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ORIGINAL ARTICLE

Evaluation of diagnostic accuracy of developed rapid SARS-COV-2 IgG antibody test kit using novel diluent system

Vani Maya[1] · Jyothi Embekkat Kaviyil[3] · Dinoop Koral Ponnambath[3] · Renjith P. Nair[2] · Anugya Bhatt[2] · Rajendra Pilankatta[4] · Lathika Valliyot[4] · Ranjeet Dungdung[4] · Ramdas Athikkal Veettil[5] · Manoj Gopi[1]

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Abstract Immunochromatographic assay kits are used in primary diagnostics which is based on the principle of antigen and antibody interaction. These kits play pivotal role in rapid surveillance of infectious diseases at early stages as well as for the surveillance of the contagious diseases. The immunochromatographic test kits lacks sensitivity and specificity with certain diseases. In this study, our intention was to develop a rapid test kit for SARS-COV-2 with a novel diluent system to enhance the efficacy of antigen–antibody binding and thereby the improvement in the sensitivity outlined. Finally, IgG antibodies against SARS-COV-2 virus peptides were analyzed using 25 positive and 25 negative confirmed clinical samples. The sensitivity of the clinical studies showed 91% sensitivity and 100% specificity. Therefore, the authors propose that this assay will be a potential tool for efficient community or sentinel surveillance of SARS-COV-2 infection and additionally, for effective monitoring of convalescent sera therapy.

✉ Manoj Gopi
dhanmanu@gmail.com

[1] Division of Artificial Internal Organs, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala, India

[2] Thrombosis Research Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala, India

[3] Department of Microbiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala, India

[4] Department of Biochemistry and Molecular Biology, Central University, Kasaragod, Kerala, India

[5] District Medical Hospital, Karattuvayal, Kanhangad, Kerala, India

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Introduction

In the immunological diagnostic/surveillance systems, antigen–antibody ligand formation plays a vital role in determining the sensitivity and specificity of a particular assay [1–3]. When a foreign antigen enters the human system, the host normally produces antibodies as part of the immune response, with subsequent formations of specific antigen- antibody complexes. Most infectious diseases are often diagnosed based on the detection of antigens and/or antibodies from the relevant biological samples [4, 5]. Due to certain conditions, such as low antibody and/or antigen titers or masking of the antibodies with other proteins, the detection of specific antibodies from the biological samples becomes complicated [6].

Timely detection of the etiologic agent is essential for appropriate diagnosis of the infectious diseases in order to aid targeted treatment. Also surveillance strategies in case of highly contagious diseases are important to contain spread of disease in population. We are currently in an era of rapid turnaround times [TAT] with immunochromatographic test kits playing a major role in aiding rapid and accurate diagnosis of some of the major infectious diseases [7]. Hence, developing newer immunochromatographic test devices incorporating novel diluent systems and other techniques that can enhance the sensitivity and specificity is essential in bringing about a better diagnostic ecosystem.

The SARS-CoV-2, causing COVID 19 infection and first isolated from China, has caused a pandemic across the globe [8, 9]. Infections with this virus can manifest with a wide range of symptoms—from asymptomatic to severe

infections. In severe cases, especially in people who are immunocompromised or with underlying chronic disorders, the virus can cause severe acute respiratory syndrome [SARS], organ failure and death [10]. In the current situation with the apparent lack of an effective vaccine, rapid detection and treatment is the best strategy that is being adopted across the world. The gold standard for SARS-CoV2 detection is real-time reverse transcriptase polymerase chain reaction [RT-PCR] due to its high sensitivity and accurate diagnosis. However, antibody-based tests are quite essential for initial mass-population screening to evaluate herd immunity and also for diagnosing asymptomatic cases [11–13]. Antibody-based tests are relatively inexpensive and less labor intensive. However, accuracy of the rapid antibody kits is relatively low due to inefficient or non-specific antigen-antibody binding, inappropriate target antigen selection or labelling methods and/or ineffective solvent systems. In this study, we aimed to develop a novel diluent composition and an immunochromatographic test device for enhancing the detection of IgG antibodies against SARS-CoV-2 in human samples.

Materials and methods

Lateral flow technology flow

In order to develop a lateral flow immunochromatographic testing device, the work was divided into 4 parts namely development of the testing strips, preparation of the diluents, quality control of the prepared strips and diluents, and the stability studies. Further, the efficacy of the developed kits were validated using clinical sera samples.

Development of membrane strips

The lateral flow device employs a membrane or a test strip through which the test sample flows. The present study used nitrocellulose membranes treated with 1% w/v Bovine Serum Albumin [BSA]. Anti- Human IgG antibody pre-coated in nitrocellulose membranes and recombinant spike protein antigen of SARS-COV-2 tagged with colloidal gold were used in test region, whereas in control, Anti-Rabbit IgG antibody precoated nitrocellulose membrane and Rabbit-IgG tagged with collodidal gold sprayed conjugates were used in control region for developing the strips [12]. The pre-coated membranes were made into 3.7 mm strips using a cutting machine [Fig. 1]. Each of these strips were further assembled into plastic cassettes and finally packed in aluminum pouches. Optimal results were achieved only when production room are to be maintained at a relative humidity of $\sim$ 50% at standard room temperature [18–22 °C, 65–72 °F].

Preparation of Diluents

Diluent buffer was prepared with the intention to maintain the optimum pH in reactions, to minimize non-specific binding, to enhance binding efficacy, and to control the flow rates. In order to achieve these desired effects, five different formulations for SARS-COV-2 lateral flow assay were made with varying concentrations of Phosphate Buffer Saline [PBS], Tween 20, BSA, Sodium dodecyl sulfate [SDS], Boric Acid, Tris HCl [Hydrochloric Acid], Sodium tetra borate, Sucrose, Thimersol, and Proclin-300 [Table 1]. All general consumables used for preparation of diluents were procured from Sigma Aldrich through institute local purchase division.

These formulations were then optimized on the basis of solubility of proteins and the hydrophobic attractions of the nitrocellulose membranes.

Quality control measures

The assembled kits were tested for quality using blood samples collected from Reverse Transcriptase Polymerase Chain Reaction [RT-PCR]—confirmed cases of COVID 19 after obtaining the necessary ethical clearance from the Institute Ethics Committee, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India [SCT/IEC/1536/MAY/2020]. Five serial dilutions of the characterized plasma samples were done with 1X PBS buffer from 1:4 to 1:128. All five combinations of the buffer were tested using these proven clinical samples to record the quality and sensitivity. PBS and samples from healthy individuals were used as the negative controls for the quality check. The testing were repeated with different batches of test kits to ensure the reproducibility of results.

Stability studies

Accelerated stability studies were performed for the SARS-COV-2 IgG rapid test and the diluents in four different batches. The batches of samples were kept in different conditions such as at 2–8 °C, room temperature [Storage temperature], 37 °C + relative humidity of 90% and 45 °C for a period of one month. Finally, all test kits were evaluated on 15th and 30th days to ensure the stability of the developed kits. Strips showing good stability till 30th day with all four sets was considered to have an approximate shelf life of 2 years [14].

Clinical validation

Clinical studies were conducted at Indian Council for Medical Research approved testing centers in the State of Kerala- SCTIMST Trivandrum and Central University,

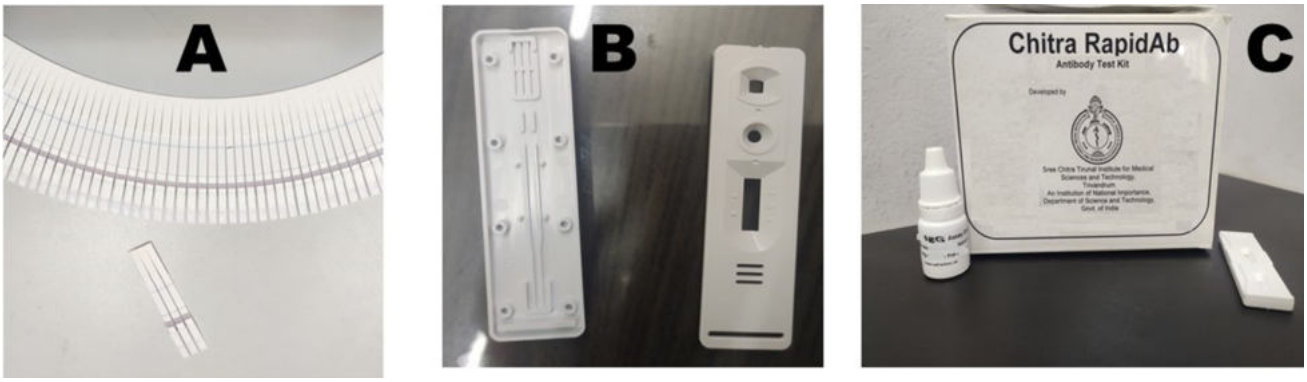

Fig. 1 Image (**A**) showing the strips developed with 3.7 mm width (**B**) showing the empty cassettes for strip assembling. (**C**) showing the fully packed strips in cassettes and diluent systems

Table 1 Composition of different diluent formulations A, B, C, D and E

Formulations	Composition
Diluent A	NaCl (80 g), Na_2HPO_4.12 H_2O (51.6 g), $NaHPO_4$.2H_2O (9.9 g), Tween 20 (5 ml), Dissolved in 1L distilled water and add the preservative Proclin 300 (0.3%), Adjust the pH at 7.4
Diluent B	Na_2HPO_4. 2H_2O (7.1 g), Triton (10 ml), BSA (10 g), Dissolved in 1L distilled water and adjust the pH at 8
Diluent C	Nacl (8.16 g), Na_2HPO_4 (1.419 g), KH_2PO_4 (0.202 g), Tween 20 (0.05%), Sucrose (20%), Trehalose di hydrate (5%), SDS (0.5 g), Triton (10 ml), BSA (10 g), Proclin 300 (0.01%), Dissolved in 1L distilled water and adjust the pH at 7.4
Diluent D	Boric acid (6.2 g), Nacl (4.4 g), Sodium tretaborate (9.5 g) Dissolved in 1L distilled water and adjust the pH at 8
Diluent E	Tris HCl (10%), NaCl (5 g),BSA (10 g), Tween 20 (0.5%), Dissolved in 1L distilled water and adjust the pH at 8

Kasaragod after obtaining the necessary ethical approvals [SCT/IEC/1536/MAY/2020]. A total of 50 samples (serum samples from 25 COVID-19 RT-PCR confirmed patients, and 25 healthy volunteers) were used for the validation process. Study enrolled only RT-PCR positive cases whose samples were collected between 7 and 14th day of onset of symptoms. It did not include asymptomatic cases.

Testing procedure

The testing protocol that was standardized to detect IgG antibodies against SARS-CoV-2 in the human sera using the lateral flow immunochromatographic test kit developed by the study team is as follows:

Peripheral blood [10–15 μL] samples were collected by finger prick by a clinician and/or trained health worker using sterile lancets. The blood samples were slowly added to the pre-prepared SARS-COV-2 test strips in the sample addition port. Immediately after that, 30–40 μL of diluent Buffer C was added into the sample port for facilitating sample movement and enhanced binding. The movement of sample and buffer across the membrane occurs due to the capillary action. Anti-SARS-CoV-2 IgG antibodies, if present in the specimen, get bounded onto the SARS-CoV-2 antigen labelled colloid gold reagent fixed on the conjugate pad. When buffer flows into the conjugate pad, gold conjugated antigen complex lifts off from the conjugate pad and moves into the nitrocellulose membrane. The complex get captured by the immobilized Anti-Human IgG antibodies as they migrate over the band and a sandwich complex is formed thereby developing a coloured test line. The remaining rabbit IgG-colloidal gold complex moves forward through the nitrocellulose membrane to form the control line on the control region. The formation of the control line indicates the validity of the test [Fig. 2].

Statistical methods

The sensitivity and specificity of the test was measured by using MedCalc Version 19.5.

Results and discussion

Kit developments and diluents

The SARS-COV-2 rapid test kit was assembled based on the standard guidelines and thus produced kits were used

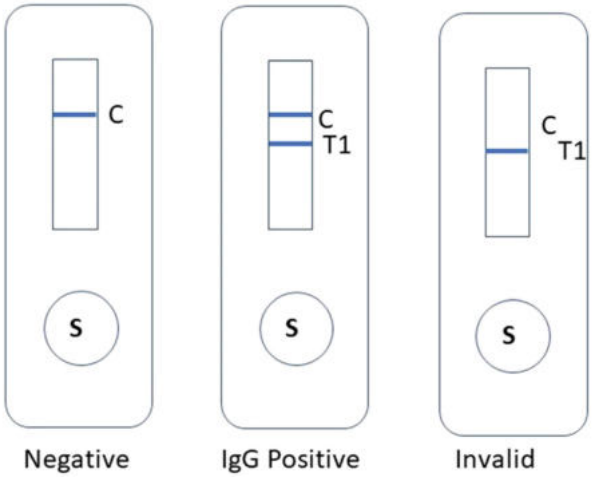

Fig. 2 Image showing the result interpretations of the rapid IgG test kit

for further laboratory validations, following which the clinical studies were undertaken. Since we used the pre-coated membranes there were no efficacy analysis performed for checking the effectiveness of monoclonal antibodies used for the developments. The effectiveness of dehumidified clean room facilities for producing the rapid test kit batches plays a key role in determining the accuracy of the developed assays. Five different buffer combinations were used to achieve the maximum binding efficiency. Efficiency of the different diluent formulations was compared by examining the band formation with different concentrations of sample. Serially diluted sample were added in the ratios of 1:32, 1:64 and 1:128 and followed by each diluent. Among the different diluents formulated, only diluent C showed a band with good intensity upto 1:64 dilution of the sample. Diluent C is having additives including sucrose, trehalose and BSA compared to other diluents which have considerable effects on the specific reactivity of anti IgG antibodies and it maintains the stability of antibodies in liquid and solid phases during storage conditions. These additives showed to reduce background signal noise in designing the kits. Moreover, diluent C contains combination of ionic and non-ionic detergents such as SDS, Triton-X and Tween 20 having amphiphilic properties which helps in preventing the non-specific binding in immunochemical assays. Diluent A, B, D and E showed positivity up to 1:32 dilution of samples [Table 2, Fig. 3]. Further clinical studies were conducted using diluent C as the diluent for testing SARS-COV-2 IgG from the patient samples.

Accelerated studies were conducted for ensuring the long-term stability of the developed kits. In-order to ensure the repeatability, the studies were done in three different production batches with three different conditions as mentioned earlier. The stability evaluations of these batches of kits were done with stored RT-PCR proven positive and negative samples. The stability studies showed consistent results and band intensity. The finally, reproducibility was tested in triplicates on three different days. The prototypes showed excellent reproducibility across different days, samples and operators.

Validations of the kits

The clinical study table indicates that 23 of 25 [92%] samples showed positive results for IgG and out of 25 negative controls, all [100%] showed negative results in the IgG rapid test kits [Table 3]. The clinical study revealed a sensitivity of 92% with a confidence interval of 73.97–99.02% and a specificity of 100% with a confidence interval of 86.28–100.00%. The test showed a positive predictive value of 100%. The rapid test kit with novel buffer-C showed increased sensitivity and specificity in clinical samples for IgG detection. Our pilot study is mainly focused in obtaining the percentage of agreement with the gold standard test (RT-PCR). Explorative studies are required for achieving the accurate sensitivity and specificities.

The diagnosis of SARS-CoV2 infection could be achieved using various diagnostics techniques currently available which includes, RNA detection by RT-PCR, antigen detection and antibody assays. Worldwide, nucleic acid detection has been considered the gold standard method in diagnosing COVID. Although RT-PCR has been used widely, it suffers from few drawbacks. In persons infected with SARS-CoV 2 viral shedding has been observed only for a transient period lasting 7–10 days post symptom-onset, after which false-negative rates are higher. RNA detection methods are costly and complex to perform requiring sophisticated equipment and trained manpower to operate. The frequency of asymptomatic COVID-19 infection has been observed in around 1/3rd of infected individuals.

These asymptomatic individuals usually do not undergo PCR testing unless indicated as primary contacts. Hence the diagnosis of most infections in the community or population level is missed. The humoral immune response to SARS-CoV2 infection exhibits 2 waves. The first wave of antibody production is by the short-lived plasma cells producing low-affinity antibodies with faster decay properties while the second wave of antibody production is by long-lived plasma cells producing high-affinity antibodies which decays slowly. The neutralizing and effector functions of these antibodies are still under question due to the reinfections observed, probable due to waning immunity or infections caused by mutant strains [15].

Table 2 Comparison of the diluents based on the band intensity produced

	Band Intensity in serially diluted clinical samples					
	1:4	1: 8	1:16	1:32	1:64	1:138
Diluent- A	+ + +	+ + +	+ +	+ +	-	-
Diluent-B	+ + +	+ + +	+	+	-	-
Diluent- C	+ + +	+ + +	+ + +	+ + +	+ +	-
Diluent-D	+ + +	+ + +	+ + +	+ +	-	-
Diluent- E	+ + +	+ + +	+ + +	+	-	-

The clinical samples used were serially diluted in the ratios 1:4, 1:16, 1:32, 1:64 and 1:128. Number of ' + " signs indicate the intensity of the band formed

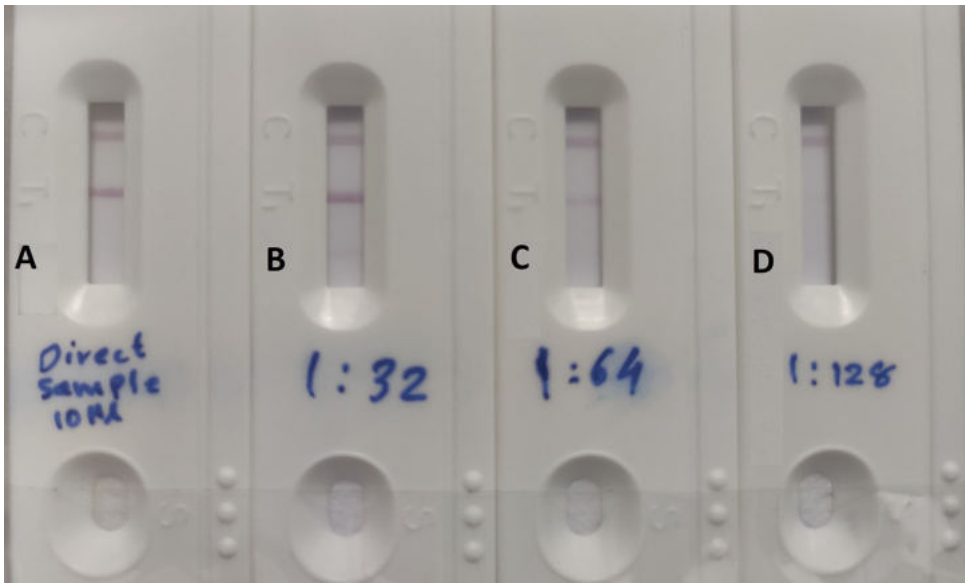

Fig. 3 Image showing the band formation using diluent C as buffer: Test **A** indicates the undiluted clinical sample and Test **B**, **C** & **D** indicate the diluted clinical samples

Table 3 Percentage of sensitivity, specificity, and Confidence Intervals of lateral flow immunochromatographic strip (LFIS) for detection of COVID IgG antibody in serum samples of studied groups

	True positive	True negative	False positive	False negative	Total	Percentage	Confidence interval
Sensitivity	23	00	00	02	25	92	73.97–99.02%
Specificity	00	25	00	00	25	100	86.28–100.00%

Clinical relevance

COVID-19 serological assays achieve good sensitivity to detect SARS-CoV-2 antibodies two weeks after the onset of symptoms. The rate of detection is almost zero during the first week. While it is variable during the second week of illness. Serologic assays although not used as first-line assays in diagnosis of SARS-CoV2 infection, addresses numerous pitfalls of molecular testing. The antibody detection methods are helpful in estimation of the population exposure and thereby accurate estimation of the fatality rate. In a recent study from Iceland, 91.1% of PCR-confirmed infection were antibody-positive. Overall, only 56% of SARS-CoV 2 infections were detected by RT-PCR, while 44% infected were not identified by RT-PCR [14% in quarantined people who had not been tested while 30% in persons who were never quarantined] [16]. This clearly indicates the diagnostic power of antibody-based assays in estimation of the true prevalence, especially in a pandemic situation. Population-level surveillance would be helpful in policy on safe reopening of schools, release of lockdown etc. Based on antibody-based assay studies, the fatality rate of COVID was found to range between 0.3 and 0.8% [0.73%—Indian serosurveillance study], which was previously estimated to be 2–10% based on only RT-PCR based

studies. In addition, these antibody-based assays are rapid, cheaper and less cumbersome to perform [15, 17].

There are numerous kits available in the market which are FDA–EUA or CE-IVD or equivalent [ICMR] approved for testing [18]. These kits have been reported to have variable sensitivity and specificity. The commercial serological tests vary in the test format [eg: EIA, LFA, CLIA, ELFA etc.], class of antibody targeted [IgM, IgG & IgA etc.], antigen used and specimen type. The targeted epitopes include nucleoprotein [N], S1 subunit of spike protein and Receptor binding domain [RBD] of spike protein. Studies show that pan-antibody [IgM + IgG + IgA] detection kits are highly sensitive than targeted immunoglobulin assays [IgM or IgG or IgA assays]. Various studies report that IgM detection did not appear earlier than IgG detection. IgG antibody assays targeting the spike protein 1 and nucleocapsid protein had comparable sensitivity, while better specificity was achieved by N protein-based kits. Cross reactivity to other endemic corona virus subtypes [OC43, NL63, 229E, HKU1] have been reported. The targeted epitope determines cross-reactivity and specificity because N is more conserved across coronaviruses than S, and within S, RBD is more conserved than S1 or full-length S [17, 19, 20].

The clinical utility of IgG assay mainly includes its role in the seroprevalence studies and assessment of the herd immunity level. For effective estimation of prevalence, an assay should ideally have specificity of more than 97%. Our kit showed high specificity [100%] and hence a potential rapid point of care tool for estimating prevalence of the infection. For SARS-CoV2 infection, whose average reproduction number [R0] is 2–3.5, herd immunity will be established when the community infection ranges between 40 and 75%. Quantitative antibody estimation [IgG levels] will be useful for choosing donors for convalescent plasma collection and to monitor immune response to newer vaccine candidates.

In this study, we investigated the importance of diluent system for rapid test kits for improving the detection limits. Immunochromatographic platforms play a vital role in the initial diagnosis and screening of diseases. The sensitivity of binding of SARS-COV-2 anti-IgG antibodies to the coated IgG antibodies in the nitrocellulose membrane is through a combination of electrostatic, hydrogen, and hydrostatic forces. These essential factors are additionally supplied through a diluent system for improving the flow, stability and sensitivity. The present study showed a 100% specificity when compared with gold standard RT PCR tested samples. The application of IgG based kit in this particular case is basically for the surveillance analysis of the population so that herd immunity in the population can be accessed at the same time. This method will be definitely useful in order to treat severe sick patients with convalescent plasma. It's important to know the antibody titration before plasma therapy. This method can be used a quick screening for the population with IgG. It is clear from the data that diluents play an important role in the sensitivity of the assay. The test missed detecting antibodies in two PCR confirmed cases [sensitivity 92%]. The RT-PCR detect the RNA of the virus and will be positive from the initial day of illness till the nucleic acid is completely eliminated from the respiratory tract. The IgG antibodies however, only start appearing in the sera after the first week of illness and peak at 21st day [21]. The clinical history about the course of infection was unfortunately not available to be analyzed, in the case of the false negative samples. Additionally, comparing an antibody detection platform with a molecular test that detects the nucleic acid of the pathogen can cause problems in accurately arriving at the diagnostic efficacy of the test device. Currently, there is a deficit of a gold standard kit that employs the detection principle of antigen -antibody binding.

The major limitations associated with lateral flow assay platforms are mainly due to the efficacy of antigen–antibody bindings and the background noises. Our results showed that more efficient diluent systems can help the immunochromatographic test device in detecting IgG better, from the clinical samples, and to rule out false positivity. The present study was carried out during the initial three months of the pandemic while a country wide lockdown was in place. The validation studies were conducted in the southern as well as the northern parts of Kerala to exclude any geographical bias that may be present. This study needs to be conducted in large cohorts to ensure the clinical utility of this buffer for commercial applications and the buffer may be validated for any other infectious diseases immunological lateral flow system development.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest to declare.

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