fatality rate still severely threaten human life and health [1]. With the increase in the probability of gene mutation and the complexity of samples during the spread of SARS-CoV-2, to effectively limit the spreading of SARS-CoV-2 and intervene in the development of the epidemic, scientists and scholars from all over the world have dedicated themselves to researching highly sensitive detection and screening methods for SARS-CoV-2 [2]. SARS-CoV-2 nucleic acid detection methods that have the advantages of high sensitivity and specificity are regarded as the main and significant methods in the pandemic [3]. The main detection methods contain complete genome sequence [4], real-time polymerase chain reaction (PCR) [5], isothermal nucleic acid amplification [6], [7], recombinase polymerase amplification [8], [9], recombinase aided amplification [10], clustered regularly interspersed short palindromic repeat (CRISPR) [11], [12], chemiluminescence assay [13], infrared absorption spectroscopy [14], and biosensor detection [15], [16]. Although the existing methods have played a crucial role in the rapid screening of SARS-CoV-2,

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it is urgent to develop a series of more accurate and highly sensitive detection methods for SARS-CoV-2 to enhance the detection efficiency and detection capacity.

Electrochemiluminescence (ECL) biosensor technology has shown broad application prospects in detecting SARS-CoV-2 due to its high sensitivity, specificity, simple operation, rapid detection rate, and anti-interference ability. Asymmetric polymerase chain reaction (asymmetric PCR) amplification strategy has the characteristics of simple operation without complex primer design. The amplified products are single-stranded that can be directly detected. Thus, the asymmetric PCR amplification strategy can replace the traditional PCR method to realize easy and efficient amplification of SARS-CoV-2. Currently, the studies of ECL biosensors based on nucleic acid amplification strategy for detecting SARS-CoV-2 are minimal. How to achieve the integration of an efficient amplification strategy and highly sensitive detection of SARS-CoV-2 genes based on ECL biosensors is a great challenge that we need to face and address. In need of developing a highly sensitive ECL biosensing method to detect SARS-CoV-2, this study has combined the highly efficient asymmetric PCR amplification strategy with the high sensitivity of the ECL biosensor. In our study, specific probes and amplification primers were designed and prepared for the particular sequences of open reading frames 1ab (ORF1ab) genes. The model of magnetic capture probes–asymmetric PCR amplification nucleic acid products-Ru(bpy)$_3^{2+}$-labeled luminescent probes was used to establish a reliable method to detect SARS-CoV-2 ORF1ab gene using ECL biosensor based on asymmetric PCR amplification technology. The feasible and reliable method has supplied a reference for the effective diagnosis of early infection of the SARS-CoV-2, timely treatment, and cutting the transmission.

## II. Materials and Methods

### A. Reagents and Instruments

Binding buffer (10 mM Tris-HCl pH = 7.4 buffer with 1 mM EDTA and 2 M NaCl) and phosphate-buffered saline (PBS, 0.01 M, pH = 7.4 and 0.01 M, pH = 8.5) were prepared in our laboratory. Ru(bpy)$_3^{2+}$-NHS ester, glycine, and bovine serum albumin (BSA) protein were purchased from Sigma, St. Louis, MO, USA. The Procell solution composed of tripropylamine (TPA) was purchased from Beijing Biolot Diagnostics Science & Technology Development Co., Ltd., Beijing, China. *N*, *N*-dimethylformamide (DMF) was purchased from Shanghai Macklin Biochemical Company Ltd., Shanghai, China. Streptavidin-modified magnetic beads were purchased from Invitrogen Life Technologies, Oslo, Norway, which are micrometer size. Influenza A (H1N1, H5N1, and H7N9), Influenza B virus, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and SARS-CoV-2 pseudovirus were purchased from Sangon Biotech, Shanghai. MiniBEST Viral ribonucleic acid (RNA)/deoxyribonucleic acid (DNA) Extraction Kit Version 5.0, 2 × PCR buffer Ex, Taq enzyme, RT Enzyme Mix, and diethyl pyrocarbonate (DEPC)-treated water were purchased from TaKaRa Bio Inc., Beijing. The 2019-nCoVRNA reference material was purchased from the China Institute of Metrology and Testing Technology, Beijing, including the SARS-CoV-2 ORF1ab gene. Saliva and urine were obtained from humans. Octect SAX2 Biosensors were purchased from Sartorius Ltd., Germany.

TABLE I
PRIMERS, TARGET ORF1ab GENE, AND PROBE SEQUENCES

| Sequences | 5'-3' |
|---|---|
| Target-ORF1ab | CTCACCTTATGGGTTGGGATTATCCTAAATGTGA TAGAGCCATGCCTAACATGCTTAGAATTATGGCC TCACTTGTTCTTGCTCGCAAACATACAACGTGTT GTAGCTTGTCACACCGTT |
| Forward primer | CTCACCTTATGGGTTGGGATTA |
| Reverse primer | AACGGTGTGACAAGCTACAACA |
| Biotin-probe | GCATGGCTCTATCACATTTAGGA-bio |
| Amino-probe | $NH_2$-TGCGAGCAAGAACAAGTGAGG |

The oscillator and UV–vis spectrophotometer were purchased from Thermo Fisher Scientific Inc., Waltham, MA, USA. The HS-3 vertical mixer was purchased from Scientz Biotechnology Company Ltd., Ningbo, China. The Eppendorf Mastercycler nexus GSX1 PCR instrument was purchased from Eppendorf China Ltd., Beijing. Octet K2 molecular interaction analysis system was purchased from Sartorius Ltd., Germany. Magnetic separation frames were purchased from Promega (Beijing) Biotech Company Ltd., Beijing. ECL biosensor was designed by our laboratory and manufactured by Xi'an Remex Analysis Instrument Company Ltd., Xi'an, China. The screen-printed gold electrode was purchased from Zensor Technology Company Ltd., Taiwan. The length of the screen-printed gold electrode is 50 mm, and the width is 12.6 mm. The screen-printed gold electrode includes the working electrode, reference electrode, and counter electrode. The working electrode and counter electrode material are gold, and the reference electrode is a silver and silver chloride mixture.

### B. Design and Synthesis of the Sequences of Primers and Probes

The conserved region fragment of the ORF1ab gene was selected as the target region using the BLAST website to compare with the sequences of SARS-CoV-2 genes. According to the BLAST results, the forward and reverse primers for asymmetric PCR amplification and biotin-labeled one complementary nucleic acid sequence of the SARS-CoV-2 ORF1ab gene (biotin-probe) and amino-modified another complementary nucleic acid sequence (amino-probe) for hybridization with target ORF1ab gene amplification products were designed by using Primer Express 5.0 software. The primers and specific probes were synthesized by Beijing Xingfangyuan Biotechnology Company Ltd., Beijing, and the sequences are shown in Table I.

### C. Preparation of Magnetic Capture Probes

Binding buffer (1 mL) washed the streptavidin-modified magnetic beads (200 $\mu$L, 10 mg/mL) using a vertical mixer

to spin them for 5 min at indoor temperature. The washing process was repeated three times via separating magnetic beads and discarding the supernatant. Then, some biotin-probes were added to the streptavidin-modified magnetic beads, the reaction was shaken for 10 min at room temperature, and washed as above. Next, the uncombined site of magnetic beads was closed by adding PBS buffer (0.01 M, pH = 7.4) that contained 1% BSA and 1% glycine, incubated for 30 min with shaking at indoor temperature, and washed as above steps. Finally, in 200 $\mu$L of binding buffer, the magnetic capture probes were distributed loosely, reserved for the next step, and stored at 4 °C.

### D. Preparation of $Ru(Bpy)_3^{2+}$-Labeled Luminescent Probes

Amino-probes were dissolved into 100 $\mu$M using 176 $\mu$L of PBS buffer (0.01 M, pH = 8.5) and mixed with 20 $\mu$L of DMF solution containing 10 mg/mL $Ru(bpy)_3^{2+}$-NHS ester in an EP tube. Then, the above mixture solution reacted with a 600 r/min oscillation rate for 12 h in an oscillator at room temperature. Next, the above solution was mixed with 1 mL of PBS buffer (0.01 M, pH = 7.4) in an ultrafiltration tube for centrifuging at 8000 g/min for 30 min, then the filtrate was discarded. This process was repeated three times to wash $Ru(bpy)_3^{2+}$-labeled luminescent probes whose final volume was fixed at 140 $\mu$L and stored at 4 °C for the next step [17].

### E. Asymmetric PCR Amplification Reaction Conditions

The total amplification reaction system volume was 20 $\mu$L, including 10 $\mu$L of 2 × PCR buffer, 0.5 $\mu$L of Ex Taq enzyme (5 U/$\mu$L), 0.5 $\mu$L of PrimeScript RT Enzyme Mix, 2 $\mu$L of 2019-nCoVRNA ORF1ab reference material as the amplified 10 $\mu$L of forwarding primer (10 $\mu$M), 0.1 $\mu$L of reverse primer (10 $\mu$M), and DEPC-treated water is replenished to 20 $\mu$L. The asymmetric PCR amplification reaction was performed with an Eppendorf Mastercycler nexus GSX1 PCR instrument. The reaction procedure adopts two steps, including reverse transcription reaction: 42 °C for 5 min, 95 °C for 10 s, one cycle; and asymmetric PCR reaction: 95 °C for 5 s, 60 °C for 20 s, a total of 44 cycles.

### F. Affinity Determination Between Probes and Target Genes

Due to bio-probe and amino-probe interacting with the ORF1ab target gene by base complementation, the affinity constant (KA) between bio-probe and ORF1ab target gene and KA between amino-probe and ORF1ab target gene can be measured, and analyzed by Octet K2 molecular interaction instrument. Bio-probe (200 nM) was immobilized on the streptavidin-modified fiber optic sensor and equilibrated. Then, the concentration of 62.5, 125, 250, and 500 nM ORF1ab target gene (PBS as negative control) were added and equilibrated. Afterward, the KA between the bio-probe probe and the ORF1ab target gene can be determined. The biotin-modified ORF1ab target gene (200 nM) was immobilized on the streptavidin-modified fiber optic sensor and equilibrated. Then, the concentration of 62.5, 125, 250, and 500 nM amino-probe (PBS as negative control) was added for equilibration. Later, the KA between amino-probe probes and ORF1ab target genes was determined.

### G. Establish an ECL Biosensor Method Based on Asymmetric PCR Amplification to Detect SARS-CoV-2

1) The asymmetric PCR amplification: The different concentrations of 2019-nCoVRNA ORF1ab reference material or nucleic acid to be tested were used, respectively, as a template for asymmetric PCR amplification, and the amplified products were stored at 4 °C for later reaction.
2) Add amplification products: Magnetic capture probes (40 $\mu$L) were mixed with different reference material's amplification products and incubated for 10 min at room temperature, washed three times using PBS buffer (0.01 M, pH = 7.4), and kept the volume to 1 mL. Products are amplified with different template concentrations.
3) Adding $Ru(bpy)_3^{2+}$-labeled luminescent probes: $Ru(bpy)_3^{2+}$-labeled luminescent probes (2.5 $\mu$L) were added to the above substances, reacted for 10 min at room temperature, and washed three times with PBS buffer. Then, the model hybridization complexes of magnetic capture probes-asymmetric PCR amplification nucleic acids products-$Ru(bpy)_3^{2+}$-labeled luminescent probes were prepared. TPA (200 $\mu$L) was added to the hybridization complexes. The hybridization complexes (10 $\mu$L) were added to the screen-printed gold electrode for the test. The ECL intensity was tested through cyclic voltammetry scanning at 0.2–1.35 V, and the scanning rate was 0.1 V/s.

### H. Reproducibility and Specificity Examination

The linear range of the method and limit of detection (LOD) were determined by establishing the method's standard curve of the ECL biosensor based on asymmetric PCR amplification for SARS-CoV-2 ORF1ab gene detection. In the linear detection range, the method's reproducibility was tested by detecting different concentrations ($10^1$, $10^3$, and $10^5$ copies/$\mu$L) of the ORF1ab target gene. In the meantime, the specificity of the method was detected by testing $10^5$ copies/$\mu$L of SARS-CoV-2 pseudovirus and non-target viruses such as influenza A (H1N1, H5N1, and H7N9), influenza B, SARS, and MERS pseudovirus.

### I. Detection of Simulated Samples

Saliva and urine (10 mL) were mixed with SARS-CoV-2 pseudovirus (0.38 $\mu$L) to prepare simulated samples, respectively. Nucleic acid was extracted from the simulated sample using a MiniBEST Viral RNA/DNA Extraction Kit to obtain SARS-CoV-2 nucleic acid extracts at a final concentration of $10^2$ copies/$\mu$L. The method of ECL biosensor based on asymmetric PCR amplification for SARS-CoV-2 ORF1ab gene detection was used to detect simulated samples. After measuring ECL intensity, the relative standard deviation (RSD) and the recovery rate can be calculated.

## III. Results and Analysis

### A. Preparation of Magnetic Capture Probes and Determination of the Optimal Immobilization Amount of Biotin-Probes

In this study, the magnetic capture probes were prepared by immobilizing biotin-probes onto the surface of streptavidin-modified magnetic particles due to the biotin that can be specifically combined with affinity. Different concentrations of the biotin probe solution were added to 200 $\mu$g of streptavidin-modified magnetic particles. The amount of biotin-probes that were immobilized on the magnetic particle surface gradually increased and saturated with the increase of the addition of the biotin-probes. Our previous work calculated the optional amount of biotin-probes immobilized on 200 $\mu$g magnetic particles, which measured the absorbance value of the biotin-probes solution at $A_{260\ \mathrm{nm}}$ before and after binding, according to the formula binding rate $= (A_{260\ \mathrm{nm\ pre}} - A_{260\ \mathrm{nm\ post}})/(A_{260\ \mathrm{nm\ pre}}) \times 100\%$, respectively. Consequently, per 200 $\mu$g of magnetic particles, the optimal addition amount of biotin-probes is 150 pmol. At this point, the immobilized amount of biotin-probes per 200 $\mu$g of magnetic particles is 71.32 pmol [18].

### B. Preparation and Characterization of $Ru(Bpy)_3^{2+}$-Labeled Luminescent Probes

$Ru(bpy)_3^{2+}$-labeled luminescent probes, made by mixing amino-probes with $Ru(bpy)_3^{2+}$-NHS esters, were analyzed by UV–vis spectrum. Our previous work measured the UV–vis spectrum of the solution before and after $Ru(bpy)_3^{2+}$-NHS ester labeled the amino-probes, shown in Appendix A. Three characteristic absorption peaks of $Ru(bpy)_3^{2+}$-NHS ester are shown in curve a at the wavelength of 247, 287, and 458 nm. The characteristic absorption peak of amino-probes is shown in curve b at the wavelength of 260 nm. $Ru(bpy)_3^{2+}$-labeled luminescent probes' characteristic absorption peaks are shown in curve c at 260, 287, and 458 nm; thus, Fig. 1 indicated that $Ru(bpy)_3^{2+}$ had been successfully achieved modified on amino-probes.

### C. Validation of the Product Validity of Asymmetric PCR Amplification

The 2019-nCoVRNA ORF1ab reference material was amplified by asymmetric PCR using the designed primers and sequenced after recovery. The sequencing results showed that the amplified products were consistent with the sequences of the detected target sequences, which could prove the validity of the amplification, and the amplified products could be used for subsequent detection. The results are shown in Fig. 2.

### D. Affinity Determination Between Probes and Target Genes

Fig. 3 shows that the ORF1ab target gene can be combined with the bio-probes immobilized on the fiber surface. The amount of ORF1ab target gene binding gradually increases and saturates with the increase of reaction time. After PBS equilibration, the addition of the amino-probe could be bound with the ORF1ab target gene addition. In contrast, the negative control (PBS buffer) did not have a binding reaction with the bio-probe and amino-probe, indicating that the bio-probe and amino-probe can specifically hybridize with the ORF1ab target gene in this study, and the binding was stable

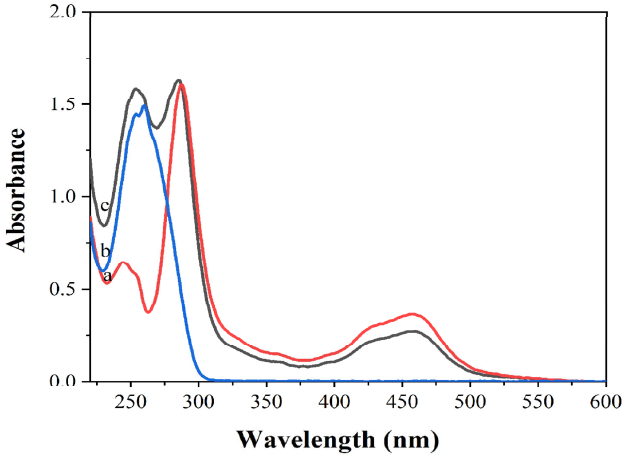


Fig. 1. UV–vis spectrum of the solution $Ru(bpy)_3^{2+}$-NHS ester labeled the amino probes. Curve a is the spectrum of $Ru(bpy)_3^{2+}$-NHS ester. Curve b is the spectrum of amino probes. Curve c is the spectrum of $Ru(bpy)_3^{2+}$-labeled signal probes [18].

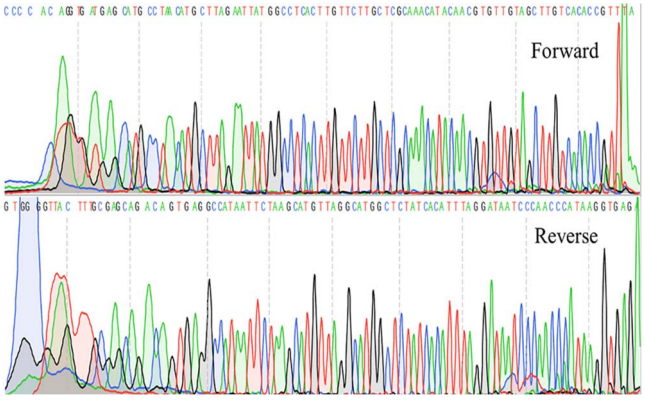


Fig. 2. Sequencing results.

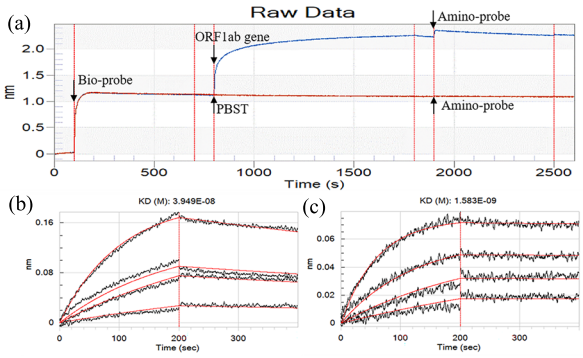


Fig. 3. Affinity determination between probes and target genes. (a) Affinity analysis of bio-probe, amino-probe, and ORF1ab target gene. (b) Determination of affinity constants between the bio-probe probe and ORF1ab target gene. (c) Determination of constants between ORF1ab target gene and amino-probe.

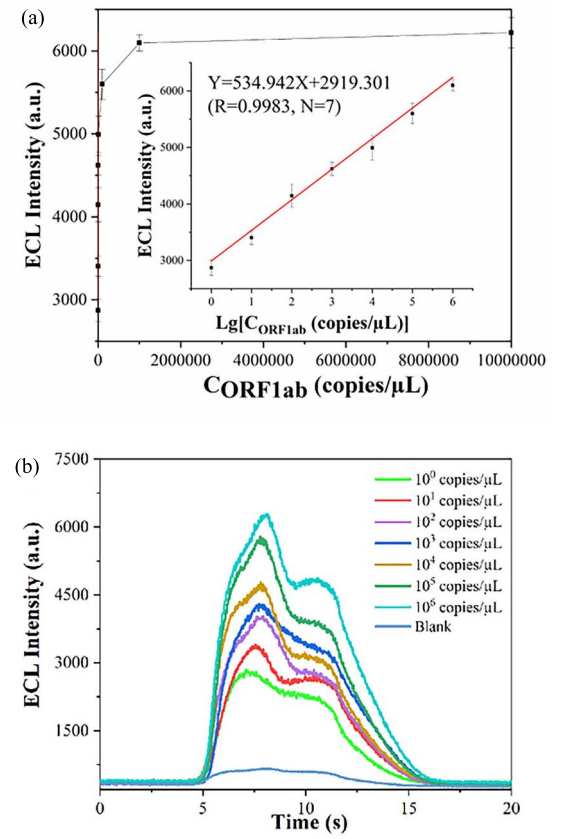


Fig. 4. (a) Standard curve and (b) response curve of ECL biosensor based on asymmetric PCR amplification techniques to detect SARS-CoV-2.

and rapid with high affinity. The affinity constant (KA = 1/KD) between bio-probe and ORF1ab target gene can reach $2.53 \times 10^7$ $M^{-1}$ and KA between amino-probe and ORF1ab target gene $6.31 \times 10^8$ $M^{-1}$.

### E. Linearity Range and LOD

The ECL sensor platform was used to test the ECL intensity of magnetic particle complexes bound to the initial concentration (1–$10^7$ copies/$\mu$L) of ORF1ab target gene reference material's amplification products. Each concentration was repeated and detected five times, and the corresponding ECL intensity values were obtained. The ECL intensity values were assayed by taking the average. When the template of 2019-nCoVRNA ORF1ab reference material concentration was in the range of 1–$10^6$ copies/$\mu$L, there was a significant linear relationship between the logarithm value of ORF1ab target gene reference material initial concentration ($X$ = LgC, copies/$\mu$L) and its ECL intensity value ($Y$, a.$\mu$.). The regression equation was $Y = 534.942X + 2919.301$ ($R = 0.9983$, $N = 7$), as shown in Fig. 4. When the concentration was 1 copy/$\mu$L, the detected ECL intensity value was 2871.40 ± 133.13, while the blank control ECL intensity value was 484.80 ± 38.22. The standard of $S/N \geq 3$ was used as the criterion to determine LOD, so LOD was determined to be 1 copy/$\mu$L (1.66 aM).

In the same conditions, utilizing the magnetic capture probes and the luminescent probe designed in this study, a method was constructed for detecting SARS-CoV-2 by ECL biosensor based on dual-probes hybridization using a detection model of magnetic capture probes-targeted nucleic acids-$Ru(bpy)_3^{2+}$-labeled luminescent probes, for which LOD was 60.2 copies/$\mu$L (100 aM) [18]. In our study, the sensitivity of the ECL biosensor based on asymmetric PCR amplification for SARS-CoV-2 ORF1ab gene detection was 60.2 times higher than the ECL biosensor based on dual-probes hybridization one.

TABLE II
COMPARISON OF DIFFERENT METHODS FOR TESTING SARS-COV-2 GENE BASED ON ECL BIOSENSOR

| Detection method | Target gene | LOD | Reference |
|---|---|---|---|
| entropy-driven Amplification | RdRp | 2.67 fM | [19] |
| CRISPR Amplification | RdRp | 12.8 aM | [20] |
| DNA walker Amplification | RdRp | 0.21 fM | [21] |
| DNA walker Amplification | RdRp | 7.8 aM | [22] |
| CRISPR Amplification | RdRp | 43.70 aM | [24] |
| CRISPR Amplification | RdRp | 32.80 aM | [25] |
| Asymmetric PCR Amplification | ORF1ab | 1.66 aM | This work |
| AuNMs and CDs | ORF1ab | 514 aM | [26] |
| Dual-probes hybridization | ORF1ab | 100 aM | [18] |

In addition, compared with the reported RdRp method for SARS-CoV-2 detection by ECL based on nucleic acid amplification technology [19], [20], [21], [22], [23], [24], [25], the sensitivity of this study was further improved, thus validating that ECL biosensor based on asymmetric PCR amplification for SARS-CoV-2 ORF1ab gene detection can not only achieve the amplification of efficiency but also improve the sensitivity of detection and so the detection of SARS-CoV-2 could be done with excellent efficiency, as shown in Table II.

### F. Reproducibility and Specificity

Within the linear detection range, the target ORF1ab gene with concentrations of $10^1$, $10^3$, and $10^5$ copies/$\mu$L was chosen to carry out the above reaction. After measuring the ECL intensity value five times and analyzing data, the average ECL intensity was obtained as 3392.80 ± 109.47, 4629.40 ± 119.01, and 5600.80 ± 183.43, respectively. The RSDs were 3.20%, 2.57%, and 3.26%, respectively, indicating that the method is of good reproducibility.

The specificity of the method was examined by using the ECL sensor to detect the target genes of SARS-CoV-2 pseudovirus compared with detecting non-target target genes of influenza A (H1N1, H5N1, H7N9), influenza B, SARS, and MERS pseudovirus, and all of the genes were at a final concentration of $10^5$ copies/$\mu$L (the ECL response curve as shown in Fig. 5). When SARS-CoV-2 pseudovirus was detected, its ECL intensity value was 5598.20 ± 181.10, with an RSD of 3.23%. When detecting non-target genes, their ECL intensity values were close to the negative values, for which ECL intensity values were 505.00 ± 23.85, 490.80 ± 19.38, 490.00 ± 21.95, 533.00 ± 14.80, 516.20 ± 32.10, and 503.40 ± 21.73, respectively, and RSDs were 4.72%, 3.95%, 4.48%, 2.78%, 6.22%, and 4.32%, respectively. Therefore, it showed that this method has good specificity in detecting the SARS-CoV-2 ORF1ab target gene, which provides a new way to identify suspected patients rapidly.

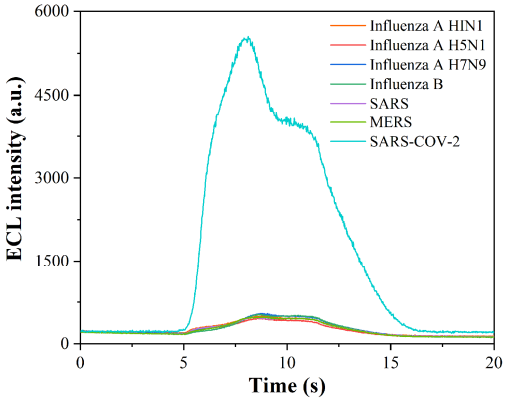


Fig. 5. Response curve of ECL biosensor based on asymmetric PCR amplification strategy for detecting different viruses.

### G. Testing of Simulated Samples

From the results, the ORF1ab gene in the human saliva and urine-simulated samples was detected, and the calculated recovery rates were 94.44% and 96.27%, and RSDs were 5.27% and 4.38%, respectively, indicating that the method can meet the analytical requirements of the saliva and urine-simulated samples with good recoveries and reproducibility.

## IV. Conclusion

This study has combined the advantages of the high efficiency of asymmetric PCR amplification with the benefit of the high sensitivity of ECL biosensors and established a reliable method for ECL biosensors based on asymmetric PCR-amplified strategy to achieve highly sensitive detection of SARS-CoV-2 ORF1ab gene. Its linear range is 1–$10^6$ copies/$\mu$L, the regression equation is $Y = 534.942X + 2919.301$ ($R = 0.9983$, $N = 7$), and the LOD is 1 copy/$\mu$L. Compared with the previously reported studies for detecting the RdRp gene of SARS-CoV-2 by ECL biosensor based on nucleic acid amplification technology, the sensitivity was improved. The method is able to meet the analytical requirements of simulated samples such as saliva and urine. It has the characteristics of high sensitivity, credible reproducibility, and specificity, which expands a new way and provides a reference basis for achieving sensitive detection of SARS-CoV-2 in complex samples in the field of epidemic prevention and control.

## Appendix

### A. Preparation and Characterization of $Ru(Bpy)_3^{2+}$-Labeled Signal Probes

See Fig. 1.

### B. Validation of the Product Validity of Asymmetric PCR Amplification

See Fig. 2.

## References

[1] A. Guillon, P. S. Hiemstra, and M. Si-Tahar, "Pulmonary immune responses against SARS-CoV-2 infection: Harmful or not?" *Intensive Care Med.*, vol. 46, no. 10, pp. 1897–1900, Oct. 2020.

[2] M. R. Benzigar, R. Bhattacharjee, M. Baharfar, and G. Liu, "Current methods for diagnosis of human coronaviruses: Pros and cons," *Anal. Bioanal. Chem.*, vol. 413, no. 9, pp. 2311–2330, Apr. 2021.

[3] C. Y. Yu, K. G. Chan, C. Y. Yean, and G. Y. Ang, "Nucleic acid-based diagnostic tests for the detection SARS-CoV-2: An update," *Diagnostics*, vol. 11, no. 1, p. 53, Jan. 2021.

[4] N. Zhu *et al.*, "A novel coronavirus from patients with pneumonia in China, 2019," *New England J. Med.*, vol. 382, no. 8, pp. 727–733, Jan. 2020.

[5] V. M. Corman *et al.*, "Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR," *Eurosurveillance*, vol. 25, no. 3, Jan. 2020, Art. no. 2000045.

[6] K. G. de Oliveira, P. F. N. Estrela, G. D. M. Mendes, C. A. D. Santos, E. D. P. Silveira-Lacerda, and G. R. M. Duarte, "Rapid molecular diagnostics of COVID-19 by RT-LAMP in a centrifugal polystyrene-toner based microdevice with end-point visual detection," *Analyst*, vol. 146, no. 4, pp. 1178–1187, 2021.

[7] K. Nawattanapaiboon *et al.*, "Colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a visual diagnostic platform for the detection of the emerging coronavirus SARS-CoV-2," *Analyst*, vol. 146, no. 2, pp. 471–477, 2021.

[8] A. A. El Wahed *et al.*, "Suitcase lab for rapid detection of SARS-CoV-2 based on recombinase polymerase amplification assay," *Anal. Chem.*, vol. 93, no. 4, pp. 2627–2634, Feb. 2021.

[9] T. R. Shelite, A. C. Uscanga-Palomeque, A. Castellanos-Gonzalez, P. C. Melby, and B. L. Travi, "Isothermal recombinase polymerase amplification-lateral flow detection of SARS-CoV-2, the etiological agent of COVID-19," *J. Virol. Methods*, vol. 296, Oct. 2021, Art. no. 114227.

[10] J. Wang *et al.*, "Multiple-centre clinical evaluation of an ultrafast single-tube assay for SARS-CoV-2 RNA," *Clin. Microbiol. Infection*, vol. 26, no. 8, pp. 1076–1081, Aug. 2020.

[11] A. Bonini *et al.*, "Advances in biosensing: The CRISPR/Cas system as a new powerful tool for the detection of nucleic acids," *J. Pharmaceutical Biomed. Anal.*, vol. 192, Jan. 2021, Art. no. 113645.

[12] X. Zhu *et al.*, "Rapid, ultrasensitive, and highly specific diagnosis of COVID-19 by CRISPR-based detection," *ACS Sensors*, vol. 6, no. 3, pp. 881–888, Mar. 2021.

[13] J. Y. Do, J. Y. Jeong, and C. A. Hong, "Catalytic hairpin DNA assembly-based chemiluminescent assay for the detection of short SARS-CoV-2 target cDNA," *Talanta*, vol. 233, Oct. 2021, Art. no. 122505.

[14] Z. Yao *et al.*, "Rapid detection of SARS-CoV-2 viral nucleic acids based on surface enhanced infrared absorption spectroscopy," *Nanoscale*, vol. 13, no. 22, pp. 10133–10142, Jun. 2021.

[15] L. Xu, D. Li, S. Ramadan, Y. Li, and N. Klein, "Facile biosensors for rapid detection of COVID-19," *Biosensors Bioelectron.*, vol. 170, Dec. 2020, Art. no. 112673.

[16] F. Cui and H. S. Zhou, "Diagnostic methods and potential portable biosensors for coronavirus disease 2019," *Biosensors Bioelectron.*, vol. 165, Oct. 2020, Art. no. 112349.

[17] X. Zhou *et al.*, "Synthesis, labeling and bioanalytical applications of a tris(2,2′-bipyridyl)ruthenium(II)-based electrochemiluminescence probe," *Nature Protocols*, vol. 9, no. 5, pp. 1146–1159, May 2014.

[18] C. Jiang *et al.*, "A study of the detection of SARS-CoV-2 ORF1ab gene by the use of electrochemiluminescent biosensor based on dual-probe hybridization," *Sensors*, vol. 22, no. 6, p. 2402, Mar. 2022.

[19] Z. Fan, B. Yao, Y. Ding, J. Zhao, M. Xie, and K. Zhang, "Entropy-driven amplified electrochemiluminescence biosensor for RdRp gene of SARS-CoV-2 detection with self-assembled DNA tetrahedron scaffolds," *Biosensors Bioelectron.*, vol. 178, Apr. 2021, Art. no. 113015.

[20] K. Zhang, Z. Fan, Y. Huang, Y. Ding, and M. Xie, "A strategy combining 3D-DNA Walker and CRISPR-Cas12a trans-cleavage activity applied to MXene based electrochemiluminescent sensor for SARS-CoV-2 RdRp gene detection," *Talanta*, vol. 236, Jan. 2022, Art. no. 122868.

[21] B. Yao *et al.*, "Rational engineering of the DNA Walker amplification strategy by using a $Au@Ti_3C_2@PEI\text{-}Ru(dcbpy)_3^{(2+)}$ nanocomposite biosensor for detection of the SARS-CoV-2 RdRp gene," *ACS Appl. Mater. Interfaces*, vol. 13, no. 17, pp. 19816–19824, May 2021.

[22] Z. Fan, B. Yao, Y. Ding, D. Xu, J. Zhao, and K. Zhang, "Rational engineering the DNA tetrahedrons of dual wavelength ratiometric electrochemiluminescence biosensor for high efficient detection of SARS-CoV-2 RdRp gene by using entropy-driven and bipedal DNA Walker amplification strategy," *Chem. Eng. J.*, vol. 427, Jan. 2022, Art. no. 131686.

[23] K. Zhang, Z. Fan, Y. Huang, Y. Ding, M. Xie, and M. Wang, "Hybridization chain reaction circuit-based electrochemiluminescent biosensor for SARS-CoV-2 RdRp gene assay," *Talanta*, vol. 240, Apr. 2022, Art. no. 123207.
[24] K. Zhang, Z. Fan, Y. Ding, and M. Xie, "A pH-engineering regenerative DNA tetrahedron ECL biosensor for the assay of SARS-CoV-2 RdRp gene based on CRISPR/Cas12a trans-activity," *Chem. Eng. J.*, vol. 429, Feb. 2022, Art. no. 132472.
[25] K. Zhang, Z. Fan, Y. Ding, S. Zhu, M. Xie, and N. Hao, "Exploring the entropy-driven amplification reaction and trans-cleavage activity of CRISPR-Cas12a for the development of an electrochemiluminescence biosensor for the detection of the SARS-CoV-2 RdRp gene in real samples and environmental surveillance," *Environ. Sci., Nano*, vol. 9, no. 1, pp. 162–172, 2022.
[26] L. Gutiérrez-Gálvez *et al.*, "Electrochemiluminescent nanostructured DNA biosensor for SARS-CoV-2 detection," *Talanta*, vol. 240, Apr. 2022, Art. no. 123203.

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