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A luciferase based automated assay for rapid and sensitive detection of SARS-CoV-2 antibodies

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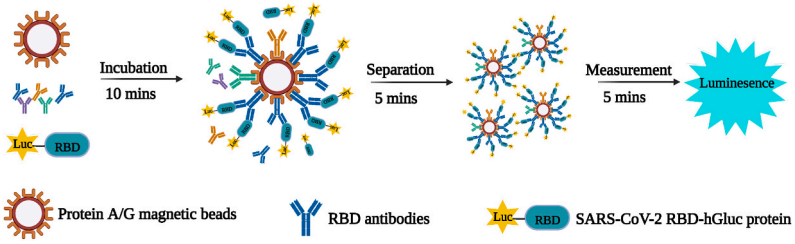
HIGHLIGHTS

- Fast and sensitive detection of SARS-CoV-2 antibodies.
- Luciferase immunoprecipitation system combined with automatic platform.
- Detecting RBD specific antibodies which can predict the viral neutralizing capability.

GRAPHICAL ABSTRACT



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ABSTRACT

The Coronavirus disease 2019 (COVID-19) pandemic brings great challenges to the public health and social economics around the world. As the pandemic continues and the mass vaccination goes on, monitoring the antibodies is particularly important for the epidemiological survey and vaccine assessment. Here, we developed a luciferase immunoprecipitation assay combined with an automated platform to detect anti-Receptor Binding Domain (RBD) antibody, where protein A and protein G modified magnetic beads were used to capture antibodies in serum samples and SARS-CoV-2 RBD was fused with Gaussia luciferase to label the captured target antibodies. The whole detection procedure can be completed within 20 min. The developed assay has proven up to 32 times more sensitive than ELISA for the detection of RBD antibodies. Furthermore, the results of the antibody detection of sera from vaccination as well as convalescence displayed good performance. The automated platform may provide a powerful tool for the control of COVID-19 pandemic by vaccination and the research of SARS-CoV-2 seroconversion.

1. Introduction

Coronavirus disease 2019, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been ongoing for two and a half years as a globe pandemic, which has caused more than five hundred millions confirmed cases and six millions deaths (https://www.who.int/emergencies/diseases/novel -coronavirus-2019, accessed on June 20, 2022).

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As a strategy against the pandemic, the vaccination has been applied worldwide. Thus the investigation of antibodies against SARS-CoV-2 from vaccination should be performed to evaluate the situation of the mass vaccination immunity and the corresponding policy. Further, as the pandemic continues and SARS-CoV-2 evolves, there are a lot of asymptomatic patients, which brings troubles for the current gold standard of the real-time reverse transcription polymerase chain reaction (RT-PCR) [1]. Therefore, many serological tests of COVID-19 have been developed and widely used to monitor the dynamics of the antibody levels for convalescent patients and vaccinated healthy people [2], as well as acted as a supplementary method for the diagnosis of COVID-19 and screening asymptomatic patients [3]. The most commonly used method for serological tests is enzyme-linked immunosorbent assay (ELISA) [4], which can qualitatively and quantitatively determine SARS-CoV-2 antibodies with excellent sensitivity and specificity. However, ELISA is labor-intensive and time-consuming, and it usually takes more than 2 h for the whole procedure. Lateral flow immunoassays (LFIA), which is a rapid and user-friendly method for point-of-care test, but the sensitivity and specificity of LFIA usually much lower than ELISA [5]. Several chemiluminescent immunoassay (CLIA) platforms are highly automated and suitable for high-throughput detection with competitive sensitivity and specificity [6–8]. However, the requirement of expensive equipment limited their wide application in developing countries. Thus, there is an urgent need to develop fast, sensitive and convenient methods to detect antibody against SARS-COV-2 to control the COVID-19 pandemic.

Here, we described an automated system with a luciferase immunoprecipitation system (LIPS) based on fusing SARS-COV-2 receptor binding domain (RBD) with Gaussia luciferase as the reporter to detect anti-RBD antibodies. The whole procedure can be finished within 20 min through performing this assay in an automatic platform with much better sensitivity compared to an indirect ELISA method. In addition, our method would not require sophisticate instruments, making it easy to perform and cost-effective. The anti-RBD antibodies were proven to have strong correlation with neutralizing antibody against SARS-CoV-2 and may be used to predict the viral neutralizing activity of sera, thus, it has a potential usage for COVID-19 diagnosis, disease stage and vaccination evaluation and the duration of neutralizing antibody.

2. Materials and methods

2.1. Samples

Anti-RBD positive serums were collected mainly from three groups: seventeen serum samples from the convalescent patients infected by SARS-CoV-2 lineage B.1, eleven vaccinated serum samples were collected from healthy volunteers at about 3 weeks after a 2nd dose of the inactivated vaccination manufactured by Sinopharm WIBP (Wuhan Institute of Biological products Co., Wuhan, China), twelve samples were from a 3rd dose vaccinated volunteers at a range from 2 to 3 months after getting vaccination, and ten serum samples collected before the pandemic were used as negative control. All sera were stored at −80 °C until use. The study has been approved by the ethical committee of Wuhan Institute of Virology, Chinese Academy of Sciences (No. WIVH17202102).

2.2. Plasmid construction and protein expression

Plasmid pCAGGS-RBD-Fc containing the gene of spike protein RBD (residues 319-541) of SARS-COV-2 B.1 strain was a generous gift from Prof. Rui Gong (Wuhan Institute of Virology, China). The humanized Gaussia luciferase (hGluc) gene contained plasmid pET28a-hGluc was constructed previously in the lab. Primers (synthesized by Sangon Biotech, Shanghai, China) (hG-forward: 5′-cagagagggatccGGCGGTGGCGGAAGCATGAAGCCCACCGAGAACAACG-3’; hG-reverse: 5′-ctgccgttcgacgatctcgagCGTCACCACCGGCCCCCTT-3’; Ph-forward: 5′-cacgcctactcagcggccgcGGAGGCGGATCCGGCGGT-3’; Ph-reverse: 5′-gagggaaaaagatctgctagcTCGAGGCTGATCAGCGGG-3′) were used to amplify hGluc gene, which was further cloned into pCAGGS-RBD-Fc to replace the Fc fragment by NotI and NheI restriction sites, the correctly constructed plasmid pCAGGS-RBD-hGluc was confirmed by sequencing. RBD-hGluc protein was then expressed in HEK293F cells. Briefly, 40 μg pCAGGS-RBD-hGluc plasmids were transfected to HEK293F cells by polyethylenimine (PEI) at a density around 10^6 cells/mL. At 72 h after transfection, cell supernatant was collected and concentrated using 5 kDa ultrafiltration tubes (Merck Millipore, Billerica, MA, USA) with centrifugation of 4000 g. The concentrated supernatant was incubated with Ni Sepharose (GE Healthcare) overnight at 4 °C with constant rotation, and then RBD-hGluc protein was purified with gradient imidazole buffer elution. SDS-PAGE and Western-blot were used to confirm the expression of RBD-hGluc protein, and the protein concentration was determined by a BCA protein assay kit (Thermo Fisher, Rockford, IL, USA).

2.3. Enzyme-linked immunosorbent assay (ELISA)

As previously described, recombinant RBD protein of SARS-CoV-2 B.1 strain (Acro Biosystems, Beijing, China) was utilized to perform ELISA assay [9]. Briefly, ELISA plates (Jet Biofil, Guangzhou, China) were coated with 100 μL/well of carbonate buffer (pH = 9.6) containing recombinant RBD proteins (5 μg/mL) and incubated at 4 °C overnight. Afterward, the plates were washed with PBS containing 0.05% Tween 20 (PBST), blocked with 330 μL/well of 5% skim milk powder (Oxoid, Altrincham, UK) for 2 h at 37 °C and then washed with PBST. Sera were diluted with PBST at 1:20 and added to the coated wells (100 μL/well). The plates were incubated at 37 °C for 1 h and then washed thoroughly with PBST. Finally, horseradish peroxidase (HRP) labeled rabbit antibodies against human IgG (Biodragon Immunotechnologies, Suzhou, China) at a dilution of 1:10,000 in PBST (100 μL/well) were added into the pates and incubated for 1 h at 37 °C. After washing with PBST, the plates were added with 100 μL/well of 3,3,5,5-Tetramethylbenzidine (TMB) (Tiangen Biotech, Beijing, China) and incubated for 10 min at room temperature. After the reaction was stopped by 2 M H_2SO_4, optical density (OD) was measured at 450 nm using a microplate reader (INFINITE 200 PRO, TECAN, Männedorf, Switzerland). The sensitivity of ELISA was determined by serially diluting an antibody-positive serum sample, which was compared with the sensitivity of the method developed in this study. The lowest dilution was looked as the limit of detection. The specificity of ELISA was tested through detecting SARS-CoV-2 RBD antibody negative samples.

2.4. Procedure of RBD-hGluc LIPS assay to detect antibody against SARS-CoV-2 RBD

The principle of RBD-hGluc LIPS assay was illustrated in Fig. 1. As displayed in Fig. 1, during the incubation, protein A/G magnetic beads captured the antibodies from serum sample and RBD-hGluc protein specifically bound with RBD antibodies. After washing to remove the free and non-specifically absorbed RBD-hGluc protein, the separated beads were used for the detection. The procedures for this assay were described as followed. Briefly, magnetic beads coated with protein A/G (Yeasen, Shanghai, China) were washed to remove preservatives then suspended in buffer A (20 mM Tris, 500 mM NaCl, 1% Triton X-100, pH 7.5). Human sera and RBD-hGluc proteins were diluted, respectively, with buffer A containing 5% skim milk powder. 10 μL protein A/G magnetic beads, 50 μL diluted human serum and 40 μL diluted RBD-hGluc proteins were mixed well and incubated for 10 min at 37 °C to form the complex of protein A/G-IgG-RBD-hGluc on the surface of magnetic beads. After thoroughly washing, 5 μL 200 μM coelenterazine (Glpbio Inc., Montclair, CA, USA) were then added in the suspended magnetic beads for the measurement of relative light unit using a SynergyTM H1 multi-mode microplate reader (Bio-Tek, now Agilent, USA).

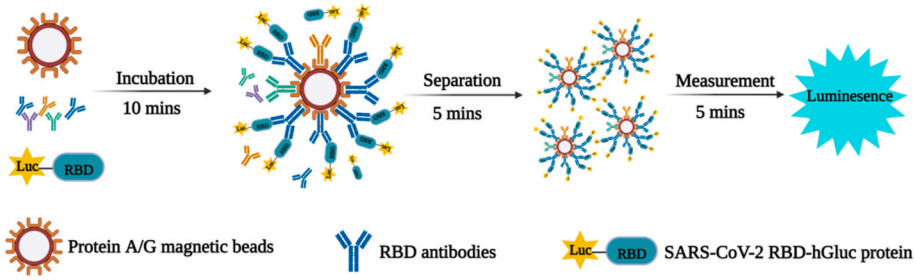

Fig. 1. The principle and the main steps of RBD-hGluc LIPS assay for RBD antibody detection. This schematic was created with biorender.com.

The incubating and washing steps were completed on an automated platform (Jifan Biotechnologies Inc, China) with 4 rows of 8-channel magnet rods could load two plates, so 32 samples could be tested simultaneously.

2.5. Statistical analysis

The unpaired Student's *t*-test was carried out to identify the differences between positive and negative groups. p value of less than 0.05 was considered to be significantly different. The cutoff value of RBD-hGluc LIPS assay was determined by maximizing the Youden's J index based on the receiver operating characteristic (ROC) analysis. All statistical analyses were performed by using GraphPad Prism 7 software.

3. Results

3.1. Characterization of recombinant RBD-hGluc protein

In order to characterize the molecular weight and the luminescent activity of the purified recombinant RBD-hGluc, SDS-PAGE, Western blot and luminescence measurement were run for the purified protein product. The calculated molecular weight of recombinant RBD-hGluc is about 50 kDa. As shown in Fig. 2A, the molecular weight of the purified recombinant RBD-hGluc protein falls the position between 50 kDa and 60 kDa on the SDS-PAGE gel, which is corresponding to the band on the polyvinylidene difluoride (PVDF) membrane at around 55 kDa (Fig. 2B), where a 3rd dose vaccinated human serum was used for Western blot analysis. The difference between the theoretical molecular weight and the realistic molecular weight was due to probable glycosylation in the Eukaryotic expression protein. The luminescence of serial diluted RBD-hGluc protein showed a linear relation with the concentration of RBD-hGluc protein (Fig. 2C), demonstrating a deserved luciferase activity of purified RBD-hGluc protein.

3.2. Optimization of the conditions for RBD-hGluc LIPS assay

We optimized four main factors that influence the detection of anti-RBD antibodies, the amount of protein A/G magnetic beads, the concentration of RBD-hGluc, incubation time and dilution times of serum samples.

The amount of protein A/G magnetic beads decided the total antibodies that could be captured. Inadequate protein A/G magnetic beads would result in not binding enough anti-RBD antibodies for signal detection, but excessive beads would cause high signal background because of nonspecific binding of RBD-hGluc protein. Here, S/N, which means signal of the luminescence of the positive samples over that of a negative sample. With RBD-hGluc protein of 12.5 μg/mL, the incubation time of 10 min and serum dilution of 100, a plot of the S/N ratio versus the amount of magnetic beads was illustrated in Fig. 3A, which showed that a good S/N ratio was obtained with 10 μL magnetic beads, so the amount of the magnetic beads of 10 μL was used for following optimization.

RBD-hGluc protein, which is used to bind most of anti-RBD antibodies in serum samples, is another important factor to affect the performance of the assay. Too low concentration of RBD-hGluc protein would weaken the sensitivity of this assay while too high concentration would lead to a significant increase of background values as the RBD-hGluc protein non-specifically adsorb on the surface of beads, and then result in high S/N. With 10 μL proteinA/G magnetic beads, the incubation time of 10 min and serum dilution of 100, serial concentrations of RBD-hGluc protein were tested. As shown in Fig. 3B, the S/N value at the concentration of 3.125 μg/mL was slightly higher than 6.25

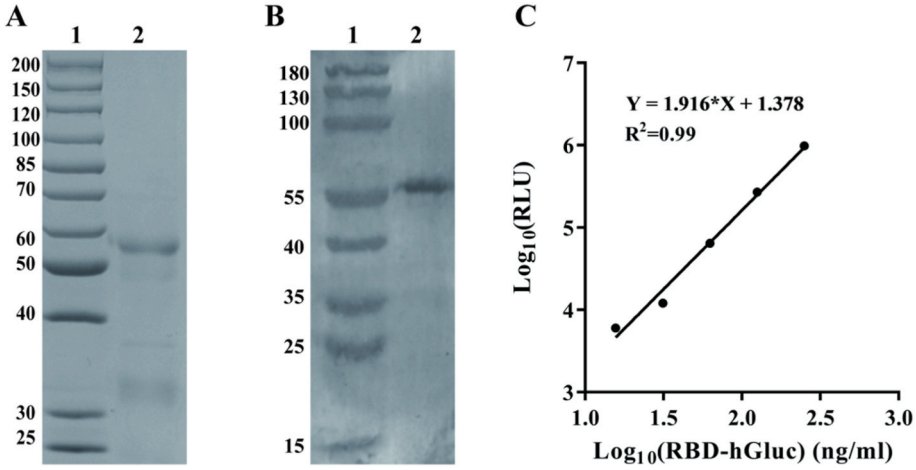

Fig. 2. Characterization of the recombinant RBD-hGluc. A. SDS-PAGE, Lane 1, protein marker (the unit is kDa); lane 2, purified RBD-hGluc with a molecular weight at around 55 kDa; B. Western-blot, Lane 1, protein marker (the unit is kDa); lane 2, RBD-hGluc hybridized with a 3rd vaccinated human serum; C. Luminescence of Gaussia luciferase versus the concentration of RBD-hGluc. Data were shown as the mean from three replications. Error bars were too small to show.

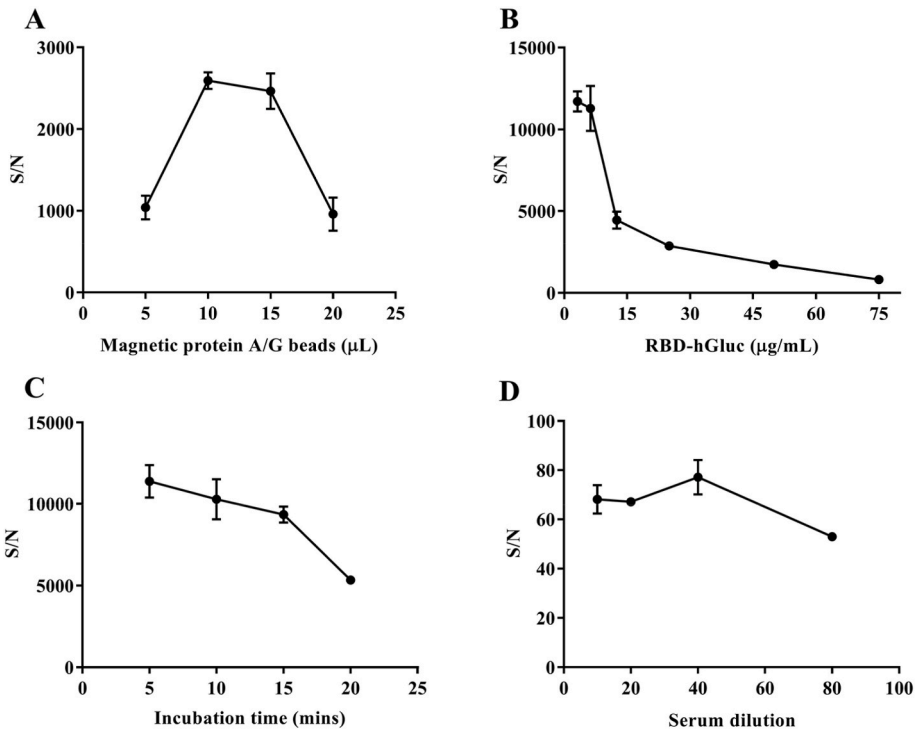

Fig. 3. Optimization of the RBD-hGluc LIPS assay conditions. The plots of S/N ratios versus the amount of protein A/G-modified magnetic beads (A), RBD-hGluc concentrations (B), incubation time (C) and dilution folds of sera (D) were displayed. Error bars represent the standard deviations from triplicates.

μg/mL because of the low negative luminescence although the luminescence at 3.125 μg/mL also decreased much. For weakly positive samples, the opportunities of RBD antibodies binding to RBD-hGluc protein decreased and less RBD antibodies were captured by the magnetic beads. In order to obtain higher luminescent values for weakly positive samples, a higher concentration of RBD-hGluc protein (6.25 μg/mL) was chosen for the following optimization.

Likewise, adequate incubation time should be set for protein A/G beads to capture antibodies and RBD-hGluc protein bind to target antibodies, but prolonged incubation time would result in the same problem of high luminescence of the negative sample as the two factors mentioned above. We used optimized the incubation time with the conditions of 10 μL proteinA/G magnetic beads and RBD-hGluc protein of 6.25 μg/mL and the serums dilution of 100. As displayed in Fig. 3C, the incubation time from 5 min to 15 min indicated a platform of S/N, so 10 min was used for the assay since fast is important for the assay.

Dilutions of the serum samples used in this assay also has impact on the S/N ratio, since the matrices of human serum is complex and varies with people. Too low dilutions of the serum may result in high luminescence of the negative samples, which would decrease the S/N ratio. However, too high dilutions of the serum samples would lead to too low target antibodies to detect, especially for the positive samples with low titer of anti-RBD antibodies. Therefore, a positive serum sample with low titer of anti-RBD antibody was used for this optimization to assure that similar low titer samples could also be detected. The three optimized conditions mentioned above were adopted to optimize the serum dilution. As shown in Fig. 3D, the dilution of 1:40 indicated a high S/N, and then the dilution of 1:40 was used for the assay of the target antibodies in the serum samples.

3.3. Determination of the cutoff value

Fifty human serum samples were used to determine the cutoff value of the LIPS method for anti-RBD antibodies detection. Thirty nine serum samples were determined as positive and others were negative by the RBD-based indirect ELISA method, which was also coordinated with the sources of each serum. As shown in Fig. 4A, the level of RBD antibodies in human serums measured by LIPS assay was represented by the relative luminescence units (RLU). There is a significant difference between positive group and negative groups ($P < 0.0001$). We determined the cutoff values of 828 based on the ROC curve (Fig. 4B) and the ROC analysis showed 100% sensitivity and 100% specificity compared with the indirect ELISA method.

3.4. Sensitivity of the RBD-hGluc LIPS assay

Serial dilutions of a 3rd vaccinated sera and a negative serum were used to compare the sensitivity of the LIPS assay with the ELISA method. As shown in Fig. 5A, the LIPS assay could still give positive results after diluting up to 655,360 times, while the ELISA method could detect up to dilutions of 20,480 times (Fig. 5B). Therefore, the LIPS assay is about 32 times more sensitive than the ELISA method.

4. Discussion

Previous studies has corroborated that the receptor binding domain of the SARS-CoV-2 spike protein could bind to the human angiotensin-converting enzyme 2 (ACE-2) receptor for the entry of the virus into the cell [10], and RBD as important immunodominant [11] contains multiple epitopes that can elicit immune system to produce neutralizing antibodies and has been used for vaccine development [12–14]. Sera containing a high level of anti-RBD antibodies could potentially prevent the invasion of SARS-CoV-2 [15,16], thus, our method has the potential to be used for evaluating the effectiveness of vaccination.

The magnetic beads based LIPS assay has shown some advantages over traditional ELISA: 1) the magnetic beads made the whole procedure easier and possible for high through-put detection easily handled by an automatic platform; 2) since the LIPS assay was performed in liquid phase, the conformation of RBD in the solution was more close to its native structure than those binding on the solid phase like the ELISA, which would make the binding reaction faster than ELISA; 3) further, humanized Gaussia luciferase we used could produce over 1000-fold

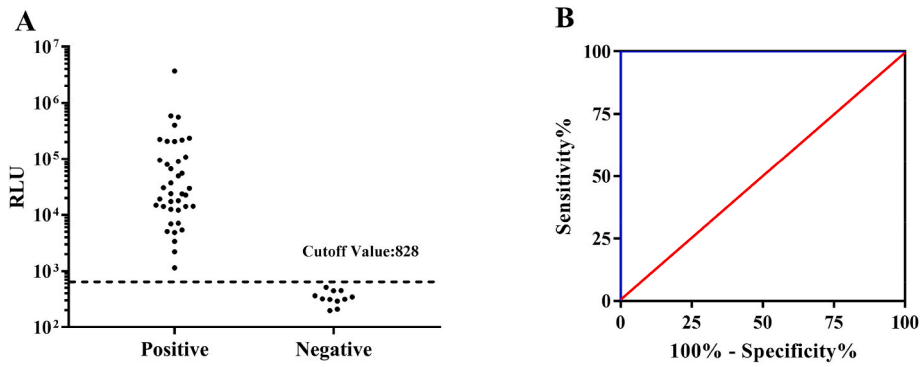

Fig. 4. Determination of the cutoff value of the LIPS assay for anti-RBD antibody detection through ROC. (A) RLU obtained by LIPS assay from a panel of the sera classified as positive or negative by ELISA. (B) ROC curve based on the data obtained.

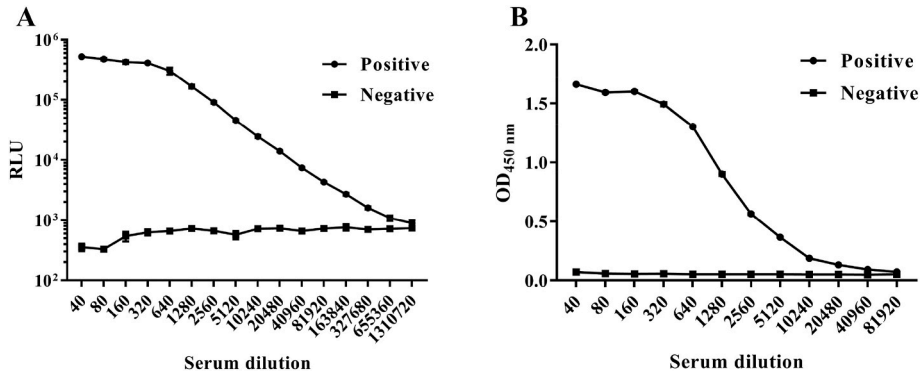

Fig. 5. The results of LIPS assay (A) and ELISA (B) for serial dilutions of positive and negative serum samples.

higher bioluminescent signal than other commonly used Renilla or Firefly luciferases according to previous reports [17], which enhanced the sensitivity of this assay, 4) in addition, the relative small molecular weight of hGluc (169 amino acids), which is much smaller than Renilla (312 amino acids) or Firefly luciferase (550 amino acids), endues it an excellent reporter for fusing antigen proteins; 5) Besides, the protein A/G non-specifically binds to the Fc fragment of antibody [18] and RBD-hGluc as the reporter allowing this assay suitable for muti-species antibodies detection without the border to prepare species-specific labeled secondary antibodies, which would contribute to the serological survey for SARS-CoV-2 susceptible animals, also the measurement of the total antibodies against RBD instead of a certain immunoglobulin meaning this method available for the diagnosis of early SARS-CoV-2 infections through detecting IgM antibodies.

There are some limitations for the RBD-hGluc LIPS assay. First, the native coelenterazine is unstable in aqueous solutions [19], and the working coelenterazine solution would auto-oxidize, resulting in a background signal of around 100 to 200 in the absence of hGluc reporter and the decrease of bioluminescence at the same concentration of hGluc. Therefore, stabilization composition of coelenterazine for high-throughput detection is indispensable to apply the assay for high throughput detection and POCT. Another issue is the rapid mutation of SARS-CoV-2 virus, especially heavy mutations on the spike protein RBD like Omicron variants, which would make most antibodies induced by early SARS-CoV-2 infection and inactivate vaccine invalid against the variants [20,21]. Therefore, for variants with heavy mutations in RBD, it is considerable to express Omicron-RBD-hGluc proteins instead of the wild-type RBD for further detection of antibodies targeting these variants.

In conclusion, we established a LIPS assay based on protein A/G magnetic beads combined with an automatic platform for rapid and sensitive detection of anti-RBD antibodies. It takes less than 20 min for the whole procedure, much faster and more sensitive than ELISA. The assay can be performed in normal laboratories because the equipment requirements are minimal. In addition, the assay needs 2.5 μL of serum, less than a drop of blood sample, therefore, the RBD-hGluc LIPS assay may provide a cost-effective solution to long-term tracking detection of RBD antibodies in convalescent and vaccinated people.

CRediT authorship contribution statement

Wenhao Zhou: Formal analysis, Conceptualization, Data curation, Methodology, Writing – original draft. **Ping He:** Data curation, Formal analysis, Methodology. **Huan Liu:** Formal analysis. **Hongping Wei:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing. **Junping Yu:** Funding acquisition, Conceptualization, Investigation, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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