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# Sandwich/competitive immuno-sensors on micro-interface for SARS-CoV-2 neutralizing antibodies

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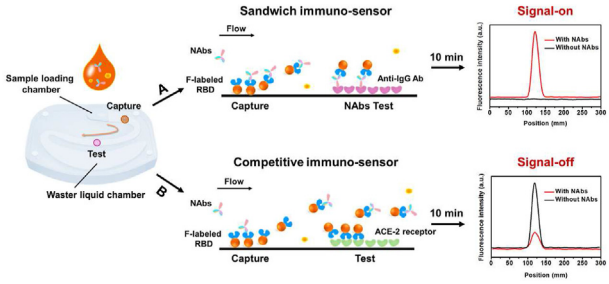
## HIGHLIGHTS

- Sandwich/competitive immuno-sensors based on lateral chromatography micro-interface was developed for quantification of SARS-CoV-2 neutralizing antibodies (nAbs) within 10 min.
- Sandwich immuno-sensor was able to detect nAbs from 4.0 ng/mL to 400 ng/mL and displayed comparable performance with existing methods.
- Sandwich immuno-sensor was applied to detect nAbs in 182 clinical serum samples from vaccinated cases.

## GRAPHICAL ABSTRACT

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## ABSTRACT

A simple, rapid and robust method to quantify SARS-CoV-2 neutralizing antibodies (nAbs) is urgently needed for determining COVID-19 serodiagnosis, vaccine development and evaluation of vaccine efficacy. In this study, we report sandwich/competitive immuno-sensors based on lateral chromatography micro-interface for accurate quantification of SARS-CoV-2 nAbs. Fluorescent microspheres (FMS) labeled receptor binding domain (RBD) antigen was prepared for detection of nAbs with high sensitivity. Sandwich and competitive immunoassay were conducted on the microfluidic-based sensor within 10 min and the fluorescent signal of immunoassay was analyzed by a portable microfluidic immunoassay instrument. The nAbs detection range of sandwich immuno-sensor and competitive immuno-sensor was 4.0 ng/mL to 400 ng/mL and 2.13 ng/mL to 213 ng/mL, respectively. Furthermore, the sandwich immuno-sensor was demonstrated to be comparable with existing methods and used to detect 182 clinical serum samples from vaccinated individuals. Sandwich immuno-sensor based on lateral chromatography micro-interface allowed reliable, fast, and low-cost detection of nAbs, which holds considerable potential for nAbs testing.

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## 1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic emerged in late 2019 is expected to continue to pose an extraordinary threat to global public health while severely disrupting societies and economies worldwide [1]. The novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [2], belongs to a betacoronavirus, which has a single-positive strand RNA genome and four structural proteins: spike (S), envelope (E), matrix (M), and nucleocapsid (N) [3]. In the process of viral infection, the spike glycoprotein (comprising a S1 subunit and S2 subunit in each spike monomer) on the SARS-CoV-2 surface is responsible for mediating receptor recognition and membrane fusion [4]. The receptor binding domain (RBD) of S1 directly binds to the cell receptor angiotensin-converting enzyme 2 (ACE-2), whereas S2 is responsible for membrane fusion [5,6]. Based on the spike glycoprotein's critical interaction with the cell receptor ACE-2, spike glycoprotein is regarded as the receptor target of neutralizing antibodies (nAbs) for vaccine design and development.

The rapid development and injection of safe and effective COVID-19 vaccine is the most advantageous weapon in the fight against the epidemic. Neutralizing antibodies (NAbs) against SARS-CoV-2 are antibodies produced by inducing an immune response after the injection of the COVID-19 vaccine that can protect against infection [7–9]. NAbs testing provide the best evidence for the evaluation of both vaccine efficacy and establishment of protective immunity. Currently, nucleic acids-based molecular diagnosis such as reverse transcription polymerase chain reaction (RT-PCR) [10] and isothermal nucleic acid amplification methods [11,12] have been developed for detection of SARS-CoV-2. Immunoassays for SARS-CoV-2 testing were also established by targeting antibodies (e.g., IgG, IgM, antigen) [13,14]. However, in order to better monitor the immune status of convalescent COVID-19 patients, herd immunity, as well as to quickly and timely evaluate the vaccine efficacy during clinical trials and after mass vaccination, there is an extreme demand for the rapid and large-scale detection of SARS-CoV-2 nAbs.

Recently, based on the principle that nAbs inhibit the binding of the spike glycoprotein RBD to ACE-2, several methods have been developed to detect SARS-CoV-2 nAbs [5,15–17]. For example, Linfa Wang and Danielle E. Anderson together reported a SARS-CoV-2 surrogate virus neutralization test without the requirement of using any live virus or cells, which could be carried out in the biosafety secondary laboratory (under the same conditions as the ordinary enzyme-linked immunosorbent assay). The authors used the purified RBD of the viral spike protein and the ACE-2 receptor of the host cell to simulate the interaction between the virus and the host on an enzyme-linked immunosorbent assay plate. The study found that this effect could be inhibited by specific nAbs in the serum of patients or animals in a way similar to traditional virus neutralization and virus neutralization test based on pseudovirus. This method also enabled to distinguish between nAbs and antibodies that bind to RBD but do not neutralize viruses [15]. However, this surrogate virus neutralization test takes 1–2 h that is unable to satisfy the demand of rapid detection. Besides, methods based on chemiluminescent immunoassay have been reported for SARS-CoV-2 nAbs testing with high throughput while limited by expensive instruments [18,19].

In this work, we explored sandwich/competitive immuno-sensors on lateral chromatography micro-interface for accurate quantification of SARS-CoV-2 nAbs. This microfluidic-based immuno-sensor platform allowed performing sandwich and competitive immunoassay for detecting nAbs in 10 min. RBD antigen-labeled with fluorescent microspheres (FMS) was used as the detection signal for nAbs, which could be simply read by a portable microfluidic immunoassay instrument. With favorable simplicity, rapidity, and low cost, this microfluidic immunoassay platform holds great promise for detecting nAbs.

## 2. Materials and methods

### 2.1. Materials

Fluoro-max dyed carboxylate-modified microparticles (fluorescent microspheres (FMS), 200 nm) and sulfo-NHS were obtained from Thermo Fisher Scientific (China) Co., Ltd. (Shanghai, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and bovine serum albumin (BSA) were purchased from Sigma Aldrich Trading Co., Ltd. (Shanghai, China). RBD antigen and ACE-2 receptor were obtained from JuKang Biotechnology Co., Ltd. (Hangzhou, China). Mouse anti-human IgG antibody was from Hangzhou ebiocore Biotechnology Co., Ltd. (Hangzhou, China). Trehalose Dihydrate was from Wako Pure Chemical Industry Co., Ltd. (Japan). Phosphate buffer saline (PBS, pH 7.4) were obtained from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Sample dilution buffer (0.1 M PBS, pH 7.4, 0.1% TX-100, 1% BSA, 3% NaCl) was from Shanghai Suchuang Diagnostic Products Co., Ltd. (Shanghai, China). FMS dilution buffer was prepared by ultrapure water containing 2% BSA and 1% trehalose. Antibody dilution buffer was prepared with 0.02 M PB and 0.02% TX-100.

Microfluidic chips and the portable microfluidic chip fluorescence instrument [14,20] were provided by Shanghai Suchuang Diagnostic Products Co., Ltd. (Shanghai, China). Clinical serum samples were collected and tested by our method in Shanghai East Hospital (affiliated East Hospital of Tongji University, Shanghai, China). 18 cases of these serum samples were also test by commercial methods based on competitive chemiluminescent immunoassay and sandwich fluorescence immunoassay, respectively. The written informed consent had been obtained since the start of the project. All experiments on clinical samples were performed in compliance with the Medical Research Ethics Committee, Shanghai Pediatric Hospital. All of clinical samples were detected directly in this assay and each extra sample was stored at −80 °C for further study.

### 2.2. Preparation of sandwich/competitive immuno-sensors

First, the modification of fluorescent microspheres (FMS) with RBD antigen was achieved by referring to previous method [14,20]. respectively. Briefly, 50 μL of FMS was dispersed in 1000 μL of MES buffer (0.05 M, pH 6.1) through ultrasound for 2 min. The FMS was activated by freshly prepared 20 μL of 1 mg/mL EDC and 50 μL 1 mg/mL sulfo-NHS at 25 °C for 30 min. Excess reagents were removed through centrifugation at 15,000 rpm for 35 min (4 °C). Carboxyl activated FMS were re-suspended in 1000 μL MES buffer through ultrasound for 2 min. 53.3 μg RBD antigen diluted with 20 μL of MES buffer was added to the activated FMS and incubated for 150 min at 30 °C. For blocking nonspecific binding sites, 100 μL of 0.5 M glycine and 100 μL of 10% BSA were added and incubated for 15 min and 30 min at 30 °C, respectively. After conjugation, FMS-labeled RBD were purified and re-suspended in PBS buffer containing 3% trehalose, 1% BSA and stored at 4 °C for further study. For sandwich immuno-sensor, 0.5 μL of 1.0 mg/mL mouse anti-human IgG antibody was patterned on the test region on the surface of bottom substrate layer; 3 μL of FMS coated RBD antigen was spotted onto capture region. For competitive immuno-sensor, 0.5 μL of 1.0 mg/mL ACE-2 receptor was patterned on the test region on the surface of bottom substrate layer; 3 μL of FMS-labeled RBD antigen was spotted onto capture region. The patterned bottom layers were then dried at 37 °C for 30 min. Finally, the immunoassay microchips

were assembled by the double-sided adhesive layer, incubated at 37 °C for 12 h, and stored at 2–8 °C for use. The prepared microchips can be stored for above 6 months in sealed and dark conditions.

### 2.3. Detection of NAbs on lateral chromatography micro-interface

The fluorescence immuno-sensors for detection of neutralizing antibodies (nAbs) on the developed lateral chromatography micro-interface was carried out as follows. 10 μL of sample was directly added to sample loading chamber and followed by 80 μL of sample dilution buffer, the mixed liquid flowed slowly through the detection channel to the waste liquid chamber, and the reaction lasted for 10 min at room temperature. After reaction, three immunoassay chips were placed in the portable immunoassay microfluidic chip instrument in a single run and centrifuged for 10 s to remove the residual liquid in the channels following fluorescence detection for 1 min. The absolute fluorescence intensity of test (defined as test value, T value) can be automatically obtained from the instrument for quantification analysis.

## 3. Results and discussion

### 3.1. Principle of sandwich and competitive immuno-sensors

Herein, a simple, washing-free, and low-cost fluorescence immuno-sensor was constructed on microfluidic platform to realize rapid and quantitative detection of SARS-CoV-2 nAbs. Two formats, sandwich and competitive immunoassay ("signal-on" system and "signal-off" system respectively) were established on this designed microfluidic chip. The microfluidic chip was designed and fabricated according to our previous work with some modification [14,20], which comprised three layers: a top layer, a bottom layer, and the double-sided adhesive layer (Fig. S1A).

Generally, IgG and IgM against SARS-CoV-2 are detected when using serological testing including neutralization testing [21,22]. It is reported that the detection of IgG show better performance than that of IgM for SARS-CoV-2 serological testing [22]. Besides, studied also have demonstrated that IgG levels could be used for predicting nAbs levels [23–26]. Because the titer of IgM antibody is usually low, and lasts for a short time, while IgG is produced in later stages along with high concentration, longer duration, and higher affinity [27]. Therefore, when sandwich-based "signal on" immuno-sensor was developed on lateral chromatography micro-interface for SARS-CoV-2 nAbs detection, IgG was selected as the target. As displayed in Fig. 1A, after the serum sample was loaded into the microchip, nAbs could be specifically recognized and captured by FMS-labeled RBD. These captured nAbs complexes continuously migrated to the test region and formed a sandwich-type immunocomplex with the anti-IgG Ab through the "antigen-antibody" affinity interaction. The microchips were detected via the portable fluorescence analyzer (Fig. S1B) to obtain fluorescence curve and the absolute fluorescence intensity (T value) increases with increasing concentration of nAbs. On the other hand, the principle of competitive-based "signal-off" immuno-sensor is illustrated in Fig. 1B, as the solution of nAbs samples migrated to the capture region, a part of F-labeled RBD was inhibit by nAbs firstly, then the uninhibited F-labeled RBD was recognized and captured by ACE-2 receptor in the test region, fluorescence signal in the test region decreases with increasing concentration of nAbs.

### 3.2. Competitive immuno-sensor for NAbs detection

Currently, the competitive-based immunoassay has been well-studied and employed for detecting nAbs [5,15,19], we thus first assess the detection performance of competitive immuno-sensor on microchips. Standard sample of nAbs (2.13 mg/mL) was diluted to be 10-fold, 100-fold and 1000-fold, and then detected by competitive immuno-sensor. A standard curve was obtained between T value and the log of the fold of dilution (Fig. 2A), which had a good detection ability with a detection limit of 2.13 ng/mL 11 cases of clinical serum samples from unvaccinated volunteers without history of COVID-19 infection (nAbs negative) were test by the competitive immuno-sensor on micro-interface to further verify this method. As shown in Fig. 2B, these negative samples could not inhibit the binding interaction between RBD and ACE-2, thereby generating very strong fluorescence signal. However, the results of negative case 1 and case 2 samples generated relatively weak signal that were very close to the results of nAbs positive samples at low levels, which may fail to distinguish between nAbs negative cases and nAbs positive cases in practice.

### 3.3. Sandwich immuno-sensor for NAbs detection

Sandwich immuno-sensor on micro-interface was investigated by detecting a series concentration of standard nAbs (400 ng/mL, 100 ng/mL, 40 ng/mL, 4 ng/mL, 0 ng/mL). The detection results were showed in Fig. 3A and B. The standard samples from 4 to 400 ng/mL generated increasing fluorescence signals at the test region (~125 mm), while very weak signal was detected when in the absence of nAbs (Fig. 3A). Fig. 3B demonstrated a good linear relationship between T value and the concentration of nAbs. Sandwich strategy is almost comparable to competitive strategy in term of the sensitivity for detecting nAbs.

To assess the feasibility of lateral chromatography micro-interface-based sandwich immuno-sensor for practical diagnosis, a total of 30 clinical serum samples including nAbs positive control ($n = 21$) and nAbs negative control ($n = 9$) were detected. These positive control samples produced fluorescence signal at different levels (Fig. 4A), while negative control samples generated almost no or very weak fluorescence peak due to the absence of nAbs (Fig. 4B). Concentrations of nAbs from the tested samples were further calculated through the standard curve in Fig. 4B according to the obtained T values. We can observe that there is a significant difference between positive control and negative control (Fig. 4C, $p < 0.0001$, using the Mann–Whitney no parametric test). The performance of sandwich immuno-sensor was further compared with two commercial methods (competitive chemiluminescence immunoassay and sandwich fluorescence immunoassay) by testing clinical serum samples ($n = 18$), the corresponding data was provided in Table S1. Our method was highly consistent with the two existing methods (Fig. 4D), suggesting that sandwich immuno-sensor has good accuracy and reliability for nAbs testing in practical application.

### 3.4. Analysis of clinical samples

This proposed lateral chromatography micro-interface-based sandwich immuno-sensor system was further applied to detect a total of 182 clinical serum samples from vaccinated individuals. These samples were collected and test in five different time periods using microchips prepared in the same batch, among them, a total of 58 cases' samples were test on the day of the first vaccination (first test). 21 days later, 40 cases' samples were test on the day of the second vaccination (second test). 28 days after the second vaccination, 29 cases' samples were test (third test). At the fourth and fifth test, 27 cases and 28 cases' samples were test at three months (90 days) and six months (180 days) after the second vaccination, respectively. At the first test, 24 cases were detected as negative (nAbs, 0 ng/mL), 34 cases (58.62%) were detected as

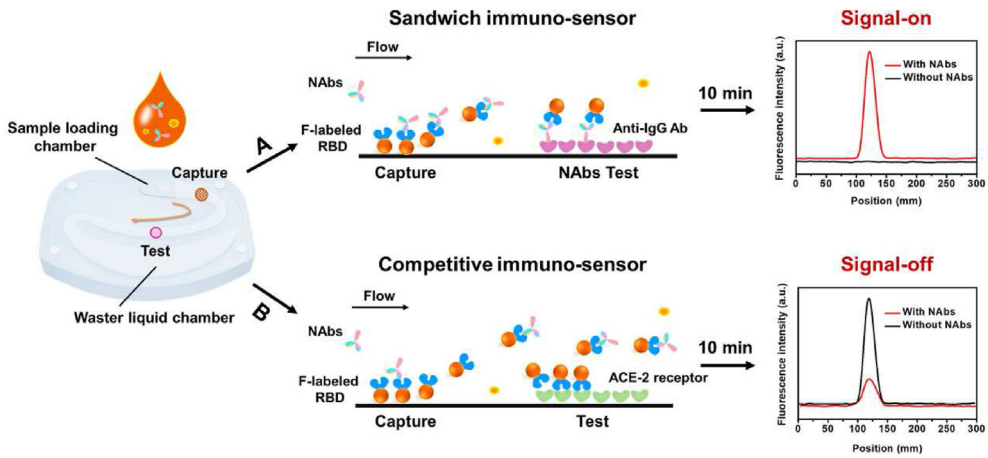


**Fig. 1.** Schematic diagram of (A) sandwich immuno-sensor and (B) competitive immuno-sensor on lateral chromatography micro-interface for nAbs detection. NAbs: Neutralizing antibodies; F-labeled RBD: fluorescent microspheres (FMS) labeled RBD antigen; Anti-IgG Ab: Anti-IgG antibody; ACE-2: angiotensin-converting enzyme 2 receptor.

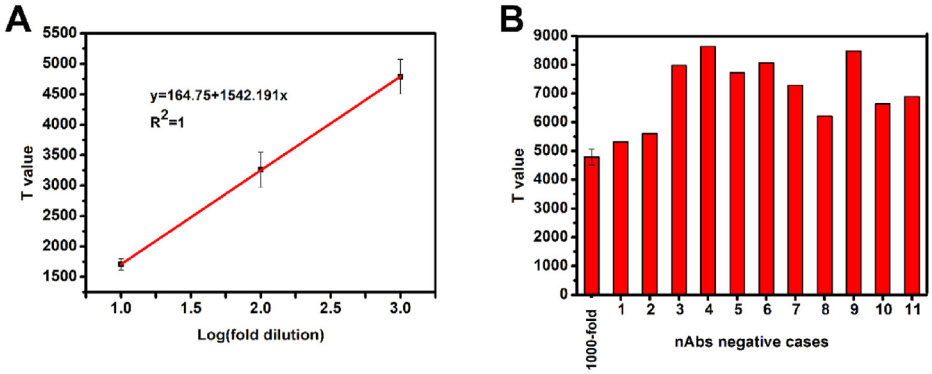


**Fig. 2.** Results of competitive immuno-sensor for nAbs detection. (A) The standard curve between the absolute fluorescence intensity (T value) and the log of fold dilution of nAbs. Standard nAbs (2.13 mg/mL) was diluted serially to 10-fold, 100-fold, and 1000-fold. (B) Practical detection results of 11 cases of clinical serum samples (nAbs negative) compared with 1000-fold diluted standard nAbs sample.

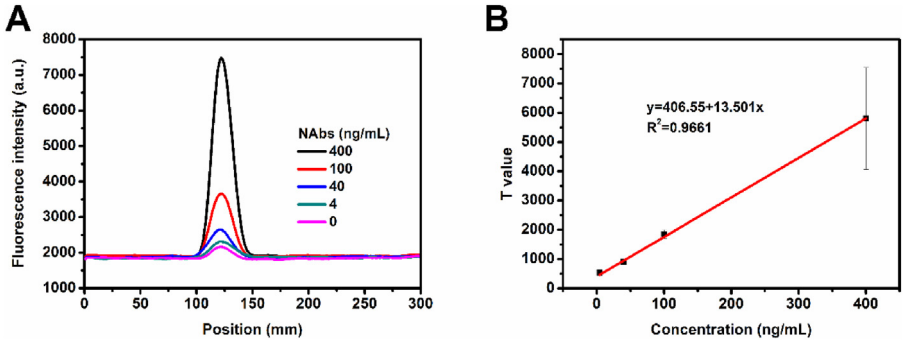


**Fig. 3.** Results of sandwich immuno-sensor for nAbs detection. (A) Fluorescence signal results of serial dilutions of standard nAbs (400 ng/mL, 100 ng/mL, 40 ng/mL, 4 ng/mL, 0 ng/mL). (B) The linearity between T value and the concentration of nAbs.

positive in different levels (Fig. 5A). At the second and third test, 18 cases and 3 cases were detected as negative respectively, 22 cases (55.00%) and 26 cases (89.66%) were detected as positive in different levels respectively (Fig. 5B and C). At the fourth and fifth test, a total of 25 cases (92.59%) and 22 cases (78.57%) were positive respectively (Fig. 5D and E). Among the five tests, the positive ratio of nAbs from the vaccinated individuals increased obviously after the second vaccination and reached 92.59% after three months, 6 months later, the positive rate of nAbs still remained 78.57% (Fig. 6A). The concentration level of nAbs sharply increased firstly and then decreased from continuously monitored individuals (n = 29) during the five tests (Fig. 6B), These findings were consistent with the results of other recent studies of neutralization by convalescent serum or serum obtained from recipients of vaccines [23,28–31]. The results together demonstrated the potential application of sandwich immuno-sensor for clinical nAbs testing.

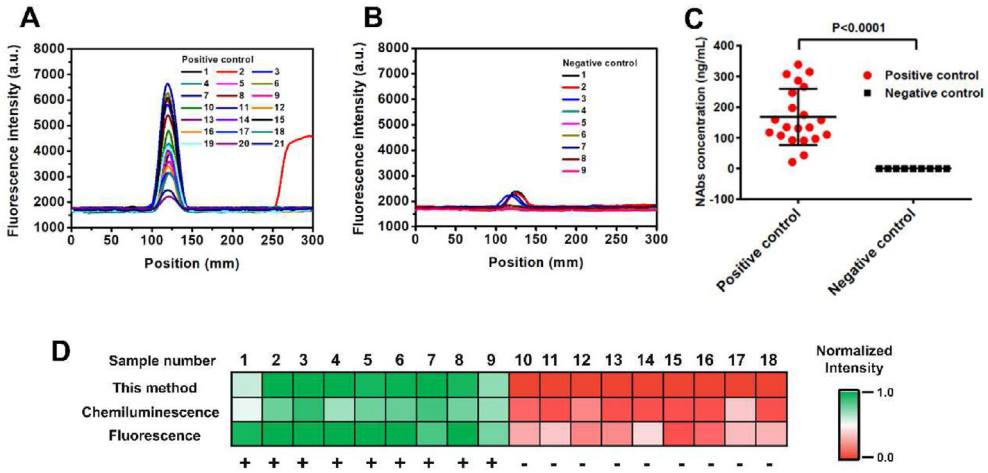


**Fig. 4.** Assessment the feasibility for practical testing using sandwich immuno-sensor. (A) nAbs positive control samples (n = 21) and (B) nAbs negative control samples (n = 9); (C) comparison of the detected concentration of nAbs between nAbs positive control samples (A) and negative control samples (B) using the Mann–Whitney no parametric test. (D) Comparison of the performance of sandwich immuno-sensor and two commercial methods (chemiluminescence indicates competitive chemiluminescence immunoassay and fluorescence indicates sandwich fluorescence immunoassay) for detecting clinical serum samples (n = 18). + indicates positive, - indicates negative.

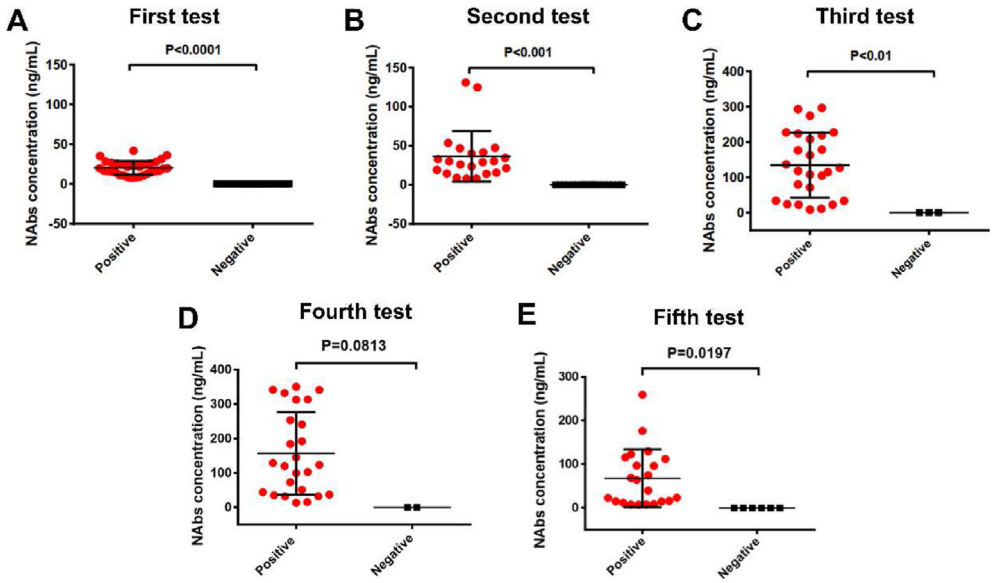


**Fig. 5.** Sandwich immuno-sensor for quantification detection of nAbs from (A) 58 cases vaccinated individuals on the day of the first vaccination (first test), (B) 40 cases vaccinated individuals on the day of the second vaccination (21 days after the first vaccination) (second test), (C) 29 cases vaccinated individuals on 28 days after the second vaccination (third test), (D) 27 cases vaccinated individuals on 90 days after the second vaccination (fourth test) and (E) 28 cases vaccinated individuals on 180 days after the second vaccination (fifth test).

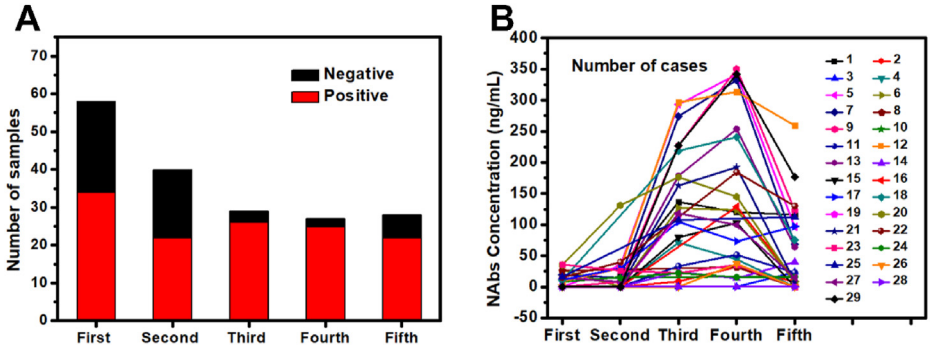


**Fig. 6.** (A) Analysis of the proportion of nAbs positive and nAbs negative of each test during the five tests from vaccinated individuals (total 182 serum samples). First: 58 cases were test on the day of the first vaccination, second: 40 cases were test on the day of the second vaccination (21 days after the first vaccination), third: 29 cases were test on 28 days after the second vaccination, fourth: 27 cases were test on 90 days after the second vaccination, and fifth: 28 cases were on 180 days after the second vaccination. (B) Continuous monitoring the trend of nAbs levels from 29 vaccinated individuals during the five tests.

## 4. Conclusion

Lateral chromatography micro-interface-based fluorescence immuno-sensor platform was established for detecting nAbs. Two formats, sandwich immuno-sensor (signal-on) and competitive immuno-sensor (signal-off) were developed and carried out on microfluidic chips. The sandwich immuno-sensor and competitive immuno-sensor was capable of detecting nAbs in the range of 4 ng/mL-400 ng/mL and 2.13 ng/mL-213 ng/mL respectively in 10 min. In addition, this sandwich-based approach was demonstrated to have good consistency with other methods for diagnosis of clinical samples. The proposed sandwich immuno-sensor assay was used to detect nAbs from 182 clinical samples, which would be useful in rapidly quantifying SARS-CoV-2 nAbs in convalescent COVID-19 patients and vaccinated individuals as well as evaluation of vaccines.

## CRediT authorship contribution statement

**Qiuyuan Lin:** Investigation, Writing – original draft, Data curation, Validation. **Jingjing Wu:** Conceptualization, Methodology, Visualization. **Liling Liu:** Investigation, Methodology. **Wenjuan Wu:** Writing – review & editing, Supervision. **Xueen Fang:** Writing – review & editing, Project administration, Supervision. **Jilie Kong:** Resources, Funding acquisition.

## 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.339144.

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