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Development and Evaluation of A Rapid Test for SARS-CoV-2 IgM/IgG Detection by Immunochromatography

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ABSTRACT The emerging of new coronavirus, SARS-CoV-2 causing COVID-19, is one of the worst pandemics in human. The viral RNA detection by using RT-PCR can indicate current and use for diagnosis of infection and case finding. Serological test can reveal previous infection and be used in multipurpose. In this study, we developed COVID-19 IgM/IgG rapid test for antibody detection in SARS-CoV-2 infection individuals. The developed test used recombinant N-protein of SARS-CoV-2 expressed in mammalian cells as gold-conjugated protein antigen and was assessed with 132 clinical samples. The results showed its sensitivity of 35.09%, 47.62% and 87.51% at 1-7, 8-14 and ≥ 15 days after onset, respectively and no cross-reactivity with other antibodies in various diseases from tested samples. In addition, the test has high specificity of 99.5%.

Keywords: SARS-CoV-2, Serological test, N-protein, Antibody detection, COVID-19 IgM/IgG Rapid test

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Introduction

Coronavirus disease 2019 (COVID-19) is an acute respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1] . The first case was reported in Wuhan, Hubei province, China in December 2019[2] and then the number of cases were increased rapidly. This disease was uncontrollable and spreading globally[3,4]. The World Health Organization (WHO) declared the outbreak as a public health emergency of international concern on 30 January 2020[5]. As of 22 June 2020, over 9 million infection cases with nearly 500,000 death cases has been reported in 216 countries[6]. The virus can spread more rapidly than severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV)[7] that responsible for SARS and MERS outbreak in 2002 and 2012, respectively[8,9]. Previous study revealed COVID-19 patients, mostly have an incubation period of 3 to 7 days, maximally 14 days and the clinical characteristics are difficult to differentiate from pneumonia caused by other common respiratory tract infections[10,11].

SARS-CoV-2 is Betacoronavirus in *Orthocoronavirinae* subfamily and Coronaviridae family shared 79.6% genome sequences identity to SARS-CoV[12]. It composes of structural proteins as of nucleocapsid (N) protein, membrane (M) protein, spike (S) protein, envelope (E) protein and several non-structural proteins. Among the four structural proteins, the spike (S) and the nucleocapsid (N) are the main immunogens. The S protein is the common target for neutralizing antibodies in therapeutic antibodies and vaccine development[13], the N protein is the most abundant with highly conserved and immunogenic phosphoprotein often uses for diagnostic marker and potential antiviral drug target[14].

Detection of viral RNA with reverse transcription polymerase chain reaction (RT-PCR) based technique is being used as a standard test for diagnosis of current SARS-CoV-2 infection and for tracking the outbreak. However, antibodies detection, not only has clinical value of host antibody response during infection, it also has advantages to understanding the epidemiology of SARS-CoV-2 since it can identify previous infection[15]. Serosurveillance using serological testing that is faster and less workload with sufficient sensitivity and specificity is urgently needed.

The serological testing has multiple purposes. For example, it can be used for contact tracing after a suspected infection for weeks or longer, and also for epidemiological studies. Serological test can be used to verify if the vaccines work as intended in clinical trials and also can be applied in development of therapeutic antibodies[16]. Lateral flow immunochromatography, which is one of the serological tests, for IgM and IgG rapid detection is based on the principle of specificity of antibody-antigen complex. There are several formats that can be developed using this principle. In this case, immunoglobulins in a specimen bind with their antigen which is readily conjugated by gold colloidal, on membrane and flow by fluid dynamics. Consequently, the complex is fixed by immobilized capture molecule, and visible positive bands are revealed. To date, the rapid test for antibody detection is widely used for viral diseases detection including Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Zika Virus (ZIKV),

depending on their specific serological markers[17-19]. In this report, we developed and evaluated COVID-19 IgM/IgG rapid test, a serological test for detection of antibodies to N protein. Cross reactivity and stability were also studied.

Materials and Methods

Expression and Purification of N protein

Recombinant N protein of SARS-CoV-2 was expressed in mammalian cells, and then purified by affinity Chromatography[20]. Briefly, FreeStyle™293-F cells (Thermo Fisher Scientific, USA) were transfected with recombinant plasmid pcDNA 3.4 : NP, expression vector pcDNA 3.4 (Thermo Fisher Scientific. USA) containing N gene encoding SARS-CoV-2 N protein tagged with FLAG® epitope, a short, hydrophilic, 8-amino acid peptide (DYKDDDDK tag) at n-terminal, using Fectopro® (Polyplus transfection, France). The transfected cell culture was incubated at 37°C and 8% CO_2 with orbital shaking at 125 rpm for 3 days, and cells were then harvested by centrifugation at 1,500x g, 10 minutes. The cell pellet was suspended in lysis buffer of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% NP-40 and 1 mM EDTA then lysed by sonication. The supernatant was collected after centrifugation at 10,000x g and applied to Anti-DYKDDDDK G1 Affinity Resin (GenScript, USA). The unbound proteins were washed with Tris-bufferd saline (TBS), pH 7.4 and the bound proteins were eluted with 0.1 M Glycine hydrochloride, pH 3.5 with neutralized by immediately adding 1 M Tris-HCl, pH 9. The eluated proteins were then dialysed in phosphate-buffered saline (PBS), pH 7.4 at 4°C overnight and finally, concentrated using Amicon Ultra-4 10 K (Merck Millipore, Germany). The obtained purified proteins were characterized by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis using anti-DYKDDDDK tag monoclonal antibody (Anti-FLAG® mAb) (GenScript, USA) and COVID-19 patient sera as probe. The protein concentration was determined by Bicinchoninic acid (BCA) assay using BCA Protein Assay kit (Pierce, Thermo Fisher Scientific. USA).

Preparation of colloidal gold conjugated N protein

Recombinant N protein was conjugated with colloidal gold particles which mean diameter of 40 nm (DCN Diagnostics, USA) as described previously[21] with some modification, Firstly, N protein was diluted to 0.1 mg/ml in 2 mM borate buffer, pH 9.0. The gold solution was adjusted to pH 9.0 with 0.2 M K_2CO_3, then mixed with the N protein at ratio 100 µg N protein: 1 ml of gold solution. The mixture was incubated for 30 minutes at room temperature with rotating, and then blocked with bovine serum albumin (DCN Diagnostics, USA) at final concentration of 1% v/v at room temperature for 30 minutes. The gold conjugated N protein was collected by centrifuge at 12,000x g for 30 minutes at 4°C. Finally, it was suspended and diluted with diluent buffer (10 mM phosphate buffer, pH 7.4 containing 0.5% BSA and 0.05% NaN_3) to 20 O.D. and measured absorbance at 540 nm using spectrophotometer (Amersham Bioscience Ultraspec®3100 Pro)

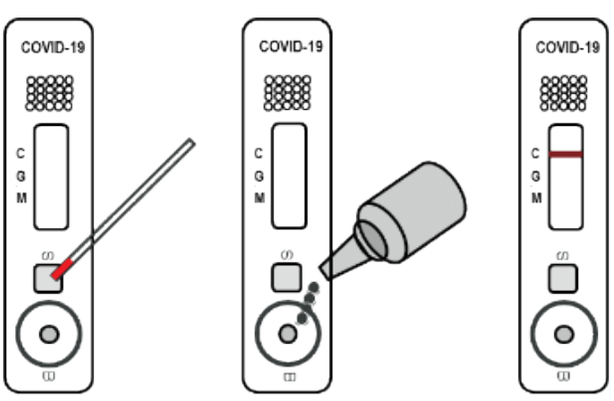
Preparation of COVID-19 IgM/IgG rapid test

The COVID-19 IgM/IgG rapid test was prepared[22] as follows. Firstly, the reagents in PBS of 0.2 mg/ml anti-FLAG® mAb, 0.8 mg/ml anti-human IgG mAb, and 0.5 mg/ml anti-human IgM (Arista Biologicals, USA) were immobilized on nitrocellulose membrane (Whatman, GE Healthcare Life Sciences, USA) at the control line (C), test line1 (G) and test line2 (M), respectively. Then, the membrane was dried for 2 hours at 37°C. The fiber glass-conjugated pad was prepared by treating it with 10 mM borate buffer pH 8.0 and let dried for 3 hours at 37°C. Then gold-conjugated N protein was sprayed and dried on for further use as a conjugated pad. The sample pad and absorbent pad consisted of untreated cotton fiber and absorbent material, respectively. For assembly, all pads were overlapped partially to ensure continuous flow of the sample and solution along the test strip and cut into 4 mm width strip. The test strips were kept in a plastic housing.

Procedure and interpretation results of rapid test for SARS-CoV-2 antibody detection

The testing procedure of COVID-19 rapid test was shown in Figure 1. Briefly, the sample of 10 µl serum/plasma or 20 µl of whole blood was applied to red cells captured sample pad at "S" well, then 5 drops approximately 180 µl of sample buffer (containing with 0.1 M Tris-HCl buffer, pH 7.7 and 0.5% Casein 0.1% NaN_3) was transferred onto sample pad at "B" well. The results can be read within 15 minutes. The results interpretation for all tests, the control line (C) should appear whether there is specific antibody or not. If it does not appear, the test is invalid. If the pink purple band appears at G line, the sample is positive for IgG. If the band appears at M line, the sample is positive for IgM. If the pink purple bands appear both at G and M lines, the sample is positive for both IgG and IgM (Figure 1).

Testing procedure



Apply sample at "S" well

Add sample buffer at "B" well

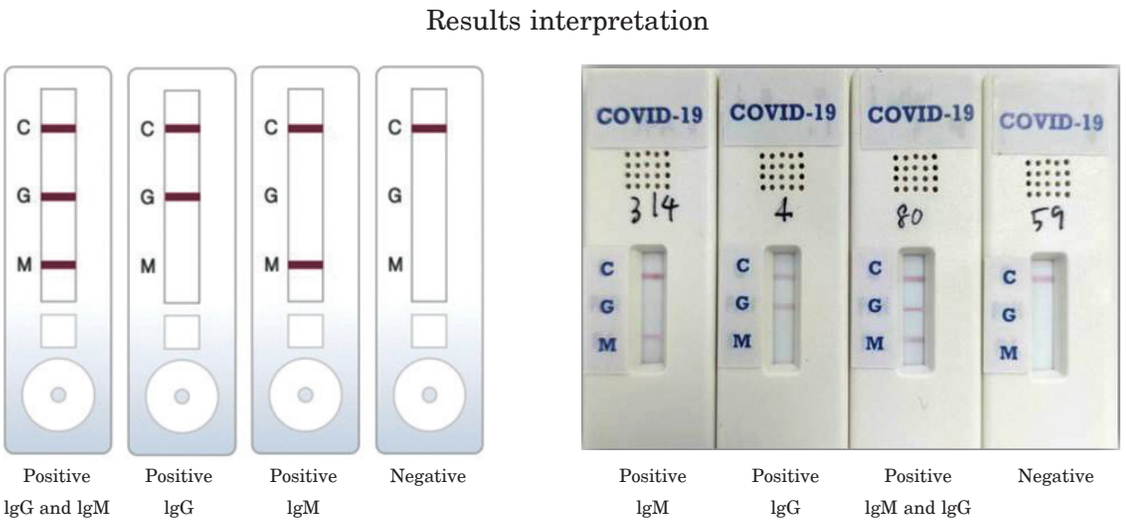


Figure 1 Procedure of testing and results interpretation of COVID-19 IgM/IgG rapid test (A) Theoretical model of the test strip (B) Actual test strip

Evaluation of COVID-19 IgM/IgG rapid test for SARS-CoV-2 antibody detection

One hundred and thirty two sera or plasma of RT-PCR confirmed COVID-19 patients, and one hundred sera from healthy donors collected during outbreak, 100 sera of blood donors collected before outbreak were used for analysis in this study. For cross-reactivity study, the antibody positive serum samples, kindly provided by National Institute of Health, Department of Medical Sciences, consisting of 9 Measles, 9 Mumps, 2 Mycoplasma, 5 Hepatitis C Virus (HCV), 5 Human Immunodeficiency Virus (HIV), 5 Enterovirus 71 (EV71) or Coxsackievirus A 10 (CA10) or Coxsackievirus A 16 (CA16), 1 Influenza virus and 5 Autoimmune diseases, were used for evaluation of the test kit. The ethics was approved by Ethical Committee of Department of Medical Sciences in 0644/EC190 by 22 June, 2020. Table 1 was used for calculation of sensitivity, specificity, and predictive values.

Table 1 Sensitivity and specificity of calculation table

		Standard Method		
		True positive	**True negative**	
Test kit	**Positive**	a	b	a + d
	Negative	c	d	c + d
		a + c	b + d	a + b + c + d

For calculation, sensitivity = a/(a + c), specificity = d/(b + d), positive predictive value = a/(a + b), and negative predictive value = d/(c + d). When a is the number of true positive samples, b is the number of false-positives samples, c is the number of false-negative samples, and d is the number of true negative samples.

Stability study

The accelerated aging test was conducted to predict the real-time shelf life. The rapid test strips were stored in drying oven (Gemmy industrial, IN-601) at 55°C for 32 days. The test strips were performed with the positive IgG and IgM, positive IgG, positive IgM and negative serum samples at day 3, 5, 11, 21, 23, and 32, and scored by intensity of the colored line from 0 to +3. The real-time shelf life was calculated based on ASTM F1980 Standard guide for accelerated aging of sterile barrier systems for medical devices, following the equations: Accelerated aging time duration = Real-time shelf life/Accelerated aging rate, Accelerated aging rate = $Q_{10}^{((TE-TA)/10)}$ where T_E is elevated temperature, T_A is ambient temperature, and Q_{10} is reaction rate which is 2 for medical devices[23].

Results

SARS-CoV-2 N protein expressed in mammalian cells

The purity and specificity of purified recombinant N protein expressed in mammalian cells was analyzed by using SDS-PAGE and western blot analysis. The results in Figure 2 showed the major band of protein with molecular weight of 46.9 kDa which corresponded to N protein was highly expressed and purified (Figure 2A). The specific band of the same molecular weight was observed by western blot analysis using anti-FLAG® mAb and COVID-19 patients serum as probe as shown in Figure 2B respectively.

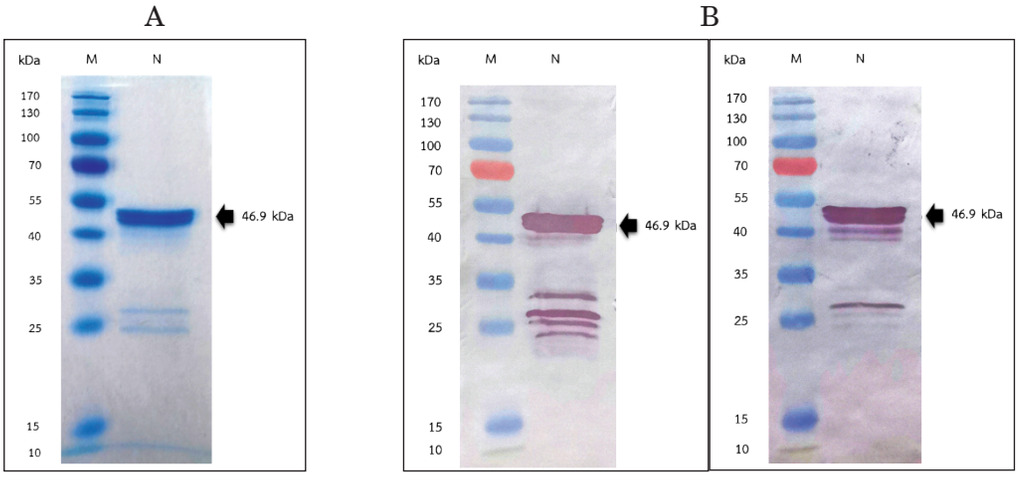


Figure 2 Characterization of purified SARS-CoV-2 N protein by SDS-PAGE (A), western blot analysis (B) probed with anti-FLAG® mAb (left) and COVID-19 patient serum (right). Lane M : Protein marker, Lane N: purified N protein.

Performance of the developed COVID-19 IgM/IgG Rapid test

The results in Table 2 showed the sensitivity of the kit for antibody to N protein detection in serum of COVID-19 patients at 35.09%, 47.62% and 87.51% for 1-7, 8-14 and ≥15 days after onset, respectively. The specificity of the test was of 99.5% (199/200). In addition, the developed

test showed no cross-reactivity with samples of various infection diseases including measles, mumps, mycoplasma, HIV, HCV, EV71, CA10, CA16, influenza virus, and autoimmune diseases.

Table 2 Performance of the COVID-19 IgM/IgG rapid test for detection of antibodies in response to SARS-CoV-2 infection at different time since onset of the disease

Days after onset	Samples (n)	Overall Sensitibity, n (%)	Detectable antibody in serum, n (%)		
			lgG	lgM	lgM/lgG
1 - 7	57	20 (35.09)	8 (14.04)	6 (10.35)	6 (10.53)
8 - 14	42	20 (47.62)	9 (21.43)	1 (2.38)	10 (23.81)
≥ 15	33	28 (87.51)	13 (39.39)	0 (0.00)	15 (45.45)

Stability of COVID-19 IgM/IgG Rapid test

The accelerated aging test result was shown in Table 3. It was found that the test strips' performance could be affected by thermal stress. The test strips stored at 55°C for 3-21 days did not show any significant change in performance, however, the test line's intensity started to drop after day 23, resulting in false negative results for some positive serum samples. According to ASTM F1980, aging at 55°C for 21 days was equivalent to 4°C for 2 years. Hence, it is recommended to store the test strips at 4°C.

Table 3 The accelerated aging test at 55°C of the test strips

Days	Positive IgG and IgM		Positive IgG		Positive IgM		Negative		Result
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	
0	+3	+2	+3	-ve	-ve	+1	-ve	-ve	Pass
3	+3	+2	+3	-ve	-ve	+1	-ve	-ve	Pass
5	+3	+2	+3	-ve	-ve	+1	-ve	-ve	Pass
11	+3	+2	+3	-ve	-ve	+1	-ve	-ve	Pass
21	+3	+2	+3	-ve	-ve	+1	-ve	-ve	Pass
23	+3	+1	+3	-ve	-ve	-ve	-ve	-ve	Fail
32	+2	-ve	+1	-ve	-ve	-ve	-ve	-ve	Fail

Note: "-ve" indicated negative

Discussion

The immunochromatographic rapid test for detection of anti-SARS-CoV-2 antibodies which are captured by N protein expressed in mammalian cells is developed. Assay sensitivity related to onset illness days according to antibody response showed sensitivity of 35.09%, 47.62% and 87.51% at 4-7, 8-14 as and ≥ 15 days after onset, respectively with high specificity at 99.5%.

The SARS-CoV-2's N-protein, an immunogenic and the most abundant viral protein, has 90% amino acid homology to previous endemic SARS-CoV's N-protein. However, there are no evidences of SARS-CoV re-emerging since 2003 or 17 years ago[15]. As follows, N-protein is applicable for being used as antigen in development of antibody detection tool. Furthermore, the level of antibody against N-protein remarkably correlates to viral neutralising antibody titer in COVID-19 patients in ELISA and $PRNT_{50}$ along with microneutralisation assay as well as anti-S protein antibodies when it is evaluated using RBD based ELISA[7,15].

In addition, the rapid test showed no cross-reactivity with blood sera of various infectious diseases and inflammatory autoimmune diseases. Despite the fact that this rapid test should be examined with SARS-CoV, MERS-CoV as well as other common human coronaviruses including HCoV-HKU1, HCoV-OC43, HCoV-NL63 and HCoV-229E to determine if it has any cross-reactivity in validation, it was not possible to perform due to lack of those specimens. It was reported that no-cross reactivity of antibody from patients with other HCoVs infection to N protein of SARS-CoV was demonstrated[15]. Likewise, the cell lines expressed-N protein is also applied as a capture molecule in the in-house indirect ELISA development since it may express the protein in the right structure. Whilst, in some commercial N-proteins, different expression systems might affect the protein configuration which possibly result in reducing or stop of antibody recognition[16].

In stability study, the rapid test is considered their stability through accelerated condition at 55 ˚C to study the shelf-life due to time constraints. Consequently, the result indicated that the test remains valid for 2 years at 4 ˚C storage[23]. For further study, three lots of manufacturing will be examined in real time and accelerated conditions and tested with the panel sera including negative, low, medium and strong titers of positive to ensure life span of the rapid test kit.

In this study, the sensitivity of antibody detection increased in the later phase of infection that may reflect seroconversion in patients. Additionally, it was reported that in severe symptomatic patients, the level of antibody in their blood is higher, compared to those mild or asymptomatic patients[15] whose convalescent plasma were also shown lower antibody level. Thus, the development of rapid test needs a large number of samples, patient's onset day, severity of symptom and other essential factors to evaluate assay sensitivity and specificity in method validation. Moreover, a prevalence of COVID-19 in Thailand is low, resulting in the requirement of high specificity of rapid tests for screening and monitoring the disease.

This lateral flow rapid test for anti-SARS-CoV-2 detection is enable to detect target analyte in serum, plasma which is portable to test in site of quarantine or remote area. Moreover, the test kit is also cost effective, non-labour intensive and non-scientific instrument requirement that is suitable for serosurveillance study.

Conclusion

The immunochromatographic rapid test for detection of anti-SARS-CoV-2 antibody (Covid-19 IgM/IgG Rapid Test) using N protein as antigen was successfully developed. The test kit

has sensitivity of 35.09%, 47.62% and 87.51% at 1-7, 8-14 and ≥15 days after onset, respectively with 99.5% specificity. The results showed non-cross reactivity with other antibodies from various diseases in tested samples. The rapid test should be stored at 4°C that can be prolong shelf life for 2 years.

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การพัฒนาและประเมินคุณภาพชุดทดสอบตรวจหาแอนติบอดี IgM/IgG ต่อ SARS-CoV-2 ชนิดรวดเร็วโดยอิมมูโนโครมาโตรกราฟี

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บทคัดย่อ โรคติดเชื้ออุบัติใหม่ที่เกิดจากไวรัสโคโรน่าสายพันธุ์ใหม่ SARS-CoV-2 เป็นสาเหตุเกิดโรค COVID-19 ซึ่งเป็นหนึ่งในโรคระบาดที่มีความรุนแรง และแพร่กระจายในวงกว้างสำหรับมนุษย์ การตรวจหาอาร์เอ็นเอของเชื้อโดยวิธี RT-PCR สามารถบ่งชี้ผู้ที่อยู่ระหว่างการติดเชื้อ และใช้เป็นวิธีมาตรฐานในการตรวจวินิจฉัยโรคและการค้นหาผู้ป่วย การทดสอบทางภูมิคุ้มกันวิทยาสามารถบอกถึงการได้รับเชื้อมาแล้ว และสามารถนำไปใช้ประโยชน์ได้หลายวัตถุประสงค์ การศึกษานี้ได้พัฒนาชุดตรวจ COVID-19 IgM/IgG ชนิดรวดเร็ว เพื่อใช้ในการตรวจหาแอนติบอดีในผู้ติดเชื้อ SARS-CoV-2 ชุดตรวจดังกล่าวใช้ N-protein ของเชื้อ SARS-CoV-2 ที่สร้างในเซลล์สัตว์เลี้ยงลูกด้วยนมเป็นแอนติเจน โดยเชื่อมกับอนุภาคทอง และนำมาประเมินผลกับตัวอย่างผู้ป่วยจำนวน 132 ตัวอย่าง ผลการทดลองพบว่ามีความไวร้อยละ 35.09, 47.62 และ 87.51 ในตัวอย่างผู้ป่วยหลังมีอาการที่ 1-7, 8-14 และ ≥15 วัน ตามลำดับ และไม่พบปฏิกิริยาข้ามกับแอนติบอดีต่อโรคอื่น ๆ ในตัวอย่างที่ทดสอบ นอกจากนี้ยังพบว่าชุดตรวจนี้มีความจำเพาะสูงที่ร้อยละ 99.5

คำสำคัญ: SARS-CoV-2, การทดสอบทางภูมิคุ้มกันวิทยา, N-Protein, การตรวจหาแอนติบอดี, ชุดตรวจ COVID-19 IgM/IgG ชนิดรวดเร็ว