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ARTICLE

Detection of SARS-CoV-2 humanized antibody with paper-based ELISA

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This paper reports the development of a rapid, simple and inexpensive colorimetric paper-based assay for the detection of Severe Acute Respiratory Symptom Coronavirus 2 (SARS-CoV-2) humanized antibody. The paper device was prepared with lamination for easy sample handling and coated with recombinant SARS-CoV-2 nucleocapsid antigen. This assay employed a colorimetric reaction, which is followed by horseradish peroxidase (HRP) conjugated detecting antibody in the presence of 3,3',5,5'-tetramethylbenzidine substrate (TMB). The colorimetric readout was evaluated and quantified for specificity and sensitivity. The characterization of this assay includes determining the linear regression curve, the limit of detection (LOD), the repeatability, and test in complex biological samples. We found that the LOD of the assay is 9.00 ng/µL (0.112 IU/mL). The relative standard deviation is approximately 10% for a sample number of n = 3. We believe that our proof of concept assay has the potential to be developed toward clinical screening of SARS-CoV-2 humanized antibody as a tool to confirm infected active cases or to confirm SARS-CoV-2 immune cases during the process of vaccine development.

1. Introduction

In the late December of 2019, an increase in atypical pneumonia patients in Wuhan, China and nearby regions was observed.[1] According to genome sequence analysis, the symptom resulted from a viral infection in Coronaviridae family, which is novel and closely related to Severe Acute Respiratory Symptom Coronavirus (SARS-CoV) and Middle East Respiratory Symptom Coronavirus (MERS-CoV) that broke out in 2002 and 2012, respectively.[2, 3] WHO declared a public health emergency of international concern in January 2020 due to the increasing confirmed and reported number of cases in many countries.[4] Later International Virus Classification Commission named the new pandemic disease as Severe Acute Respiratory Symptom Coronavirus-2 (SARS-CoV-2), 2019 novel coronavirus (2019-nCoV) or COVID-19 in March 2020.[5] By the time of the submission of this manuscript (26th August 2020), the total casualty are 23.9 million confirmed cases of SARS-CoV-2 infection and 820,000 confirmed deaths.[6]

SARS-CoV-2 is a single-stranded and positive-sense RNA constituting 30 kb, which is more than 89% similar to SARS-CoV.[5, 7] The infected patients have a wide range of symptoms from mild flu-like symptom to severe bilateral pneumonia and death.[8] Moreover, some patients are confirmed to have a potential to spread the virus silently in the early stage without any symptom.[9] Therefore, unlike all previous pandemics, it is extremely difficult for government agencies to control and prevent the spread of this disease. As a result, many nations have made a decision to lockdown their country for a few weeks, to enforce social distancing measures and to encourage people wearing facial masks to minimize the spread of the disease.[7, 10] Subsequently, diagnostic tests for detecting COVID-19 are urgently needed for identifying infected patient and implementing quarantine procedure to separate those who are infected from the community to limit the spread.

The diagnosis of SARS-CoV-2 infection requires a timely and accurate testing method. Currently, reverse transcription polymerase chain reaction (RT-PCR) is one of the most accurate and sensitive method and being recommended by the World Health Organization (WHO) to determine SARS-CoV-2 infection, because this test directly detects viral RNAs in the sample.[11] However, sample collection and processing for performing the diagnosis require well-trained medical staff and high-level biological safety facilities.[12] These factors are huge obstacles particularly for developing countries due to limited capacity to carry out timely and sufficient number of tests. Another promising diagnostic method for SARS-CoV-2 infection is serological testing based on Enzymatic-Linked Immunosorbent Assay (ELISA). Serological assay relies on antigen and antibody resulting from protein enriched on a viral membrane or host immune response.[7] For immune response, the antibodies in blood which is immunoglobin G (IgG) and immunoglobin M (IgM) are produced to detect a specific

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invading pathogen. Thus, some serological assays take advantage of measurable specific SARS-CoV-2 antibody concentration in human blood to identify whether the patient get infected or not. Nevertheless, the immune response is based on individuals because the antibodies produced by the immunity mechanism can only be found at a detectable level only from 5 days to 2 weeks after the infection.[11, 13] Thus, a serological test is not suitable for early diagnosis. The other mean for SARS-CoV-2 detection is using lateral flow immunological assay (LFIA) or rapid diagnostic tests (RDTs). LFIA and RDTs provide fast response and are user-friendly. However, their sensitivity and specificity are still controversial. These tests also faced the same issues as ELISA, because they are based on specific antibodies generated from the immune response.[14, 15]

The best practice to avoid misinterpretation of the result is to consider both common laboratory tests: nucleic acid and serological assays. The subsequent clinical manifestation determines the period of infection. Moreover, in the period of urgent vaccine development, the efficacy of vaccine in a large number of human trials also requires serological assay to determine the humanized antibody specific to SARS-CoV-2 [16, 17]. With this regard, a relatively rapid, sensitive and inexpensive platform could help to relieve the above mentioned issues especially in developing countries. Paper-based ELISA is promising for diagnostic applications due to the low cost, ease of use, and the small sample volume [18]. In a low-resource setting, colorimetric assay is useful and easy to interpret and can be detected with naked eyes. One of the mostly used colorimetric reactions is 3,3',5,5'-tetramethylbenzidine substrate and horseradish peroxidase (TMB/HRP). This reaction generates a colour product in the presence of the target, where the target biomolecules are conjugated with HRP [19]. Therefore, an inexpensive and accessible serological assay remains challenging for detecting SARS-CoV-2 antibody. The present study performed a simple, rapid and inexpensive paper-based ELISA assay for SARS-CoV-2 antibody detection. The target was captured with specific antigens and formed immunological complex on the paper. The colorimetric readout based on the TMB/HRP reaction was observed in the presence of HRP conjugated with the detecting antibody. The assay can be completed within 30 minutes, which is faster than a conventional ELISA that generally takes around 1-2 hours. Moreover, as the assay benefits from the high surface-to-volume ratio of the paper matrix, it only requires a few microliters to perform each test. This assay has been successfully performed in human serum, demonstrating its suitability for complex biological samples.

2. Material and methods

2.1 Reagents and instrumentations

Recombinant SARS-CoV-2 nucleocapsid (MBS355892, MyBioSource, USA) and SARS-CoV-2 nucleocapsid humanized antibody (MBS355887, MyBioSource, USA) was used for the paper-based ELISA assay. 10% Bovine Serum Albumin (BSA, A1595, Sigma Aldrich, USA) was used as a blocking buffer. Phosphate Buffer Saline (PBS, 10388739, Fisher Scientific, USA) was prepared from a tablet by dissolving in DI water (Milli-Q, Merck, USA). Tween-20 detergent (655204, EMD Millipore, USA) was used as an additive surfactant for preparing PBST containing 0.05% Tween-20 in PBS. Secondary antibody used in this study is Rabbit anti-human IgG (ab97156, Abcam, UK) was conjugated with horseradish peroxidase (HRP) by HRP Conjugation kits (ab102890, Abcam, UK). TMB substrate (002023, Thermo Fisher Scientific, Germany) was used for TMB/HRP reaction. For complex biological sample, human serum (H4522, Sigma Aldrich, USA) was used as a mocked human sample. For image acquisition system, the 1.3 megapixels TOUPCAM camera (UCMOS01300KPA, Touptek, China) with 1/3" Aptina CMOS sensor was assembled with 6.2 mm focal lens (58428, Edmund Optics Inc, USA). For software, the image system was connected via USB and controlled by Toupview software (Version 4.8, Touptek, China). Image processing and quantification were performed with programming code in numerical computing software (MATLAB R2018b, The MatkWorks Inc, USA).

2.2. Paper fabrication and assay preparation

Whatman chromatography filter paper (CHR, Whatman, UK) was cut into 5-mm diameter and aligned at the centre between two laminate films with a 4-mm diameter hole. The laminated films with sandwiched paper (L-CHR) was fed into a laminator at 130°C at a speed of 10 mm/s. Next, L-CHR was used to prepare the paper-based ELISA. Fig. 1 shows the preparation of the paper platform. Briefly, 5 µL of 0.2 mg/mL recombinant SARS-CoV-2 nucleocapsid in 2% BSA was immobilized on the L-CHR. The antigen was incubated in a humidified box for 10 min before drying in the incubator at 37°C for 10 min. The washing solution (PBS and PBST) was added on L-CHR surface and removed by putting blotting paper underneath to absorb excessive reagents. 10 µL 1xPBS was added twice to remove unbound antigen. Then, L-CHR was dried for 10 min in the incubator and kept in a petri dish at room temperature until further use.

2.3 SARS-CoV-2 humanized antibody colorimetric detection assay

The assay principle used in this study is a paper-based indirect ELISA. A volume of 5 µL of the sample was added onto the paper platform, and incubated for 10 min. Subsequently, the paper device was washed as mentioned above with 10 µL 1× PBST for 5 times and 1×PBS for 1 time. A volume of 3 µL of 0.1 ng/µL Rabbit anti-human IgG conjugated with HRP was incubated for 3 minutes to capture humanized antibody and to form a complex in the presence of SARS-CoV-2 humanized antibody. It is worth noting that the HRP mixture is freshly prepared in every independent experiment as enzymatic activity decays over time. Subsequently, the paper device was washed with 10 µL 1× PBS thrice. To obtain colorimetric readout, 5 µL of TMB was added onto the paper device. The negative sample with only 1×PBS will be performed for background signal because some HRP may not be completely

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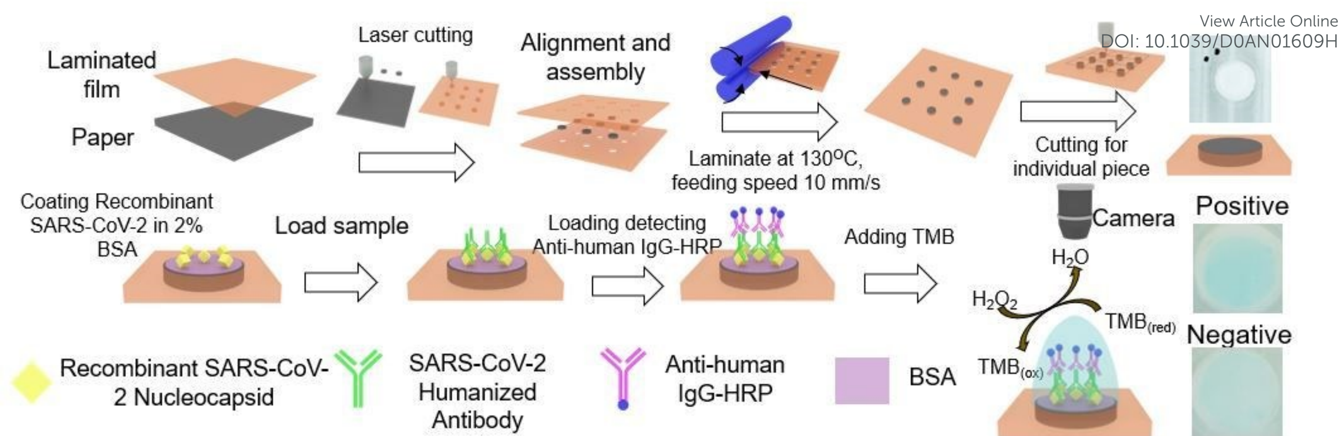

Fig. 1 Schematic diagram of the fabrication and preparation process of the paper-based device for SARS-CoV-2 humanized antibody detection assay. The paper was prepared and coated with recombinant SARS-CoV-2 antigen to capture SARS-CoV-2 humanized antibody in the sample. Subsequently, HRP/TMB reaction facilitated the naked-eye readout to validate the detection assay.

removed from the assay. The control sample used in this assay is the blank paper with TMB solution.

2.4 Colorimetric readout and data processing

After adding TMB onto the paper, the assay was placed under the camera and captured at 1st, 3rd, 5th, 10th, 15th, and 20th minute. Qualitative data were observed as the change in colour due to the TMB/HRP reaction. For quantitative data, all images were quantified and processed with MATLAB. Briefly, the individual image was imported by MATLAB. The area of interest was defined by cropping the image. The original RGB colour image was split into R, G and B channels as grayscale images. Next, the mean grey value ΔRGB was estimated for each channel as:

$$\Delta RGB = \sqrt{(R-R_0)^2 + (G-G_0)^2 + (B-B_0)^2}, \quad (1)$$

where *R*, *G* and *B* are the mean grey value from arbitrary images of red, green, blue channel respectively. The mean grey values R_0, G_0 and B_0 are of the red, green, blue channels of the control sample, respectively. To make sure that the colour change only results from the assay, the RGB value reported in this study was subtracted by background signal from negative samples as:

$$RGB\ value = \Delta RGB - \Delta RGB_{neg} \quad (2)$$

where ΔRGB is the mean grey value of the sample and ΔRGB_{neg} is the mean grey value of the negative sample, calculated from Eq. (1). All experiments were performed with at least a sample number of *n* = 3. The error bars were determined by the standard deviation.

3. Result and Discussion

Fig. 1 shows the assay protocol. SARS-CoV-2 humanized antibody was detected using TMB/HRP-based colorimetric reaction. In the presence of SARS-CoV-2 humanized antibody, the target antibody with HRP enables the oxidation of TMB and generates the blue-colour complex. This process facilitates the naked-eye observation. Moreover, the intensity of the blue colour is proportional to the concentration of SARS-CoV-2 humanized antibody in the sample. The colour intensity was quantified and processed with MATLAB. To demonstrate the specificity of the assay, we performed the assay with a sample containing SARS-CoV-2 humanized antibody at a concentration of 50 ng/µL (Positive Control), while the sample without SARS-CoV-2 humanized antibody was anti-human CA-125 (CA-125), which is non-specific to recombinant SARS-CoV-2 nucleocapsid antigen (Fig. 2). In addition to the negative control experiment, there is no colour or very low response on colour change in the absence of detecting antibody conjugated with HRP. The other negative control, where no recombinant SARS-CoV-2 nucleocapsid antigen was coated on the paper (No-target

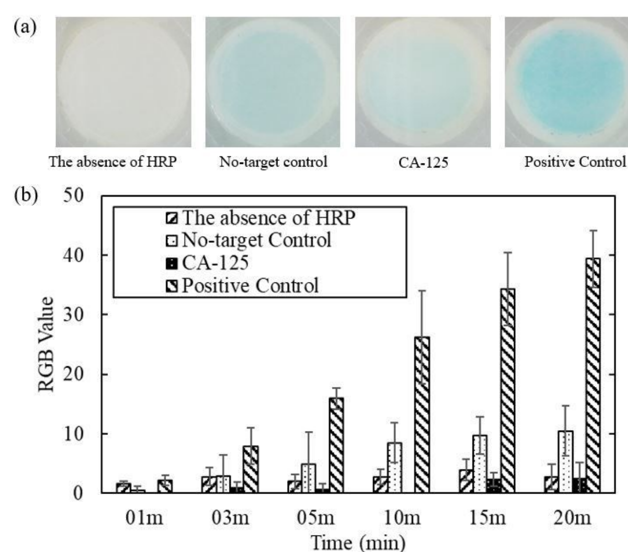

Fig. 2 Specificity of the assay. (a) Images of colorimetric assay obtained for the assay without HRP in the detecting step (The absence of HRP), the paper without recombinant SARS-CoV-2 antigen (No-target Control), the sample without SARS-CoV-2 humanized antibody (CA-125) and the sample with SARS-CoV-2 humanized antibody (Positive Control) 50 ng/µL at 20th min (b) The bar diagram for RGB value observed in 1st, 3rd, 5th, 10th, 15th, and 20th min from various conditions with known SARS-CoV-2 humanized antibody concentration at 50 ng/µL

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Control), also shows low response on colour change due to the remaining HRP in the assay. These studies suggest that our assay depends on the presence of recombinant SARS-CoV-2 nucleocapsid antigen and SARS-CoV-2 humanized antibody. Additionally, the data showed that this assay is not vulnerable to non-specific antibody sample, resulting in high specificity of the assay. The specificity result corresponds to available commercial ELISA kits, which shows a high specificity. [20, 21] It is worth noting that background signals for CA-125 and no-target controls were higher than the absence of HRP (Fig. 3a). It could result from the residue of HRP left on the paper. Many previous paper-based ELISA studies using colorimetric reaction also found the development of colour in negative samples [22-24]. There are several ways to minimize this relatively high background signals. Blocking steps including concentration of blocking solutions have to be optimized and selected[22]. In terms of quantification, the signal from the negative sample could easily be subtracted from the individual quantitative data to obtain the relative quantitative signal for a given concentration of sample. [23, 24]For the analysis of clinical samples (e.g., human serum, plasma, etc.), the background signal could be reduced via diluting the samples using specific diluent. Further details are given below.

Conventional ELISA IgG/IgM kits available in the market now are used as a qualitative assay (Yes/No assay), depending on result interpretation in each laboratory. However, our assay can evaluate the working range of antibody concentration in the sample by colour intensity generated by the assay. Furthermore, colorimetric readout can be processed and quantified by MATLAB programming which is possibly embedded in point-of-care diagnostic device. To determine the sensitivity of the assay, known SARS-CoV-2 humanized antibody concentrations were prepared in PBS (1, 5, 10, 25, 50, and 100 ng/µL). Fig. 3a depicts the naked-eye readout of the intensity of the blue colour versus the increasing antibody concentration. For negative sample, it also showed the light blue colour resulting from the remaining HRP solution. The clear observation of colour change between the positive and the negative sample is more than 10 ng/µL.

Following the above observation, the colour changes on the paper from TMB/HRP reaction were quantified via image processing. The observation agrees well with the quantitative result that the ΔRGB value of the positive sample is approximately twice than that of the negative sample. An increasing trend of the RGB value corresponds to the increasing concentration of SARS-CoV-2 humanized antibody in the range from 1 to 100 ng/µL (Fig. 3b). The increase of colorimetric responses comes from a higher concentration of SARS-CoV-2 antibody. These captured antibodies can seize anti-human IgG antibodies conjugated with HRP, which accelerate TMB/HRP reaction leading to an increase of blue colour intensity (Fig. 3a). The linear equation for colorimetric assay was fitted to be $y = 0.8338x + 2.8888$, with $R^2 = 0.9981$ with the data set of SARS-CoV-2 humanized antibody concentration from 1 to 50 ng/µL due to a linear region, the inset photo of Fig. 3c. The detection limit was evaluated by linear equation and the ratio of ΔRGB value of the positive sample to that of negative sample was about two times. These data suggest that the limit of detection (LOD) of the assay is 9.00 ng/µL or 0.112 IU/mL. Our LOD is also significantly lower than that of some conventional ELISA IgG kits available in the market (0.112 vs 5 IU/mL).[25] We have also investigated the repeatability of the assay. Fig. 4a depicts that colorimetric readout of independent tests at 20th min with 100 ng/µL SARS-

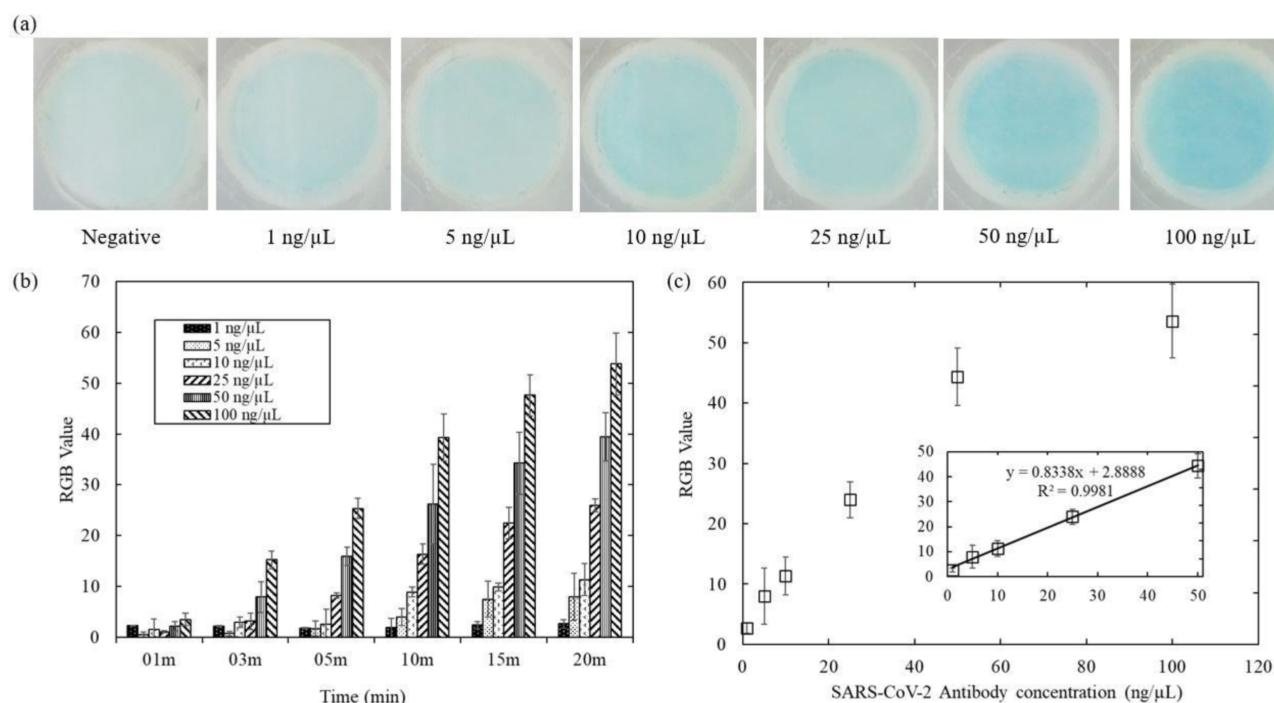

Fig. 3 Sensitivity of the assay. (a) Images of colorimetric assay of SARS-CoV-2 humanized antibody detection from a known concentration of 1 to 100 ng/µL at 20th min; (b) The bar diagram for RGB value observed in 1st, 3rd, 5th, 10th, 15th, and 20th min from a known SARS-CoV-2 humanized antibody concentration from 1 to 100 ng/µL; (c) Corresponding RGB value and SARS-CoV-2 humanized antibody concentration curve at 20th min. The inset shows the analogous linear calibration plot from the range of 1 to 50 ng/µL. Error bars represent the standard deviation of three independent experiments

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CoV-2 humanized antibody was consistent and quantified. The quantification data showed that the assay has a relative standard deviation (%RSD) of around 10% for sample $n = 3$ (Fig. 4b). The repeatability and %RSD of the assay also comparable with that of the conventional ELISA IgG kit based assay (10% vs ~ 15-20%) [25].

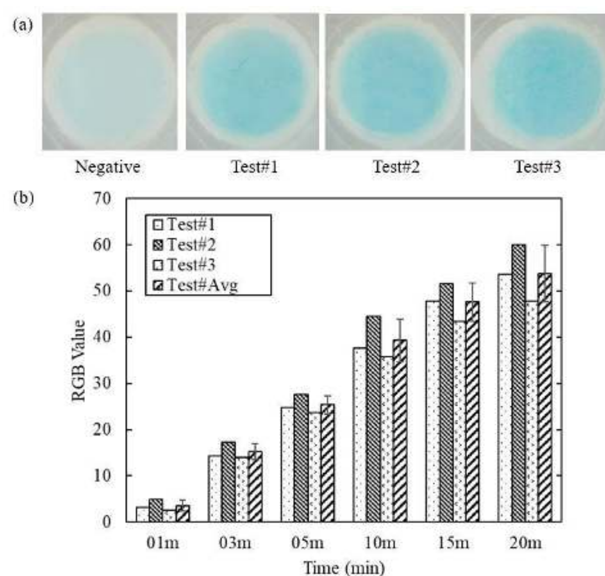

Fig. 4 Repeatability of the assay. (a) Images of colorimetric assay obtained for independent assay experiments (Test#1, Test#2 and Test#3) with 100 ng/µL SARS-CoV-2 humanized antibody at 20th min (b) The bar diagram for RGB value observed in 1st, 3rd, 5th, 10th, 15th, and 20th min from various conditions with known SARS-CoV-2 humanized antibody concentration at 100 ng/µL

To date, there is no report on colorimetric paper-based ELISA for SARS-CoV-2 humanized antibody detection. Nonetheless, there are a few reports on paper-based ELISA working with protein and antibody detection[18, 26-28]. The linear dynamic ranges of the concentration of our study (1.0 – 50 ng/µL) is similar or better than those of the reported values of other paper-based ELISA systems: NC16A autoimmune antibody (1.0 - 50 ng/µL) [26], α-fetoprotein (AFP) (0.1 – 11.2 pg/µL) [28], and neuropeptide Y (NPY) (14.04 – 64.68 fg/µL)[23]. Different types of protein could be detectable in various range of concentration thanks to protein-to-protein variation[29]. Therefore, antibody or protein detection based on paper-based ELISA needs to be optimized to obtain the working range of concentrations.

To demonstrate the suitability of our assay for complex biological samples, we performed experiments with spiked serum samples. Briefly, SARS-CoV-2 antibody was spiked into the human serum with a ratio of 1:1 between human serum and SARS-CoV-2 antibody solution. Fig. 5a depicts the readout by the intensity of blue colour with the SARS-CoV-2 humanized antibody concentration at 50 ng/µL. However, the bar diagram displayed the RGB value obtained from SARS-CoV-2 humanized antibody in serum. The blue colour for negative sample in the serum is relatively higher than the negative sample in PBS. This could be explained by the fact that commercially available healthy human serum used in this study may contain HRP or any biological impurity, which could show enzyme-like activity and thus accelerate TMB reaction resulting in colour change. Nevertheless, performed with our assay, 50 ng/µL (0.62 IU/mL) of SARS-CoV-2 humanized antibody concentration in human serum is still lower than LOD of conventional IgG ELISA kits (0.62 vs 5 IU/mL). Therefore, to improve clearer observation of SARS-CoV-2 antibody spiked in human serum, sample preparation may be required to remove irrelevant biological substances or impurities which may lead to a high background noise. Our study also demonstrated that after 50 ng/µL SARS-CoV-2 antibody was spiked in diluted (1:100) human serum, the background signal was reduced, and the quantitative result provided significantly higher RGB value than diluted (1:1) human serum (Fig. 5b). Consequently, most conventional ELISA kits working with human serum suggest that human serum is diluted at ratio 1:10 or 1:100 in a specific diluent before adding into the well plate to minimize background noise [18, 25, 30].

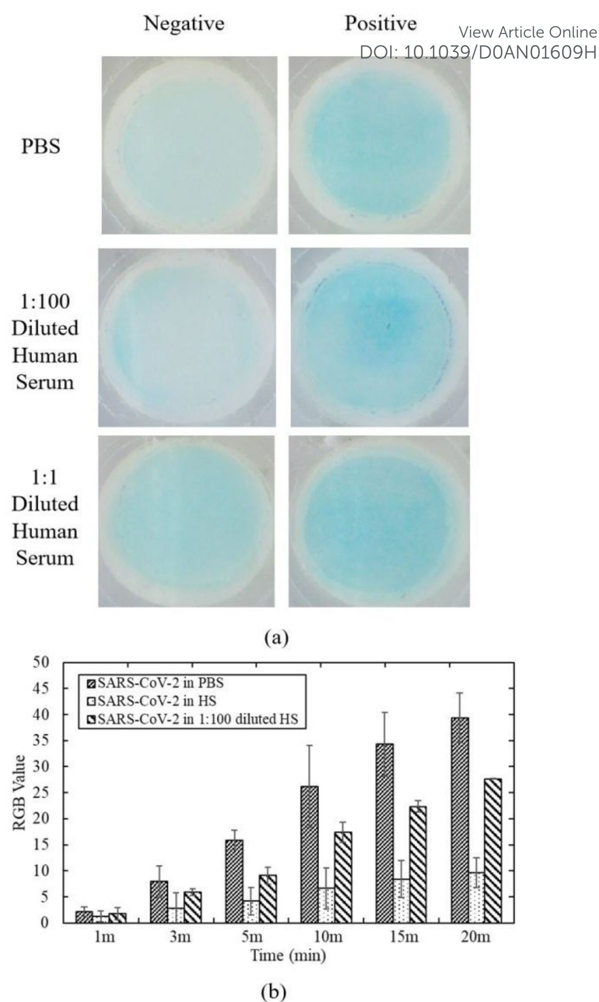

Fig. 5 Spike sample analysis. (a) Images of colorimetric assay comparing the result when 50 ng/µL SARS-CoV-2 humanized antibody was spiked in PBS (Upper row) and in human serum (Lower row) at 20th min (b) The bar diagram for RGB value observed in 1st, 3rd, 5th, 10th, 15th, and 20th min from different spike medium with known SARS-CoV-2 humanized antibody concentration at 50 ng/µL

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4. Conclusions

We have developed a rapid and inexpensive colorimetric paper-based assay. The colorimetric readout facilitates naked-eye observation and quantitative data by digital image processing. With this proof-of-concept assay, the obtained experimental data suggest that this assay can detect SARS-CoV-2 humanized antibody at 10 ng/μL (0.124 IU/mL) with %RSD ~ 10%. Whereas, LOD and reproducibility of commercial ELISA kits available in the market are 5 IU/mL with %RSD ~ 15 – 20%. There are some distinct advantages in our assays. First, this assay can be done within 30 min, which is more rapid than conventional ELISA. Second, this assay can detect SARS-CoV-2 humanized antibody in human sample, demonstrating its high potential to be an alternative way to perform serological testing in the laboratory especially in limited-resource environment. Third, the cost of an assay is around 1.45 – 1.65 USD, as shown in the cost breakdown (Table 1). However, sample preparation is required because human serum cause a relatively high background signal, which reduces the sensitivity of the assay. We believe that our assay has a potential to be developed to a point-of-care diagnostic device and can achieve sensitivity, specificity, and reproducibility similar or better to the more promising colorimetric assays of serological testing. However, using serological test for preliminary screening is still debatable because immune response to SARS-CoV-2 of infected patients was more lagging than the following symptom[31]. Thus, our paper-based assay could be used as a tool to confirm the infected active cases or to confirm SARS-CoV-2 immune cases. This paper platform could also contribute to the evaluation of vaccination efficacy in a large number of human trial samples due to its rapid, inexpensive and friendly-usage platform.

Table 1 Cost breakdown of paper-based ELISA for SARS-CoV-2 humanized antibody detection

Material list	Price/Quantity	Cost per 1000 assay
Whatman Chromatography filter paper	0.8 - 1$/20x20 cm	0.5 – 0.6$
Laminated film	0.1 - 0.2$/48 assay	2.1 – 4.2$
Recombinant SARS-CoV-2 nucleocapsid	700 - 800$/1 mg	1400 - 1600$
Rabbit Anti-human IgG	140 – 160$/0.5 mg	0.008 – 0.009$
HRP conjugation kits	70 - 80$/kit	42 - 48$
TMB solution	200 - 250$/500 mL	4 - 5$
	Total	1449 - 1658$
	Cost per assay	1.45 – 1.65$

Conflicts of interest

There are no conflicts to declare.

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