

A Review of Present Measures of Covid-19 Diagnosis and Crispr Base Diagnosis as Future Direction

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Abstract. COVID-19 caused by SARS-CoV-2 seems to come to an end, some societies have already prepared to coexist with COVID and some are still pursuing zero COVID. qRT-PCR as a gold standard played the main role in the diagnosis of SARS-CoV-2 with a complement of antigen tests but the high cost of machines, time-consuming and false results due to inappropriate sample collecting questioning the gold standard. Novel CRISPR technology provides alternatives to the present diagnosis, measures such as CREST, SHERLOCK and one-step version-STOPCovid can be vital to the reopening and efficient running of society in the post-pandemic era.

Keywords: COVID-19, qRT-PCR, GICA, CRISPR.

1. Introduction

COVID-19 spread throughout the world and cause millions of deaths with its unprecedented capability of transmission among humans especially its ability of transmission during the incubation period. Countermeasures such as quarantine and isolation are effective and have immediate control over the outbreaks, but it's not a practical long-term solution, they come with enormous economic costs and a negative attitude from people. The general policies toward the virus in the post-pandemic era had therefore switched from strictly control to surveillance and suppression, for which diagnosis of COVID-19 is significantly important, especially in shortening the window period detecting asymptomatic patients and protecting possible risk groups.

China's dynamic zero-Covid policy greatly relies on the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) test and colloidal gold immunochromatographic assay (GICA). There is no existing ideal testing measure but those existing measures each have their emphasis, the combination of present diagnostic measures provides a not bad detection of infected cases under a variety of real-life occasions.

RT-qPCR also known as the molecular test using real-time quantitative reverse transcription polymerase chain reaction, is the most widely used tool of diagnose COVID-19 worldwide, the sample is taken by a long nasopharyngeal swab into the nostril or an oropharyngeal swab for the throat to get the fluid which may be loaded with the virus, and based on the position of the sample wanted to test there are also mid-turbinate swab which has shorter length or a very short anterior nares swab. In the lab test, RT-qPCR has a sensitivity at about 0.777 (95% CI: 0.715, 0.849), whilst specificity at 0.988 (95% CI: 0.933, 1.000) [1] and the result for the test can be produced within 60min. However, in the reality, the testing is more of a giant screening and there are many factors that can affect the accuracy of the result (period of infection, quality of the sample collected, contamination etc.), and the strict thermal cycling required by RT-qPCR is one of the main drawbacks for COVID testing in large scale.

In addition to the commonly used RT-qPCR diagnosis, GICA has an absolute dominant in rapid testing, the testing also required a wet sample just like the fluid taken by swab in the nostril or throat in the RT-qPCR test. GICA detects antibodies present in our immune system after infection of SARS-CoV-2 to determine whether a person is infected. The GICA result of measuring anti-S-RBD (a humoral-type immune response following contact with SARS-CoV-2.) antibody showed a nice correlation to the result measured by ELISA, indicating the potential of GICA to be the rapid test tool for COVID-19 infection. Researchers did the GICA on the blood serums of 40 subjects fully vaccinated with the COVID-19 vaccine and 20 unvaccinated subjects, and those clinical results show

high sensitivity and specificity of GICA, supporting the efficacy of using GICA to measure the protection rate and effectiveness of the vaccine [2].

A combined diagnosis had been operating for a considerable time in China, there are problems showing up, the time taken for an approved result of RT-qPCR test is about 6h, this is already considered fast as there are giant-scale of samples waiting to be diagnosed in China, but it is not a short waiting for individuals. Regular testing is very time-consuming and put a lot of resistance on people's lives, therefore a better solution is to look forward to replacing current measures. High specificity, efficiency, preciseness and easy-operate make CRISPR-based assay one of the solutions, but more about this technic is waiting to be learned and the closed diagnostic system is unbuilt. A better diagnostic measure of SARS-CoV-2 can give us a better sense of security under the pandemic, protect those risked populations and better preparedness for the emerging or reemerging virus.

2. qRT-PCR

2.1. Gold standard

The qRT-PCR is designated as the gold standard of COVID-19 diagnosis by the WHO and indeed the most common way of diagnosis since the pandemic. The qRT-PCR is based on reverse transcription and polymerase chain reaction with the addition of the ability to present the result in a quantitative way, by florescent dye method (SYBR Green I, PicoGreen, BEBO) or probe method. Apart from the accuracy and specificity inherited from the basic RT-PCR measure, the quantitative result presentation of the qRT-PCR assay makes it able to measure the viral loads and easy to build a standardized procedure and corresponding system which is important for the public health surveillance of COVID-19.

2.2. Mechanism

The sample is collected by a long nasopharyngeal swab into the nostril or an oropharyngeal swab for the throat to get the fluid which may be loaded with the virus, and based on the position of the sample wanted to test there are also shorter nasal swabs (mid-turbinate swab) or a very short swab (anterior nares swab). The principle of qRT-PCR is not complicated, the COVID-19 ssRNA is reverse transcribed into cDNA, and this cDNA as the template amplified by the process of qPCR. In the earlier version, cDNA first undergoes DNA denaturation at 94-98 Celsius degrees where the dsDNA is separated, then the primer binds to the 3' end of the ssDNA in the annealing step at 50-65 Celsius degrees, and eventually, DNA polymerase copy the ssDNA from the free nucleotides in the solution under 75-80 Celsius degrees during elongation, repeating the thermal cycling. There is also one step qRT-PCR apart from normal RT +qPCR two steps reaction, in one step qRT-PCR, the reverse transcriptase and DNA polymerase are premixed into a single tube to ensure the reverse transcription and the amplification step can be performed in a single reaction.

The spectrofluorometric is done by adding dye or probe into the mixture solution including cDNA, referring to the fluorescent dye method and probe method. The fluorescent dye (e.g., cyber green) is non-specific, they are very sensitive and bind to DNA's minor gap to become fluorescent and can be detected by the fluorometer. On the other hand, the probe method is specific and the general COVID-19 qRT-PCR assay is done using the probe called TaqMan. The probe is an ssDNA and has a fluorophore and a quencher at the 5' and 3' respectively. When the fluorophore is closely held with the quencher by the ssDNA stand, the fluorescent emitted by the fluorophore is absorbed by the quencher and can't be detected. During the denaturation, the probe can attach to its specific complementary sequence of the separated DNA and be degraded by the DNA polymerase and release the fluorophore away from the quencher and free to move in the solution so the result shows fluorescent. This happens during every replication, once a strand of DNA is copied by the polymerase.

The result is further turned into quantitative by programmed machines recording the cycle threshold (Ct) values of tested samples, this value is the number of cycles required for a detectable

(above the back ground signal) amount of florescent PCR product appears, and the initial viral load is decisive to the Ct value, in general, higher the viral load in the sample smaller the Ct value.

2.3. Problems

At beginning of the outbreak, the Ct value for COVID-19 released by CDC China for positive is <37 , negative is 40 or Ct can't obtain, and any value between 37 and 40 is suspected and can be diagnosed as positive if the retested Ct value still <40 with a peak shown in the curve of expansion, however, the component of sample mixture and the value of the threshold is ungiven, caused some misdiagnose at the beginning of the pandemic, revealing the problem that threshold and the procedure of sample preparing were ununiform and a diagnostic system with strict protocol should be built.

Ct values can be easily affected by multiple hematological and biochemical markers. On the other hand, pre and post analytical variables such as collection transport and storage of sample, class of specimen, sampling time, viral kinetics, nucleic acid extraction, viral load, primer, real-time PCR efficiency, and Ct value should also take into account [3]. Also, the Ct value changes with time, according to the research conducted by Phillips, Quintero, Wald-Dickler, Holtom & Butler-Wu in 2022 [4], significantly lower Ct values were observed (indicating higher levels of viral RNA) during the surge of COVID-19 in LA., the conclusion made is that higher viral load cause this increase in transmission. On the other hand, the mutation of the virus causes changes in viral load that can result in various Ct values and the standard of qRT-PCR diagnosis should be adjusted at different periods of the pandemic to produce more reliable results.

The successful carryout of the qRT-PCR assay is determined by many factors. Demand requirements of the lab and sophisticated machines pose a lot of resistance to the large-scale social screening of the SARS-CoV-2. As well as properly trained personnel, because the sample collecting and testing mostly rely on human laboring, and strict protocol should be followed to prevent the contamination from environment and cross-contamination. The inhibitor and gDNA are the main cause of failed tests. The inhibition can result in a decrease in sensitivity of qRT-PCR, common examples are salt and detergents which are carried over from earlier steps of preparation, and strict adherence to the sample preparation process can reduce the chance of inhibition. During the amplification, the enzyme may act on both cDNA and gDNA, and the fluorescent will be presented by both DNA and unable to classify, therefore the result may be false-positive. The reduction of gDNA is important, this is done by using DNase to degrade gDNA before the reverse transcription of RNA, and the result will be more reliable.

3. GICA

3.1. GICA as a complement

GICA is a much simpler COVID-19 diagnosis than qRT-PCR and played an important role as a complement to the qRT-PCR in the early screening of the pandemic. Antigens and antibodies are two kinds of target types for the GICA. The antibody test detects previous SARS-CoV-2 viral infections or the efficacy of the vaccine. For COVID-19 diagnosis the most commonly detected target is the nucleocapsid protein antigen of SARS-CoV-2 in the upper respiratory tract during the infection. Negative results should be treated as presumptive and confirmed, however, positive results may be due to bacterial infection or simultaneous infection of viruses other than SARS-CoV-2. There is presence of viral antigen if a positive result is presented, and infection status should be determined according to the history of the patient and other clinical diagnostic information.

There are sandwich format and blocking formats for the GICA-based COVID-19 Antigen Test Cassette, the positive result for the sandwich format is two lines while for the blocking format is one line. During the lockdown, sandwich format rapid test kit is delivered to every household to facilitate self-testing, but the utilization of this antigen test came to an end due to underlying problems including false results.

3.2. Mechanism (sandwich format)

Anterior nasal swab or a nasopharyngeal swab is used to collect the sample from the secretion in the respiratory tract containing the target nucleocapsid protein. Then the sample swab is put into an extraction buffer and incubation for 10 minutes to extract the antigen, contents of the buffer in the COVID-19 antigen rapid test kit include Tris-HCL/PBS to keep biological molecules intact and Tween-20/Brij 58 for lysing the capsid and spike.

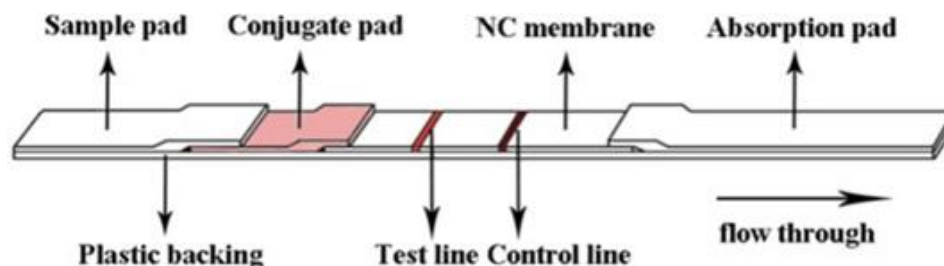


Figure 1. Schematic diagram of the immunochromatographic strip [5].

The sample with the buffer solution containing presented nucleocapsid protein antigens is dropped on the sample pad (Figure 1) and flows through the conjugation pad containing colloidal gold anti-SARS-CoV-2 antibody complex with chicken IgY antibodies-colloidal gold complex as a purpose of quality control. The nitrocellulose (NC) membrane with two separated lines coated with immobilized antiSARS-CoV-2 antibodies and mouse monoclonal anti-chicken IgY antibody. During the capillary reaction, if the sample contains a nucleocapsid protein antigen, the SARS-CoV—2 nucleocapsid protein antigen will bind to the colloidal gold anti-SARS-CoV-2 antibody complex and diffuse forward along the NC membrane and bind with the immobilized anti-SARS-CoV-2 antibody, while chicken IgY bind with the mouse monoclonal anti-chicken IgY antibody. colloidal gold shows a visible red cooler at where they gathered, so two lines are presented if the tested sample contains SARS-CoV-2 nucleocapsid antigen.

3.3. Problems

GICA antigen test has a lower sensitivity and higher but higher specificity when compares with gold standard PCR diagnosis, so there is a considered false negative rate which can cause a lot of problems when many people are self-testing. Following the examples in Spain and U.S, assuming a 90% sensitivity, if the entire population is tested and there is a 5.2 million infected, 520,000 of them would be falsely negative [6]. False negative will result in further spreading of COVID-19 and a delay in treatment and medication may result in worsen symptoms.

According to the U.S. Food and Drug Administration (FDA), the risk of false negative of COVID-19 antigen test can be reduced by several testing in the same period of time, and the reduction in the false negative rate is important to prevent the spreading of SARS-CoV-2 by people unknowing their infection.

In over 500 work places in Canada from 1.2021 to 10.2021, Researchers from the University of Toronto's Rotman School accessed nine million results of rapid antigen tests, resulting in 462 false positives (0.05%). False positives may be explained by tests at a late infectious state or individuals not following the right procedure of self-testing [7]. False positives can be misleading, and result in unwanted panic and a change in attitude toward COVID-19 over a long period of time.

4. CRISPR-based diagnosis

4.1. Potential alternatives to qRT-PCR

CISPR is developed to a strong gene editing technology as well as the treatment of cancer and other genetic diseases. Basically, the CRISPR done by a specific protein attach to the complementary piece of target gene via guide RNA. Different Cas proteins have various characteristic. Among them,

the Cas9 protein is known for the tool of gene editing whilst Cas12a and Cas13a are more specialists in the diagnosis. Cas12a is good at cutting specific DNA and Cas13a is specific to the RNA, making it a highly designated SARS-CoV-2 detection tool. Many CAS13-based diagnosis technologies had been developed recently, including Cas13-based, rugged, equitable, scalable testing (CREST); Specific High-sensitivity Enzymatic Reporter unlocking (SHERLOCK) and SHERLOCK Testing in One Pot (STOP).

4.2. CREST

CREST (Figure 2) is a diagnosis technology achieved by combining reverse transcription, polymerase chain reaction, in vitro transcription and detection based on CAS 13 cutting fluorescent poly(U) cleavage reporter, producing a qualitative result florescent if the sample contains viral RNA. CREST has high sensitivity and is thus easily contaminated, the work area of preamplification steps and application/post-amplification steps should be separated and the items should not be cross-use between two areas.

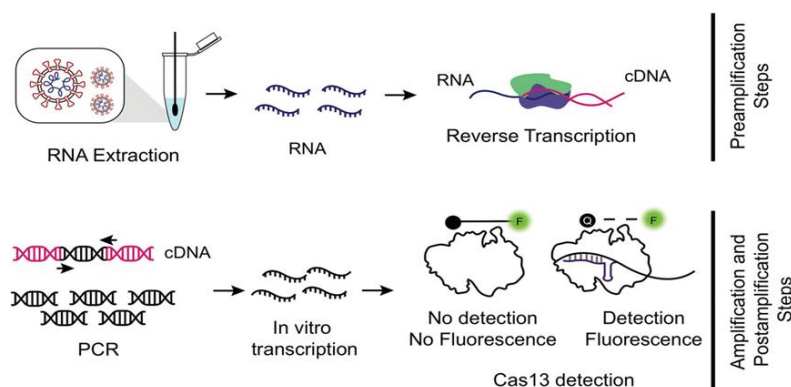


Figure 2. Steps of CREST assay [8]

4.3. SHERLOCK

SHERLOCK (Figure 3) is a rapid sensitive CRISPR-based diagnosis measure. According to the protocol from Zhang Lab, there are three steps to detect SARS-CoV-2 viral RNA:

- 1, Isothermal amplification reaction for 25 min at 42C (incubation of RNA extracted)
- 2, 30 min at 37C (incubation with CAS12, guide RNA and reporter)
- 3, Dip the test strip into the reaction from step 2 (shows the result <5min)

In the data from Thailand, the SHERLOCK was 100% specific and 96% sensitive with the fluorescence readout on samples taken by swabs from nasopharyngeal and throat, and 88% sensitive of the lateral-flow readout, almost competing the gold standard-qRT-PCR, revealing it's potential to become a major test approach [9].

However, the translocation of fluid and open-air test strip greatly increases the chance of contamination, affecting the reliability of the result. Therefore, the STOP is developed, and only demanded a single temperature and one fluid handling process. The STOP assay for COVID-19 is called STOPCovid.

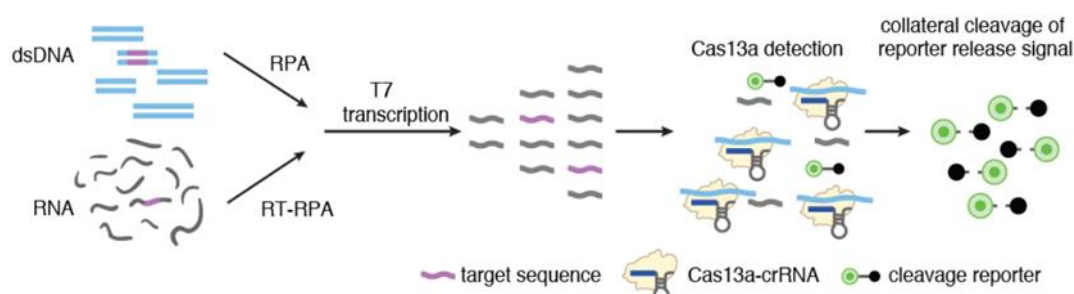


Figure 3. Steps of SHERLOCK [10]

4.4. STOPCovid

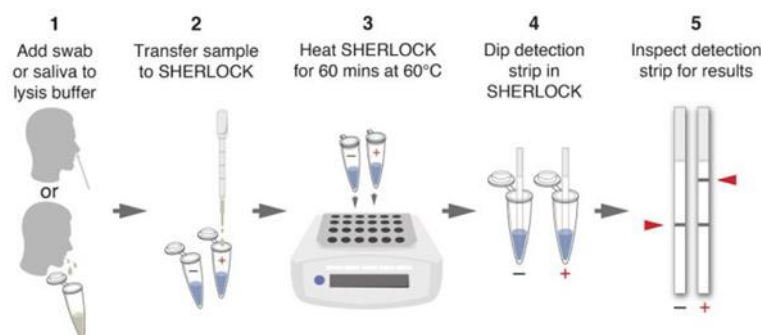


Figure 4. Procedures of STOPCovid [11]

STOPCovid assay (Figure 4) combines isothermal amplification and CRISPR-based detection in one step. The viral RNA is amplified at 55 to 70 °C via loop-mediated isothermal amplification (LAMP), and a thermostable CAS enzyme- AapCas12b with AapCas12b guide RNAs is used to locate the SARS-CoV-2 nucleocapsid protein. However, using QuickExtract lysis virus-containing patient samples to release viral RNA requires 10 mins at 22 °C; 10 mins at 60 °C; Or 5 mins at 95 °C and a visually readout of the detection result by eye using a commercially-available paper dipstick requires a 2min at 22°C.

Clinically validated tests are used to compare the result of nasopharyngeal swabs taken from COVID-19 patients. In these lateral comparisons, STOPCovid detect the virus with 97 percent sensitivity and 100% specificity. The result can be given within one hour and no specialized equipment is required although a thermocycler machine is required to maintain the temperature.

4.5. Limitation on CRISPR-based diagnosis

qRT-PCR is the gold standard had a mature system of diagnosis, and CRISPR-base diagnosis should have extended advantages over qRT-PCR to become the mainstream of diagnosis. RT and PCR are still used in the processing of raw RNA samples. Although the CRISPR have higher specificity than using the dye method or the probe method, a mature CRISPR system is waiting to be built to replace qRT-PCR, including registration and lab protocols.

5. Conclusion

The CRISPR-based diagnosis is showing its ability in high specificity and high sensitivity testing with a cheap cost and fast time required. CRISPR-based diagnosis is still a novel technology, more about safety and performance is to be tested, it has great potential to replace the qRT-PCR and become the most common form of broad diagnosis. even as a complementary addition to the qRT-PCR, CRISPR-based diagnosis is still a powerful weapon added to our public health toolkit, and be very important not only during COVID-19 but future upcoming pathogens.

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