Detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in clinical samples using Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

A Atikana1,*, L Sukmarini1,*, Hariyatun1, A M Ridwanulah1, H A Nugroho2, P S Sushadi2, A B Juanssilfero1, D F Agustiyanti1, M Y Putra1, R A Ningrum1, and P Lisdiyanti1

1 Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI), Jl. Raya Bogor KM. 46, Cibinong, Bogor, Indonesia
2 Research Centre for Biology, Indonesian Institute of Sciences (LIPI), Jl. Raya Bogor KM. 46, Cibinong, Bogor, Indonesia

*Email: A Atikana: akhirta.atikana@lipi.go.id; L Sukmarini: linda.sukmarini@lipi.go.id

Abstract. The coronavirus disease 2019 (COVID-19) is caused by an infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which has infected more than 400,000 people, causing a worldwide pandemic. As of August 15, 2020, as many as 111,455 cases of COVID-19 have been confirmed in Indonesia with 6,071 deaths that have been recorded thus far. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) has been used as a gold standard to detect the infection of SARS-CoV-2 in many clinical samples due to its sensitivity and specificity. In Indonesia, one of the recommended and commonly used diagnostic kit for COVID-19 was MiRXES Fortitude Kit 2.0E under the license of Singapore's Agency for Science, Technology, and Research (A*STAR), Singapore. Aiming to assess the basic analytical and clinical performance of the MiRXES Fortitude Kit 2.0E, this study prepares serial dilutions of the provided positive control to establish PCR efficiency and to estimate the limit of detection. Moreover, a panel of SARS-CoV-2-positive-clinical samples (nasopharyngeal swab specimens) was tested to preliminarily evaluate the kit’s sensitivity on clinical samples. This study is expected to provide valuable assessment information for laboratories that develop routine diagnostic protocols for COVID-19, especially those using the MiRXES Fortitude Kit (A*Star, Singapore).

1. Introduction
Since the end of 2019, a novel coronavirus called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has emerged and caused a new pandemic disease called coronavirus disease 2019 (COVID-19). The virus was reported to be transmitted via droplets, and until around August 2020 there was no cure or vaccine available yet to treat COVID-19. Although originally concentrated in Hubei, China, the COVID-19 has been spread around the world with more than 400,000 people infected worldwide in August 2020. During its early outbreaks in China in December 2019–February 2020, Indonesia reported no single case of infection yet. Only on March 2, 2020, nearly a month after the World Health Organization (WHO) officially declared the outbreak of COVID-19, the first two cases of COVID-19 were then reported in Indonesia [1]. As of August 15, 2020, as many as 111,455 cases of COVID-19 have been confirmed, spread to all 34 provinces in the country (figure 1), with 6,071 deaths that have been recorded thus far [2].
Due to its sensitivity and specificity, the reverse-transcription polymerase chain reaction (RT-PCR) has been used as a gold standard to detect the infection of SARS-CoV-2 in many clinical samples as recommended by the United States (US) Centers for Diseases Control and Prevention (CDC) [3]. In clinical settings, the RT-PCR assay has been routinely used to detect RNA viruses by amplification of viral nucleic acids [4]. Firstly, the reverse transcriptase enzyme converts the viral RNA to its complementary DNA via reverse transcription (RT) process, followed by PCR amplification process. Depending on the detection strategy, the RT-PCR method can be divided as quantitative real-time RT-PCR (qRT-PCR), in which the target gene can be monitored through fluorescent signals in real-time during the PCR amplification [4] and nested PCR in which two sets of primers were used to avoid the amplification of non-specific targets [5]. Primers were designed to target the SARS-CoV-2 genes, including the RNA-dependent RNA polymerase (RdRP) gene, nucleocapsid (N) gene, envelope (E) gene, spike protein (S) gene, and large open reading frame (ORF1ab) gene [6].

Highly sensitive methods are desirable in a public health emergency, including the COVID-19 pandemic. More than 50 commercial RT-PCR diagnostic kits for detecting COVID-19 have been listed and recommended by the World Health Organization (WHO) [7] and the Indonesian government-mandated Task Force for COVID-19 [8]. Additionally, more than 100 nucleic acid detection-based kits have been reviewed by Pokhrel et al. [6] that can fast and accurately diagnose COVID-19, as well as to contain and control the virus outbreak in the future. The CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time Reverse Transcriptase (RT)–PCR Diagnostic Panel that can detect the SARS-CoV-2 virus in upper and lower respiratory specimens was also included in the review list. According to Pokhrel et al. [6], the choice of methods and diagnostic kits to detect the SARS-CoV-2 is important to reduce non-specific target amplification in clinical samples, thus leading to increased specificity and sensitivity. The qRT-PCR diagnostic kits developed by the US-CDC has a detection limit as low as 4-10 RNA copies per μL for X gene, while the protocols used by German scientists showed as few as 5.6 RNA copies per reaction for the E gene and 3.8 RNA copies per reaction for the RdRP gene. The protocols by Hongkong scientists preferred to target the N gene and the ORF1ab gene, while the France used the RdRP and the E genes to detect SARS-CoV-2. Meanwhile, the researchers at Singapore's Agency for Science, Technology, and Research (A*STAR) and the Department of Laboratory Medicine at Tan Tock Seng Hospital (TTSH), Singapore, have also developed a qRT-PCR-based COVID-19 diagnostic test kit, called the MiRXES Fortitude Kit 2.0E.

Figure 1. The confirmed cases of COVID-19 in Indonesia and map distribution. The data was derived from the Ministry of Health, Republic Indonesia, as of August 15, 2020, showed the highest case was in East Java [2].

Confirmed cases by province

- 100–499
- 500–999
- 1,000–4,999
- 5,000–9,999
- 10,000–49,999
[9]. Under the license of A*STAR Singapore, interestingly, the kit has been claimed to have high accuracy by targeting the non-structural protein 1 (\(NSP1\)) gene. Moreover, it has shown an assay efficiency at 99% via a dilution series of 150,000 to 10 copies per reaction and a limit of detection (LoD) of 25 copies per reaction.

The use of MiRXES Fortitude Kit 2.0E to detect SARS-CoV-2 has been reviewed previously in a number of studies [10-12]. However, information on the basic analytical and clinical performances of this kit in Indonesia is still limited. Aiming to establish the PCR efficiency and LoD of the MiRXES Fortitude Kit 2.0E, this study performed serial dilutions of positive control provided by the kit manufacturer. Moreover, a panel of SARS-CoV-2-positive-clinical samples (nasopharyngeal swab specimens) were also tested to preliminarily evaluate the kit’s sensitivity to detect viral RNA in clinical specimens. This study is expected to provide a valuable assessment information that is useful for any of the referral laboratory that develops protocols for routine diagnostics of COVID-19, especially to those who used the MiRXES Fortitude Kit (A*Star, Singapore).

2. Methodology

2.1. Specimen collections and viral RNA extraction

Clinical specimens from the nasopharyngeal swab from the collection of the Biosafety Level-3 (BSL-3) Laboratory, Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI), were used in this study. Viral RNA were isolated from the swab specimens collected from people who were suspected to have COVID-19. In the present study, total viral RNA was extracted from 200 \(\mu\)L of swab specimens using the Viral Nucleic Acid Extraction Kit II (Geneaid Biotech Ltd., Taiwan) according to the manufacturer’s instructions in the BSL-3 Laboratory, LIPI. The total RNA of all samples were stored at -80°C until further use.

The extracted viral RNA was used as a template for qRT-PCR. To evaluate the sensitivity of the diagnostic kit to detect viral RNA in clinical samples, serial dilutions of SARS-CoV-2-positive clinical samples RNA were prepared and used as template for the qRT-PCR. In addition to that, a two-fold serial dilution of positive control provided by the kit was also prepared to evaluate the LoD of the kit as well as to assess the kit performance.

2.2. qRT-PCR for COVID-19 detection

This study used the one-step qRT-PCR diagnostic kit, MiRXES Fortitude Kit 2.0E (MiRXES, Pte. Ltd., Singapore) to qualitatively detect SARS-CoV-2 specific RNA in nasopharyngeal swab samples. The kit targeted the \(NSP1\) genes of SARS-CoV-2 which probe is labelled with the FAM fluorophore. For internal control (IC), the probe is labelled with HEX fluorophore. A total of 25 \(\mu\)L PCR mix reaction was prepared for each sample, containing 2.5 \(\mu\)L of template RNA, 8.4 \(\mu\)L of nuclease-free water, 12.5 \(\mu\)L of the universal probes reaction mix, 1 \(\mu\)L of the primer-probes mix, 0.5 \(\mu\)L of reverse transcriptase enzyme, and 0.1 \(\mu\)L of IC. The master mix was prepared on ice. The PCR condition to detect the COVID-19 using MiRXES Fortitude Kit 2.0E was performed as follows: reverse transcription (RT) (48°C for 15 min), followed by a cycle of the inactivation of RT process or the initial denaturation (95°C for 2 min 30 sec), and 42 cycles of denaturation (95°C for 20 sec) and annealing (59°C for 42 sec). The qRT-PCR was performed on a CFX96™ Real-Time System combined with C1000™ Touch Thermal Cycler (Bio-Rad Laboratories, Inc., USA).
Figure 2. Amplification plot window to detect SARS-CoV-2 using the One-Step qRT-PCR kit MiRXES Fortitude Kit 2.0E (MiRXES, Pte. Ltd.). Anomalous PCR amplification profile in red color indicated a signal of non-specific amplification, meaning the cycle threshold (Ct) values obtained from such curves were invalid. PCR amplification profile in blue indicated the result of specific/target amplification, meaning the Ct values obtained from such curves were valid [9].

In each qRT-PCR run, at least one positive control and one negative control were used. A positive result for SARS-CoV-2 was indicated if the cycle threshold (Ct) value of target gene (SARS-CoV-2, FAM) was < 40 and the presence of growth sigmoidal curves in the PCR amplification step was detected (figure 2). On the other hand, a negative result for SARS-CoV-2 was indicated if an undetermined Ct value of the target gene (SARS-CoV-2, FAM) was shown and there was absence of sigmoidal curves in the PCR amplification step. The test was valid if the positive control showed the Ct target gene < 40 with the presence of growth sigmoidal curves in the PCR amplification step and if the negative control showed undetermined Ct target gene with the absence of sigmoidal curves in the PCR amplification step. The internal control (IC, HEX) should show a Ct value of < 40 to pass the test quality control. The test was deemed invalid if it showed undetermined Ct for both target gene and IC, thus repeat qRT-PCR is needed.

3. Results and Discussion

More than 100 commercial nucleic acid-based diagnostic kits for detecting COVID-19 have been reviewed so far [10-12], including MiRXES Fortitude Kit 2.0E, which was also recommended in Indonesia. This study investigated the efficiency, LoD, and sensitivity of the kit to detect the SARS-CoV-2 in clinical samples from the collection of the BSL-3 Laboratory, Research Centre for Biotechnology, LIPI. Unlike the majority of molecular diagnostic tests that target ORF1ab, N, E, or RdRP genes, the Fortitude Kit 2.0E targeted a single region-specific to SARS-CoV-2, the NSP1 gene positioned on the ORF1ab region, and was reported to be less prone to mutation [13]. Moreover, recent improvements have been made for MiRXES Fortitude Kit 2.1E by targeting two different regions of ORF1ab gene.
Figure 3. A standard curve retrieved from a two-fold serial dilution of MiRXES Fortitude Kit positive control. The amplification target was the NSP1 of SARS-CoV-2 (FAM).

The MiRXES Fortitude Kit 2.0E (MiRXES, Pte. Ltd.) has shown satisfactory performance based on its PCR efficiency for targeted SARS-CoV-2 viral sequence, the NSP1 gene. The assay of a duplicate two-fold dilution series of positive control demonstrated a high efficiency >95% and R square was 0.95 (figure 3). Moreover, the manufacturer has claimed that the kit has the LoD at 25 copies per reaction, which was correlated to the Ct value 35. In this present study, the Ct values of the two-fold serial dilution of positive control were consistently detected until the ¼ fold dilution with the Ct 35.54 – 36.27. However, the RNA of SARS-CoV-2 could still be detected at the 1/8 fold (Ct 36.72) and 1/10 fold (Ct 37.51) dilutions, while no viral RNA could be observed at the dilutions higher than 1/10 fold (table 1).

Table 1. The Ct values of a two-fold serial dilution of the Fortitude Kit 2.0 positive control.

| Content                  | Dilution  | Ct values |
|--------------------------|-----------|-----------|
| Positive control (PC)    | undiluted | 34.47     |
|                          | 1/2       | 35.33     |
|                          | 1/4       | 35.54     |
|                          | 1/8       | 36.72     |
|                          | 1/10      | NA        |
| Negative control (NC)    |           | NA        |
| Non-template control (NTC)|          | NA        |

aNA = not available

To assess the LoD and sensitivity of the kit in the clinical samples, a two-fold serial dilution of SARS-CoV-2-positive clinical samples was performed. Using a selected clinical sample with a high initial concentration of SARS-CoV-2 RNA (33.64 ng µL⁻¹), the kit was able to positively identify the sample and consistently detect until the 1/8 fold dilution, though it could still detect viral RNA at the 1/10 fold dilution (figure 4, table 2). On the other hand, the previously confirmed SARS-CoV-2 clinical sample with a lower concentration (14.76 ng µL⁻¹) was only consistently detected until the ¼ fold dilution, though viral RNA could still be observed at the 1/8 fold dilution (figure 5, table 3). It is suggested that the kit is considered sensitive enough to detect SARS-CoV-2 RNA in a clinical sample with a minimum concentration of 1.476 ng µL⁻¹ (RNA yield ≈ 3.75 ng) and the Ct ≤ 38. Moreover, in
In this present study, we tested 139 clinical samples that resulted in 16 positively confirmed SARS-CoV-2 samples (Figure 6). Fifteen out of 16 individuals testing positive for the NSP1 gene showed Ct values within range 31.40-37.36, only one positive sample with Ct 38.5 was found (data not shown). These samples were within the linear regression of the standard curve as mentioned above (Figure 3).

**Figure 4.** The amplification of NSP1 gene of SARS-CoV-2 (FAM probe) from a two-fold serial dilution of SARS-CoV-2-positive clinical sample with a high concentration (33.64 ng µL⁻¹).

**Table 2.** The Ct values of a two-fold serial dilution of a positive clinical sample with a high initial concentration of SARS-CoV-2 RNA (33.64 ng µL⁻¹). The qRT-PCR amplification target was the NSP1 gene of SARS-CoV-2.

| Content                        | Dilution (fold) | Ct values | Ct values |
|--------------------------------|-----------------|-----------|-----------|
| Positive control (PC)          | undiluted       | 33.26     | 33.06     |
| Clinical sample (33.64 ng µL⁻¹)| 1/2             | 33.16     | 34.03     |
|                                | 1/4             | 34.34     | 35.27     |
|                                | 1/8             | 36.32     | 35.71     |
|                                | 1/10            | NA        | 37.75     |
| Negative control (NC)          |                 | NA        | NA        |
| Non-template control (NTC)     |                 | NA        | NA        |

*NA = not available

**Figure 5.** The amplification of NSP1 gene of SARS-CoV-2 (FAM probe) from a two-fold serial dilution of SARS-CoV-2-positive-clinical-sample with lower initial concentration (14.76 ng µL⁻¹).
Table 3. The Ct values of a two-fold serial dilution of a positive clinical sample with a lower initial concentration of SARS-CoV-2 RNA (14.76 ng µL⁻¹). The qRT-PCR amplification target was the NSPI gene of SARS-CoV-2.

| Content                        | Dilution (fold) | Ct values |
|--------------------------------|-----------------|-----------|
| Positive control (PC)          | undiluted       | 32.94     |
| Clinical sample (14.76 ng µL⁻¹)| 1/2             | 34.38     |
|                                | 1/4             | 36.36     |
|                                | 1/8             | 37.21     |
|                                | 1/10            | NA        |
| Negative control (NC)          | NA              | NA        |
| Non-template control (NTC)     | NA              | NA        |

*NA = not available

In diagnostic virology, qRT-PCR is widely used because it is the most robust technology to detect the presence of viral RNA. However, every diagnostic kit has its limitations, depending on factors such as the use of different specific target genes in each kit. Thus, considering the LoD of each diagnostic tool is an important step before developing protocols for routine COVID-19 detection in diagnostic laboratories. This is important to reduce non-specific target amplification in clinical samples, as well as to increase the specificity and sensitivity [6]. In May 2020, a total 139 samples were tested for COVID-19, using the MiRXES Fortitude Kit 2.0E. Among all samples tested in this study, a total of about 10% specimens were detected positive for SARS-CoV-2 infection (Figure 6). Considering our findings, this preliminary study not only demonstrated the robustness of qRT-PCR, but also confirmed the LoD and sensitivity of the MiRXES Fortitude Kit 2.0E, to detect the SARS-CoV-2 viral RNA in clinical samples. However, further studies using more clinical samples are important and needed to validate the detection assays.

![Figure 6](image-url)

**Figure 6.** The total percentage of SARS-CoV-2 RNA confirmed in clinical samples during May 2020 (n=139) analysed in this study. Positive samples were 16 out of 139 clinical samples (about 10%).
The viral nucleic acid detection by qRT-PCR is considered as a gold standard for COVID-19 detection. However, it still has limitations in analysis and determination of the viral load. A combination of qRT-PCR and droplet digital PCR (ddPCR) can be used to explore the changes in viral load in different samples, since the ddPCR offers better sensitivity on absolute quantification than RT-PCR [14].

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Conflict of Interest
The authors declare no conflict of interest. This study was not in any way endorsed by the MiRXES Fortitude Kit 2.0E (A*Star, MiRXES, Pte. Ltd., Singapore).

Author contributions
AA and LS are the main authors who conceived the study design, the writing of original first draft of the manuscript, and the preparation of data presentation. AA, LS, and H, and AMR contributed to data analysis and verification. AA, LS, and H performed the real-time PCR. HAN and PSS performed the viral RNA extraction. ABJ, DFA, and RAN provided the clinical samples and performed their maintenance. MYF, RAN, and PL provided chemicals reagent and consumables, as well as the supervision process. All authors reviewed and edited the final manuscript.