Landscape Coronavirus Disease 2019 test (COVID-19 test) in vitro

-- A comparison of PCR vs Immunoassay vs Crispr-Based test

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Abstract

The outbreak of COVID-19, caused by 2019 novel coronavirus (2019-nCoV), has been a global public health threat and caught the worldwide concern. Scientists throughout the world are sparing all efforts to explore strategies for the determination of the 2019-nCoV virus and diagnosis of COVID-19 rapidly. Several assays are developed for COVID-19 test , including RT-PCR, coronavirus antigens-based immunoassays, and CRISPR-based strategies (Cas13a or Cas12a), etc. Different assays have their advantages and drawbacks, and people should choose the most suitable assay according to their demands. Here, we make a brief introduction about these assays and give a simple overview of them, hoping to help doctors and researchers to select the most suitable assay for the   Coronavirus Disease 2019 test (COVID-19 test) .

Background

In Dec of 2019, one kind of novel viral pneumonia broke out in Wuhan of China and aroused worldwide concern. This virus was temporarily named as 2019 novel coronavirus (2019-nCoV) by the World Health Organization (WHO) on Jan 7th 2020 [1]. Then, this virus was re-termed as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) based on its sequence similarity to that of 2002-2003 SARS, and the disease it caused was called *Coronavirus Disease 2019* (COVID-19) by WHO on Jan 12th, 2020.

SARS-CoV-2 is 60～200nm in diameter and encapsidates a large single-stranded RNA virus (26-32kb) with many spikes on the virus capsid (Fig. 1A). Several characteristic genes typical for coronaviruses (ORF) are observed in the genomes of SARS-CoV (Fig. 1B), such as spike (S), envelope (E), and nucleocapsid (N). Among them, the receptor-binding domain (RBD) of Spike subunit 1 (S1) is indispensable for the viral infection. To date, the mechanisms about how SARS-CoV transduces human cells have not been completely elucidated yet. Whereas a report shows SARS-CoV 2 infects human with similar processes to SARS by binding to the angiotensin-converting enzyme 2 (ACE2) receptor of target cells [2-4], such as respiratory epithelial cells. Once humans are infected with SARS-CoV-2, they will have the following symptoms: ① having fever and feeling fatigue systematically; ② sneezing, runny nose, sore throat, dry cough, and shortness of breath in the respiratory system; ③ diseased function in kidney; ④ diarrhea in intestines; ⑤ deceased white blood cells.
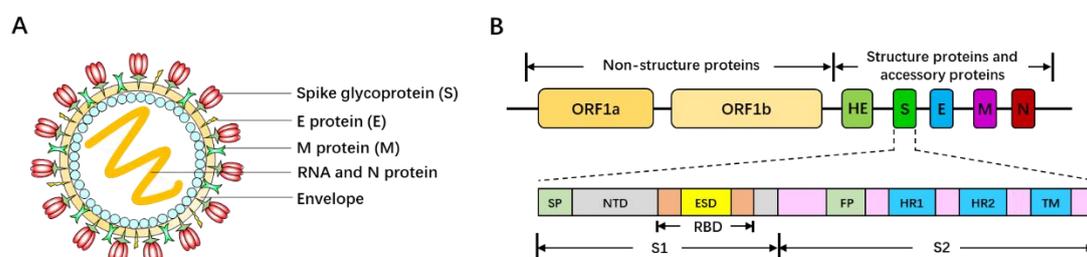

Figure 1. SARS-CoV-2 capsid structure and genome map. (A) Three-dimensional structure diagram of SARS-CoV-2. (B) Genome organization of SARS-CoV-2 [5]. ORF: open reading frame. E: envelope. M: membrane. N: nucleocapsid. HR1: heptad repeat 1. HR2: heptad repeat 2. SP: signal peptide. NTD: N-terminal domain. RBD: receptor binding domain. S: spike. S1: subunit 1. S2: subunit 2. TM: transmembrane domain.

It has been reported that person-to-person transmission from infected COVID-19 patients is really rapidly [5-7]. Although China government is basically in control of COVID-19 pathophoresis by separating patients from normal, forcing citizens to wear masks, and controlling traffic, now the viral pneumonia is globally threatening the health of people all over the world, especially in Europe and North America. Since no specific therapeutic drugs or vaccines are available for patients with COVID-19, it is

really necessary and noteworthy to determine whether the patient is infected early and separate the infected patients from the healthy population immediately to avoid the widespread of SARS-CoV-2. To date, there are several strategies for COVID-19 test: ① real-time PCR (RT-PCR) method; ② immunoassay; ③ Crispr-Cas13a (SHERLOCK)-based test.

RT-PCR (Real time -PCR) in Coronavirus Disease 2019 (COVID-19) test

1) Principles for diagnostics

RNA samples from the upper and lower respiratory specimens of patients can be extracted and reverse transcribed into single-stranded cDNA. If the patient was infected with COVID-19, the COVID-19 cDNA would be carried in the patient's cDNA sample and can be detected via real-time quantitative polymerase chain reaction (RT-PCR) with primers or probes specifically targeting COVID-19 genome. For RT-PCR, the system contains a pair of primers, a probe, and DNA polymerase. The probe is a single oligonucleotide chain complementary to the target DNA with a reporter and a quencher at each end of the probe. When the probe is complete, the fluorescence signal emitted from the reporter is absorbed by the quencher, thus no fluorescence can be detected. During PCR amplification, DNA polymerase cleaves and degrades the probe based on its 5'- 3' exonuclease activity, which will release the reporter from the quencher and emit fluorescence. When a DNA strand is synthesized, a probe would be cut off and a unit of fluorescence signal would be emitted (Fig. 2). Thus, the intensity of the fluorescence is proportional to the amount of newly synthesized DNA strands. Based on this principle, the amount of 2019-nCoV cDNA in patient's specimen can be quantified.
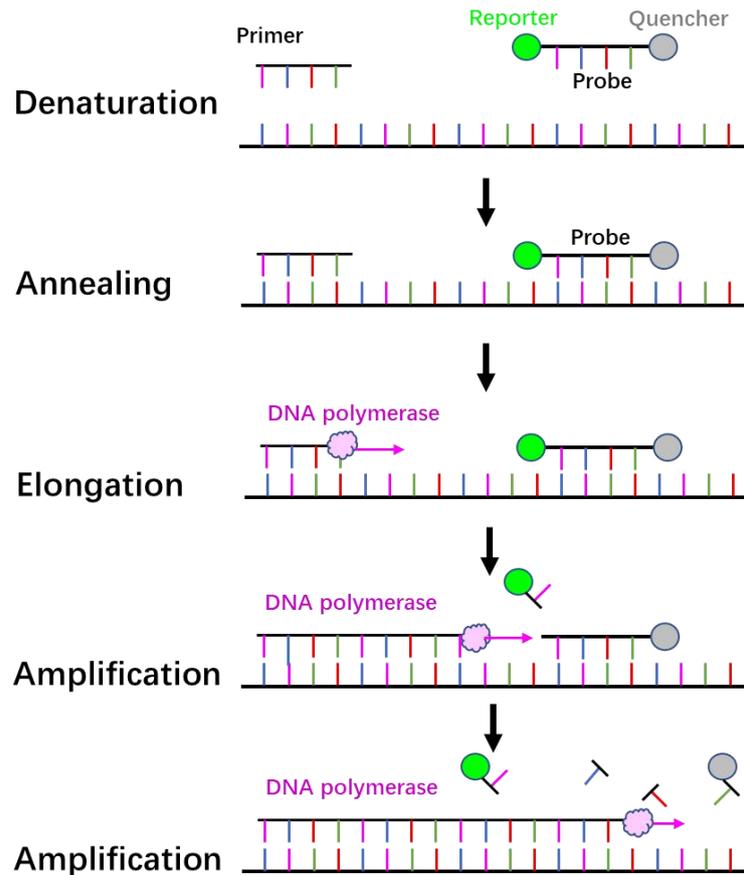
Figure 2. Principle of qRT-PCR.

2) Advantages and disadvantages

Advantages:

① High sensitivity.

② Easy to be operated on a large scale.

Disadvantages:

① Professional technician and special apparatus are required to perform RT-PCR experiments and analyze the data, which costs so much.

② The standard positive control affects the experimental accuracy, and a false negative result may occur due to improper handling.

3) Optional Targets, primers, and probes from different departments

Different departments have their own systems for the determination of COVID-19 by targeting various open reading frames of SARS-CoV-2, which are shown as follows. Primers and probes of Orf1ab and N (nucleocapsid gene) are commonly used for COVID-19 test in China. Three pairs of primers and probes of N (nucleocapsid gene,

N1, N2, N3) are applied in the US CDC for COVID-19. And primers and probes of E (envelope gene) are utilized for COVID-19 test in Europe.

Organisation	Target	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')
University of Hong Kong, Beijing Center for Disease Prevention and Control, Capital Medical University Beijing Research Center for Preventive Medicine	orf1b	TGGGGYTTTACRGGT AACCT (Forward; Y=C/T, R=A/G),	AACRCGCTTAACAAA GCACTC (Reverse; R=A/G)	TAGTTGTGATGC WATCATGACTAG (Probe in 5'-FAM/ZEN/3'-IBF Q format; W=A/T)
	N	TAATCAGACAAGGA ACTGATTA	CGAAGGTGTGACTTC CATG	FAM-GCAAATTG TGCAATTTGCGG-IBFQ
Charité Virology, Berlin, Germany; Olfert Landt, Tib-Molbiol, Berlin, Germany; Erasmus MC, Rotterdam, The Netherlands; Public Health England, London	RdRP	GTGARATGGTCATGT GTGGCGG	CARATGTTAAASACA CTATTAGCATA	FAM-CAGGTGGA ACCTCATCAGGA GATGCBBQ
	E gene	ACAGGTACGTTAATA GTTAATAGCGT	ATATTGCAGCAGTAC GCACACA	FAM-ACACTAGC CATCCTTACTGC GCTTCGBBQ
	N	CACATTGGCACCCGC AATC	GAGGAACGAGAAGA GGCTTG	FAM-ACTTCCTCA AGGAACAACATT GCCABBQ
The US Centers for Disease Control and Prevention (CDC), Integrated DNA Technologies	N1	GACCCCAAAATCAGC GAAAT	TCTGGTTACTGCCAG TTG AATCTG	FAM-ACCCCGCA T TACGTTTGGTGG ACC-BHQ1
	N2	TTACAAACATTGGCC GCAAA	GCGCGACATTCCGAA GAA	FAM-ACAATTTGC CCCCAGCGCTTC AG-BHQ1
	N3	GGGAGCCTTGAATAC	TGTAGCACGATTGCA	FAM-AYCACATT GGCACCCGCAAT

		ACCAAAA	GCATTG	CCTG-BHQ1
	RNAse P	AGATTTGGACCTGCG AGCG	GAGCGGCTGTCTCCA CAAGT	FAM –TTCTGACCTGAA GGCTCTGCGCG– BHQ-1

Table 1. Optional Targets, primers, and probes from different departments

Immunoassay in Coronavirus Disease 2019 (COVID-19) test

1) Point-of-care lateral flow test

A. Principles for Diagnostics

Once entry into host cells, SARS-CoV-2 will provoke the adaptive immune system in the body of the host. 3-6 days post-infection, IgM antibody will be produced, while IgG antibody will be generated 8 days post-infection. Thus, these antibodies against SARS-CoV-2 can be detected in patient's blood. Moreover, IgM tends to be an indicator of the recent exposure of virus, while IgG indicates earlier virus infection. The IgM and IgG can be determined based on highly specific antibody-antigen interactions. In brief, add 10-15μl blood specimens and 70μl sample dilution buffer to the sample port. When the blood specimen flows through the SARS-CoV-2 recombinant antigen (receptor binding domain of SARS-CoV-2 spike protein) labeled by 40nm gold nanoparticle (AuNP) colloids via chromatographic lateral flow, a complex of IgM-antigen-AuNP/IgG-antigen-AuNP will form. Then this complex continues to move on and passes through the coated mouse anti-human IgM/G antibody at M/G coating line, they will form a double antibody sandwich colloidal gold complex, showing a color band at the coating line (red/purple). The excessive fluid continues to flow through the Control line, Rabbit IgG conjugated AuNP will interact with Goat anti-Rabbit IgG antibody, exhibiting a color band at the control line [8]. This point-of-care lateral flow test utilizes a colloidal gold as an indicator to determine the IgM/G against SARS-CoV-2, which is a reliable and visual index for the rapid screening of SARS-CoV-2 carriers (Fig. 3).
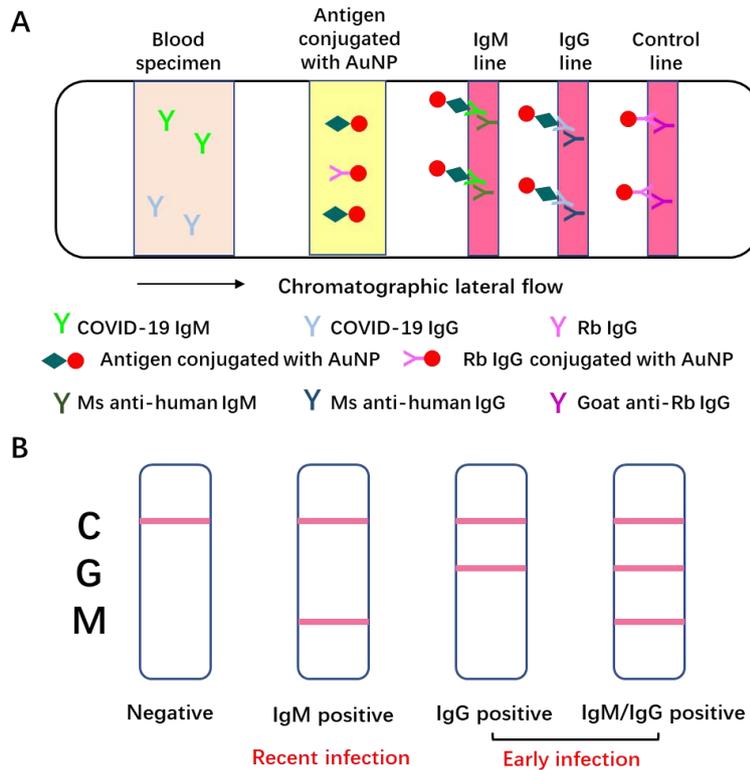
Figure 3. Schematic illustration of Point-of-care lateral flow immunoassay [8]. A: Schematic diagram of rapid SARS-CoV-2 IgM-IgG combined antibody detection device; B: An illustration of different testing results. C: control line, G: IgG line, M: IgM line.

B. Advantages and disadvantages

Advantages:

① Easy to use and operate on a large scale.

② No requirement for additional equipment.

③ High sensitivity (88.66%) and specificity (90.63%).

④ Cost little time, less than 15 minutes.

Disadvantages:

Long window period. It's difficult to detect the early infections, for IgM antibody could only be detected in patient's blood 3-6 days post-infection, while IgG can be detected 8 days post-infection.

C. Optional Targets

The targets of point-of-care lateral flow tests are human antibodies against SARS-CoV-2 antigens. SARS-CoV-2 mainly has four structural proteins with great genetic similarity to those of SARS-CoV, including S (76.0%), E (94.7%), M (90.1%), and N (90.6%). Based on the antigen epitope analysis of SARS-CoV and SARS-CoV-2 [9], N protein and S protein are expected to be potential antigen targets

with a large number of epitopes for the induction of both T cell response and B cell response, which might show promising prospects for vaccine development and induction of long-term immune responses. Additionally, several structural shreds of evidence show S protein is crucial for the entry of SARS-CoV-2 into host cells [2-4, 10] and administration of antibody against SARS-CoV S protein blocks SARS-CoV-2 S mediated entry into cells, which might give a blueprint for COVID-19 vaccine development targeting S protein epitopes [3].

2) ELISA and the other immunoassay in Coronavirus Disease 2019 (COVID-19) test

Besides the above Point-of-care lateral flow test, ELlSA also can be utilized to quantify SARS-CoV-2 antigens via the double antibody sandwich method. Briefly, coat the plate with capture antibody, which can interact with antigens and pull the antigens down once sample is added. Then detection antibody, conjugated with biotin or fluorophores, is added to bind to the other epitope of antigens. Thus, antigen can be detected with capture antibody and detection antibody and labeled by biotin or fluorophores. Additionally, when SARS-CoV-2 infects humans, the immune system will generate a pile of cytokines, including IL2, IL7, IL10, GSCF, IP10, MCP1, MIP1A, and TNFα [11]. These cytokines can also be detected by ELISA or other immunoassays, such as Western blot or immunostaining.

3)   Core for COVID-19 immunoassay: recombinant 2019nCoV antigens

All of these immunoassays for COVID-19 test are based on the antigens of 2019nCoV. To date, GeneMedi has produced many recombinant antigens, including Nucleocapsid (N protein), Spike protein (S protein, S1+S2 ECD), Spike protein (S1 protein), spike RBD protein, Envelope (E protein), 3C-like Proteinase. These recombinant antigens are available and ready-to-use on GeneMedi:
https://www.genemedi.net/i/recombinant-2019-ncov-antigens-reagents

.CRISPR-based tools for Coronavirus Disease 2019 (COVID-19) test

1) Principles for Diagnostics

As RT-PCR assay for COVID-19 requires several reagents, specific instruments and complicated processes, CRISPR-based tools have been developed to test COVID-19 within 30 minutes, which is really easy and simple to operated and saves a lot of time.

A. CRISPR-Cas13a (SHERLOCK)

Based on CRISPR (clustered regularly interspaced short palindromic repeats) technology, single-effector RNA-guided ribonucleases (RNases), such as Cas13a (previously known as C2c2) [12, 13], can be activated to engage in "collateral" cleavage of nearby nontargeted RNAs once recognizing its RNA targets. Recombinase polymerase amplification (RPA) can efficiently amplify DNA from single-molecule to 10^{12} level at a low isothermal state with no need to break the double-stranded structure of DNA [14]. Combined with RPA and T7 RNA polymerase, the CRISPR-Cas13a system can detect RNA rapidly with attomolar (10^{-18})

sensitivity. This technology can be applied for real-time detection of the presence of target RNAs in vitro with signal amplification by non-specific collateral cleavage of nearby nontargeted reporter RNA (Fig. 4) [15]. Therefore, this system is called Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) based on the amplification of nucleic acid and Cas13a-mediated collateral cleavage of a reporter RNA.
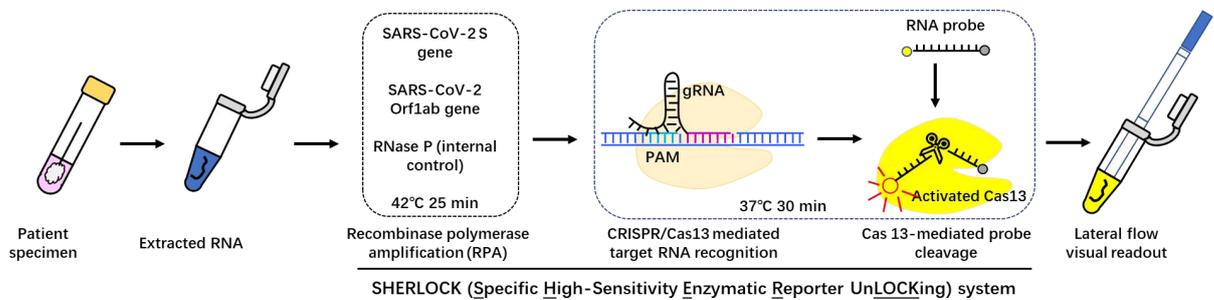

Figure 4. General principle of SHERLOCK tool. dsDNA, double-stranded DNA; RPA, recombinase polymerase amplification, RT-RPA, reverse transcriptase–RPA [15].

For COVID-19 determination, two specific gRNAs targeting Orf1ab and S gene are used and the sequences are as follows. The SHERLOCK COVID-19 detection protocol only requires 3 steps: ① 25min incubation for isothermal amplification of the extracted nucleic acid sample with RPA kit; ② 30 min incubation for the determination of pre-amplified viral RNA sequence using Cas13 protein; ③ 2 min incubation for visual read out of the detection result with a commercially-available paper dipstick.

S gene crRNA	5'- GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACGCAGCACCAGCUGUCCAACCUGAAGAAG-3'
Orf1ab-crRNA	5'-GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACCCAACCUCUUCUGUAAUUUUUAAACUAU-3'
Reporter RNA	5'-/56-FAM/mArArUrGrGrCmAmArArUrGrGrCmA/3Bio/-3'

Table 2. crRNA for SHERLOCK COVID-19 detection

B. CRISPR-based DETECT Lateral Flow Assay

Similarly, another diagnostic tool based on CRISPR is DETECTR (DNA endonuclease-targeted CRISPR trans reporter) system [16, 17]. DETECTR system carry out reverse transcription as well as DNA amplification simultaneously with loop-mediated amplification (RT-LAMP) [18]. The gRNAs are designed to target E gene and N gene of SARS-CoV-2 with the protospacer adjacent motif (PAM) sequence for Cas12 (also known as Cpf1) enzyme [19]. When Cas12 specifically recognizes and binds to the target single-stranded DNA (ssDNA), it will be activated

to completely degrade ssDNA molecules non-specifically. DETECTR system is based on the isothermal amplification of target DNA and Cas12-mediated to collateral cleavage the ssDNA probe. The detailed workflow of DETECTR system-based COVID-19 test is shown in Fig. 6 [20], similarly to SHERLOCK system.
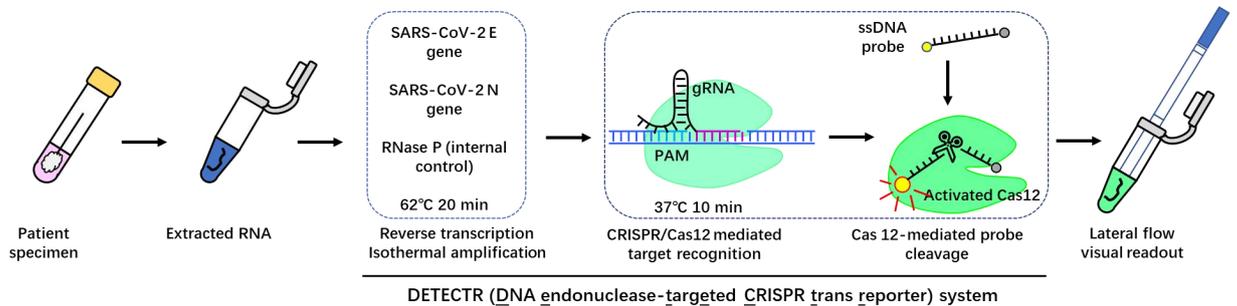

Figure 5. Flow diagram of COVID-19 test based on DETECTR system [20].

N gene crRNA	5'-CACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGG-3'
E gene crRNA	5'-CTTGCTTTCGTGGTATTCTTGCTAGTTACACTAG-3'
Reporter	5'-/56-FAM/TTATTATT/3Bio/-3', IDT

Table 3. crRNA for DETECTR system COVID-19 detection

2) Advantages and disadvantages

Advantages:

① Easy to use and operate in a large scale.

② No requirement of additional equipment.

③ High specificity and sensitivity. Both of the two molecular diagnostic technologies, SHERLOCK and DETECTR, can be used to detect specific RNA/DNA at the attomolar level [16, 17].

④ Cost little time, within 1h.

⑤ Can detect early infection of SARS-CoV-2.

Disadvantages:

There may exist some off-target effects.

Summary

Among the three methods for COVID-19 test, IgM/IgG point-of-care lateral flow test is the simplest and most rapid regardless of professional skill, and cost the least time with no requirement of additional instruments. Based on the detection of viral RNA, CRISPR-based tests are simpler and easier than RT-PCR test with no need for bulky instruments and complicated operations.

Methods	RT-PCR	Immunoassay based point-of-care lateral flow test	CRISPR-based test
Specificity	High	High	High
Consumption time	4-6 hours	Within 15 min	Within 1 hour
Bulky instrumentation	Required	No requirement	No requirement
Disadvantage	False negative result may occur due to improper handling	Long term needed for antibody generation after infection	Off-targets may exist
Cost	High	Low	Low
Target	Different departments have various targets, such as Orf1ab, N gene, E gene	IgM/IgG against SARS-CoV-2	SHERLOCK system: Orf1ab, S gene; DETECTR system: E gene and N gene.
Sample	Urine, blood, saliva, specimen from respiratory, etc.	Blood	Urine, blood, saliva, etc.

Table 4 A summary and comparison of different strategies for COVID-19 test.

Reference

1.   Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382:727-733.

2.   Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020.

3.   Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. Cell. 2020.

4.   Cai X. An insight of comparison between COVID-19 (2019-nCoV disease) and SARS in pathology and pathogenesis. OSFPreprints. 2020.

5.   Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet. 2020;395:514-523.

6.   Bai Y, Yao L, Wei T, Tian F, Jin DY, Chen L, et al. Presumed Asymptomatic Carrier Transmission of COVID-19. JAMA. 2020.

7.   Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, et al. Transmission of 2019-nCoV Infection from an Asymptomatic Contact in Germany. N Engl J Med. 2020;382:970-971.

8.   Li Z, Yi Y, Luo X, Xiong N, Liu Y, Li S, et al. Development and Clinical Application of A Rapid IgM-IgG Combined Antibody Test for SARS-CoV-2 Infection Diagnosis. J Med Virol. 2020.

9.   Ahmed SF, Quadeer AA, McKay MR. Preliminary Identification of Potential Vaccine Targets for the COVID-19 Coronavirus (SARS-CoV-2) Based on SARS-CoV Immunological Studies. Viruses. 2020;12.

10.   Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020;367:1260-1263.

11.   Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395:497-506.

12.   Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science. 2016;353:aaf5573.

13.   East-Seletsky A, O'Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, et al. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature. 2016;538:270-273.

14.   Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. PLoS Biol. 2006;4:e204.

15.   Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science. 2017;356:438-442.

16.   Petri K, Pattanayak V. SHERLOCK and DETECTR Open a New Frontier in Molecular Diagnostics. CRISPR J. 2018;1:209-211.

17.   Gronowski AM. Who or What is SHERLOCK? EJIFCC. 2018;29:201-204.

18.   Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28:E63.

19.   Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science. 2018;360:436-439.

20.   James P. Broughton, Xianding Deng, Guixia Yu, Clare L. Fasching, Jasmeet Singh et al. Rapid Detection of 1 2019 Novel Coronavirus SARS-CoV-2 Using a CRISPR-based DETECTR Lateral Flow Assay. medRxiv preprint doi: https://doi.org/10.1101/2020.03.06.20032334