Biomaterials Translational

Plant-produced recombinant SARS-CoV-2 receptor-binding domain; an economical, scalable biomaterial source for COVID-19 diagnosis

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Key Words:
COVID-19; lateral flow immunoassay; *Nicotiana benthamiana*; point-of-care testing; recombinant protein; SARS-CoV-2

From the Contents

ABSTRACT

The outbreak of the novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spread rapidly causing a severe global health burden. The standard COVID-19 diagnosis relies heavily on molecular tests to detect viral RNA in patient samples; however, this method is costly, requires highly-equipped laboratories, multiple reagents, skilled laboratory technicians, and takes 3–6 hours to complete. To overcome these limitations, we developed a plant-based production platform for the SARS-CoV-2 receptor-binding domain as an economical source of detection reagents for a lateral-flow immunoassay strip (LFIA) which is suitable for detection of IgM/IgG antibodies in human samples. Further, we validated the plant-produced SARS-CoV-2 receptor-binding domain-based LFIA as a useful diagnostic tool for COVID-19. A total of 51 confirmed COVID-19 serum samples were tested using the LFIA, and the obtained results were consistent with those from polymerase chain reaction assays, while providing sensitivity and specificity of 94.1% and 98%, respectively. The developed LFIA is rapid, scalable, user-friendly, and relatively inexpensive with a simple test procedure, making it useful for the routine monitoring of COVID-19 in clinical settings. This study was approved on March 19, 2020 by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (COA No. 354/2020 and IRB No. 236/63).

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Introduction

Coronaviruses belong to the family Coronaviridae, subfamily Coronavirinae that can infect a wide variety of animals, including humans, bats, camels, and birds, and cause respiratory diseases. In December 2019, clusters of patients with pneumonia-like symptoms were admitted to the hospitals, which were later reported to be caused by a novel coronavirus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2). By January 30, 2020, the outbreak had been declared a public health emergency of international concern by the World Health Organization.[1] The global pandemic coronavirus disease 2019 (COVID-19) outbreak had caused > 47 million confirmed infections and > 1.2 million deaths by November 2020.[2] Additionally, the virus was reported to be transmitted from human to human by close contact. Currently, rapid and accurate diagnosis is essential for the prevention of extensive COVID-19 outbreaks.

Implementation of effective screening procedures and diagnosis of infected individuals is indispensable for disease management during epidemics. Current diagnostic tests for SARS-CoV-2 include real-time reverse transcriptase-polymerase chain reaction-based assays which require high-end facilities and considerable time.[3, 4] In light of the rapid spread of the disease, the current diagnosis based on molecular tests is not field-usable, is expensive, and is not rapid enough to screen infected individuals. In addition the results might vary based on the viral load and quality of the sample.[5] There is great interest in using point-of-care serology tests that are easily portable, easy to use, and cost-effective, to overcome these limitations.

Lateral flow immunoassays (LFIAs), or dipstick tests, offer all these advantages. Like in-home pregnancy tests, LFIAs are single-use point-of-care diagnostic tests that can be used outside a laboratory.[6] In COVID-19, specific IgM antibodies are produced early in the infection, whereas specific IgG antibodies develop in the later stages.[7] Hence, the detection of IgM/IgG antibodies against SARS-CoV-2 using LFIA could help assess the epidemiology of the disease and evaluate the infection and immune status of the human population.[8]

The receptor-binding domain (RBD) of the spike protein located on the surface of SARS-CoV-2 is responsible for viral entry into the host cell and eliciting the neutralizing antibody response; hence it is considered a promising antigen to use as a vaccine candidate and also for the serological diagnosis of COVID-19.[9, 10] Production of recombinant RBD protein to use as a diagnostic reagent in LFIA could be expensive in mammalian or insect cell cultures, representing the major drawback for large-scale diagnostic tests, especially for low-income countries. A practical, accurate diagnostic test can be affordable only if the reagents used for testing are cost-effective. Likewise, if the expression platform produces recombinant RBD protein rapidly at a low cost, the cost of an LFIA would be reduced significantly.

Recently, plants have attracted attention as a potential alternative means of producing recombinant proteins over other traditional expression systems due to the system's affordability, reliability, scalability, and flexibility. Recent advances in the development of transient expression systems in plants offer rapid, large-scale methods of producing biopharmaceuticals that can be used either as diagnostic or therapeutic agents.[11-18] Considering the advantages of the plant expression platform, we expressed recombinant RBD antigen in *Nicotiana benthamiana*.[19] The purified plant-produced antigen was then used to develop a diagnostic reagent to establish and validate the LFIA.

Considering the speed of the spread of the virus and the urgent need for a rapid, cost-effective method to screen large populations, we developed and evaluated a rapid LFIA using plant-produced recombinant RBD protein to detect IgM and IgG antibodies against SARS-CoV-2 in clinical samples from patients in Thailand. We tested and validated the efficacy of the assay using serum from patients in whom real-time reverse transcriptase-polymerase chain reaction confirmed SARS-CoV-2 infection.

Methods

Expression of SARS-CoV-2 RBD in *N. benthamiana*

The RBD of SARS-CoV-2 spike protein was transiently expressed in *N. benthamiana* plants and purified by affinity column chromatography. The procedures were performed as previously reported.[19] The schematic representation of recombinant protein expression in plants was shown in **Figure 1**. Briefly, the gene encoding the SARS-CoV-2 RBD construct was incorporated into a geminiviral vector (pBY2e) (**Figure 2**). The pBY2e was obtained from Professor Hugh Mason (Arizona State University, USA). The recombinant pBY2e-SARS-CoV-2-RBD vector was transformed into *Agrobacterium tumefaciens* strain GV3101 (Gold Biotechnology® Inc., Olivette, MO, USA), which was then used to infiltrate *N. benthamiana*. The SARS-CoV-2 RBD was harvested and purified from *N. benthamiana* using Ni–nitriloacetic acid affinity resin (Expedeon, Cambridge, UK).

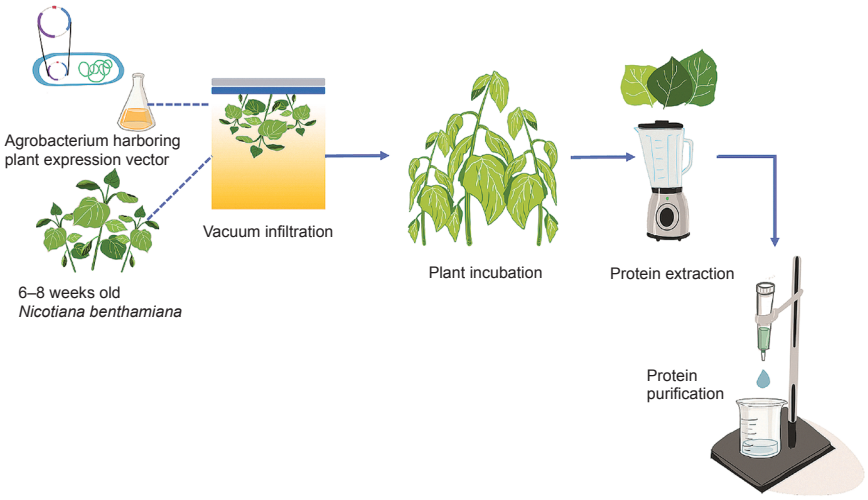

Figure 1. Schematic representation for the transient expression of SARS-CoV-2 RBD protein in plants. RBD: receptor-binding domain; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

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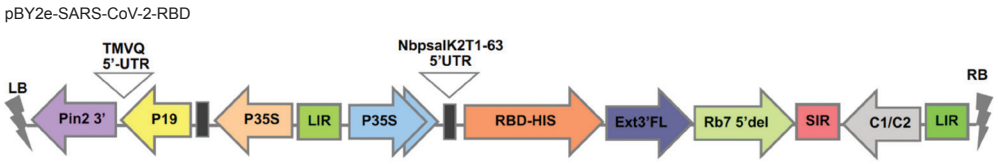

Figure 2. Schematic diagram of the T-DNA region of the geminiviral vector used in this study. pBY2e: geminiviral vector; RBD: receptor-binding domain; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; UTR: untranslated region.

Conjugation of colloidal gold nanoparticles with detecting reagents

The RBD and chicken IgY were conjugated with colloidal gold nanoparticles (AuNPs) and deposited at the conjugate pad as the detecting reagent. AuNPs, 40 nm in diameter, were obtained from Kestrel BioSciences Thailand Co. Ltd. (Pathumthani Province, Thailand). The pH of the AuNP suspension was adjusted to pH 8.0 with 0.2 M K_2CO_3. The plant-produced SARS-CoV-2 recombinant protein and chicken IgY (10 μg each, Kestrel BioSciences Thailand Co. Ltd.) were then both added to 1 mL AuNP colloid. After incubation for 10 minutes at room temperature, 10% (w/v) bovine serum albumin (0.1 mL, HiMedia Laboratories, Mumbai, India) was added to the mixture to block the AuNP surface. After incubation for 3 hours at room temperature, the mixture was kept at 4°C overnight. Next day, the AuNP–RBD–IgY conjugates were recovered after centrifuge, 9660 × *g*, 30 minutes, 4°C. The supernatant was discarded, and 1 mL of 1 mg/mL bovine serum albumin in borate buffer was added to the AuNP conjugate and re-suspended. Before dispensing to the conjugate pad, the AuNP–RBD–IgY conjugates were supplemented with 10% (w/v) sucrose and 5% (w/v) trehalose. The AuNP–RBD–IgY conjugates were then sprayed onto the conjugate pad at a rate of 10 μL/cm, then the conjugate pad was dried at 37°C for one hour. The resultant conjugate pad was stored at room temperature, in a dry place, and protected from light.

Preparation and assembly of the LFIA

The LFIA framework was demonstrated (**Figure 3**). The goat anti-human-IgM (1 mg/mL; Kestrel BioSciences Thailand Co. Ltd.), goat anti-human-IgG (1 mg/mL; Lampire Biological Laboratories, Pipersville, PA, USA), and goat anti-chicken IgY (1 mg/mL, Lampire Biological Laboratories) antibodies were immobilized on the IgM test line (M), IgG test line (G), and control line (C), as appropriate. The CN 140 backed nitrocellulose membrane (Unisart®, Sartorius Stedim Biotech GmbH, Goettingen, Germany) was striped with the abovementioned antibodies, at a rate of 0.1 μL/cm. The striped membrane was dried in a convection oven (Memmert UN160 Universal Laboratory, East Troy, WI, USA) at 37°C for 1 hour. The striped membrane was then assembled together with an AuNP–RBD–IgY conjugate pad, sample pad (Ahlstrom 1662, Ahlstrom-Munksjö CytoSep® 1662, Helsinki, Finland), and wick pad (Whatman grade 470, Sigma-Aldrich, St. Louis, MO, USA). Finally, the LFIA was assembled and defined as the Baiya 'Rapid COVID-19' IgM/IgG test kit.

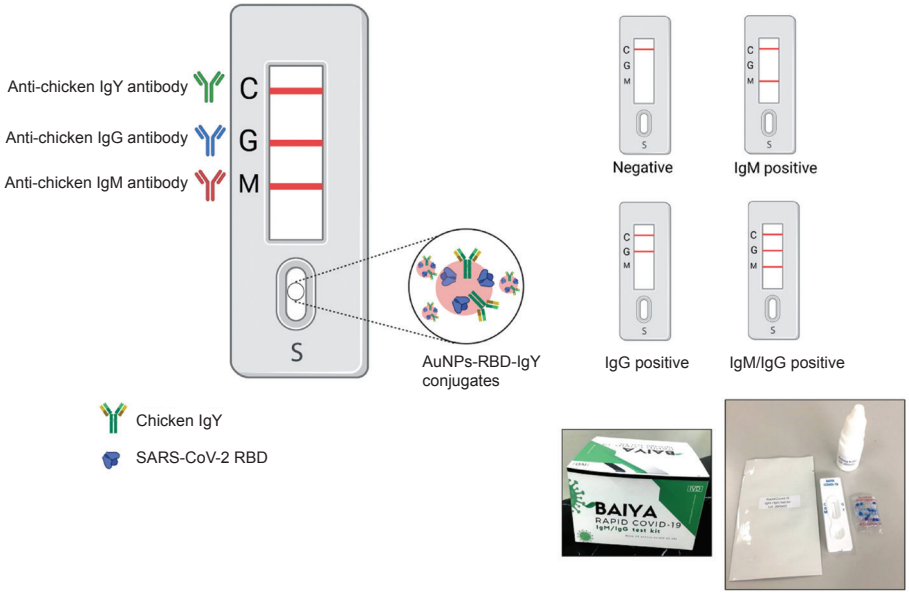

Figure 3. Schematic illustration of the developed Baiya' Rapid COVID-19' IgM/IgG test kit. Different test results are shown. AuNPs: gold nanoparticles; C: control line; G: test line for human IgG; M: test line for human IgM; RBD: receptor-binding domain; S: Sample pad; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

The purified SARS-CoV-2 RBD was tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (**Figure 4**). The bands of protein were detected visually using InstantBlue® (Expedeon, Cambridge, UK) staining. The specific analysis of SARS-CoV-2 RBD was done via western blotting. After transferring proteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the membrane was treated with skim milk solution. The SARS-CoV-2 RBD was probed with anti-His antibody (HRP conjugate, ab1187, Abcam, Cambridge, UK). Finally, SARS-CoV-2 RBD was detected with enhanced chemiluminescence plus detection reagent (Abcam).

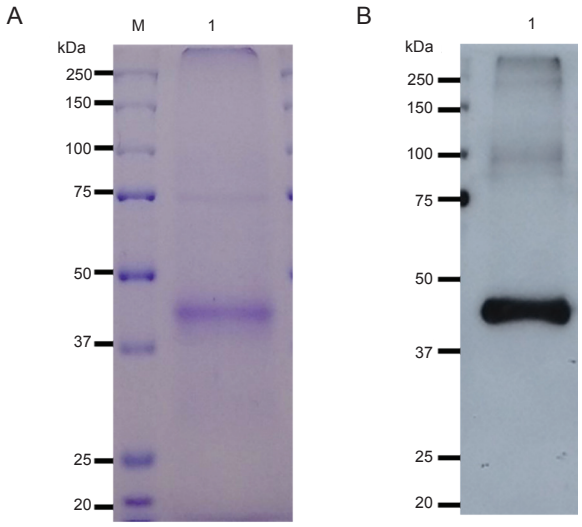

Figure 4. Western blot analysis of purified SARS-CoV-2 RBD protein from *Nicotiana benthamiana* agroinfiltrated with pBY2e-SARS-CoV-2-RBD. (A, B) The purified SARS-CoV-2 RBD protein was loaded at 4 μg/lane under reducing conditions and visualized with InstantBlue® (A) The purified SARS-CoV-2 RBD protein was loaded at 200 ng/lane under reducing conditions and detected with a horseradish peroxidase-conjugated rabbit anti-His antibody (B). M represents the protein molecular weight ladder, and lane 1 shows the purified SARS-CoV-2 RBD. pBY2e: geminiviral vector; RBD: receptor-binding domain; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Testing of COVID-19 samples using the LFIA system

Serum samples from SARS-CoV-2-infected patients (20 males and 31 females with averages ages of 33 and 42 years, respectively) and negative serum samples were tested using the developed Baiya' Rapid COVID-19' IgM/IgG test kit. The Ethics Committee (the Faculty of Medicine, Chulalongkorn University) approved this human sample research (COA No. 354/2020 and IRB No. 236/63) since March 20, 2020. The LFIA strip was taken from the sealed pouch immediately before use. Ten microliters of a patient's serum were added onto the sample-loading area, followed by two drops of phosphate-buffered saline with 0.05% (v/v) Tween-20. The results were read visually and interpreted 15 minutes after testing. The 51 serum samples from patients confirmed with SARS-CoV-2 infection were tested to evaluate the sensitivity of the LFIA system. The specificity of the LFIA was evaluated using 150 residual serum samples presumed negative for SARS-CoV-2 infection, which were obtained from the Thai Red Cross blood bank. The specificity and sensitivity of the rapid test kits were calculated as follows:

Sensitivity (%) = True positive/(True positive + False negative) × 100

Specificity (%) = True negative/(True negative + False positive) × 100

Results

Expression and purification of the SARS-CoV-2 RBD

Expression of the SARS-CoV-2 RBD in *N. benthamiana* was successfully achieved. As expected from the corresponding amino acid sequence, the purified SARS-CoV-2 RBD was observed to have a molecular weight of approximately 38 kDa. The SARS-CoV-2 RBD was applied as a diagnostic reagent for the COVID-19 LFIA .

Testing of COVID-19 samples using the LFIA system

The Baiya 'Rapid COVID-19' IgM/IgG test kit was designed and manufactured by Baiya Phytopharm Co., Ltd., Bangkok, Thailand. The test kit was made available in cassette form. The protocol was based on a lateral flow qualitative immunoassay for the rapid and simultaneous detection of anti-SARS-CoV-2 IgM and anti-SARS-CoV-2-IgG antibodies in human samples. When a patient is infected with SARS-CoV-2, anti-SARS-CoV-2 IgM and anti-SARS-CoV-2-IgG are produced. The antibodies used reacted with the RBD of AuNP–RBD–IgY conjugates in the conjugate pad. Then the AuNP–RBD–IgY conjugates bound to anti-SARS-CoV-2 IgM/IgG antibodies were captured by the antibody deposited on the test line, resulting in the appearance of a red line. If a serum sample was negative for anti-SARS-CoV-2 IgM/IgG antibodies, the test line(s) would not be observed. The control line

was always observed because AuNP–RBD–IgY conjugates could be captured by the anti-IgY antibody deposited as the control line. The Baiya 'Rapid COVID-19' rapid IgM/IgG test kit is shown in **Figure 3**. A total of three detection lines are present on the strip, the IgM (M), IgG (G), and control (C) lines. Development of the red line in the control position shows that the test is valid. If no colour develops on the control line, the test is invalid, and the test should be repeated with another cassette. The schematic interpretation of the result is presented in **Figure 3**. Representative test strips showing the results of serum samples collected from different Thai patients are shown in **Figure 5**. The developed IgM/IgG test kit has a sensitivity and specificity of 94.1% (48/51) and 98% (148/150), respectively. The performance of the LFIA using plant-made SARS-CoV-2 RBD is similar to those achieved with SARS-CoV-2 RBD produced using other expression systems.[20, 21] Overall, the plant-based SARS-CoV-2 RBD was effective for the development of a COVID-19 diagnostic assay.

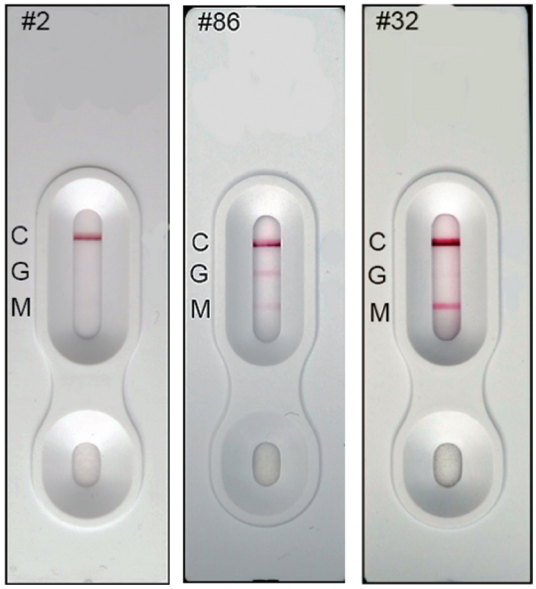

Figure 5. Representative test strips showing the results of serum samples collected from different patients in Thailand. The test sample is added to the sample well of the test cassette, where AuNP–RBD–IgY conjugates react with human anti-SARS-CoV-2 IgG and IgM in the sample. The sample then migrates through capillary action through the test and control lines. Sample #2 was found to be negative for both IgM and IgG, #86 was positive for IgM and IgG, while #32 was positive for IgM only. AuNPs: gold nanoparticles; C: control line; G: test line for human IgG; M: test line for human IgM; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Discussion

The serological testing of COVID-19 is crucial for disease management, and will help identify asymptomatic cases that could spread the virus further. As current vaccines or therapeutic measures are inadequate to control or treat the disease, prevention is the only option available to check and prevent the spread of infection.[22, 23] Current control measures aim at reducing the outbreaks of COVID-19. However, accurate, early diagnosis of the disease is critical to minimize further spread of the virus and resultant human losses. Consequently there is an urgent need to detect acute and chronic diseases in a simple, affordable, and rapid manner, which was the primary objective of the present study. Hence, an LFIA was developed using a recombinant plant-produced RBD antigen coupled with AuNPs as the detection label.

Plant expression systems offer many advantages for the production of recombinant proteins, which can be used as diagnostic reagents, vaccines, and drugs.[24] The main benefits include cost-effectiveness, the speed of transient expression for rapid large-scale production of recombinant proteins, and the system's safety as plants do not host any known human pathogens.[25] The plant-purified antigen was further used to make an LFIA cassette. The developed LFIA kit is intended for the *in vitro* qualitative detection of IgG and IgM antibodies against SARS-CoV-2 in human samples. The test results can be obtained in less than 15 minutes, and the results assessed visually. The LFIA developed here could make a considerable economic impact in reducing the costs of testing and reducing human mortality by identifying asymptomatic cases and containing the further spread of the virus. Usually, healthcare workers in hospitals and laboratories, especially in developing and under-developed countries, have to screen samples for COVID-19 mainly by polymerase chain reaction testing, which incurs a high cost. In contrast, the LFIA is comparatively cost-effective, can be performed even without professional help and could serve as a preliminary screening test to screen susceptible samples.[26, 27] Thus, the presented study demonstrates the potential of a plant expression system for the rapid large-scale production of recombinant proteins during epidemic situations and the efficiency of LFIA technology for achieving rapid non-laborious point-of-care diagnosis of COVID-19. To the best of our knowledge, this is the first diagnostic kit that uses a plant-produced recombinant protein against SARS-CoV-2 for the diagnosis of COVID-19. The developed assay could represent

a considerable improvement in COVID-19 diagnosis.

In summary, we evaluated a rapid point-of-care COVID-19-specific LFIA developed using a plant-produced recombinant RBD antigen to detect SARS-CoV-2-specific IgG and IgM. The specific recognition of RBD-SARS-CoV-2 by the IgG/IgM antibodies of COVID-19-infected patients emphasizes the potential of the plant-produced RBD protein for COVID-19 serodiagnosis. The developed LFIA device will help healthcare workers detect the presence of antibodies in patient serum samples in less than 15 minutes. This rapid screening test offers many advantages, including a reduced requirement for sample processing requirement, cost-effectiveness, and ease of use, as well as being easily portable, time-efficient, and technologically feasible, making these assays compatible with clinical application in Thai hospitals. Because patients with a post-dengue virus infection or with rheumatoid factor IgM possibly produced false results of LFIA.[28, 29] Baiya' Rapid COVID-19' rapid IgM/IgG test kit should be the further test to identify these interferences from various patient conditions.

Author contributions

KR, GY, BS and WP performed the expression and analysis of SARS-CoV-RBD protein. KK, PS, EP and ST performed the analysis and collected the data. ST and WP conceived and designed the analysis and managed the research project. All authors wrote, revised, and approved the manuscript.

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Conflicts of interest statement

Suthira Taychakhoonavudh and Waranyoo Phoolcharoen from Chulalongkorn University are co-founders/shareholders of Baiya Phytopharm Co., Ltd.

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