



Optimization of Da An Gene Kit for SARS-CoV-2 Detection in Real-Time RT-PCR

Seprianto¹, Muhammad Arreza¹, Titta Novianti¹, Febriana Dwi Wahyuni¹, Oktaviani Naulita Turnip¹ Roaslein Putri², Henny Saraswati^{1,*}

¹ Program Studi Bioteknologi, Fakultas Ilmu-ilmu Kesehatan, Universitas Esa Unggul, Jakarta, Indonesia 11510;

² PT. Ecosains Hayati, Jakarta, Indonesia 13550

* Correspondence: hennysaraswati@esaunggul.ac.id

Abstract

Background: SARS-CoV-2 is a new type of coronavirus of the genus Betacoronavirus and the family Coronaviridae that causes a respiratory disease called COVID-19. The virus has a sheath and genetic material in the form of single-chain RNA. The genome structure of this virus is divided into two types, namely genes that encode non-structural proteins consisting of the ORF1a / ORF1b gene and genes that encode structural proteins consisting of spike glycoprotein (S), envelope (E), membrane glycoprotein (M), and nucleocapsid protein (N). **Methods:** The method of detecting SARS-CoV-2 with real time RT-PCR is the most recommended method because it has high specificity and accuracy. The specificity of a method is necessary to be able to specifically recognize the pathogen that causes the disease. Real time RT-PCR requires sampling with a swab on the oropharynx or nasopharynx to be examined in the laboratory which later the presence of viral RNA becomes a molecule that is assessed for diagnosis results. In this study, volume optimization was carried out on the Da An Gene kit used for the detection of SARS-CoV-2 with Reverse Transcription Polymerase Chain Reaction (Real time RT-PCR) with the aim of saving the use of reagents from available kits but with amplification results remaining optimal and accurate. **Results:** There were three SARS-CoV-2 RNA samples used consisting of N62, N63, and N79 samples and three types of total volume used were 20 μ l, 15 μ l, and 10 μ l. The results of this study showed that the three positive samples contained SARS-CoV-2 with a Cq value of < 40. **Conclusion:** A volume of 20 μ l is the optimal volume, which is more efficient than the manufacturer's recommended volume of 25 μ l.

Keywords: COVID-19; Da An Gene Kit; Optimization; Real Time RT-PCR; SARS-CoV2



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Introduction

At the end of 2019, an outbreak of pneumonia emerge from Wuhan, Hubei Province, China. The etiology of this disease is SARS-CoV-2 virus which is a new virus in the Coronavirus group (Prastyowati, 2020). Based on the phylogenetic and taxonomy studies, the International Committee on Taxonomy of Viruses (ICTV) named this new virus as SARS-CoV-2, a virus that is closely related to human and bats Coronaviruses (ICTV, 2020). The World Health Organization (WHO) named the disease as COVID-19 (Coronavirus Disease 2019) referring to the year it was discovered, in 2019.

SARS-CoV-2 is a virus of the genus Betacoronavirus and the family Coronaviridae. This virus has genetic material in the form of single-stranded RNA. Under observation with an electron microscope, on the surface of this virus, there are three types of proteins embedded in the lipid bilayer, namely spike glycoprotein (S), membrane glycoprotein (M), nucleocapsid protein (N) and, envelope (E) (Kumar et al., 2020). The structure of the

SARS-CoV-2 genome when sequenced from the 5' to 3' end is divided into two parts, namely genes that encode non-structural proteins consisting of the ORF1a/ORF1b gene, and genes that encode structural proteins consisting of spike glycoprotein (S), envelope (E), membrane glycoprotein (M), and nucleocapsid protein (N) (Hu et al., 2021)

COVID-19 symptoms in patients vary widely from mild to requiring hospitalization. Dry cough, fever and fatigue are symptoms that common. Patients with severe symptoms experienced acute respiratory distress syndrome that requiring mechanical ventilation support. These patients had an increase in the production of pro-inflammatory cytokines (cytokine storm) associated with multi-organ failure and death (Coperchini et al., 2020).

It is known that the natural host of the coronavirus, especially SARS-CoV-2, are bats and some wild animals such as pangolins, rats, and ferrets act as intermediate hosts that have the potential to be intermediaries in the process of transmission of this virus to humans (Cui et al., 2019). This is also reinforced by several studies showing that the genome sequence between SARS-CoV-2 and the bat coronavirus named RaTG13 has a similarity of more than 95% (Hu et al., 2020; Yadav & Saxena, 2020; Yuliana, 2020). SARS-CoV-2 that has entered the host will recognize and attach to the host cell receptor with the help of a pointy S protein on the surface of the virus that serves to mediate the entry of the virus into the host cell. The receptor recognized by SARS-CoV-2 is an ACE2 enzyme that can generally be found in epithelial cells of the nose, mouth, nasopharynx, lungs, kidneys, and liver. In the lungs, ACE2 receptors are very abundant in the alveolus, precisely in alveolar cells type II (Kumar et al., 2020b; Yadav & Saxena, 2020).

An accurate diagnosis is very important and crucial for detecting SARS-CoV-2. The real-time RT-PCR is the most recommended molecular test infection detection method due to its high sensitivity and accuracy (Lu et al., 2020). Real-time RT-PCR requires sampling with a swab on the oropharynx or nasopharynx at the laboratory that later the presence of viral RNA into molecules that are assessed for diagnosis (Yanti et al., 2020). Viral RNA is converted to cDNA which is generated by the reverse transcriptase enzyme before being replicated with a PCR machine. The presence of the virus will be detected by a PCR machine with the help of biomarkers in the form of fluorescence (Yusuf, 2010).

The right use of the PCR technique and according to the procedure can provide results as expected. In addition, optimization is needed to get optimal PCR results and minimize the occurrence of false-positive results. Optimization can be done by varying the current conditions of the PCR process, such as DNA polymerase, temperature, time, PCR buffer, reagent volume, and concentration (Park et al., 2020). The COVID-19 PCR kit consists of reagents used for In Vitro Diagnostic (IVD) purposes and aims to detect the genes present in SARS-CoV-2 such as the N gene, as well as the ORF1ab gene. The Da An Gene kit consists of reagents such as NC (ORF1ab/N) PCR reaction solution A, NC (ORF1ab/N) PCR reaction solution B, NC (ORF1ab/N) negative control, and NC (ORF1ab/N) positive control. The purpose of this study is to optimize the volume of Da An Gene kits by using smaller volumes than from the kit protocol (25 ul) to save the use of Da An Gene kits during SARS-CoV-2 detection with real-time RT-PCR technique.

Methods

Tools and Materials

This research was conducted at the Molecular Biology Laboratory, Universitas Esa Unggul. The tools used in this study was Eco48 PCR machine (PCRmax, UK) with EcoStudy software V5.2.16 (PCRmax, UK) for RT-PCR result analysis. The Detection Kit for 2019 Novel Corononavirus (2019-nCoV), which was later referred as Da An Gene Kit (Da An Gene Co., Ltd. of Sun Yat-sen University, China), was used for RT-PCR. This kit has several components i.e NC (ORF1ab/N) PCR reaction solution A, NC (ORF1ab/N) PCR reaction solution B, NC (ORF1ab/N) negative control and NC (ORF1ab/N) positive control (Co, Gene., 2019).

This kit was probe-based RT-PCR kit with 3 reporters: FAM (Flourescence Amidites), VIC and Cy5. FAM channel was used for N gene detection, VIC channel for ORF1ab gene detection, while Cy5 channel for internal controls. Negative controls QC was non-amplification result for FAM and VIC channel, while Cy5 channel's Ct value < 35. Quality control for positive control was obvious amplification curves for FAM and VIC channel with Ct value < 32, and amplification or no amplification curve for Cy5 (Co, Gene., 2019).

Positive result was determined by obvious amplification curve on FAM and VIC channel with Ct value < 40, while negative results was determined by no amplification curve in FAM and VIC channel and obvious amplification curves in Cy5 channel (Co, Gene., 2019).

Sampling

This study used RNA samples from symptomatic patients with nasopharyngeal swab test. Ethical clearance was obtained from Research Ethical Committee, Universitas Esa Unggul no. 0375-21.375/DPKE-KEP/FINAL-EA/UEU/X/2021. This 3 samples used mention as N62, N63, and N79.

Amplification with Real-time PCR

Reagent NC (ORF1ab / N) PCR reaction solution A and B was thawed at room temperature, then homogenized with vortex. Samples, positive and negative controls were prepared with this 2 (NC (ORF1ab/N) with composition shown in Table 2. The composition of the reaction mixture recommended by manufacturer is shown in Table 1.

Table 1. Reaction mixture recommended by manufacturer

| Volume | NC (ORF1ab/N) PCR reaction solution A | NC (ORF1ab/N) PCR reaction solution B | Positive/negative sample or control |
|--------|---------------------------------------|---------------------------------------|-------------------------------------|
| 25 µl | 17 µl | 3 µl | 5 µl |

Table 2. Reaction mixture used in this research

| Volume | NC (ORF1ab/N) PCR reaction solution A | NC (ORF1ab/N) PCR reaction solution B | Positive/ negative sample or control |
|--------|---------------------------------------|---------------------------------------|--------------------------------------|
| 20 µl | 13.6 µl | 2.4 µl | 8 µl |
| 15 µl | 10.2 µl | 1.8 µl | 3 µl |
| 10 µl | 6.8 µl | 1.2 µl | 2 µl |

RT-PCR methods carried out with Eco 48 PCR machine (PCRmax, UK) with conditions shown in Table 3.

Table 3. RT-PCR condition and steps

| Phase | Cycle | Temp. | Duration |
|-----------------------|-------|-------|------------|
| Reverse Transcription | 1 | 50°C | 15 minutes |
| Initial Denature | 1 | 95°C | 15 minutes |
| Amplification | 45 | 94°C | 15 seconds |
| | | 95°C | 45 seconds |

Analysis of RT-PCR result

The results of the RT-PCR were analyzed by software EcoStudy software V5.2.16 (PCRmax, UK) which shows a graph of the formation of double-stranded DNA and shows the Cq value for each sample used.

Result

Amplification Results

Da An Gene volume optimization is done with final volumes 20 μ l, 15 μ l, and 10 μ l using 3 reporters i.e FAM (blue), VIC (green), and Cy5 (red) (Figure 1). Positive and negative controls show amplification curve on Cy5 channel ≤ 35 . There is no obvious amplification on FAM and VIC channel for negative controls, while positive controls have obvious amplification curves ≤ 32 (Figure 1 and Figure 1a for easier reading). These result met the criteria which was set by manufacturer as a valid reaction.

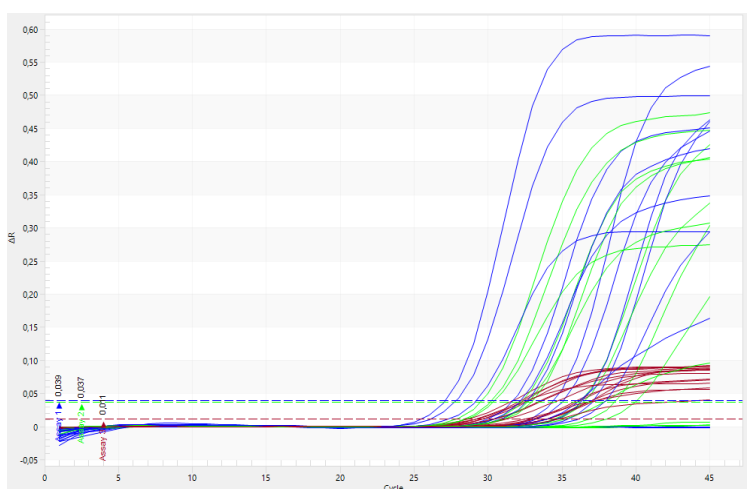


Figure 1. Amplification curve for all samples, positive and negative samples; blue line (FAM), green line (VIC) and red line (Cy5).

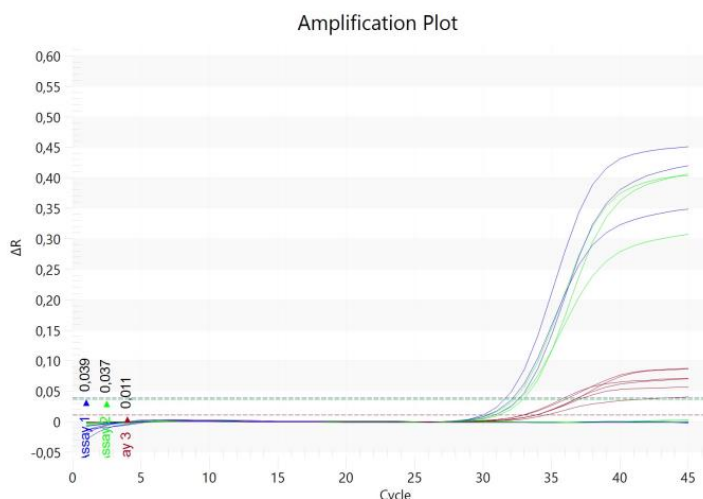


Figure 1a. Positive and negative control curve; figure legend same as Figure 1.

Cq value for all samples were shown in Tables 4 and 5.

Table 4. Cq value with FAM

| Sample | Cq Value (FAM) | | |
|--------|----------------|-------|-------|
| | 20 µl | 15 µl | 10 µl |
| N62 | 34.38 | 35.05 | 36.15 |
| N63 | 37.09 | 36.36 | 37.93 |
| N79 | 27.09 | 27.79 | 29.16 |

Table 5. Cq value with VIC Assay

| Sample | Cq Value (VIC) | | |
|--------|----------------|-------|-------|
| | 20 µl | 15 µl | 10 µl |
| N62 | 36.21 | 36.44 | 37.77 |
| N63 | - | 38.54 | 39.94 |
| N79 | 29.45 | 30.14 | 30.32 |

Discussion

The conditions of the RT-PCR reaction carried out in this study were in accordance with the criteria set by the manufacturer. This can be seen from the amplification results in positive and negative controls. FAM and VIC used in the Da An Gene Kit, are a type of dye label that is part of the TaqMan probe. The TaqMan probe itself is designed to attach to certain DNA sequences during the annealing stage to give a signal that will later be read by a PCR machine. The TaqMan probe consists of two types of molecular components at each end: reporter and quencher. The reporter is located at the end of the 5' probe which specifically serves to provide a fluorescence signal that will later be read by the PCR machine. This reading signal intensity can be measured as the cycle conditions run, where the greater the intensity, the more DNA is amplified. While quencher is located at the end of a 3' probe that specifically serves to prevent the reporter from fluorescent before being cut by the activity of the enzyme Taq DNA polymerase during the extension process which separates the reporter and quencher. Technically, if the reporter and quencher are still in a unified state, then when the reporter is excited, what happens is that the energy will move to the quencher. This event is called FRET (Fluorescence Resonance Energy Transfer). Therefore, for a reporter to be able to give a signal it must be in a state of separation from the quencher (Navarro et al., 2015; Soheili & Samiei, 2005).

There are three samples used: samples N62, N63, and N79. The study used reagents plus samples with a total volume of 20 µl, 15 µl, and 10 µl. In the sample N62 (FAM) obtained the smallest Cq value at a volume of 20 µl with a value of 34.48. The same is also found in VIC assay where the smallest Cq value is at a volume of 20 µl with a value of 36.21. While in the sample N63 with ASSAY FAM at volume 15 µl has the smallest Cq value with a value of 36.36, while at volume 20 µl only obtained Cq value 37.09. Then in the same sample as the VIC assay at a volume of 20 µl unfortunately did not get a Cq value and the curve was also flat. Furthermore, the sample of N79 with FAM assay obtained the smallest Cq value at a volume of 20 µl, which is with a value of 27.09, while for assay VIC also obtained the smallest Cq value at volume 20 µl, which is with a value of 29.45. Overall, the three volumes used in the study showed that most samples with a total volume of reagents plus a sample of 20 µl produced the smallest Cq value, making it the optimal volume. The results of the tests are expected to save the use of reagents for the detection of SARS-CoV-2. In the Wink et al., 2021 study it was argued that regardless of the reliable results of SARS-CoV-2 infection detection testing with reagent volume plus optimized samples, this test can save the use of available reagents.

Although the Cq values of all three samples and the three volumes tested showed a value of < 40 and were considered positive to contain SARS-CoV-2, most of its Cq values are > 25 which can be interpreted that viral load in the samples were low (Faico-Filho et al., 2020; Kampf et al., 2021). In addition, the absence of Cq value and flat curve in the N63 (VIC) sample with a volume of 20 µl can be caused by several factors including human error during the sample preparation process such as the presence of bubbles formed in the well when pipetting or it could also be due to external factors with some examples such as contamination, reagents that are not mixed with the sample, or unreadable by PCR machines (Jalali et al., 2017; Life Technologies, 2012).

The value Cq itself stands for cycle quantity which in the result of the amplification curve is the intersection point between the amplification curve of the sample tested and the threshold line. In addition, the Cq value in the PCR amplification cycle indicates the point at which the fluorescence level exceeds the threshold limit. In some protocols, the Cq value can be written with Ct which stands for cycle threshold. The Cq value provides information about the cycle of several PCR machines that can detect signals from fluorescence that are fluorescent during the PCR process, where the smaller the Cq value, the more DNA copies there are in the sample. So in this study, it can be interpreted that the smaller the Cq value, the greater the viral load in the sample (Faico-Filho et al., 2020). Then for the threshold value on the amplification curve is the smallest limit where the PCR machine can distinguish the signal from fluorescence with the background noise (Butler, 2012). Analysis of amplification curve data is done on EcoStudy software. Threshold values can be changed as needed, but in this study, the threshold value is not changed whatsoever and the value is set automatically by EcoStudy software.

Other study by Mohammed et al (Mohammed et al., 2022) showed that Da An Gene kit remains a reliable diagnostic tool even though it used a total volume differ from the manufacturer's recommendations. In this research, solution A was titrated 0.25X, 0,375X and 0,5X with total volume was 10 ul.

The lack of Cq value data with the total volume of reagents plus samples of 25 µl (according to the kit protocol) is an obstacle due to the maximum capacity of reagent volume on the PCR well machine so that there is no comparison data for Cq value data from optimized volumes. In the Da An Gene kit protocol, the total volume of reagents plus samples used is 25 µl, while the PCR machine (PCRmax Eco 48) used in this study in each well is only able to accommodate reagents plus samples up to a maximum of 20 µl. Therefore, under such conditions, it can also be a reason to optimize the volume on the kit so that it can still do SARS-CoV-2 detection.

Conclusions

We tested 3 different total volumes of Da An Gen Kit to detect SARS-CoV-2. From these 3 volumes, we found that volume 20 ul was the most optimal volume in this study, since it produces the smallest Cq value compared to the other two total volumes. The laboratory with limited-resource setting should do their optimization of kit performance to produce a valid test.

Declaration Statement

The authors reported no potential conflict of interest.

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