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Optimization of *Real-Time* PCR Conditions for COVID-19 Diagnosis with Logix Smart Reagent™

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ABSTRACT
Corona Virus Disease 2019 or COVID-19 is a new type of virus that attacks the respiratory tract and can cause death. Laboratory examinations play an essential role in diagnosing COVID-19 with a set of reagents or kits. Sampiand sampling is carried out with a nasopharyngeal swab or oropharyngeal swab. Positive samples of COVID-19 patients used in this study were converted into RNA at the COVID-19 Referral Clinic in Bekasi, after which volume optimization was carried out with a total volume of 5 µl, 8 µl, and 10 µl with the Logix Smart™ kit. The method in this study uses One-Step Real-time PCR. This method is the best method for carrying out several bear tests because it can reduce the possibility of sample contamination. The procedure is fast and has high sensitivity. The fluorescence detection used in this study was FAM with a specific target of COVID-19 RNA and ROX with a particular DNA target of RNase-P. This research was conducted to obtain optimal volume conditions under the manufacturer's standards in detecting the SARS-CoV-2 virus. The results of this study indicate that a total volume of 5 l is the optimal total volume for detecting the presence of the SARS- CoV-2 virus in samples taken from patients.

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1. INTRODUCTION

In December 2019, there was an increase in pneumonia cases in Wuhan, Hubei, China. On February 11, 2020, the International Committee on Taxonomy of Ice Virus (ICTV) announced the name of the new virus as SARS-CoV-2 (Harcourt et al., 2020). The World Health Organization (WHO) also reported that pneumonia caused by SARS-CoV-2 is COVID-19 (Chan et al., 2020)(Setiati & Azwar, 2020). COVID-19 disease has zoonotic properties, which means it can be transmitted from animals to humans, and it is currently known that this new type of virus has been known to be transferred from humans to humans. CoVs disease is the cause of some respiratory diseases that are deadly for humans (Valitutto et al., 2020). In addition to the SARS and MERS outbreaks in 2002 and 2012, the current Covid-19 explosion is also caused by this virus. The spread of the disease due to the SARS-CoV-2 virus is so fast with an easy transmission mechanism through the splash of saliva (droplets) and then into the respiratory tract through The mucus glands and then enters the respiratory tract (Ember et al., 2022). In addition, transmission can also be through the air aerosols inhaled into the respiratory tract. The difference between droplets and aerosols is in the size of the particles. Droplet particle sizes are greater than or equal to 5 microns, while aerosols are less than 5µm (Ember et al., 2022).

Logix Smart™ Coronavirus Disease 19 (COVID-19) reagent (kit) is a Real-Time PCR (RT- PCR) test that aims for qualitative detection in vitro of nucleic acids in the nasopharynx and oropharynx. The Logix Smart™ COVID-19 kit uses humans' RNase-P gene (Ribonuclease P) as an internal inhibitor control. Positive results indicate the presence ofSARS-CoV-2 RNA and negative results do not preclude SARS-CoV-2 virus infection

and cannot be used as a clinical decision in patients. Adverse effects should be observed with clinical observations, patient history, and epidemiological information. The reagent RT-PCR volume of manufacturers used in diagnosing COVID-19 with this Logix Smart™ COVID- 19 kit is 10µl (Co-Diagnostics Inc., 2020)(Santini, 2021)(Dramé et al., 2020).

This study aims to get the optimal volume of detecting the COVID-19 virus with Logix smart kit™ COVID-19. This study used the One-Step Real-Time PCR (RT-PCR) method. The one-step RT-PCR method is sensitive and specific for detecting the SARS-CoV-2 virus. The RT-PCR targets are several genes from the SARS-CoV-2 virus, namely genes N, E, S, and RdRP. This method has advantages in reducing the use of pipetting, reducing contamination in samples, and fast procedures.

2. RESEARCH METHOD

The research was conducted at the Laboratory of Molecular Biology of the Faculty of Health Sciences, Kholiq Raus Building 3rd floor, Esa Unggul University. Tools used in this study include a tube, vortex laboratory shaker MX-S, mini centrifuge, micropipette for volumes from 2 to 20 µL, Laminar Air Flow (LAF, real-time PCR (PCRmax). eco48), well-plate reaction adhesive plastics, laptops, and sample reagent racks.

The materials used in this study are consumables, among others, disposable gloves, lab coats, pipette tips with volumes of 10µl, 20µl, 200µl, sodium hypochlorite fluid, RNA samples of patients who positively confirmed COVID-19, Logix Smart™ Coronavirus Disease 2019 (COVID-19) Kit, and sample reagent storage (Co-Diagnostics Inc., 2020). This study used samples of established positive COVID-19 patients who had been isolated into RNA at the COVID-19 Referral Clinic in Bekasi.

First, we cleaned all workplaces with 70% alcohol, removed each component from the diagnostic kit, positive sample COVID-19 patients, and placed it on a reagent rack at room temperature. Details and samples that have been liquid are temporarily twisted down for 3 seconds before being used to ensure the reagent is mixed and remove condensation or residue from the lid (Aoki et al., 2021)(Tahamtan & Ardebili, 2020).

Each prepared reaction should include all the reagents for the sample, at least one positive control and one negative control. All piping should be done on ice, if possible. Positive control piping is recommended to be done last to avoid contamination events. Replace the pipette tips after grabbing each component (Aoki et al., 2021).

Proper volume optimization can also determine effective and efficient results for reagents. Generally, the optimal volume required to test the Logix Smart COVID-19 kit is 10 µL. At this stage, the importance used to conduct the Logix Smart COVID-19 test kit is 5µL, 8 µL, and 10 µL (Co-Diagnostics Inc., 2020).

The pipette tips with each take the component were replaced. The process of pipetting negative control is carried out at the initial stage according to the required volume, for volume 5 µL required 2,5 µL µL negative control, for volume 8 µL required 4 µL negative control. Volume 10 µL required 5 µL negative control, then added Master Mix according to the rest of the volume for optimization. The positive and negative patient samples were done the same and Master Mix. The positive control stage is done at the end to avoid contamination, and the volume needed is the same as the control of the negative and the sample. All reagents and samples are pipette to a well-plate PCRmax reaction and are spaced to distinguish each volume. The well-plate was covered by adhesive until tight. The well-plate was placed into the real-time PCR instrument with the correct orientation and then set the temperature, assays, and samples on the PCRmax software (Tahamtan & Ardebili, 2020).

3. RESULTS AND DISCUSSION

The results of Cq (Cycle quantity) were analyzed using EcoStudy software. The software will detect fluorescence signals in the sample, negative and positive control, experiment name, well-plate settings, sample naming, gene amplification, and values. Samples of COVID-19 patients tested positive if the fluorescence signal detected was below cycle 45 and the Cq value showed below 30, while it was possible at AQ value above 30. The sars- CoV-2 virus is still detected even though the patient has no symptoms (Valitutto et al., 2020). Samples of COVID-19 patients test negative if the fluorescence signal is not seen for the SARS-CoV-2 virus, but sometimes the fluorescence signal indicated above cycle 45 can occur in the RNase-P gene. If the fluorescence signal is detected and has a Cq value on the negative control, the result is invalid and should be retested (Kobayashi et al., 2021).

Optimizing the total volume of 5 µl in 3 positive samples, positive control, and negative control indicates the presence of detected fluorescence signals. Based on fluorescence signals detected in figure 1 showing that all three positive-control on the amplification of the RdRP gene detected the presence of SARS-CoV-2 virus in the sample used (Kobayashi et al., 2021)(Ember et al., 2022). Positive control, sample N148, and sample N160 have a Cq value below 30. Sample N148 has a Cq value of 28.11, sample N160 has a Cq value of 28,91, while sample N159 has a Cq value of 26,05. Cq is above 30, which is 30,54. The fluorescence signal could detect the three positive controls on the amplification of the RNase-P gene. The Cq value of the three positive controls in

the RNase-P gene showed a value below 30; sample N159 had a Cq value of 26,05, sample N148 had a value of 25,83, and sample N160 had a Cq value of 25. The full results can be seen in table 1.

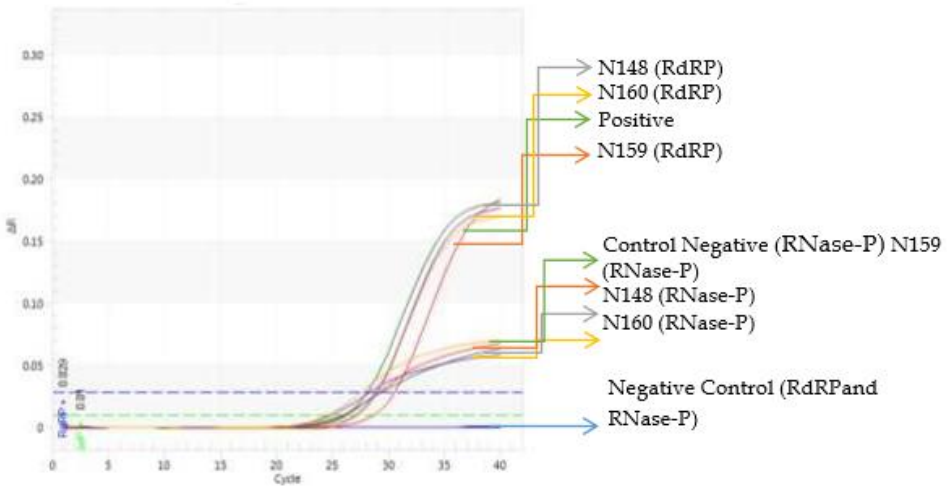

Figure 1. Graph of Fluorescence signal of amplification RNA of RdRP dan RNase-P gene with 5µl of volume reaction RT-PCR

Table 1. Cq value in each sample with a total volume of 5 µl

No.	Sample	Gene Amplification	Cq	Cq mean
S1.	N159	RdRP	30.54	30.54
		RNAseP	26.05	26.05
2.	N148	RdRP	28.11	28.11
		RNAseP	25.83	25.83
3.	N160	RdRP	28.91	28.91
		RNAseP	25	25

Figure 1 shows that negative control fluorescence signal and positive control were also detected at 5µl. On positive control with amplification of the RdRP gene shows a value of Cq 29,04 while amplification of the RNase-P gene offers a Cq-value is 24,57. Negative controls with RdRP gene amplification and RNase-P gene amplification showed no Cq value. The full results can be seen in table 2.

Table 2. Cq values on positive control and negative control

No.	Control	Gene Amplification	Cq	Cq Mean
1.	Negative	RdRP	-	-
		RNAseP	-	-
2.	Positive	RdRP	29.04	29.04
		RNAseP	24.57	24.57

A total volume optimization of 8 µl detected fluorescence signals in all three positive control and negative controls. Figure 2 shows that all three positive-control with RdRP gene amplification and amplification of the RNase-P gene detected the presence of SARS-CoV-2 virus in the sample used. Both treatments with RdRP gene amplification showed AQ values below 30, namely N148 samples with Cq values of 28,87 and N160 samples with Cq values of 27,85, while in the sample N159 points to NQ above 30, which is 31.03. The Cq value on the amplification of the RNase-P gene indicates that all three samples have a Cq value below 30, namely the sample N159 with a value of Cq 26,36 and the sample N148 with a value of Cq 26,74. And a sample of N160 with a value of Cq 24,26. More results can be seen in Table 3.

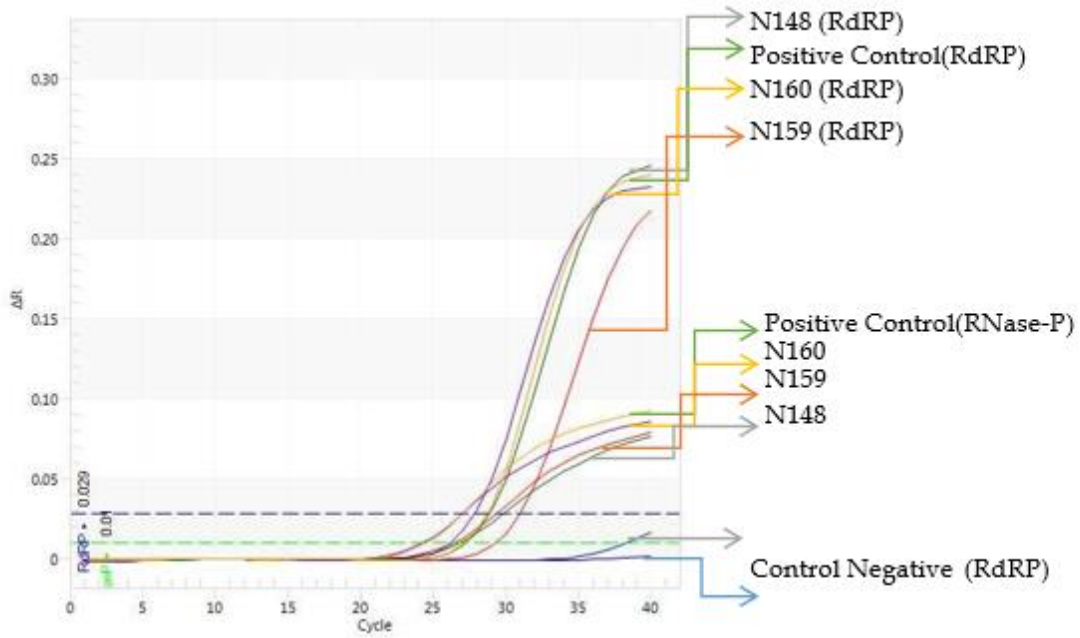

Figure 2. Fluorescence signal detected at a total volume of 8 µl

Table 3. Cq value in each sample with a total volume of 8 µl

No.	Sample	Gene Amplification	Cq	Cq Mean
1.	N159	RdRP	31.03	31.03
		RNase-P	26.36	26.36
2.	N148	RdRP	28.87	28.87
		RNase-P	26.74	26.74
3.	N160	RdRP	27.85	27.85
		RNase-P	24.26	24.26

Figure 2 shows that fluorescence signals on positive controls were detected, and fluorescence signals on negative controls with amplifying the RNase-P gene showed an upward curve. In contrast, negative control with amplification of the RdRP gene indicates a flat- curve. The Cq value on positive control with RdRP gene amplification suggests a Cq-value of 28,69 and on amplification of the RNase-P gene indicates a value of 24,48. In the negative control, amplification of the RdRP gene does not show the presence of a Cq value, but the amplification of the RNase-P gene indicates the Cq-value, which is a value of 38,23. The full results can be seen in table 4.

Optimization with a total volume of 10 µl indicates the detection of fluorescence signals in all three positive samples, positive control, and negative control. Figure 3 shows that all three specimens positively detected the presence of the Sars-Cov-2 virus. Cq values in all three samples with RdRP gene amplification and RNase-P gene amplification had different values. In amplifying the RdRP gene, two specimens have a value below 30. Namely, sample N148 has a value of Cq 28.02, sample N160 has a value of Cq 28.44, while The N159 sample has a Cq value above 30, which is 31.61. In the amplification of the RNase-P gene, all three specimens had a Cq value below 30, i.e., the N148 sample showed a value of Cq 26.53, the N160 specimens showed a value of Cq 27,04, and The Cq value of N159 is 27,7. These results can be seen in table 3.

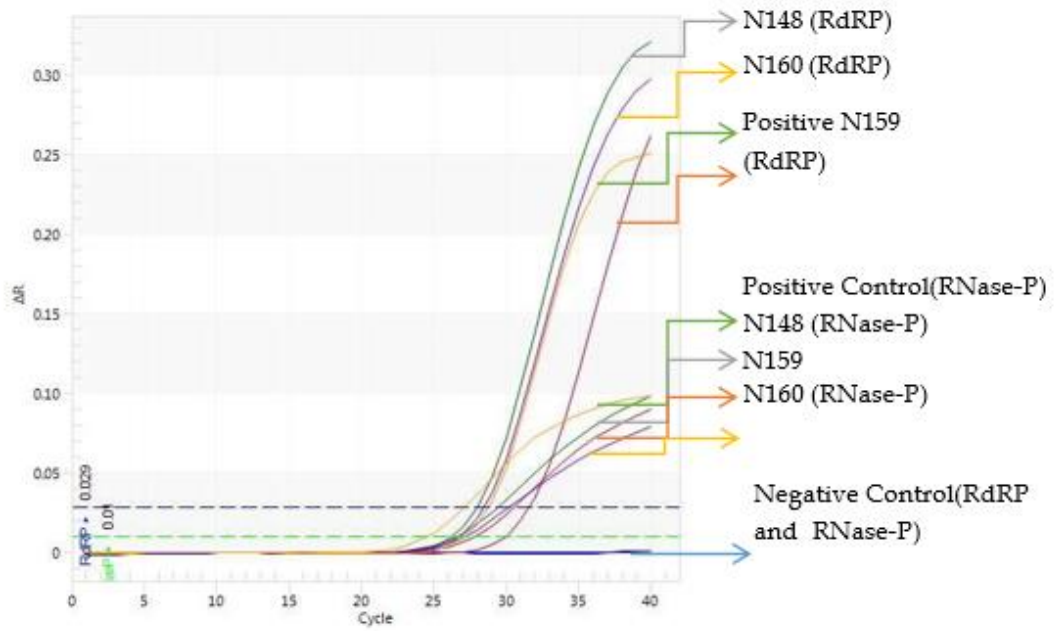

Figure 3. Fluorescence signal detected at a total volume of 10 µl

Table 3. Cq value in each sample with a total volume of 10 µl

No.	Sample	Gene Amplification	Cq	Cq Mean
1.	N159	RdRP	31.61	31.61
		RNase-P	27.7	27.7
2.	N148	RdRP	28.02	28.02
		RNase-P	26.53	26.53
3.	N160	RdRP	28.44	28.44
		RNase-P	27.04	27.04

Figure 3 shows fluorescence signals detected on positive and negative control. The positive control showed a Cq value of 28.76 in RdRP gene amplification and a Cq value of 24.53 in RNase-P gene amplification. In negative controls, the amplification curve shown is flat and offers no Cq value. The full results can be seen in table 4.

Table 4. Cq values on positive controls and negative controls with a total volume of 10 µl

No.	Control	Gene Amplification	Cq	Cq Mean
1.	Negative	RdRP	-	-
		RNase-P	-	-
2.	Positive	RdRP	28.76	28.76
		RNase-P	24.53	24.53

Table 5. Cq Value Comparison

Volume	5 µl	8 µl	10µl	5 µl	8 µl	10µl
N148	28.11	28.87	28.02	25.83	26.74	26.53
N159	30.54	31.03	31.61	26.05	26.36	27.7
N160	28.91	27.85	28.44	25	24.26	27.04
Range (ΔCq)	**2.43**	3.18	3.59	**1.05**	2.48	1.17

The Cq values in the 5 µL volume of the RT-PCR reaction for amplifying the RdRP gene and RNase-P-gene were 2.43 and 1.05, respectively. The value of ΔCq with a total of 10 µl in RdRP gene amplification indicates the Cq value of 2.48, 3.59, and amplification of the RNase-P-gene shows indigo 1.17. Meanwhile, the value of Cq is high at the reaction volume of 8 µL and 10 µL. More results can be seen in table 5.

4. DISCUSSION

The sample used in this study was a positive sample of patients confirmed to be COVID-19. Until now, research on volume optimization with Logix Smart kits™ COVID-19 in diagnosing COVID-19 has only been conducted to meet the needs in the optimal use of Logix Smart™ COVID-19 Kits in saving reagents. This reaction volume optimization is done with a total volume of 5µl, 8µl, and 10µl. Logix Smart™ COVID-19 kit uses CoPrimer technology to detect RNA from SARS-CoV-2 in the lower respiratory tract (Tahamtan & Ardebili, 2020).

Logix Smart kit™ COVID-19 uses the RNase-P-gene (Ribonuclease P) in humans as an internal target control inhibitor to see if sampling patients by swab method is successful or not. If gen RNase-P is not detected, then the swab should be repeated for patient sampling. Viral RNA genes are measured by cycle threshold (Ct) values, where amplification cycles are needed to detect fluorescent signals (Hou et al., 2020).

Samples of COVID-19 patients tested positive if the fluorescence signal detected was below cycle 45 and the Cycle quantity (Cq) value showed below 30 (Santini, 2021). Each specimen at each total volume has a different Cq value in fluorescence signals: the lower the Cq value, the more SARS-CoV-2 virus in the sample. In contrast, at a Cq value above 30, The possibility of the Sars-Cov-2 virus may still be detected even though the patient already has no symptoms. The condition is because there are still remnants of the carcass of the SARS-CoV-2 virus that was carried during sampling but could not infect (Aoki et al., 2021)(Singh et al., 2021).

The complete volume selection aims to determine whether the use of total volumes under the manufacturer's standards can detect the presence of the SARS-CoV-2 virus in samples taken from patients by the swab method (Kobayashi et al., 2021). The selection of volume total is 5µl, 8µl, and 10µl is intended to find the optimal total volume under the manufacturer's standard of 10 µl and aims to reduce the use of reagents. The delta value Cq (ΔCq) at a total volume of 5 µl is the lowest, so the total volume of 5 µl is the optimal total volume in this study. It can be used as a diagnostic of COVID-19 Logix Smart kit™ in patient *swab* samples to reduce reagents use (Valitutto et al., 2020).

In the negative control, the total volume of 8 µl with amplification of the RNase-P gene detects the presence of fluorescence signals. It indicates that the Cq value on each negative control has no Cq value, and No fluorescence signal was detected (Aoki et al., 2021). The results were declared invalid and had to be retested. The fluorescence signal detected on negative control total volume of 8 µl is known because the Logix Smart™ COVID-19 kit has previously been used in detecting patients' negative samples so that amplifying the RNase-P gene can be used (Santini, 2021) (Dramé et al., 2020). It was seen on the negative volume control of 8 µl. Another possibility can be caused because, in the replacement tips of each component retrieval, there is a sample of patients who fell in a negative control *well-plate* at a total volume of 8 µL (Singh et al., 2021).

5. CONCLUSSION

The minimal volume resulting in optimal COVID-19 Diagnosis test with the Logix Smart kit™ RT-PCR reagent is at 5 µL. Two genes, RNase-P and RdRP genes that characterize SARS COV-2 genes, were successfully amplified at a volume of at least 5 µL.

6. ACKNOWLEDGEMENT

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7. REFERENCES

Aoki, K., Nagasawa, T., Ishii, Y., Yagi, S., Kashiwagi, K., Miyazaki, T., & Tateda, K. (2021). Evaluation of clinical utility of novel coronavirus antigen detection reagent, Espline® SARS-CoV-2. *Journal of Infection and Chemotherapy*, *27*(2), 319–322. https://doi.org/10.1016/j.jiac.2020.11.015

Chan, J. F. W., Yuan, S., Kok, K. H., To, K. K. W., Chu, H., Yang, J., Xing, F., Liu, J., Yip, C. C. Y., Poon, R. W. S., Tsoi, H. W., Lo, S. K. F., Chan, K. H., Poon, V. K. M., Chan, W. M., Ip, J. D., Cai, J. P., Cheng, V. C. C., Chen, H., … Yuen, K. Y. (2020). A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *The Lancet*, *395*(10223), 514–523. https://doi.org/10.1016/S0140-6736(20)30154-9

Co-Diagnostics Inc. (2020). *Logix Smart ™ Coronavirus Disease 2019 (COVID-19) Kit*. *2019*(March), 21.

Dramé, M., Tabue Teguo, M., Proye, E., Hequet, F., Hentzien, M., Kanagaratnam, L., & Godaert, L. (2020). Should RT-PCR be considered a gold standard in the diagnosis of COVID-19? *Journal of Medical Virology*, *92*(11), 2312–2313. https://doi.org/10.1002/jmv.25996

Ember, K., Daoust, F., Mahfoud, M., Dallaire, F., Ahmad, E. Z., Tran, T., Plante, A., Diop, M.-K., Nguyen, T., St-Georges-Robillard, A., Ksantini, N., Lanthier, J., Filiatrault, A., Sheehy, G., Beaudoin, G., Quach, C., Trudel, D., & Leblond, F. (2022). Saliva-based detection of COVID-19 infection in a real-world setting using reagent-free Raman spectroscopy and machine learning. *Journal of Biomedical Optics*, *27*(02), 1–24. https://doi.org/10.1117/1.jbo.27.2.025002

Harcourt, J., Tamin, A., Lu, X., Kamili, S., Kumar, S., Murray, J., Queen, K., Tao, Y., Paden, C., Zhang, J., Uehara, A., Wang, H., Goldsmith, C., Bullock, H., Wang, L., Whitaker, B., Lynch, B., Gautam, R., Schindewolf, C., … Thornburg, N. (2020). Isolation and characterization of SARS-CoV-2 from the first us COVID-19. *Bio Rxiv*, *114*(June), e00146. https://doi.org/10.1016/j.sciaf.2019.e00146

Hou, T., Zeng, W., Yang, M., Chen, W., Ren, L., Ai, J., Wu, J., Liao, Y., Gou, X., Li, Y., Wang, X., Su, H., Gu, B., Wang, J., & Xu, T. (2020). Development and evaluation of a rapid CRISPR-based diagnostic for COVID-19. *PLoS Pathogens*, *16*(8), 1–12. https://doi.org/10.1371/journal.ppat.1008705

Kobayashi, R., Murai, R., Asanuma, K., Fujiya, Y., & Takahashi, S. (2021). Evaluating a novel, highly sensitive, and quantitative reagent for detecting SARS-CoV-2 antigen. *Journal of Infection and Chemotherapy*, *27*(6), 800–807. https://doi.org/10.1016/j.jiac.2021.01.007

Santini, A. (2021). Optimising the assignment of swabs and reagent for PCR testing during a viral epidemic. *Omega (United Kingdom)*, *102*, 102341. https://doi.org/10.1016/j.omega.2020.102341

Setiati, S., & Azwar, M. K. (2020). *COVID-19 and Indonesia*. *April*.

Singh, A. K., Nema, R. K., Joshi, A., Shankar, P., Gupta, S., Yadav, A. K., Nema, S., Mathew, B. J., Shrivas, A., Patankar, C., Raghuwanshi, A., Pandey, R., Tripathi, R., Ansari, K., Singh, K., Yadav, J., Biswas, D., & Singh, S. (2021). Testing of four-sample pools offers resource optimization without compromising diagnostic performance of real time reverse transcriptase-PCR assay for COVID-19. *PLoS ONE*, *16*(5 May), 1–10. https://doi.org/10.1371/journal.pone.0251891

Tahamtan, A., & Ardebili, A. (2020). Real-time RT-PCR in COVID-19 detection: issues affecting the results. *Expert Review of Molecular Diagnostics*, *20*(5), 453–454. https://doi.org/10.1080/14737159.2020.1757437

Valitutto, M. T., Aung, O., Tun, K. Y. N., Vodzak, M. E., Zimmerman, D., Yu, J. H., Win, Y. T., Maw, M. T., Thein, W. Z., Win, H. H., Dhanota, J., Ontiveros, V., Smith, B., Tremeau-Brevard, A., Goldstein, T., Johnson, C. K., Murray, S., & Mazet, J. (2020). Detection of novel coronaviruses in bats in Myanmar. *PLoS ONE*, *15*(4), 1–11. https://doi.org/10.1371/journal.pone.0230802