

## Coronavirus-SARS-CoV-2: Biology and Problems in rRT-PCR Detection

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### Abstract

Coronavirus disease 2019 (COVID-19) first appeared in China in December 2019 and was declared a pandemic by the World Health Organization. COVID-19 is caused by Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2), a new virus previously unknown to humans. Here we look at what is known about this virus, the main method for detecting the presence of this virus in a person who is used as a golden standard, and the problems that could arise in this detection method. Understanding the biology of the virus and the strengths and weaknesses of the detection method are important for patient management and for overcoming the pandemic.

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## INTRODUCTION

In December 2019, a severe respiratory disease appeared in China, which is now named coronavirus disease 2019 (COVID-19) ([World Health Organization, 2020a](#)). The World Health Organization has declared the COVID-19 outbreak a pandemic. This disease has spread to 215 countries, infecting more than six million people and causing more than 370 thousand deaths as of May 31<sup>st</sup>, 2020 ([Worldometer, 2020](#)). COVID-19 is a disease caused by Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) infection with symptoms of cough, fever, fatigue, headache, and shortness of breath ([Guan et al., 2020](#); [Wang et al., 2020](#)).

The diagnosis of COVID-19 is made through observing the clinical manifestations that arise and the diagnostic examination of SARS-CoV-2. The SARS-CoV-2

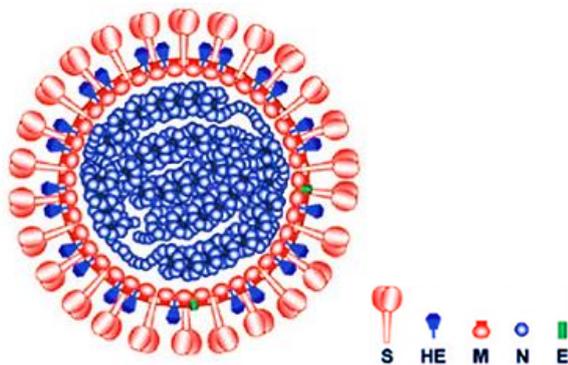
diagnostic tests are carried out through Reverse Transcription polymerase chain reaction (RT-PCR) testing as a method to confirm the diagnosis of cases of COVID-19 according to WHO guidelines ([World Health Organization, 2020b](#)). In this paper, we discuss the biology of SARS-CoV-2 and the principles of RT-PCR as the basis to understand the method of detection of SARS-CoV-2 and problems that may occur using this detection method.

## SARS-CoV-2 BIOLOGY

The SARS-Cov-2, an agent causing a disease called COVID-19 is a new species of coronaviruses. The disease was first reported in Wuhan, China in 2019 ([World Health Organization, 2020a](#)). Coronavirus belongs to the order of Nidovirales, identified by its envelope

characteristics and positive-sense RNA as genetic material. The length of the coronavirus genome is about 26,4–31,7 kb. Coronaviridae and Roniviridae family are the largest RNA virus among other viruses (King *et al.*, 2011). The SARS-CoV-2 genome is 29.9 kb (Forster *et al.*, 2020).

As a member of Nidovirales, coronavirus is classified into the family of Coronaviridae which usually has three or four envelope proteins. Viruses that belong to Coronaviridae family have the most abundant membrane protein (M) among other proteins, i.e. spike glycoprotein (S), nucleocapsid protein (N), an envelope protein (E), dan hemagglutinin-esterase protein (HE) as visualized in **Figure 1** by King *et al.* (2011).



**Figure 1.** Envelope structure of coronavirus. The outer layer of coronavirus consists of S, HE (only found in Betacoronavirus cluster), M, N, and E protein (King *et al.*, 2011)

Coronavirus has a few E proteins on the outer layer of its envelope. It is a pentameric integral membrane protein that facilitates the movement of molecules passing through the ion channel and/or viroporin. Even though there is only a small number of this protein, it was identified as an essential element of virion morphogenesis and a virulent factor of Severe Acute Respiratory Syndrome Virus (SARS)-CoV (King *et al.*, 2011). Wan *et al.* (2020) reported that SARS-CoV-2 and bat SARS-like coronavirus were in the same  $\beta$  genus. Both were also originated from the same ancestral line. Human SARS-CoV and bat SARS-CoV can infect a target cell through angiotensin-converting enzyme 2 (ACE2)

receptor. Alignment of Receptor-Binding Domain (RBD) of SARS-CoV and SARS-CoV-2 showed that SARS-CoV-2 RBD had a mutation on 105 residues. It was predicted that the mutation can enhance the affinity of SARS-CoV-2 RBD to the ACE2 receptor (Wan *et al.*, 2020).

Spike glycoprotein (S) is one of the targets of T cell response in the immune system. It acts as an inducer in the neutralization of virus infectivity by antibodies (King *et al.*, 2011). The S protein also promotes the binding of the virus envelope to the ACE2 receptor and the entry of the virus into the cell target (Wan *et al.*, 2020).

Angiotensin-converting enzyme 2 genes are expressed in several human cells including endothelial cells of small and large arteries and veins, smooth muscle cells, enterocytes of the small intestine (duodenum, jejunum, and ileum), alveolar epithelial cells, basal cell layer of the epidermis hair follicle, cardiac myofibroblast and fat cell membrane in several organs, smooth muscle cells surrounding the sebaceous glands in the skin, endothelial and smooth muscle cell of the brain, parietal epithelial cell of the kidney, and proximal tubular cell of the kidney. Epithelial cells of lungs and small intestine contain an abundant amount of ACE2 protein. However, ACE2 protein is not identified in the upper respiratory tract, such as an oral cavity, nasal mucosa, and nasopharynx. It is also not identified in enterocytes of the stomach and colon, endothelial lining of the sinusoids liver, spleen, lymph nodes, thymus, immune cells (B cell, T lymphocyte, and macrophage), bone marrow, and kidney (Hamming *et al.*, 2004). Despite ACE2 proteins are scattered all over endothelial and smooth muscle cells, not every organ is infected by a coronavirus. SARS-CoV-2 may need a co-receptor as in found in HIV. The HIV requires surface receptor CD4 and chemokine co-receptor for infecting and entering the cell target (Zhang *et al.*, 1996).

## POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is a DNA amplification technique that was invented by Karl Mullis in 1984 (Mullis, 1987). This technology is commonly used for pathogen identification, forensic analysis (Schaad & Frederick, 2002), gene identification, and genetic engineering (Garibyan & Avashia, 2013). The principle of the PCR process is the alteration of temperature using DNA polymerase used in DNA replication in the living cells. The temperature is altered to facilitate denaturation (separating DNA double-stranded), annealing (primer attachment to DNA template), and elongation (DNA amplification) process. There are several main components required for amplification in the PCR process: DNA sample, DNA polymerase enzyme, primer, and buffer. The reaction is catalyzed by DNA polymerase using a DNA sample as a template (Joshi & Deshpande, 2011).

Primers are short pieces of single-stranded DNA that can be designed for restricting the segment of DNA target (Schaad & Frederick, 2002). In the process of amplification, the sample is used in a small amount. Therefore, PCR becomes a sensitive technique for DNA identification (Garibyan & Avashia, 2013).

Molecular biologists have developed variations of the original PCR process such as Reverse Transcriptase PCR (RT-PCR), Real-time PCR, and Quantitative PCR (qPCR). The RT-PCR is a PCR technique that uses RNA as the original template. It uses reverse transcriptase for DNA synthesis from the RNA template followed by PCR. Real-time PCR can observe the amplification product in real-time, during the PCR process by using a fluorescent label that binds to the final PCR product (Higuchi *et al.*, 1993; Deepak *et al.*, 2007). The qPCR can be used to quantify nucleic acids at a specific time. For gene expression studies, RT-PCR, real-time PCR, and qPCR are combined into one process called RT- qPCR or rRT-PCR. This

process includes DNA synthesis from an RNA template using reverse transcriptase, followed by PCR, detection, and quantification. This method is used for the diagnosis of the Coronavirus disease or COVID-19 since SARS-CoV-2 is an RNA virus. This test is accurate and sensitive enough for detecting SARS-CoV-2 in samples (Long *et al.*, 2020; Tahamtan & Ardebili, 2020).

## RT-PCR KITS

Real-time PCR is the most sensitive method to detect the presence of the virus that causes COVID-19. Real-time PCR can detect viruses with a detection limit (analytical sensitivity) of 1000 copies/mL, this analytical sensitivity depends on sample volume, elution volume, and RNA extraction methods, and other factors (Tahamtan & Ardebili, 2020). Many RT-PCR kits can be used as diagnostic tools for COVID-19. van Kasteren *et al.* (2020) compared the performance of seven RT-PCR kits. The kits that were assessed are shown in **Table I**.

**Table I.** RT-PCR kits for COVID-19 diagnosis (van Kasteren *et al.*, 2020)

Manufacturer	Country	Storage Condition	Regulatory Status	Target Gene(s)
Altona Diagnostics	Germany	-20°C <sup>c</sup>	RUO <sup>a</sup>	E <sup>b</sup> , S
BGI	China	-20°C	CE-IVD	ORF1ab
CerTest Biotec	Spain	Room Temp.	CE-IVD	ORF1ab, N
KH Medical	Korea	-20°C	CE-IVD	RdRp, S
PrimerDesign	England	-20°C	CE-IVD	RdRp
R-Biopharm AG	Germany	-20°C	RUO <sup>d</sup>	E
Seegene	Korea	-20°C	CE-IVD	N, E <sup>b</sup>

E: Envelope protein of SARS Cov-2

CE-IVD: European conformity label-in Vitro diagnostics

RdRp: RNA dependent RNA polymerase of SARS Cov-2

N: Nucleocapsid protein of SARS-Cov-2

ORF1ab: Open Reading Frame 1a and b of SARS Cov-2

RUO: Research Use Only

S: Spike protein of SARS Cov-2

<sup>a</sup>According to the manufacturer's website the kit is RUO, the FindDx website states CE-IVD certification for this kit.

<sup>b</sup>These E-gene assays are specific for bat (-related) beta coronaviruses. They detect both SARS-CoV-1 and -2.

<sup>c</sup>Shipment is performed at room temperature.

<sup>d</sup>According to the manufacturer, CE-IVD certification will be applied shortly.

All RT-PCR kits performed splendid results with PCR efficiency  $\geq 96\%$ . All RT-PCR were able to identify positively 10 out of 13 selected clinical samples with various concentrations, with the highest concentrations of SARS-CoV-2 RNA with Ct less than 34.5 in an in-house E-gene PCR. None of the assays showed cross-reactivity with a confirmed non-coronavirus respiratory viral infection. When performing diagnostics in a low viral loads condition such as in mild or no symptoms or patients during the later stage of infection or health-care workers (Zou *et al.*, 2020), RT-PCR kits from R-Biopharm AG, BGI, KH Medical, and Seegene had the best performance in identification of the clinical samples. All the RT-PCR kits assessed in the study may be used for routine diagnostics of COVID-19 in patients by experienced molecular diagnostic laboratories.

## RT-PCR TEST SAMPLES

In general, most patients only develop mild (40%) or moderate (40%) disease, 15% develop in the severe condition that requires oxygen support, and 5% have a critical disease with complications such as respiratory failure, acute respiratory distress syndrome (ARDS), sepsis and septic shock, thromboembolism, and/or multiorgan failure, including acute kidney injury and cardiac injury (World Health Organization, 2020c). RT-PCR test samples for initial diagnosis come from the upper respiratory tract (URT) in the form of nasopharyngeal (NP) and oropharyngeal (OP) swabs, nasopharyngeal wash/nasopharyngeal aspirate, nasal swab, or nasal mid-turbinate swab. If the URT specimen shows a negative result, then the specimen is taken from the lower respiratory tract (LRT) consisting of sputum,

aspirate, or lavage (Center for Disease Control and Prevention, 2020a; World Health Organization, 2020b). In other considerations, stools can be used as specimens for RT-PCR samples (World Health Organization, 2020b). Some patients show negative swabs, but viral RNA can be detected in the stool (Hindson, 2020; Lo *et al.*, 2020; Wölfel *et al.*, 2020). The specimen is collected as soon as possible in the initial onset (Center for Disease Control and Prevention, 2020b). Research showed that the viral loads in throat swab and sputum peaked in 5-6 days after symptom onset (Pan *et al.*, 2020). Another research indicated that viral load was higher in the nose (NP and OP swabs) than in the throat (Zou *et al.*, 2020).

The use of NP swabs as a specimen is preferred over OP swabs due to higher detection rates. This is consistent with research that demonstrates early viral development in the nasal/NP (Wang *et al.*, 2020). Sampling techniques through swabs must be performed at the right anatomy and time so that negative-false can be avoided. Besides, only synthetic fiber swabs with plastic or wire shafts are used (Center for Disease Control and Prevention, 2020b). In NP swabs, mini tip swabs are inserted into the nose parallel to the palate until they reach the tip, namely the nasopharynx. Gently rub and roll the swab and wait a few seconds to absorb the secretion. Then slowly remove the swab while rotating it. The technique for collecting specimens in the form of an NP swab is illustrated in **Figure 2**. The OP swab is done by inserting the swab into the posterior pharynx and tonsillary areas (Center for Disease Control and Prevention, 2020b; World Health Organization, 2020b). Each swab is put into a sterile container containing 2-3 mL viral transport medium (VTM), Amies transport medium, or sterile saline. The specimens for LRT are placed in dry and sterile containers (Center for Disease Control and Prevention, 2020b).

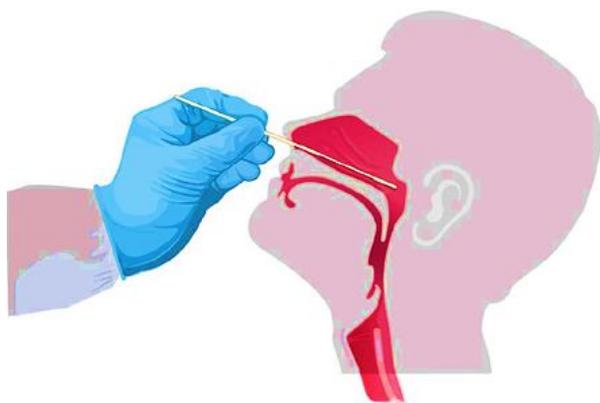


Figure 2. Nasopharyngeal Swab (Center for Disease Control and Prevention, 2020b)

Collected specimens are stored at 2-8°C for up to 72 hours. If a delay in testing or shipping is expected, store specimens at -70°C or below (Center for Disease Control and Prevention, 2020b). Complete information about specimen collection and storage is in **Table II**.

Table II. Specimen collection and storage (Center for Disease Control and Prevention, 2020b; World Health Organization, 2020a)

Specimen type	Collection materials	Storage temperature until testing in-country laboratory	The recommended temperature for shipment according to expected shipment time
Nasopharyngeal and oropharyngeal swab	Dacron or polyester floccated swabs*	2-8°C	2-8°C if ≤ 12 days -70°C (dry ice) if > 12 days
Bronchoalveolar lavage	Sterile container*	2-8°C	2-8°C if ≤ 2 days -70°C (dry ice) if > 2 days
(Endo)tracheal aspirate, nasopharyngeal or nasal wash/aspirate	Sterile container*	2-8°C	2-8°C if ≤ 2 days -70°C (dry ice) if > 2 days
Sputum	Sterile container	2-8°C	2-8°C if ≤ 2 days -70°C (dry ice) if > 2 days
Stool	Stool container	2-8°C	2-8°C if ≤ 5 days -70°C (dry ice) if > 5 days

For transport of samples for viral detection, use viral transport medium (VTM) containing antifungal and antibiotic supplements. Avoid repeated freezing and thawing of specimens. If VTM is not available sterile saline may be used instead (in which case, duration of sample storage at 2-8°C may be different from what is indicated above).

Low viral load (World Health Organization, 2020b) is one of the things that can negatively impact the results of the

RT-PCR analysis. It is recommended that NP be collected twice from both sides of the nostrils or that OP and NP be combined in one VTM to maximize test sensitivity and limit the use of test resources (Center for Disease Control and Prevention, 2020b). The second factor is that the specimen was collected late or very early in the infection. Hence, it is very important to take specimens at the right time. The third is that the specimen was not properly handled and shipped. Another factor is the technical reasons inherent in the test, e.g. virus mutation or inhibition of PCR (World Health Organization, 2020b).

## STEPS IN INVESTIGATION OF COVID-19 SPECIMEN SAMPLES

In Indonesia, The Ministry of Health has issued a direct procedure for handling the COVID-19 sample at the National Board of Health Research and Development. Specimen investigations have followed WHO standards and were carried out at the Biosafety Level 2 (BSL-2) Laboratory. The investigation procedure is divided into three stages, consisting of acceptance, examination, and reporting. At the acceptance stage, specimens are taken from patients at the referral hospital and then sent to the National Board of Health Research and Development. To ensure further accuracy, not only one, but three specimens should be obtained from one patient. A swab sample from a COVID-19 patient is taken by a sampling officer with a Personal Protective Equipment (PPE) to be mixed with the VTM (Ministry of Health of the Republic of Indonesia, 2020a).

The VTM can be used to collect specimens from the throat and nasal swabs from human patients. This solution can be prepared locally based on the WHO recommendation. The VTM contains infusion broth, bovine albumin fraction V, gentamicin sulfate, amphotericin B, and sterilized distilled water. Commercial VTM is also available such as COPAN

Universal Transport Medium and Eagle Minimum Essential Medium (E-MEM) (World Health Organization, 2006).

Continuing to the examination stage, at this stage, the specimens received by the National Board of Health Research and Development are extracted for their RNA (Ribonucleic Acid) or viral genetic material. After the RNA is obtained, it is then used as a template for Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR) to detect the presence of specific SARS CoV2 genes. COVID-19 diagnoses of patients are based on the RT-PCR test results (Ministry of Health of the Republic of Indonesia, 2020b).

In the RT-PCR process, there are negative and positive controls. In the negative control, the amplification of viral genes must not be found while in the positive control there must be an amplification of viral genes that are marked by curves. If the RT-PCR negative control is found to be positive, then the PCR process must be repeated (Bustin & Nolan, 2004). The final stage is the reporting, there was indeed a special channel that needed to be done first to report or confirm the results. After interpretation of the results by RT-PCR experts, these results were sent to doctors who will carry out the next stage of verification and validation to report to the hospital and the health service (Espy *et al.*, 2006).

## FALSE POSITIVE RESULTS IN THE RT-PCR TEST

The false-positive result of the RT-PCR test means that the test is positive even if no virus is present in the sample tested. False-positive results of the RT PCR diagnostic tests are very unlikely. Positive results depend on the sequences of the primers and probes used, and possible cross-reactions with non-target sequences have usually already been ruled out during the design phase (Bustin & Nolan, 2020).

For example, the SARS-CoV-2 rRT-PCR Diagnostic Panel primer and probe sequences were tested for homology with human genome sequences, other coronaviruses, and human microflora to prevent potential false-positive results (Center for Disease Control and Prevention, 2020a). No homology was found with the human genome and microflora sequences, but one of the primers or probes had homology with SARS-CoV and Bat SARS-like coronavirus genome. Since homologies with these coronavirus genomes were not present for the whole set of primers and the RT-PCR reaction probe, a false positive outcome would be unlikely (Corman *et al.*, 2020). For a false-positive result to happen, the sample could have been contaminated with the virus (Tahamtan & Ardebili, 2020). Contamination from equipment and reagent could be a source of false-positive results. Usually, this type of potential false positive would be detected by the use of negative control reactions. If the negative control reaction was positive, the test result would not have been valid and the test had to be repeated. This occurred in a test facility in Yala, Southern Thailand, where the negative controls of 40 specimens showed positive results. When the problematic specimens were sent to two other laboratories for analysis, the results were consistently negative. Machine error was suspected to be the cause of false-positive results (Thai Public Broadcasting Service World, 2020).

## FALSE NEGATIVE RESULTS IN THE RT-PCR TEST

False-negative results from RT-PCR are much more common compared to false-positive results and have more serious consequences in the epidemiological management of the disease (Kalifarhood *et al.*, 2020). Every RT PCR kits have a limit of detection, ranging from 200-1000 copies/mL, and this is the limit of detection

under controlled laboratory conditions. Under real-life conditions, the limit of detection could become higher. Therefore, a low viral load could cause false-negative results. Mistakes during specimen collection, storage, and transportation could result in false-negative results (Jagodziniski *et al.*, 2020).

Variations in the target sequence could cause false-negative results. The SARS-CoV-2 has evolved into three central variants (Forster *et al.*, 2020). Even though the design of primers and probes has taken into account variations in the SARS-CoV-2 genome sequences, new mutations could occur in the target sites.

The viral load would fluctuate during disease progression. From 72 nasal swabs and 72 throat swabs obtained from 18 patients in Zhuhai, Guangdong, China, the Ct value from 0 to 21 days after symptom onset fluctuated. A Ct value of 40 was considered to be negative and several patients Ct values decreased (positive results) after a previous Ct value of 40 (negative results), and some patients had inconsistent results (one positive and one negative) from nasal and throat swabs taken on the same day (Zou *et al.*, 2020). Negative test results that later became positive were also observed in other longitudinal studies. According to Kucirka *et al.* (2020), from samples collected from the upper respiratory tracts, no virus could be detected with RT-PCR on the day of infection. On the day of symptom onset, the median false-negative rate was 38%, and the lowest false-negative rate of 20% was obtained from samples collected three days after symptom onset. Then the false-negative rate would increase again, reaching 66% at 16 days after symptoms onset.

## CONCLUSION

COVID-19 is caused by SARS-CoV-2, an enveloped RNA virus with a large genome (29.9 kb) closely related to human SARS-CoV and bat SARS-CoV. The S protein

on the surface of SARS-CoV-2 binds to the ACE2 receptor of the host and promotes entry of the virus into the cell target. ACE2 receptors are present in cells of the arteries, veins, smooth muscles, small intestine, alveoli of the lungs, hair follicles, cardiac myofibroblasts, skin, brain, and kidney, thus SARS-CoV-2 could potentially infect these tissues. The gold standard for detection of SARS-CoV-2 is by rRT-PCR and kits for SARS-CoV-2 detection are available commercially. Proper sampling site, sampling method, the timing of sampling, treatment, and handling of specimens during shipping and storage are all important in obtaining correct test results. Ongoing research is needed to select a better sampling site, timing, and treatment of samples to minimize false results which have serious consequences for the management of the disease.

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