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Rapid and Sensitive Detection of anti-SARS-CoV-2 IgG, Using Lanthanide-Doped Nanoparticles-Based Lateral Flow Immunoassay

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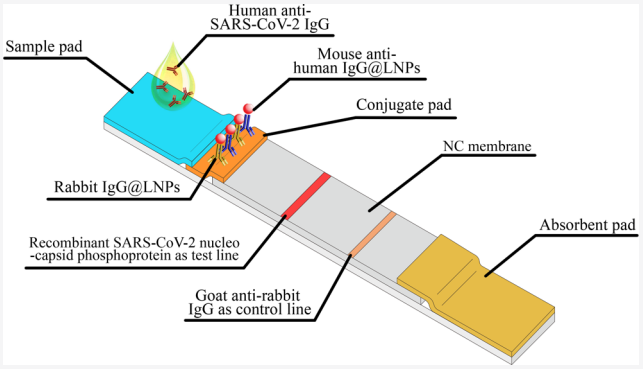
ABSTRACT: The outbreak of 2019 coronavirus disease (COVID-19) has been a challenge for hospital laboratories because of the huge number of samples that must be tested for the presence of the causative pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Simple and rapid immunodiagnostic methods are urgently needed to identify positive cases. Here we report the development of a rapid and sensitive lateral flow immunoassay (LFIA) that uses lanthanide-doped polysterene nanoparticles (LNPs) to detect anti-SARV-CoV-2 IgG in human serum. A recombinant nucleocapsid phosphoprotein of SARS-CoV-2 was dispensed onto a nitrocellulose membrane to capture specific IgG. Mouse anti-human IgG antibody was labeled with self-assembled LNPs that served as a fluorescent reporter. A 100-μL aliquot of serum samples (1:1000 dilution) was used for this assay and the whole detection process took 10 min. The results of the validation experiment met the requirements for clinical diagnostic reagents. A value of 0.0666 was defined as the cutoff value by assaying 51 normal samples. We tested 7 samples that were positive by reverse-transcription (RT-)PCR and 12 that were negative but clinically suspicious for the presence of anti-SARS-CoV-2 IgG. One of the negative samples was determined to be SARS-CoV-2 IgG positive, while the results for the other samples were consistent with those obtained by RT-PCR. Thus, this assay can achieve rapid and sensitive detection of anti-SARS-CoV-2 IgG in human serum and allow positive identification in suspicious cases; it can also be useful for monitoring the progression COVID-19 and evaluating patients' response to treatment.



In early December 2019, the cases of severe acute respiratory syndrome coronavirus (SARS-CoV-2)-infected pneumonia were identified in Wuhan City, Hubei Province, China.[1] Since then, the 2019 coronavirus disease (COVID-19) outbreak has rapidly spread throughout the country and around the world.[2] Despite emergency measures, the current situation is grim. According to the COVID-19 report published by the National Health Commission of the People's Republic of China, over 70 000 cases of COVID-19 have been diagnosed in China. As our knowledge of the clinical features of COVID-19 and route of transmission of SARS-CoV-2 has grown, it has been determined that the virus has an incubation period that may be as long as 24 days, along with a high basic reproductive number (R_0 = 3.77).[3,4] Early diagnosis and treatment are critical for controlling the COVID-19 outbreak.

A rapid and accurate laboratory method for the diagnosis of COVID-19 is desired,[5] but the huge number of samples and complex situations in hospitals pose major challenges.[6] Establishing standards for specimen collection and nucleic acid extraction as well as the proper testing environment, and correctly interpreting test results are the major problems faced by hospital laboratories, especially those in rural areas, where resources are inadequate. Many factors could contribute to false negative results by reverse transcription (RT-)PCR, and the positive rate of the first RT-PCR test for SARS-CoV-2 was just 70%.[7]

Chest computed tomography (CT) is being used as a confirmatory diagnostic method for samples with high clinical suspicion that were found to be negative for SARS-CoV-2 by RT-PCR.[8] A simple and rapid immunodiagnostic method for COVID-19 would be a useful adjunct or more practical alternative to chest CT. Immunodiagnostic methods such as lateral flow immunoassay (LFIA) have the advantages of being convenient and rapid. However, traditional colloidal gold-based LFIAs do not have optimal performance or quantification capability. In recent years, many fluorescence-based LFIAs

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have been developed for quantitative or semiquantitative detection,[9−12] but conventional fluorescent dyes are not the ideal reporter because they are prone to photobleaching and have poor stability or quantum yield. Lanthanide-doped polysterene nanoparticles (LNPs) can overcome these problem while maintaining the advantages of simple and rapid detection.[13,14]

COVID-19 diagnosis based on the detection of anti-SARS-CoV IgG in serum has been reported.[15,16] Monitoring changes in serum SARS-CoV IgG concentration can also be useful for evaluating response to treatment and prognosis.[17] High-throughput sequencing has revealed a high degree of similarity between SARS-CoV-2 and SARS-CoV, the causative pathogen in the 2003 SARS outbreak.[18] Based on this finding, quantitative detection of anti-SARS-CoV-2 IgG for the diagnosis of COVID-19 is entirely feasible. Given the absence of a specific medicine, convalescent serum and plasmapheresis may be used to treat COVID-19, as in the case of SARS.[19]

In this study, we report the development of a LFIA that uses a recombinant nucleocapsid phosphoprotein and self-prepared LNPs to detect anti-SARS-CoV-2 IgG in human serum.

■ EXPERIMENT

Reagents, Samples, and Instrumentation. Tris(1,3-diphenyl-1,3-propanedionato)(1,10-phenanthroline)europium(III) ($Eu(DPP)_3Phen$), styrene (St), acrylic acid (AA), 2,2′-azobis(isobutyronitrile) (AIBN), and cetyltrimethylammonium (CTMA) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 2-(*N*-morpholino) ethanesulfonic acid (MES), surfactant (including polyvinylpyrrolidone (PVP) and TritonX-100), carboxyl activating agents (including 1-ethyl-3-(3-(dimethylamino)propyl) carbodimide hydrochloride (EDC) and *N*-hydroxy-sulfosuccinimide (sulfo-NHS)), and Proclin-300 were obtained from Sigma−Aldrich (St. Louis, MO, USA). Functional block polymer, Tetronic 1307 (surfactant S9), was obtained from BASF Corp. (Florham Park, NJ, USA). Rabbit IgG and goat anti-rabbit IgG were purchased from Rockland Immunochemicals (Limerick, PA, USA). Mouse anti-human IgG was obtained from Abcam (Cambridge, MA, USA). Nitrocellulose (NC) membranes, glass fiber conjugate pads, and cellulosic absorbent pads were purchased from Millipore (Hong Kong, China). Cellulosic sample pads were from Jieyi Biotechnology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was obtained from Roche Diagnostics (Hong Kong, China). Other chemicals used in this study were of analytic grade and used without further treatment. Ultrapure water, obtained using a Milli-Q Advantage A10 purification system (Millipore), was used in all experiments.

The following buffer solutions were used: coating buffer (0.3% trehalose [w/v] and 0.1% NaN_3 [w/v] in 100 mM citrate-buffered saline [CBS], pH 8.5); sample pad treatment buffer (100 mM $Na_2B_4O_7{\cdot}10H_2O$, 1% PVP [w/v], 0.2% casein-Na [w/v], 1% TritonX-100 [v/v], and 0.1% NaN_3 [w/v]); conjugate pad treatment buffer (0.5% poly(vinyl alcohol) [PVA] [w/v], 0.5% BSA [w/v], and 1% TritonX-100 [v/v] in 50 mM phosphate buffer, pH 7.4); activating buffer (50 mM MES, pH 5.1); binding buffer (10 mM phosphate buffer, pH 7.0); washing buffer (0.2% Tween-20 [v/v], 0.9% NaCl [w/v], and 0.05% Proclin-300 [v/v] in 25 mM Tris-HCl, pH 7.8); storage buffer (1% BSA [w/v], 5% trehalose [w/v], 20% sucrose [w/v],and 0.05% Proclin-300 [v/v] in 25 mM Tris-HCl, pH 9.0); and assay buffer (7.5% BSA [w/v], 0.3% TritonX-100 [v/v], and 0.1% surfactant S9 [w/v] in 10 mM PBS, pH 7.4). All solutions in this study were freshly prepared before use.

Serum samples were obtained from Guangzhou Eighth People's Hospital and Nanfang Hospital and stored at −20 °C until use. This study was approved by the Ethics Committee of the Science and Technology Department of Southern Medical University (Guangzhou, China).

Expression of Recombinant SARS-CoV-2 Nucleocapsid Phosphoprotein. The RNA genome of SARS-CoV-2 was kindly provided by DAAN Gene Co. (Guangzhou, China). Reverse transcription was performed to generate cDNA using PrimeScript II first Strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan; Catalog No. 6210A). The full-length nucleocapsid phosphoprotein gene was amplified from the cDNA by PCR using the following primers: 5′-ATG TCT GAT AAT GGA CCC CAA AAT C-3′ (forward) and 5′-TTA GGC CTG AGT TGA GTC AGC −3′ (reverse), which were designed based on sequences in GenBank (MN908947.3). PCR products were sequenced and cloned into the pMD-19T backbone vector; the resultant pMD-19T-N plasmid was fused with the linear pET-32a vector (Not I), using a Seamless Cloning and Assembly Kit (Beijing Zoman Biotechnology, Beijing, China; Catalog No. ZC231), from which the nucleocapsid phosphoprotein gene was amplified using the primers 5′-AG CTC CGT CGA CAA GCT TGC ATG TCT GAT AAT GGA CCC CAA AAT-3′ (forward) and 5′-TG GTG GTG GTG GTG CTC GAG TGC GGC CGC GGC CTG AGT TGA GTC AGC ACT-3′ (reverse). The target protein was expressed in *Escherichia coli* BL21 competent cells by induction with isopropyl *β*-D-1-thiogalactopyranoside, with the induction time optimized to ensure maximal expression. Positive serum samples were used to confirm the antigenicity of the purified recombinant protein by Western blotting.

Preparation of LNPs. The LNPs were synthesized by miniemulsion polymerization. Briefly, 200 mg of $Eu(DPP)_3Phen$ and 20 mg of AIBN were dissolved in 2 g of St in a glass vial; and 60 mg of CTMA and 100 mg of AA were dissolved in 15 mL of deionized water. The aqueous and monomer solutions were mixed with a magnetic stirrer for 10 min and miniemulsions were prepared with an ultrasonicator for 20 min in an ice bath. Nitrogen gas was bubbled for 2 h at room temperature to remove oxygen from the mixture, and the emulsion was then stirred at room temperature for 20 h. The product was ultrasonically washed 4 times with water and centrifuged. The resultant LNPs were dispersed in water (5% w/v) and stored as stock at 4 °C.

Functionalizing LNPs with Protein Molecules. LNPs were functionalized with mouse anti-human IgG antibody (M-HIgG) and rabbit IgG (RIgG) as previously described,[20] with some modifications. A two-step procedure was used to prevent aggregating between LNPs via cross-linking of antibody molecules. Briefly, 20 μL of EDC (1% EDC [w/v] in activating buffer) and 180 μL of sulfo-NHS (1% sulfo-NHS [w/v] in activating buffer) solutions were mixed and added to 2 mg of LNPs resuspended in 0.8 mL of activating buffer. The mixture was incubated for 30 min with gentle stirring at room temperature. To remove unreacted components, the LNPs was washed three times by centrifugation at 18000*g* for 10 min with binding buffer after NHS-ester was formed. The pellet was ultrasonically resuspended in 0.95 mL of binding buffer. The antibodies were purified and diluted in binding buffer at a concentration of 1 mg/mL; 50 μL of as-prepared antibody

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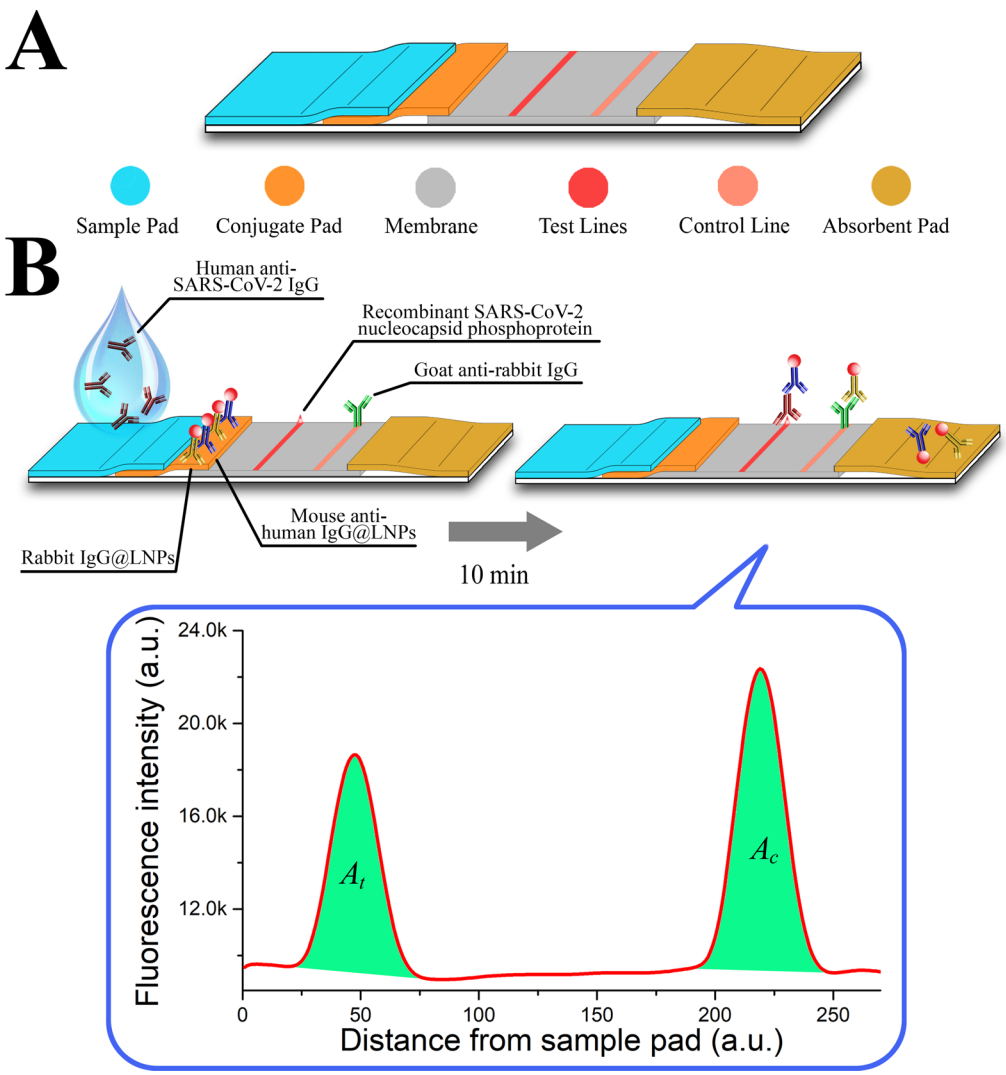

Figure 1. Design and fabrication of the developed assay. (A) Lateral flow test strip. (B) Assay.

solution (M-HIgG or RIgG) was added into the activated LNPs suspension. A 4-h incubation with gentle rotation was applied, then 1.5 μL of ethanolamine was added and the admixture was further incubated for 10 min. In order to purify the functionalized LNPs, the washing process was repeated three times before adding blocking buffer (2% BSA in 25 mM phosphate buffer, pH 7.4). The functionalized LNPs were ultrasonically resuspended in blocking buffer, and a 30-min incubation with gentle mixing was employed to block any remaining unreacted hydrophobic sites on the surface of LNPs and to form a thin hydration layer to prevent aggregation. The antibody-enameled LNPs were cleansed three times with washing buffer and were resuspended in storage buffer.

Fabrication of LFIA Strips. LFIA strips were prepared as described in our previous work.[21] The sample pad was immersed in a sample pad treatment buffer for 1 h and dried at 37 °C for 24 h. The conjugate pad was saturated with a conjugate pad treatment buffer at room temperature for 1.5 h and also dried at 37 °C for 24 h. M-HIgG@LNPs and RIgG@LNPs were diluted in storage buffer (0.125 and 0.05 mg/mL, respectively) and combined; the mixture was dispensed onto the conjugate pad at 1 μL/cm using a Biolet Quanti dispenser (BioDot, Shanghai, China), and dried at 37 °C before being stored in a dry cabinet at room temperature until use. Recombinant nucleocapsid phosphoprotein and goat anti-rabbit IgG were diluted with coating buffer at concentration of 2 mg/mL, then dispensed onto an NC membrane (Millipore; Catalog No. HF13502XSS) at 1 and 0.8 μL/cm, respectively, as test and control lines. The membrane was dried at 37 °C for 4 h. The NC membrane, absorbent pad, conjugate pad and sample pad were sequentially laminated together onto a backing card, as shown in Figure 1. Strips with 4 mm wide were cut and placed into disposable plastic housings for later use. The assembled test strips were stored in a moisture-proof cabinet.

Analytical Procedure and Statistical Analysis. As shown in Figure 1, serum samples were diluted with assay buffer (1:1000), and 100 μL of diluted sample was added to the sample well of a LFIA strip. As the liquid migrated from the sample pad toward the absorbent pad, functionalized LNPs were captured at the test and control lines. After a 10-min incubation, the strip was loaded into the portable fluorescence reader for detection of fluorescence at excitation and emission wavelengths of 365 and 615 nm, respectively, with the delay time set at 20 μs. Excitation of the fixed LNPs produced a bright fluorescent zone on the membrane. The results are denoted as the fluorescence peak area of the test line (At) and control line (Ac). The At/Ac ratio (R) was calculated to

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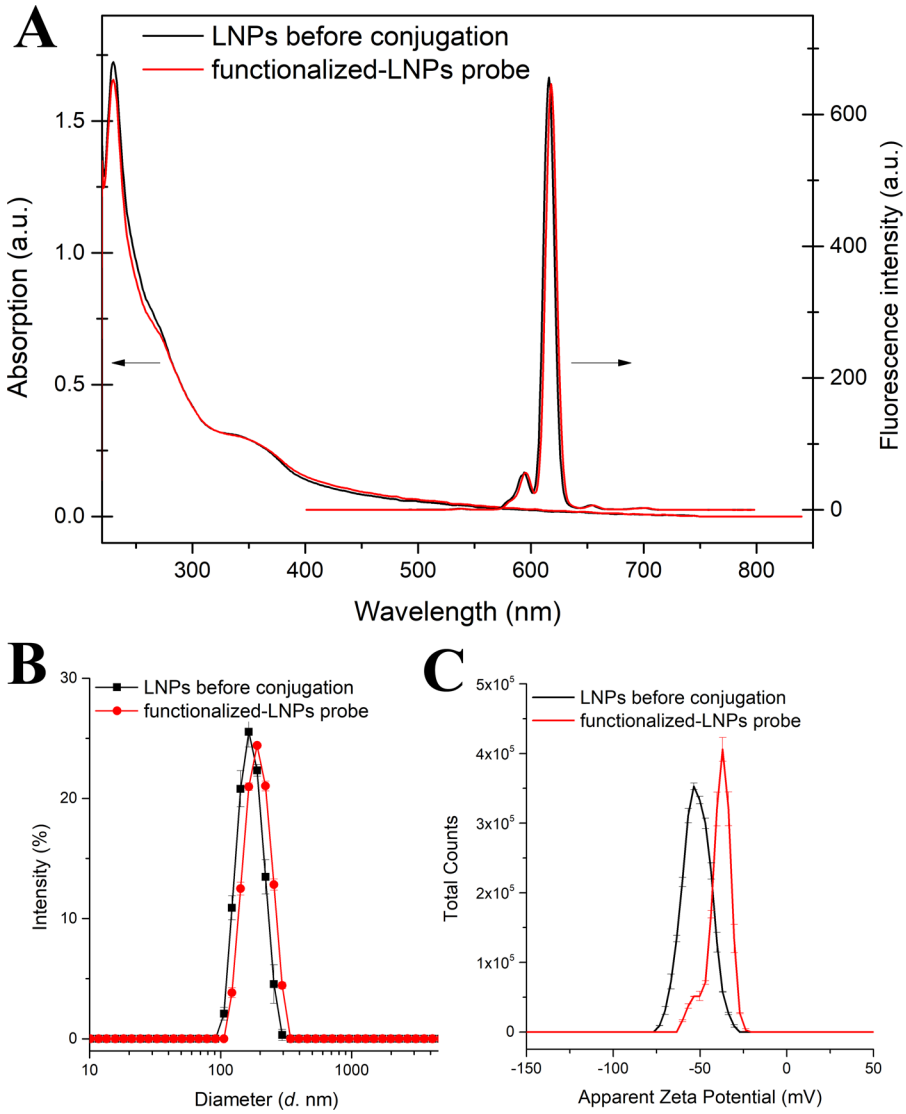

Figure 2. Physical properties of LNPs and functionalized LNP probe: (A) absorption and emission spectra, (B) size distribution of naked and conjugated LNPs, and (C) zeta potential distribution.

determine the anti-SARS-CoV-2 IgG concentration in the serum.

RESULTS AND DISCUSSION

Characterization of LNPs and Functionalized LNP Probe. Most biochemical assays are performed in solution. In aqueous environments, the emission of lanthanide chelates is strongly suppressed due to energy loss through the non-radiative activation of the $5D_0$ state to the O−H vibration of water molecules.[22] Thus, it is preferable to encapsulate lanthanide chelates in water-dispersible spheres. The maximal fluorescence emission peak of LNPs was obtained at ∼615 nm; the peak was narrow and symmetric (Figure 2a). Dynamic light scattering analysis was performed for physical characterization of LNPs before and after functionalization. The change in the size and surface zeta potential of LNPs (Figures 2b and 2c) indicated that the M-HIgG and RIgG antibodies were successfully conjugated.

Optimization. Immunoreaction time significantly influences fluorescence signals. In this assay, a positive sample was measured to establish the optimal assay time. The At/Ac ratio (R) was measured every 2 min over a period of 0−16 min from the start of incubation. R increased rapidly over the first 4 min and reached a plateau at 10 min (see Figure 3). Based on this result, the total assay time was determined as 10 min.

Reproducibility. The reproducibility of our assay was evaluated based on intra-assay and inter-assay variations. Four samples were measured five times a day for five consecutive days, and the coefficient of variation (CV) values were calculated. The intra-assay and inter-assay CVs were 7.71%−9.69% and 11.51%−14.63%, respectively (see Table 1). Since the CVs were all <15%, we concluded that our assay is reproducible.

Clinical Sample Tests. In order to define the appropriate cutoff value for our assay, we tested 51 normal serum samples and 7 samples that were determined to be positive by RT-PCR. The cutoff value of R (the mean of normal samples plus 3 times the standard deviation) was calculated as 0.0666. All 7 samples that were positive by RT-PCR had an R value of >0.0666 (Figure 4), indicating that the developed assay can detect anti-SARS-CoV-2 IgG in positive samples.

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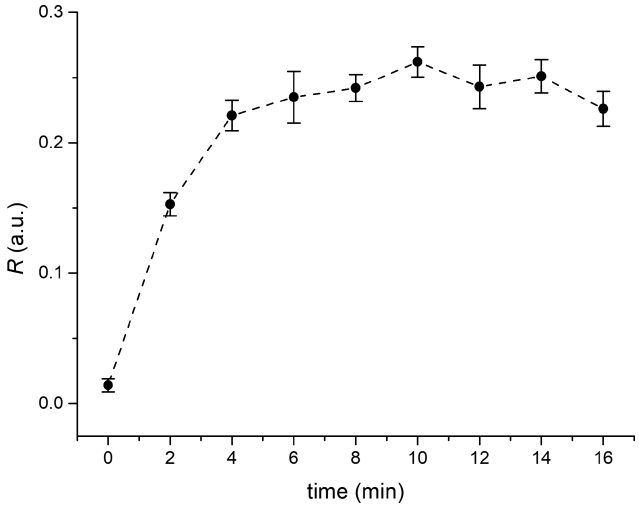

Figure 3. Optimization of immunoreaction time.

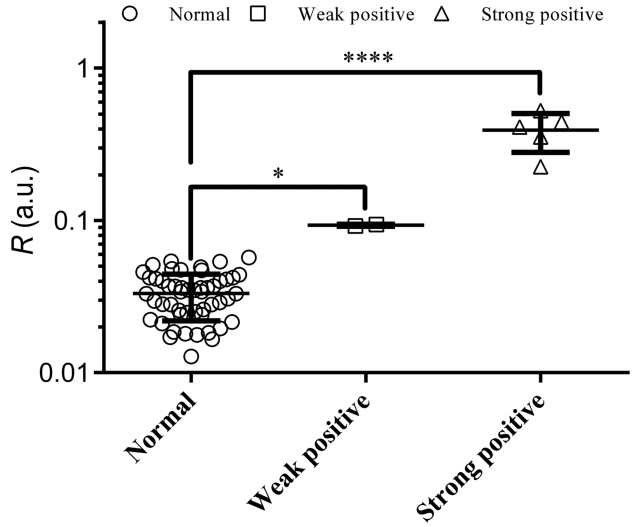

Figure 4. Test results for 58 serum samples, including 51 normal and 7 positive samples. [Symbol legend: (*) $P < 0.05$, (****) $P < 0.0001$ (one-way analysis of variance and Fisher's least significant difference test).]

To confirm the diagnostic accuracy of our assay, we used it to test 7 samples that were positive by RT-PCR and 12 that were negative but clinically suspect. The χ^2 test showed that the P-value of the McNemar test was 1.0, and the value obtained with the kappa test was 0.890 ($P < 0.001$) (see Table 2). Thus, there was no statistically significant difference between the results obtained with our developed assay versus those obtained by RT-PCR.

One of the 12 clinically suspicious samples that were negative by RT-PCR was found to be SARS-CoV-2 IgG positive with our assay. Unlike other suspected cases, this case did not only have the fever and came with high clinical suspicion. The negative result of RT-PCR test raised clinical concerns about the management of this case. Base on our IgG test result, the RT-PCR test result was more likely to be a false negative. At present, prevention of cross-infection is very essential in outbreak control; timely isolation and treatment should be adopted for this case.

Table 2. χ^2 Test for Diagnostic Results of the Developed Assay and RT-PCR

	RT-PCR Test		
	positive	negative	total
developed LFIA			
positive	7	1	8
negative	0	11	11
total	7	12	19

CONCLUSION

The present study describes a simple and rapid LNP-based immunoassay for detecting anti-SARS-CoV-2 IgG in human serum. Because there is presently no official anti-SARS-CoV-2 IgG standard available, the assay cannot be improved from semiquantitative to accurate quantification. However, the results of the validation experiment meet the requirements for clinical diagnostic reagents. Our assay can be used to monitor the progression of COVID-19, as well as responses to treatment. We expect this assay to be highly useful for helping to contain the COVID-19 outbreak by allowing timely diagnosis through early detection of SARS-CoV-2.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c00784.

Supplemental Figures S1−S4 (PDF)

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Table 1. Reproducibility Test of the Developed Assay

	Intra-assay			Inter-assay		
sample	mean, R	standard deviation, SD	coefficient of variation, CV (%)	mean, R	standard deviation, SD	coefficient of variation, CV (%)
A	0.029	0.0028	9.66	0.034	0.0048	14.12
B	0.039	0.0037	9.49	0.053	0.0061	11.51
C	0.256	0.0208	8.13	0.231	0.0338	14.63
D	0.390	0.0301	7.72	0.468	0.0680	14.53

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Notes

The authors declare no competing financial interest.

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Supporting Information

Rapid and sensitive detection of anti-SARS-CoV-2 IgG using lanthanide-doped nanoparticles-based lateral flow immunoassay

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Fig. S1. Result of PCR of nucleoprotein gene.

Fig. S2. SDS-PAGE analysis of nucleoprotein expressed in *E. coli*.

Fig. S3. SDS-PAGE and western blotting analysis of purified nucleoprotein.

Fig. S4 Optimization of sample dilution.

Supplemental Data Section

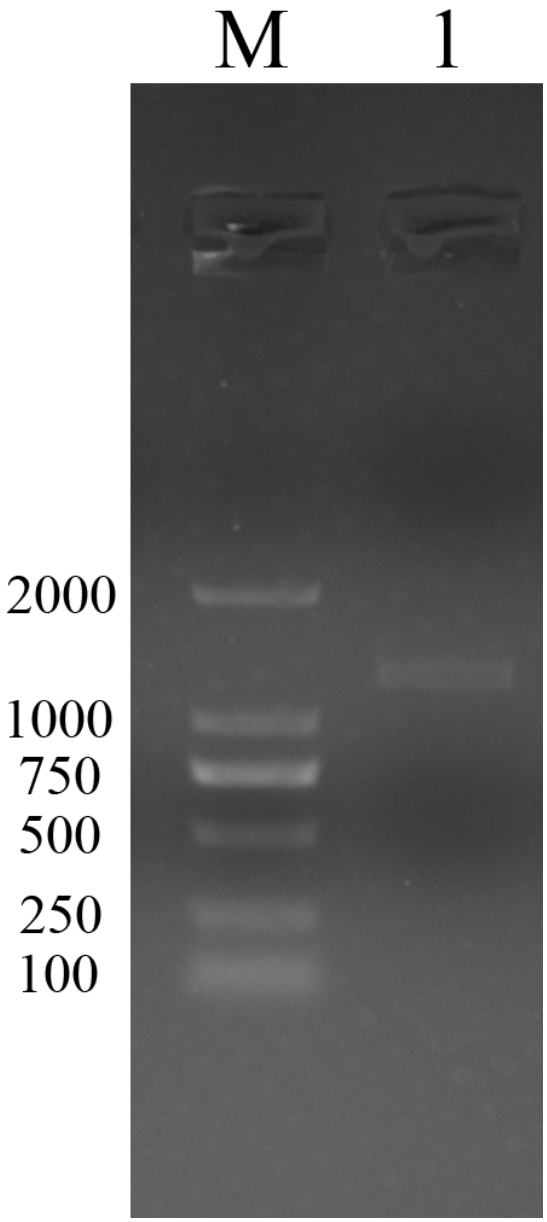

Fig. 1S. Result of PCR of nucleoprotein gene: (lane M) marker. (lane 1) PCR product

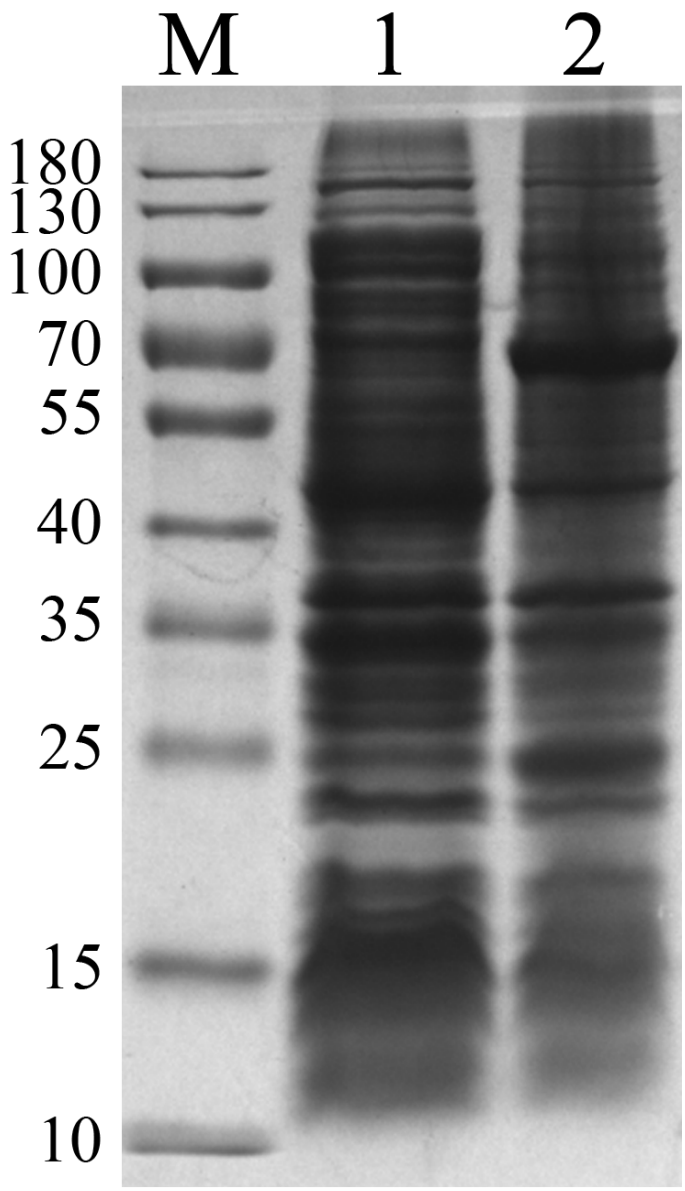

Fig. S2. SDS-PAGE analysis of nucleoprotein expressed in *E. coli*: (lane M) Protein marker. (lanes 1) pET-32a/N E. coli lysate before IPTG induction. (lanes 2) pET-32a/N *E. coli* lysate after induced with IPTG for 4 hours.

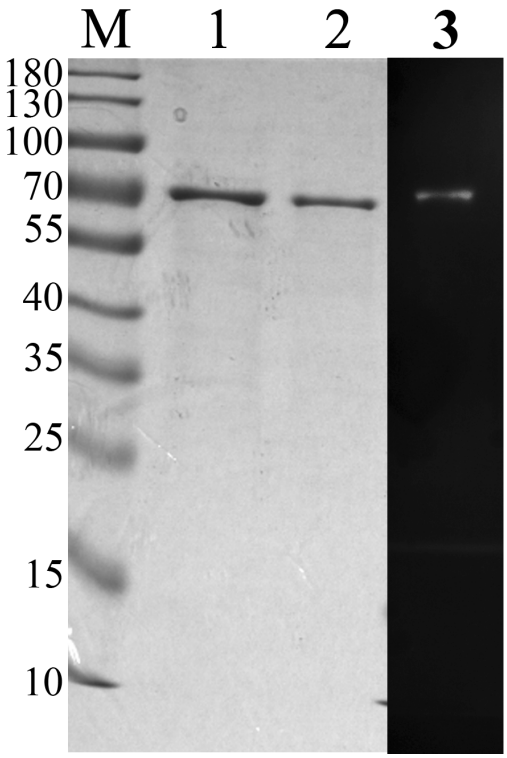

Fig. S3. SDS-PAGE and western blotting analysis of purified nucleoprotein: (lane M) Protein marker. (lanes 1: 3.6 μg and 2 μg) purified N protein. (lane 3) western blotting analysis of purified nucleoprotein.

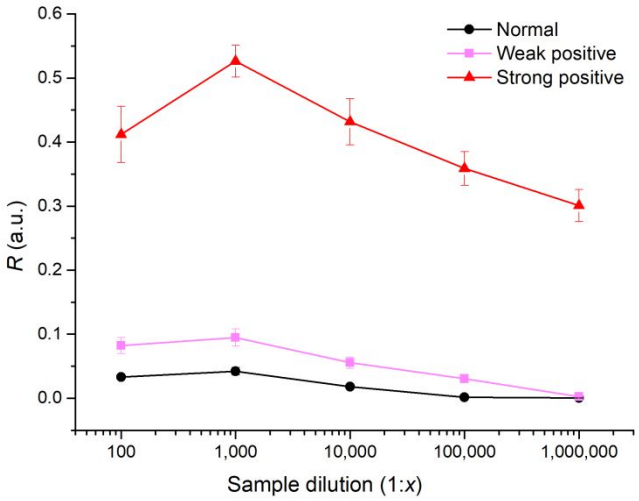

Fig. S4 Optimization of sample dilution.