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ORIGINAL ARTICLE

# Determination of human COVID-19 total antibodies in serum using a time-resolved fluorescence immunoassay

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## Abstract

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is spreading rapidly around the world. Antibody detection plays an important role in the diagnosis of COVID-19. Here, we established a new time-resolved fluorescence immunoassay (TRFIA) to determine COVID-19 total antibodies. A double-antigen sandwich TRFIA was optimized and established: recombinant nucleocapsid phosphoprotein (N protein) and spike protein (S protein) of COVID-19 immobilized on 96-well plates captured human COVID-19 antibodies and then banded together with the N/S proteins labeled with europium(III) ($Eu^{3+}$) chelates, and finally, time-resolved fluorometry was used to measure the fluorescence values. We successfully established a TRFIA method for the detection of human COVID-19 total antibodies, and the cutoff value was 2.02. There was no cross-reactivity with the negative reference of the National Reference Panel for IgM and IgG antibodies to COVID-19. The CV of the precision assay was 3.19%, and the assay could be stored stably for 15 days at 37°C. Compared with that of the colloidal gold method and chemiluminescence method, the sensitivity of the TRFIA method was higher, and the false positive/negative rate was lower. This established TRFIA has high sensitivity, accuracy, and specificity, which indicates that this method provides a new detection method for the high-throughput routine diagnosis of COVID-19.

**KEYWORDS**
COVID-19, double-antigen sandwich, nucleocapsid phosphoprotein, spike protein, time-resolved fluorescence immunoassay, total antibodies

**Abbreviations:** COVID-19, corona virus disease 2019; SARS-CoV-2, corona virus 2; TRFIA, time-resolved fluorescence immunoassay; N protein, nucleocapsid phosphoprotein; S protein, spike protein; Eu3+, Europium(III); RT-PCR, real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; Fs, Fluorescenceserum sample; Fb, Fluorescenceblank control; SD, standard deviation; CV, coefficient of variation.

## 1 | INTRODUCTION

A novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is causing the rapid spread of coronavirus disease 2019 (COVID-19) worldwide. As of April 2021, there were over 150 million confirmed cases, 3 million confirmed deaths, and 216 countries/areas or territories with COVID-19 cases.[1] Accurate and fast diagnosis of SARS-CoV-2 is important to isolate patients with COVID-19 in a timely manner and stop the pandemic. Currently, viral nucleic acid assay using real-time polymerase chain reaction (RT-PCR) assay is the standard for COVID-19 diagnosis.[2] Additionally, serologic tests to identify COVID-19 antibody dynamics and response to SARS-CoV-2 have been developed.[3,4]

Human COVID-19 antibody detection, as a complementary approach to viral nucleic acid assays, has attracted attention.[5] In China, rapid colloidal gold, enzyme-linked immunosorbent assay (ELISA), and chemiluminescence assay approaches using a recombinant antigen to detect IgM, IgG, or total antibodies have been established and widely used in the clinic.[5–8] However, due to the limitations of colloidal gold, ELISA, and chemiluminescence methods themselves, their sensitivity, accuracy, and specificity are not high enough, and there are high false-positive and false-negative rates.[9] A variety of new detection methods are constantly being researched and explored.[5,9,10]

In this study, we optimized and established a time-resolved fluorescence immunoassay (TRFIA) for human COVID-19 total antibody detection. Compared with the colloidal gold method and chemiluminescence method, the TRFIA method has better detection performance, and it could represent a complementary approach to the COVID-19 nucleic acid assay.

**Highlights**

- The double antigen sandwich time-resolved fluorescence immunoassay (TRFIA) method has high sensitivity, accuracy, and specificity for COVID-19 total antibodies detection.
- TRFIA provides a new detection method for COVID-19 total antibodies.
- TRFIA may be one of the complement approaches for COVID-19 nucleic acid assay.

## 2 | MATERIALS AND METHODS

### 2.1 | Antigen, antibody, and samples

Recombinant SARS-CoV-2 nucleocapsid phosphoprotein (N protein, *Escherichia coli*) of SARS-CoV-2 was prepared by the School of Laboratory Medicine and Biotechnology, Southern Medical University (Guangzhou, China).[8] Recombinant SARS-CoV-2 spike protein (S protein, *E. coli*) of SARS-CoV-2 was prepared by Guangzhou Youdi Biotechnology Co., Ltd. (Guangzhou, China). A total of 225 healthy people serum samples were obtained from the First Affiliated Hospital of Guangzhou Medical University and were stored at −80°C. Serum samples of eight patients (Table S1) diagnosed with COVID-19 were obtained from the First Affiliated Hospital of Guangzhou Medical University and were stored separately at −80°C. The National Reference Panel for IgM and IgG antibodies to COVID-19 came from the National Institutes for Food and Drug Control of China. The Institutional Review Board of the First Affiliated Hospital of Guangzhou Medical University approved this study, and all participants gave written informed consent.

### 2.2 | Reagents, solutions, and instrumentation

A europium(III) ($Eu^{3+}$) labeling kit was purchased from PerkinElmer (Norwalk, USA). Tween-20, bovine serum albumin (BSA), Triton X-100, $\beta$-naphthoyltrifluoroacetate, tri-*n*-octylphosphine oxide, and Tris were procured from Sigma-Aldrich (St. Louis, USA). A Sephadex G50 column was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). A COVID-19 viral nucleic acid assay kit (real-time PCR method) was purchased from DAAN GENE (Guangzhou, China, Registration certificate no. 20203400063). A COVID-19 total antibody detection kit (colloidal gold method) was purchased from Wondfo (Guangzhou, China, Registration certificate no. 20203400176). A COVID-19 total antibody detection kit (chemiluminescence method) was purchased from InnoDx (Xiamen, China, Registration certificate no. 20203400198). Ninety-six-well plates and a time-resolved analyzer were purchased from PerkinElmer (PerkinElmer, DELFIA1235, USA). Other chemicals and reagents used were analytical grade. The washing buffer was 20 mmol/L Tris-HCl (pH 8.0) supplemented with 0.9% NaCl and 0.05% Tween-20 (v/v). The blocking buffer was 50 mmol/L PBS (pH 7.4) supplemented with 5% BSA (m/v). The labeling buffer was 50 mmol/L $Na_2HCO_3$-$Na_2CO_3$ (pH 9.0) supplemented with 0.9% NaCl. The assay buffer was 50 mM Tris-HCl (pH 7.8) supplemented with 0.02% BSA, 0.05% Tween-20, and 0.02% Proclin300. The enhancement solution was 0.1 mol/L acetate-phthalate buffer (pH 3.2) containing

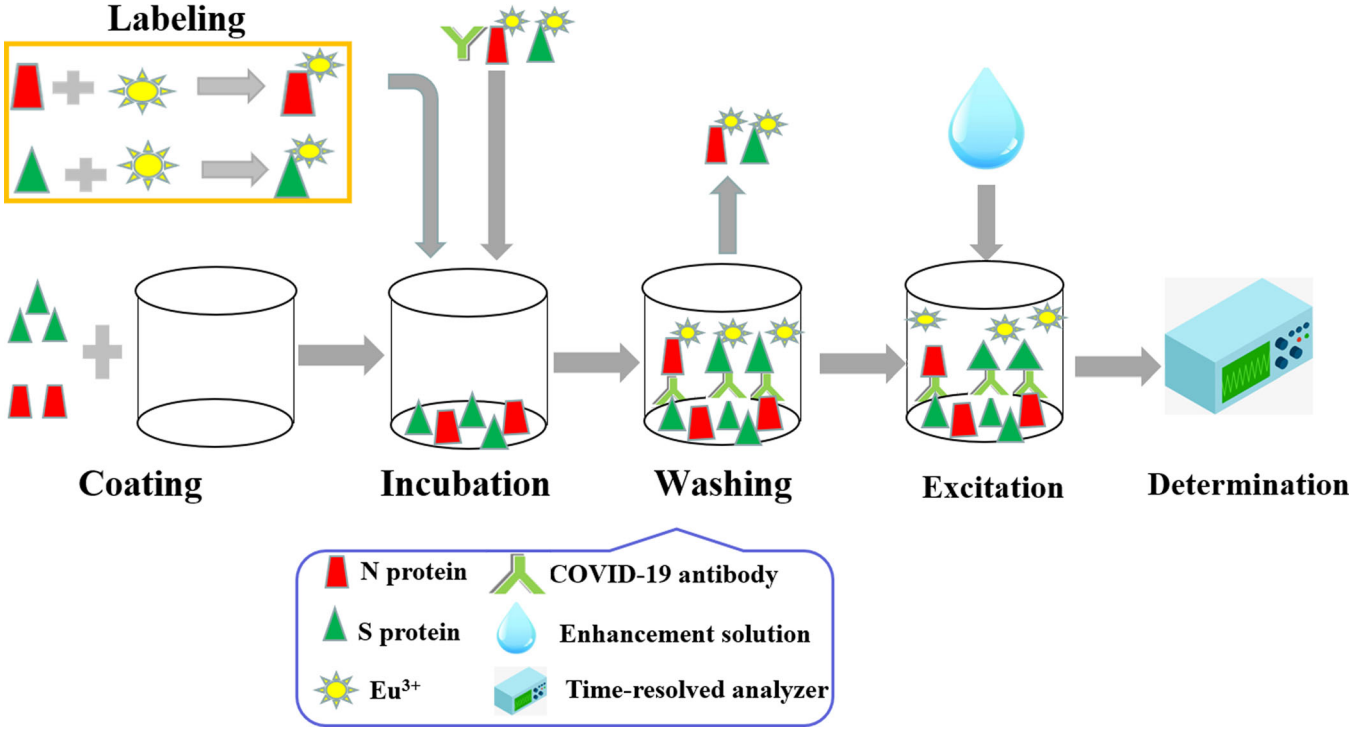


**FIGURE 1** Scheme of the present TRFIA for human COVID-19 total antibody detection

0.1% Triton X-100, 20 $\mu$mol/L $\beta$-naphthoyltrifluoroacetate, 50 $\mu$mol/L tri-n-octylphosphine oxide, and 0.5% glacial acetic acid.

## 2.3 | Optimized coating procedure of N and S proteins

The N protein and S protein solutions were prepared at concentrations of 5 $\mu$g/ml to optimize the optimal N:S coating ratio. The coating procedure was as follows: different volumes of SARS-CoV-2 N protein and S protein were added to 96-well plates to coat them at 37°C for 2 h. After washing three times using washing buffer, the plates were blocked with 250 $\mu$l/well blocking buffer for 1.5 h at 37°C. Then, the blocking buffer was removed, dried under vacuum, and finally stored in 96-well plates at −20°C.

## 2.4 | Optimized labeling procedure of N(S) protein

According to the protocol provided by the $Eu^{3+}$ labeling kit, the N protein and S protein were labeled with $Eu^{3+}$ chelates. Briefly, 2 mg N(S) protein was washed with labeling buffer three times, and then, 300, 500, 800, or 1000 $\mu$g $Eu^{3+}$ chelates were added. The mixture was gently shaken for 16 h at room temperature, and then, the $Eu^{3+}$-labeled N(S) protein was purified using a Sephadex G50 column and monitored with a fluorescence analyzer. Finally, purified $Eu^{3+}$-labeled N(S) protein was dried under vacuum and stored at −20°C.

## 2.5 | Optimized assay procedure

A one-step assay procedure was established by optimizing the following parameters: the volume of the standards/samples, coated N and S protein, $Eu^{3+}$-labeled N protein and $Eu^{3+}$-labeled S protein, enhancement solution, and the total reaction time. Briefly, serum samples or standards and $Eu^{3+}$-labeled N(S) protein were added to coated 96-well plates and then incubated at room temperature. After washing the wells, enhancement solution was added to the wells and shaken gently for a few minutes. Finally, a time-resolved analyzer was used to measure the fluorescence. The scheme of the present TRFIA for human COVID-19 total antibody detection is shown in Figure 1.

## 2.6 | Qualitative criteria

This TRFIA method was used to detect 225 healthy people serum samples. The fluorescence values of the serum samples and blank control (background values)

were recorded, and the ratio of $Fluorescence_{serum\ sample}$/$Fluorescence_{blank\ control}$ (Fs/Fb) of each sample was calculated.

## 2.7 | Specificity assay

A specificity assay was performed using the negative/positive reference of the National Reference Panel for IgM and IgG antibodies to COVID-19, including influenza A IgM-positive serum, influenza A/B IgM-positive serum, *Legionella pneumophila* IgM-positive serum, *Chlamydia pneumoniae* IgM-positive serum, rheumatoid factor-containing serum, *Mycoplasma pneumoniae* IgM-positive serum, respiratory syncytial virus IgM-positive serum, *Chlamydia pneumoniae* IgG-positive serum, measles virus IgG-positive serum, mumps virus IgG-positive serum, normal healthy people serum, and COVID-19-diagnosed patient serum.

## 2.8 | Precision and stability assay

Precision assay: The precision reference of the National Reference Panel for IgM and IgG antibodies to COVID-19 was mixed at a ratio of 1:1 for precision evaluation. The optimized assay procedure determined the mixed precision reference 10 times. Stability assay: The positive reference of the National Reference Panel for IgM and IgG antibodies to COVID-19 was mixed at a ratio of 1:1 for stability evaluation. These TRFIA kits were incubated at 37°C for 5, 10, 15, 20, and 30 days. Then, these kits were used to detect the mixed positive reference 10 times. The fluorescence values were recorded, and the mean, standard deviation (SD), and coefficient of variation (CV) were calculated.

## 2.9 | Sensitivity and accuracy comparison with COVID-19 nucleic acid assay, colloidal gold assay, and chemiluminescence immunoassay

For sensitivity comparison, the limit of detection reference of the National Reference Panel for IgM and IgG antibodies to COVID-19 was mixed at a ratio of 1:1 for sensitivity evaluation. The mixed limit of detection reference was diluted with matrix serum by two-fold dilution, and a total of 10 dilution series were performed, which were sequentially labeled L1–L10. Then, this TRFIA method, colloidal gold test strip, and chemiluminescence immunoassay simultaneously measured the L1–L10 references and judged the negative/positive results, and the four sets of results were compared.

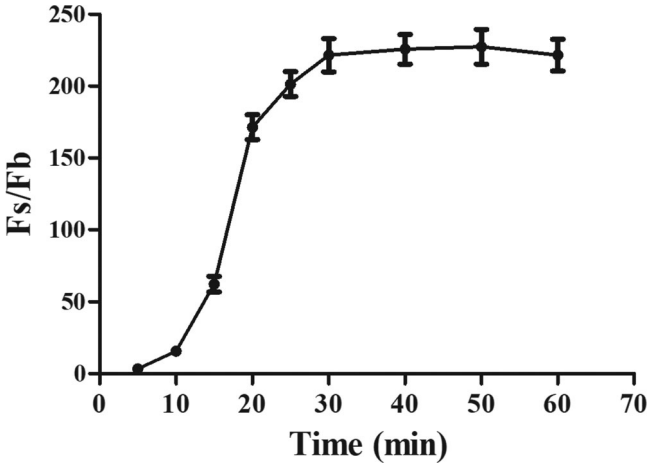


**FIGURE 2** Optimization of immunoreaction time

For accuracy comparison, throat swab samples of 225 healthy people and eight patients diagnosed with COVID-19 were measured by the COVID-19 nucleic acid assay kit (real-time PCR method). Corresponding serum samples of a total of 225 healthy people and eight patients diagnosed with COVID-19 were measured simultaneously by this TRFIA method, colloidal gold test strip, and chemiluminescence immunoassay, and then judged them positive and negative according to their corresponding qualitative criteria and compared the four determination results.

## 2.10 | Statistical analysis

SPSS 19.0 was used in the statistical analysis. The data are expressed as the mean ± SD. Data were graphed using GraphPad Prism 5 (GraphPad Software, USA).

# 3 | RESULTS

## 3.1 | Assay procedure optimization

A double-antigen sandwich TRFIA method was developed with a volume reaction system under the following conditions: N and S protein coating volume: 80 μl N protein (5 μg/ml) + 120 μl S protein (5 μg/ml), total 200 μl/well (Table S2); optimal ratio of $Eu^{3+}$ labeling reagent and N protein: 500 μg:2 mg (5.62 $Eu^{3+}$ ions on each N protein); and optimal ratio of $Eu^{3+}$ labeling reagent and S protein: 800 μg:2 mg (7.86 $Eu^{3+}$ ions on each S protein) (Table S3). The reaction system consisted of 25 μl standard or sample + 110 μl/well $Eu^{3+}$-labeled N protein (4 μg/ml) + 90 μl/well $Eu^{3+}$-labeled S protein (4 μg/ml) (Table S4) + 200 μl/well enhancement solution (Table S5). The optimized immunoreaction time was 30 min (Figure 2).

**TABLE 1** Specificity results of TRFIA

| National reference | No. | Results |
|---|---|---|
| Influenza A IgM-positive serum | N1 | – |
| Influenza A/B IgM-positive serum | N2 | – |
| Influenza A IgM-positive serum | N3 | – |
| *Legionella pneumophila* IgM-positive serum | N4, N5 | Both− |
| *Chlamydia pneumoniae* IgM-positive serum | N6, N7 | Both− |
| Rheumatoid factor-containing serum | N8 | – |
| *Mycoplasma pneumoniae* IgM-positive serum | N9, N10 | Both− |
| Respiratory syncytial virus IgM-positive serum | N11–N13 | Both− |
| *Chlamydia pneumoniae* IgG-positive serum | N14–N16 | All− |
| Measles virus IgG-positive serum | N17 | – |
| Mumps virus IgG-positive serum | N18 | – |
| Normal healthy people serum | N19–N25 | All− |
| COVID-19-diagnosed patients serum | P1–P10 | All+ |
| Conformance rate of negative reference | 100% | |
| Conformance rate of positive reference | 100% | |

*Note:* "−" means negative; "+" means positive.

The optimized assay procedure was as follows: 25 $\mu$l COVID-19 antibody calibrators, serum samples or standards and 200 $\mu$l $Eu^{3+}$-labeled N(S) protein were added to coated 96-well plates and then incubated for 30 min at room temperature. After washing the wells, 200 $\mu$l/well enhancement solution was added to the wells and shaken gently for 2 min. Finally, a time-resolved analyzer was used to measure the fluorescence.

## 3.2 | Qualitative criteria

After performing a normality test with SPSS 19.0, the Fs/Fb value of COVID-19 total antibodies was found to be normally distributed, so the calculation was performed using the one-sided upper limit of the 95% reference interval range. For 225 healthy human serum samples, the mean Fs/Fb value was 1.63, and the SD was 0.119. The cutoff values were calculated using the following formula: cutoff value = mean + 1.96 SD.[11] The cutoff value of this TRFIA was 2.02, which meant that when the Fs/Fb value was greater than 2.02, the sample was positive for COVID-19 total antibodies.

## 3.3 | Specificity results

The negative/positive references of the National Reference Panel for IgM and IgG antibodies to COVID-19, as potential interferents, were detected using the present TRFIA method, and the results are shown in Table 1. There was no cross-reactivity with the negative reference, and all the positive references were positive, which showed that this TRFIA method had high specificity for human COVID-19 antibodies.

## 3.4 | Precision and stability results

The precision and stability results are shown in Table 2. Ten repeated determinations were performed for the same precision reference, the determination results were all positive, and the CV was 3.19%, which is less than the national standard (15%). The 37°C accelerated stability test found that before 15 days, all the positive references were positive, and there was almost no significant change in the Fs/Fb value. After 20 and 30 days, the Fs/Fb value was significantly reduced and could not be detected to be positive for the positive reference. The precision and stability results indicated that this TRFIA method had high precision, and its stability was good.

## 3.5 | Sensitivity and accuracy comparison with COVID-19 nucleic acid assay, colloidal gold assay, and chemiluminescence immunoassay

The sensitivity comparison results are shown in Table 3. The limit of detection reference was diluted with matrix serum by two-fold dilution; L7 (dilution: $1:2^6$) was positive and L8 (dilution: $1:2^7$) was negative by the TRFIA method, L2 (dilution: $1:2^1$) was positive and L3 (dilution: $1:2^2$) was negative by the colloidal gold test strip method, and L4

**TABLE 2** Precision and stability results of TRFIA

| Assays | Samples | | Results |
|---|---|---|---|
| Precision ($n = 10$) | CV (%) | | 3.19% |
| | Qualitative results | | All+ |
| Stability ($n = 10$) | 37°C 5 days | Fs/Fb | 7.40 ± 0.33 |
| | | Qualitative results | All+ |
| | 37°C 10 days | Fs/Fb | 7.35 ± 0.30 |
| | | Qualitative results | All+ |
| | 37°C 15 days | Fs/Fb | 7.33 ± 0.32 |
| | | Qualitative results | All+ |
| | 37°C 20 days | Fs/Fb | 3.19 ± 0.92 |
| | | Qualitative results | + or − |
| | 37°C 30 days | Fs/Fb | 1.24 ± 0.64 |
| | | Qualitative results | All− |

*Note:* "−" means negative; "+" means positive.

Abbreviation: Fs/Fb, $\text{Fluorescence}_{\text{serum sample}}/\text{Fluorescence}_{\text{blank control}}$.

**TABLE 3** Sensitivity comparison of TRFIA method, colloidal gold assay, and chemiluminescence assay

| No. | TRFIA | Colloidal gold assay | Chemiluminescence assay |
|---|---|---|---|
| L1($1:2^0$) | + | + | + |
| L2($1:2^1$) | + | + | + |
| L3($1:2^2$) | + | − | + |
| L4($1:2^3$) | + | − | + |
| L5($1:2^4$) | + | − | − |
| L6($1:2^5$) | + | − | − |
| L7($1:2^6$) | + | − | − |
| L8($1:2^7$) | − | − | − |
| L9($1:2^8$) | − | − | − |
| L10($1:2^9$) | − | − | − |

*Note:* "−" means negative; "+" means positive.

(dilution: $1:2^3$) was positive and L5 (dilution: $1:2^4$) was negative by the chemiluminescence kit method. The comparison results indicated that the sensitivity of the TRFIA method was higher than that of the colloidal gold method and chemiluminescence method.

The accuracy comparison results are shown in Table 4. Similar to the COVID-19 viral nucleic acid assay, all 225 healthy people serum samples were negative by the TRFIA method. However, there were three false positives by the colloidal gold method, and there was one false positive by the chemiluminescence method. For the serum samples of the eight patients diagnosed with COVID-19, similar to the COVID-19 viral nucleic acid assay, all of them were positive by the TRFIA method. However, there were two false negatives by the colloidal gold method, and there was one false negative by the chemiluminescence method. The comparison results indicated that the accuracy of this TRFIA method was higher than that of the colloidal gold method and chemiluminescence method.

## 4 | DISCUSSION

Antibody responses begin to appear over a period of days to weeks after SARS-CoV-2 infection, which, to some extent, are dependent on the sensitivity of detection and the viral protein used as the antigen.[3] The detection sensitivity is closely related to the detection method. This TRFIA method may be a promising detection approach for SARS-CoV-2 IgM and IgG antibodies. Due to different emission wavelengths and kinetics, lanthanide chelates ($Sm^{3+}$, $Eu^{3+}$, $Tb^{3+}$, etc.) have no nonspecific background (interferents), which results in the TRFIA showing better performance in antibody detection in serum.[12,13] Compared

**TABLE 4** Consistency results of COVID-19 nucleic acid assay, colloidal gold assay, chemiluminescence assay, and TRFIA method

| Methods/kits | Samples | Positive | Negative |
|---|---|---|---|
| Nucleid acid assay kit | 225 Healthy human throat swabs | All− | |
| | 8 COVID-19-diagnosed patients throat swabs | All+ | |
| Colloidal gold kit | 225 Healthy human serum | 3+ | 222− |
| | 8 COVID-19-diagnosed patients serum | 6+ | 2− |
| Chemiluminescence kit | 225 Healthy human serum | 1+ | 224− |
| | 8 COVID-19-diagnosed patients serum | 7+ | 1− |
| TRFIA method | 225 Healthy human serum | All− | |
| | 8 COVID-19-diagnosed patients serum | All+ | |

*Note:* "−" means negative; "+" means positive.
Abbreviation: N/A, not applicable.

with the colloidal gold method and chemiluminescence method, the TRFIA method has a higher sensitivity and wide dynamic range.[14] Importantly, the double-antigen sandwich method has higher sensitivity than the double-antibody sandwich method and indirect ELISA method.[15] Based on the above advantage of this method, the double-antigen sandwich TRFIA method established has a lower false-negative rate and can be useful for monitoring the progression of COVID-19 and evaluating patients' response to treatment.

The coronavirus nucleocapsid (N) is a structural protein that forms complexes with genomic RNA, interacts with the viral membrane protein during virion assembly, and plays a critical role in enhancing the efficiency of virus transcription and assembly.[16] The spike (S) glycoprotein is a determinant of host range and is the target of neutralizing antibodies and subunit vaccine development.[17] The key atomic-level interactions between the SARS-CoV S protein receptor-binding domain (RBD) and its host receptor angiotensin converting enzyme 2 (ACE2) can regulate both the cross-species and human-to-human transmissions of SARS-CoV.[18] The latest research found that the SARS-CoV-2 N protein, S protein, and papain-like protease are relatively nonconservative; in particular, to date, the N protein has had more than half its genes changed in the past few months.[19] This explanation may be one of the reasons for missed detection, and it also shows that a single N protein or S protein coating will cause a higher false-negative rate and missed detection rate. In ELISA-based assays on the recombinant viral N protein, the positive rates of IgM antibodies were 75.6% and 93.1% for the confirmed and probable cases, respectively.[4] It has been reported that combining N protein and S protein as coated antigens can increase the sensitivity.[20] This TRFIA method was designed for simultaneous coating of N protein and S protein to increase the sensitivity and decrease the missing detection rate.

Using simultaneous coating of N protein and S protein, we established COVID-19 total antibody detection. For the TRFIA method itself, there was no cross-reactivity with the negative reference of the National Reference Panel for IgM and IgG antibodies to COVID-19, and all the positive references were positive. The CV of the precision assay was 3.19%, and the assay could be stored stably for 15 days at 37°C. The sensitivity of the TRFIA method reached L7 ($1:2^6$) for the limit of detection reference of the National Reference Panel for IgM and IgG antibodies to COVID-19. There were no false-positive or false-negative cases for serum samples of 225 healthy people and eight patients diagnosed with COVID-19. Moreover, the sensitivity of the TRFIA method was higher than that of the colloidal gold method (L2) and chemiluminescence method (L4). The accuracy of this TRFIA method was also higher than that of the colloidal gold method (false positives: 3/225; false negatives: 2/8) and chemiluminescence method (false positives: 1/225; false negatives: 1/8). In summary, the optimized immunoreaction time of TRFIA is only 30 min, and more than 90 samples can be tested at one time. The detection efficiency was much higher than that of the nucleic acid detection method (2–3 h) and the ELISA method (2–3 h). Additionally, its detection accuracy and sensitivity are higher than those of the colloidal gold method and chemiluminescence method. Therefore, this TRFIA is suitable for large-scale human COVID-19 total antibody testing.

## 5 | CONCLUSION

In conclusion, we developed a COVID-19 total antibody detection method using a double-antigen sandwich TRFIA. This double-antigen sandwich TRFIA detection has high sensitivity, specificity, and accuracy, and it takes only 30 min to generate results and can detect more than 90 samples (96-well plate) at a time. This TRFIA method

for total antibody detection represents a new approach for the high-throughput routine diagnosis of COVID-19.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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