Development of a lateral flow immunoassay with HRP enhancement for spiked SARS-CoV-2 protein N detection in human saliva

Minh Hieu Vu[1, 2], Doan Hong Ngoc Tran[2, 3], Minh-Anh Dang-Trinh[2], Huynh Chan Khon[3, 4, 5*]

[1]School of Biotechnology, International University, Vietnam National University - Ho Chi Minh City, Quarter 6, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam
[2]Institute of Malariology, Parasitology and Entomology, Ho Chi Minh City, 699 Tran Hung Dao Street, Ward 1, District 5, Ho Chi Minh City, Vietnam
[3]School of Biomedical Engineering, International University, Vietnam National University - Ho Chi Minh City, Quarter 6, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam
[4]Research Centre for Infectious Diseases, International University, Vietnam National University - Ho Chi Minh City, Quarter 6, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam
[5]Vietnam National University - Ho Chi Minh City, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam

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Abstract:

The Coronavirus (SARS-CoV-2) is an enveloped RNA virus that caused a dangerous COVID-19 pandemic following an outbreak in Wuhan, China in 2019. In response to the pandemic, the development of lateral flow assays (LFAs) has been crucial for the detection of viruses, commonly targeting the spike (S) or nucleocapsid (N) protein in nasopharyngeal swab (NS) specimens. COVID-19, caused by the SARS-CoV-2 virus, predominantly manifests as a respiratory tract infection-like illness, characterized by symptoms such as fever, dry cough, upper airway congestion, runny nose, sore throat, myalgia, headache, and exhaustion. This study presents the development of an LFA targeting the N protein to detect the coronavirus in saliva specimens, using a sandwich format. The use of 80 nm gold nanoparticles (AuNPs) has been investigated, and the application of horseradish peroxidase (HRP) and TMB substrate has been employed to enhance the limit of detection (LOD). The results demonstrate a significant 200-fold improvement in LOD, from 10 to 50 pg/ml, for N protein spiked in saliva samples after the application of HRP-TMB. This finding highlights an important advancement towards the utilization of saliva samples in diagnostic applications.

Keywords: **horseradish peroxidase, lateral flow assay, mixed-sized gold nanoparticles, SARS-CoV-2 nucleocapsid protein.**

Classification numbers: **3.5, 3.6**

1. Introduction

The Coronavirus is an enveloped RNA virus belonging to the Coronaviridae family and primarily affects humans, as well as other species such as mammals and birds, causing various respiratory problems. Researchers have classified the Coronaviridae family into four genera, namely α, β, γ, and δ, based on the structure of the genome and phylogenetic analysis. The γ and δ genera mainly infect birds, while the α and β genera are commonly reported to infect mammals and humans [1]. Prior to the COVID-19 outbreak, two pandemics caused by β-CoV occurred: severe acute respiratory syndrome coronavirus (SARS-CoV, 2002-2003) and Middle East respiratory syndrome coronavirus [2]. COVID-19, caused by the SARS-CoV-2 virus, predominantly manifests as a respiratory tract infection-like illness, characterized by symptoms such as fever, dry cough, upper airway congestion, runny nose, sore throat, myalgia, headache, and exhaustion. While diarrhea has been recorded in a few patients, dyspnoea is a potential symptom in certain individuals. Severe cases of COVID-19 can rapidly

*Corresponding author: Email: hckhon@hcmiu.edu.vn

progress to septic shock, acute respiratory distress syndrome, and coagulation dysfunction [3].

Currently, there are two main groups of clinically and commercially available COVID-19 tests. The first category comprises molecular assays for detecting SARS-CoV-2 viral RNA using PCR-based or nucleic acid hybridization-related methods. The second group consists of immunological and serological tests that focus on identifying antibodies and antigens in infected individuals [3, 4]. However, these techniques require specialized equipment, are time-consuming, and labour-intensive, highlighting the urgent need for rapid, convenient, and affordable technologies. Consequently, there has been increased interest in immunogold labelling technology, which offers excellent sensitivity, a simple procedure, and rapid detection in immunoassays. LFA production has been developed to address these considerations. As of June 2021, 1,102,383 RT-qPCR tests had been performed, compared to 36,663,990 antigen quick tests (AQT) used by citizens in Europe [5].

Both the spike glycoprotein (S-protein) and the nucleocapsid (N-protein) can serve as primary immunoassay targets for SARS-CoV-2 [3]. The coronavirus spike protein, a trimeric single-pass membrane protein, plays a crucial role in the early stages of the virus infection cycle by facilitating the fusion of viral and host cell membranes. It consists of a long ectodomain, a single-pass transmembrane anchor, and a short intracellular tail. The ectodomain comprises the receptor-binding domain (RBD) subunit S1 and the membrane-fusion subunit S2. The S1 subunit attaches to a receptor on the host cell surface, while the S2 subunit merges the host and viral membranes to enable viral entry into host cells [6, 7]. The coronavirus nucleocapsid protein, which interacts with the viral membrane protein during virion assembly and forms complexes with genomic RNA, serves as a structural protein with multiple functions. It plays a crucial role in enhancing virus transcription, translation, and assembly [8]. Additionally, the nucleocapsid protein elicits a highly antigenic response and is frequently the primary target of the immune response, particularly with immunoglobulin G-dominated antibodies [9]. Moreover, the nucleocapsid protein employs strategies to evade the host's innate immune system by interfering with interferon production, which is essential for the viral pathogenesis response [10]. The detection of the SARS-CoV-2 antigen has predominantly focused on the receptor-binding domain of the S1 subunit and the nucleocapsid N protein, considering their significance in the pathophysiology and immunogenicity of the SARS-CoV-2 virion in the human body.

Since the onset of the pandemic, nasopharyngeal swabs (NS samples) have been widely used as the primary samples for SARS-CoV-2 diagnosis due to the high viral loads and early expression in the nasopharyngeal region, with viral loads ranging from approximately 9.9×10^2 to 1.2×10^8 copies/ml within the first 7 days [11]. However, the use of deep nasopharyngeal swabs has been reported as uncomfortable for both adults and children. As a result, based on user feedback and complaints, saliva specimens have been employed for COVID-19 LFAs throughout the United States, even though the reported viral load in saliva specimens is lower than that in nasopharyngeal swabs, ranging from 10^2 to 10^3 copies/ml [12]. Saliva samples have shown lower positivity rates (88%, 95% CI: 81 to 93%) compared to nasopharyngeal swabs [13]. Despite the challenges posed by saliva components, such as viscosity and enzyme activity, saliva remains a promising and comfortable sample type for SARS-CoV-2 LFAs.

Gold nanoparticle technology has been extensively utilized in LFA production. Various sizes of gold nanoparticles have been investigated for colorimetric analysis in LFAs. Larger gold nanoparticle sizes over 60 nm can lead to unstable outcomes in LFAs, although they exhibit a significant increase in colour [14, 15]. In recent years, with advancements in LFA development and the application of the enzyme-linked immunosorbent assay (ELISA) principle in LFAs, researchers have explored the

use of HRP enzymes as labels to build competitive LFAs for the multiplexed study of carbaryl and endosulfan, comparing the results obtained with different systems [16]. The application of the TMB substrate in HRP amplification involves a chemiluminescence reaction where HRP reduces hydrogen peroxide and oxidizes TMB, resulting in a colour change from colourless to blue, facilitating visual observation. Furthermore, HRP-TMB amplification has shown a 100-fold improvement in signal compared to conventional AuNP-based methods [17, 18].

Based on the information mentioned above, this study aims to investigate the use of 80 nm AuNPs in LFA production for the detection of SARS-CoV-2 N protein using human saliva samples. Additionally, the study explores the use of HRP as an enhancer to improve the LOD in AuNP-based LFAs and compares the results to a commercial LFA from China.

2. Materials and methods

2.1. Materials

Gold Conjugation Kit (80 nm, 20 OD) and HRP Conjugation Kit - Lightning-Link® were purchased from Abcam (Cambridge, UK). Hi-FlowTM Plus 180 nitrocellulose membrane cards, C083 Cellulose Fiber Sample Pad Strips, and Glass Fiber Diagnostic Pad were purchased from Merck Millipore (Burlington, Massachusetts, USA). Saliva was collected from volunteers. SARS-CoV-2 Nucleocapsid Recombinant Protein (RP-87665, Invitrogen) was selected as the target antigen for all experiments. SARS-CoV-2 Nucleocapsid Recombinant Rabbit Monoclonal Antibody (3E8A5, Invitrogen) was used as the capture antibody, and Anti-SARS-CoV-2 Nucleocapsid antibody produced in mouse (7B3, Sigma-Aldrich) was used as the detection antibody, while Anti-Rabbit IgG (whole molecule) antibody produced in goat (R1131, Sigma Aldrich) was used as the control antibody. Healthy saliva samples were collected from volunteers.

2.2. Methods

2.2.1. Preparation of detection antibody-gold nanoparticle (Ab-AuNPs) conjugates

The detection antibody (detection Ab) was conjugated to 80 nm AuNPs using the Gold Conjugation Kit (ab154876, Abcam) following the user manual. The UV-Vis spectra of bare AuNPs and detection Ab-AuNPs conjugates were analysed using a microplate reader (Synergy HT, Biotek). Absorbance spectra from 450-600 nm with 1 nm-step intervals were recorded for both solutions. A successful conjugation was expected to result in a right shift in the UV-Vis spectra due to the change in the local refractive index of the nanoparticles.

2.2.2. Preparation of detection antibody-horseradish peroxidase (Ab-HRP) conjugates

The detection antibody (detection Ab) was conjugated to HRP using HRP Conjugation Kit - Lightning Link ® (ab102890, AbCam) according to the user manual.

2.2.3. Optimization of different lateral flow assay parameters

Prior to the optimization process, the functionality of both capture and control antibodies, as well as the detection Ab-AuNPs conjugation, was tested on the half-strip model. The two antibodies (capture Ab and control Ab) were diluted in PBS pH 7.4 buffer and dotted on the nitrocellulose membrane to create a test line (T-line) and a control line (C-line). The membrane was then blocked with 1% (w/v) BSA for 15 minutes to minimize nonspecific binding, followed by complete air-drying before testing. Detection Ab-AuNPs conjugates were diluted in PBS pH 7.4 buffer containing 1% BSA. For testing the half-strip models, detection Ab-AuNPs conjugates were premixed with SARS-CoV-2 N protein at 500 ng/ml, which was further diluted in 1X PBS.

Three parameters of the LFA strips were optimized in this study: The concentration of capture antibody,

the concentration of Ab-AuNPs conjugate, and the concentration of Ab-HRP conjugate.

(1) Capture antibody concentration: Different dilution rates of capture antibody (0.5, 0.2, and 0.1 mg/ml) were prepared using 1X PBS pH 7.4 buffer and dotted on the membrane to create the T-line. The C-line was prepared at a concentration of 0.5 mg/ml, followed by dropping a mixture of detection Ab-AuNPs conjugates and diluted SARS-CoV-2 N protein onto the nitrocellulose membrane.

(2) Conjugate concentration: The intensity increased with higher conjugate concentrations. Detection Ab-AuNPs conjugate was prepared at different dilution rates of 1:2, 1:5, and 1:10, and then applied onto the nitrocellulose membrane after mixing with diluted SARS-CoV-2 N protein.

(3) Ab-HRP concentration: The Ab-HRP was serially diluted at different ratios ranging from 1:1 to 1:5000 by 1X PBS pH 7.4 buffer.

2.2.4. Test-strip performance with HRP enhancement

The full strip model of the LFA was assembled based on the established optimal conditions. SARS-CoV-2 N protein was resuspended and serially diluted in saliva samples, ranging from 500 ng/ml to 50 pg/ml. Each extracted sample (500 µl-1 ml) was added to the extraction buffer of the COVID-19 Antigen Rapid Test Kit (eDiagnosis). Subsequently, 100 µl of each extracted sample was applied to the developed test strips, and the signals were observed. To enhance the band intensity, all strips were washed with 1X PBS pH 7.4 buffer at least once. An optimal concentration of Ab-HRP (70 µl) was directly dropped onto the sample pad for absorbance. After 10 minutes in dark conditions, 5 µl of TMB substrate was added directly to the T-line and C-line of all strips for colour appearance. The reaction was stopped by washing the unbound Ab-HRP with 1X PBS.

2.2.5. LOD and comparison with the commercial test kit

Spiked saliva samples (100 µl) were dropped onto the Easy Diagnosis Antigen Kit Test to estimate the LOD. The results were compared with our developed test strip, which included HRP enhancement. Band intensities were observed visually and further analysed using ImageJ software. The LOD was determined as the lowest antigen concentration at which the test line was still visible.

3. Results

3.1. Establishment of a lateral flow assay for detecting SARS-CoV-2 nucleocapsid protein

The working principle of our LFA design is illustrated in Fig. 1. Initially, the extracted sample is applied to the sample pad. The antigens present in the sample migrate towards the conjugation pad and bind with the detection Ab-AuNPs conjugates. The N protein-detection Ab-AuNPs complexes are captured by the immobilized capture antibody (capture Ab) at the T line, forming a sandwich structure. Any excess detection Ab-AuNP conjugates that do not bind to antigens are captured at the C line by anti-rabbit IgG, indicating a positive signal and confirming the proper functioning of the assay.

To evaluate the functionality of the assay, we first assembled a half-strip model, which consisted of only the nitrocellulose membrane and absorbent pad, and pre-mixed the sample with the detection Ab-AuNPs conjugates. The half-strip models were used to test samples containing N protein at a concentration of 1 µg/ml. A valid outcome of the strip should show the appearance of both the T line and C line in red colour (Fig. 1A), while the absence of the C line or the appearance of both lines indicates an invalid result (Fig. 1D). Subsequently, detection Ab-HRP was applied to the valid strips to enhance the signal (Fig. 1B). For signal examination, TMB substrate was directly added to the T line and C line, resulting in the appearance of blue colour in both lines for a positive result and only the C line appearance for a negative result (Fig. 1C).

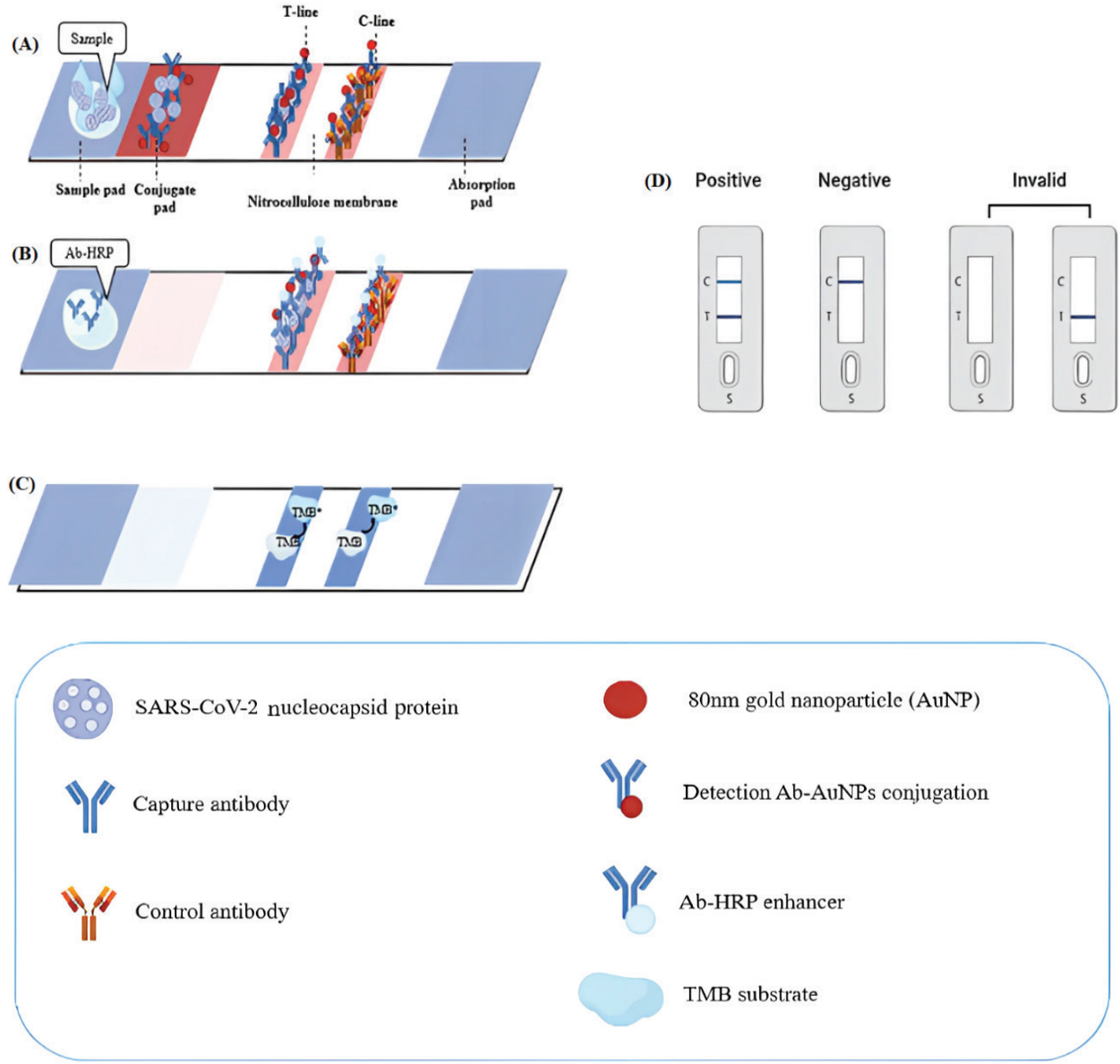

Fig. 1. The designed LFA strips for SARS-CoV-2 detection and its principle. (A) The principle of LFA using AuNP technology; **(B)** The binding of Ab-HRP into the based-AuNPs LFA strips; **(C)** The colour changes of HRP with TMB substrate reaction; **(D)** Interpreting results of LFA strips.

3.2. Full strip model

After confirming the functionality of the assay using the half-strip models, we developed the full strip model of the LFA for SARS-CoV-2 N protein detection. Experiments were conducted to determine the optimal assembly conditions to achieve a good signal-to-noise ratio. A high level of background noise would hinder the subsequent HRP enhancement, as it would result in the entire strip turning blue, reducing the colour intensity observed visually. Through optimization experiments, we determined that the optimal dilution concentration of the capture Ab was 0.2 mg/ml, the dilution rate of the detection Ab-AuNPs conjugate was 1:5, and the dilution rate of the detection Ab-HRP conjugate was 1:1000. Fig. 2 illustrates the appearance of the developed full strip, including the sample pad, conjugate pad, nitrocellulose membrane, and absorption pad, after being tested with N protein at a concentration of 500 ng/ml.

Fig. 2. The result of the developed full-strip, including the sample pad, conjugate pad, nitrocellulose membrane, and absorption pad, after being tested with 500 ng/ml SARS-CoV-2 N protein.

3.3. Evaluation of LOD of LFA strip

To determine the LOD of the LFA strips, SARS-CoV-2 N proteins were prepared at different concentrations ranging from 1 µg/ml to 50 pg/ml. After preparing complete LFA strips, samples with different concentrations were applied to the strips. Visual examination of the strips revealed the development of a positive signal at the T line for samples with concentrations ≥10 ng/ml (Fig. 3). For samples with concentrations lower than 10 ng/ml, the T line either showed a faint red line or no signal. Strong positive signals were observed at the C line in all test strips, indicating that they were functioning properly.

3.4. Enhancement of LFA strip sensitivity by HRP enhancement

To enhance the LOD of the LFA strip for the N protein, the HRP enhancement method was applied after the strips showed certain signals. Specifically, LFA strips from the LOD experiments mentioned above were washed with 1X PBS buffer and treated with detection Ab-HRP, followed by 10 minutes of incubation in dark conditions. Visual examination of the signal intensities showed that the band intensities changed from the red colour of AuNPs to the blue colour due to the oxidation of the TMB substrate by HRP. As a result, the LOD was improved to 50 ng/ml through HRP enhancement, which represented a 200-fold increase compared to the AuNP method. The signal intensities before and after signal enhancement were further analysed using the ImageJ densitometry function (Figs. 4, 5).

3.5. Comparison of the LOD of the LFA strip with the commercial test kit

Two types of tests were validated based on the appearance of the C line in the negative control strips. With the sample extracted using the commercial buffer provided in the eDiagnosis Antigen Test Kit, the commercial strip showed an LOD of 100 pg/ml (Fig. 6A) [19], while our developed LFA strip demonstrated an LOD of 50 pg/ml (Fig. 3B) after applying HRP enhancement. The ImageJ results confirmed the successful appearance of the T line in the LFA strip (Fig. 6B).

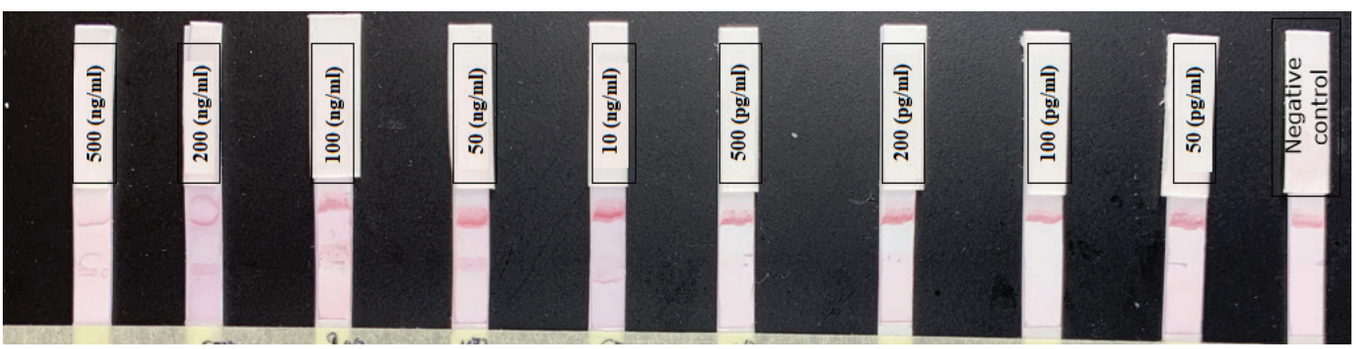

Fig. 3. Visual examination of results from LFA strips with 80 nm AuNPs-Ab conjugates.

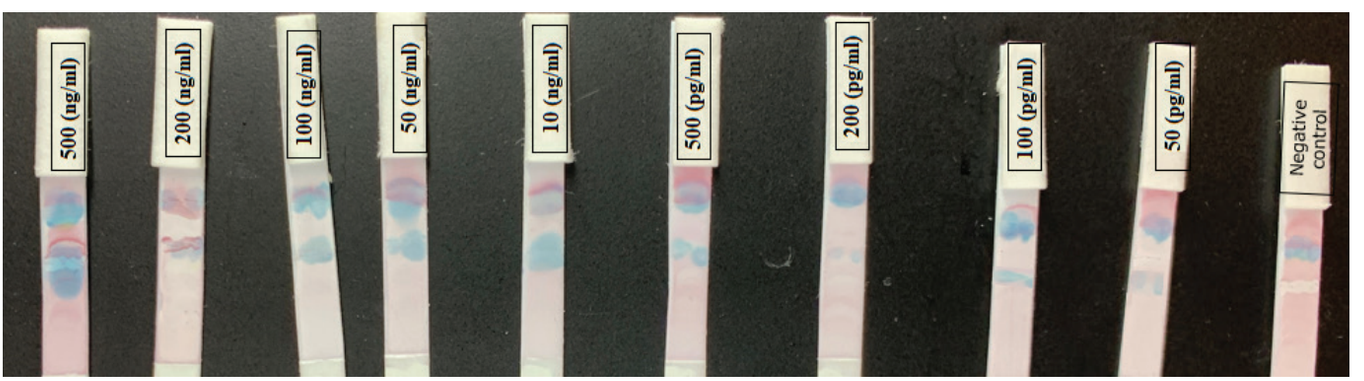

Fig. 4. Visual examination of results from LFA strips after HRP signal enhancement.

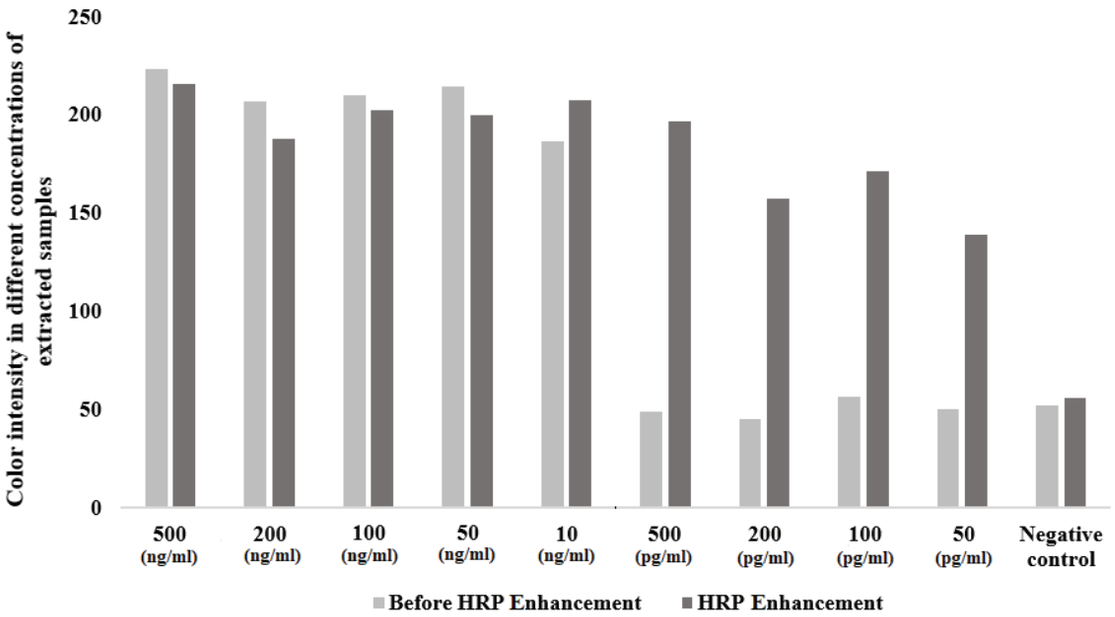

Fig. 5. ImageJ analysis to compare band intensities of LFA strips before and after HRP signal enhancement.

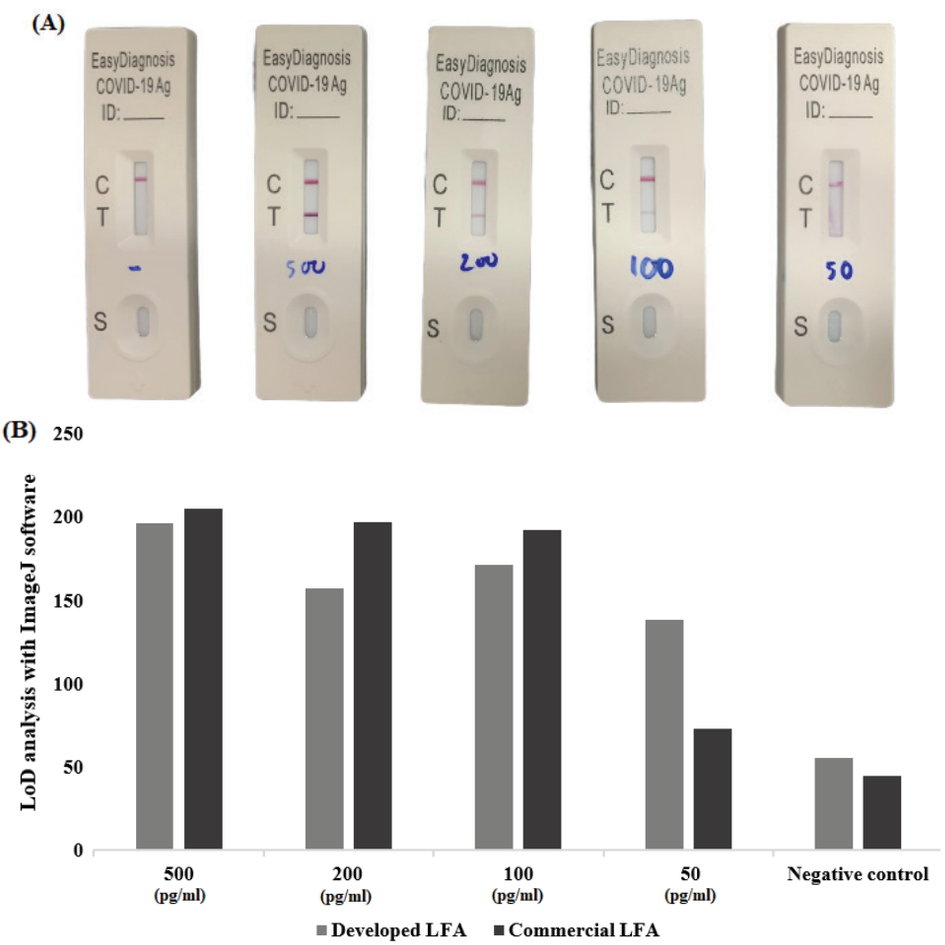

Fig. 6. Commercial test kit analysis. (A) LOD of the commercial test kit; **(B)** Data analysis of commercial test kit with the developed LFA strips using ImageJ software.

4. Discussion

LFAs are widely used for point-of-care diagnostics due to their simplicity, speed, and cost-effectiveness. In LFAs, AuNPs are commonly employed due to their unique optical properties, allowing for visual detection of target analytes. Typically, AuNPs with diameters ranging from 10 to 100 nm are used in LFAs, with sizes of 20, 30, and 40 nm being the most common. The size of the gold nanoparticles can affect the colour and intensity of the signal produced, as well as their stability and interaction with other components of the LFA. Previous studies have reported that the use of colloidal gold with a diameter ≥60 nm leads to unstable detection on the strip [20-24]. However, larger gold nanoparticles tend to produce stronger colour signals, which can impact assay sensitivity. In this study, we selected 80 nm AuNPs to enhance the colour signal in the LFA assays. The appearance of test lines on the LFA strips after sample loading indicated successful assay development, although the LOD was not yet optimal.

Various labelling approaches have been explored to enhance the sensitivity of LFAs, such as dual AuNPs, silver staining, enzyme amplification, surface-enhanced Raman scattering, and photothermal methods [25]. Each method has its own limitations, such as high cost, the need for additional signal readers, and dependence on dye stability. In this study, we employed HRP and its substrate TMB for signal enhancement in the LFA strips. HRP catalyses the conversion of TMB to a blue-coloured product, which is then converted to a yellow-coloured product by adding an acidic stop solution. In enzyme-linked immunosorbent assays, HRP is often conjugated to a detection antibody and used to detect the presence of specific antigens or antibodies in biological samples. HRP is widely available and relatively inexpensive compared to other enzymes used in assays. By applying HRP-antibody amplification and TMB substrate for signal enhancement, the sensitivity of the assay was significantly improved by 200-fold, with the LOD being enhanced from 10 ng/ml to 50 pg/ml. This improvement surpassed the sensitivity of the commercial test kit, which had a LOD at 100 pg/ml. These results demonstrate the potential of HRP enhancement as a means to increase signal intensity and develop novel LFAs.

5. Conclusions

This study highlights the promising role of HRP in enhancing LFAs. Through the application of HRP enhancement, there was a 200-fold improvement in the visual LOD, which increased from 10 ng/ml to 50 pg/ml of SARS-CoV-2 N protein in saliva when using the commercial buffer of the eDiagnosis COVID-19 Antigen Test Kit. Compared to other pathogen detection methods such as conventional culture, qRT-PCR, and ELISA, this assay demonstrated completion within approximately 15-20 minutes, with a simple procedure that can be visually interpreted and at a low cost. These characteristics position this assay as a significant step towards the development of future SARS-CoV-2 test kits targeting the N protein using HRP enhancement.

CRediT author statement

Minh Hieu Vu: Algorithm, Experiment, Writing, and Editing; Doan Hong Ngoc Tran: Experiment, Writing - Original draft preparation; Minh-Anh Dang-Trinh: Reviewing and Editing; Huynh Chan Khon: Writing - Reviewing and Editing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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