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In-house Multiplex rRT-PCR Assay for Sars-Cov2 Detection in Indonesia using a new primer design

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Abstract

During the COVID-19 pandemic, we attempted to develop an in-house rRT-PCR kit, utilizing custom primers targeting NSP14 and RdRp, with the RPP30 gene as an internal control. This kit will support Indonesian independence in enhancing COVID-19 diagnostics. The primer and probe were designed by a bioinformatics tool, determining primer specificity and sensitivity, optimizing probe concentrations, establishing LoD (Limit of Detection), LoQ (Limit of Quantification), and rRT-PCR efficiency, multiplex testing of the rRT-PCR kit on clinical samples, and testing the kit's stability. The in-house rRT-PCR kit can detect NSP14, RdRp, and RPP30 genes. The optimal concentrations for the NSP14, RdRp, and RPP30 probes are 1 μM, 1.5 μM, and 1.5 μM, respectively. The LoD and LoQ for the NSP14 are 0.22 ng/μL and 0.67 ng/μL, and for the RdRp are 1.08 ng/μL and 3.28 ng/μL. The rRT-PCR efficiencies for the NSP14, RdRp, and RPP30 are 80.3%, 100.6%, and 106%, respectively. Detection of ten clinical samples, comprising seven true positive and three true negative samples, showed Ct (Cycle threshold) values of 28–31 for the RPP30 gene, Ct 21–27 for the RdRp gene, and Ct 30–34 for the NSP14 gene. Stability testing of the rRT-PCR kit demonstrated promising results, where the kit stored at -20°C for seven days showed almost no difference in Ct values. This in-house multiplex rRT-PCR will support Indonesian independence in enhancing COVID-19 diagnostics, providing a dependable method for detecting SARS-CoV-2 in clinical samples.

Keywords: *COVID-19, SARS-CoV-2, in-house multiplex rRT-PCR, NSP14, RdRp*

Introduction

Detection of SARS-CoV-2 using the Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) method is considered the gold standard, as it is a sensitive and specific quantitative test capable of detecting the virus in the early stages of infection [1–3]. Additionally, rRT-PCR is more sensitive than conventional RT-PCR tests, as it accurately determines virus titers with a high level of sensitivity [4]. The World Health Organization (WHO) has issued protocols for rRT-PCR testing to confirm COVID-19, targeting two fragments of the RdRp gene (IP2 and IP4) developed by the National Reference Center for Respiratory Viruses, Institute Pasteur, Paris, and the E gene (E-Sarbeco) [5]. The Korea Centers for Disease Control & Prevention (KCDC) also uses the RdRp gene as a detection target for COVID-19 confirmation testing [6]. In addition to the RdRp gene, the US Centers for Disease Control and Prevention (CDC) uses the N gene as a detection target for COVID-19 confirmation testing [7].

Establishing a diagnosis of COVID-19 is essential for early treatment and breaking the transmission chain [8,9]. It caused the demand for RT-PCR tests to be very high, especially in the early days of the pandemic. Furthermore,

several researchers have developed other diagnostic tools for simpler and faster testing, such as antigen-based rapid tests, a portable surface plasmon resonance (SPR) sensor, or an electrochemical [10–12]. While this test is less sensitive than the rRT-PCR, the test can be performed at point-of-care, which is very useful for the surveillance and early detection of COVID-19 [13]. However, PCR is still necessary because of its high sensitivity and as a gold standard in developing other diagnostic tools. In addition, PCR technology continues to develop; for example, RNA samples from patients can be extracted using automatic machines with high throughput, simplifying the RNA extraction process using a heat-based without the centrifugation step or using lysate directly in a simulated nasal matrix to remove the extracted RNA, so that the PCR technique that was initially complex and time-consuming becomes simpler and lower cost [14–16]. However, during the pandemic, demand for PCR equipment was very high, and only a few countries were producers. As a result, hospitals and laboratories often need help meeting the need for PCR reagents, especially in non-manufacturing countries. Therefore, producing your PCR kit in-house is one way to overcome limitations and does not require high costs.

This study aims to develop a low-cost, in-house multiplex rRT-PCR kit. We design different primer and probe sequences from the existing ones. We used the RdRp target gene because, based on our experience testing COVID-19 samples with rRT-PCR, the RdRp gene is easy to amplify. We chose the NSP14 gene for the second target, noting its absence in commercial kits. The NSP14 gene exhibits stability across SARS-CoV-2 variants and is critical in viral replication and proofreading, rendering it less susceptible to mutations than structural genes [17,18]. NSP14 encodes a bifunctional enzyme important for maintaining the accuracy of viral genome replication, a function that places strong evolutionary pressure to conserve its sequence. Previous studies have successfully employed NSP14 in tests for clinical and environmental samples, demonstrating its reliability across diverse conditions [19,20]. By focusing on conserved regions such as NSP14, our approach aims to minimize the risk of diagnostic failure, even as new variants continue to emerge.

Developing an in-house kit can be more cost-effective than purchasing commercial kits, especially for high-volume testing. It helps avoid problems if the supply chain is disrupted so that testing can continue during outbreaks. An in-house kit can be optimized for specific requirements, such as various sample types, local variants, or and experimental conditions. It allows adjustments to enhance the detection of emerging SARS-CoV-2 variants, which may not be promptly addressed in fixed commercial assays. Consequently, this in-house multiplex rRT-PCR kit will improve the efficiency and outcome of detection compared to commercial alternatives.

Experimental

Equipment

The KingFisher™ Flex Purification System was used to isolate RNA. Equipment for real-time PCR (AriaMx Real-Time PCR System), electrophoresis gel agarose (Biorad), isofreeze PCR rack 96-well system, micropipette (Thermo Scientific), microplate spectrophotometer (Thermo ScientificTM Multiskan Sky), microcentrifuge, optical PCR 8-cap strip (GenFollower), PCR plate 96-well plate (GenFollower), PCR tube stripe (GenFollower), microtube rack (Biologix), sterile microtube 1.5 mL, and tips for micropipettes.

Material

RNA samples from COVID-19 patients were obtained from the C.29 laboratory (National Reference Laboratory, Covid-19 Testing No. 29), Parasitology Laboratory, Advanced Biomedical Laboratory, Faculty of Medicine, Universitas Padjadjaran, Indonesia. All samples utilized in this study were confirmed as either positive or negative using the Sansure Biotech Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing).

SensiFAST SYBR No-ROX One-Step kit, SensiFAST Probe No-ROX One-Step kit and MyTaqTM One-Step RT-PCR kit were purchased from Bioline, Meridian Bioscience,

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agarose (Sigma-Aldrich), TE buffer (Tris-Cl 1 M pH 7.4, EDTA 0.5 M pH 8.0), TAE buffer (Tris base, glacial acetic acid, EDTA 0.5 M pH 8.0), loading dye buffer, DNA marker 100 bp (Thermo Fisher), and nuclease-free water (Thermo Fisher). Integrated DNA Technologies (IDT) synthesized the designed primers and probes. All RNA sequences of COVID-19 patients were obtained from the Global Initiative on Sharing All Influenza Data (GISAID).

Methods

Primer and Probe Design

A total of 98 RNA sequences from Indonesian COVID-19 patients obtained from GISAID were cleaved based on the target gene and aligned with the Wuhan reference genome (Acc: NC_045512) obtained from the National Center for Biotechnology Information (NCBI/https://www.ncbi.nlm.nih.gov/) using the SeqMan program (Lasergene). Sequences that were conserved and did not contain mutations were used as templates for designing primers and probes using the Primer3plus program (https://www.primer3plus.com/). The primer design parameters were set with PCR product sizes of 100-200 bp, melting temperature (Tm) between 55ºC - 60ºC, and a maximum 3ºC difference between the two primers. The primers and probe were analyzed for secondary structures, such as hairpin, homo-dimer, and hetero-dimer, using the OligoAnalyzer on the IDT website (https://www.idtdna.com/pages/tools/oligoanalyzer) and Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer Testing Using MyTaq™ One-Step RT-PCR Kit

The reaction mixture consists of 12.5 µL 2X MyTaq one-step Mix; 0.25 µL reverse transcriptase, 0.5 µL RiboSafe RNase inhibitor; each forward and reverse primer (10 µM) 1 µL; 2.5 µL RNA template (10 ng/µL), DEPC-treated water to reach a total volume of 25 µL. The PCR cycle conditions are: temperature at 45°C for 20 minutes (reverse transcription), initial denaturation at 95°C for 1 minute followed by 40 cycles; 95°C for 10 seconds (denaturation), 60°C for 10 seconds (annealing), and 72°C for 30 seconds (extension). PCR products were characterized using 2% agarose gel electrophoresis in 1x TAE buffer, utilizing the mini-sub cell GT system (Biorad) at 80 V for 45 minutes. The electrophoresis results were visualized under UV light.

Optimization of Probe Concentration with Singleplex rRT-PCR

Each probe was prepared at 1, 1.5, 2, 2.5, and 3 µM concentrations. The reaction mixture consists of 10 µL 2X SensiFAST Probe No-ROX One-step mix; 0.2 µL reverse transcriptase, 0.4 µL Ribosafe RNase inhibitor; 1 µL each of forward primer, 1 µL probe, 2.5 µL RNA template (10 ng/µL), and DEPC-treated water to reach a total volume of 25 µL. The experiment was conducted in triplicate for each probe concentration. The thermal cycle was set as follows: 45ºC for 10 minutes (reverse transcription), 95ºC for 2 minutes (denaturation), followed by 40 cycles at 95ºC for 5 seconds (denaturation), and 60ºC for 20 seconds (annealing and elongation) using the AriaMx Real-Time PCR System.

Multiplex rRT-PCR assay

Multiplex rRT-PCR was conducted by combining all reaction components as described above, including the probes (NSP14, RdRp, and RPP30 at optimum concentrations) and 2.5 µL RNA template (10 ng/µL) to achieve a total reaction volume of 25 µL. The experiment was done in triplicate using the same cycles as the singleplex rRT-PCR.

Determination of LoD, LoQ, and PCR Efficiency

The concentration of RNA samples was determined using nucleic acid quantification on the Multiscan Sky Microplate Spectrophotometer (Thermo Scientific) ELISA reader. The concentration of RNA templates used in the reaction mixture were at 50, 10, and 1 ng/µL concentrations. Each sample was replicated three times and subjected to singleplex rRT-PCR. Subsequently, standard curves were plotted to determine PCR efficiency, and the limit of detection (LoD) and

the limit of quantification (LoQ) were calculated by using regression analysis. The limit of detection (LoD) was determined based on the equation LoD = (3.3 x δ) / S, and the limit of quantification LoQ = (10 x δ) / S [6]. Here, S represents the slope value of the standard curve, and δ denotes the standard deviation. The slope value was obtained from the linear regression equation y = bx + a. PCR efficiency was determined using the standard curve method with the equation: E = 100 x [10 (− 1/ S)] − 1. Efficiency values between 90% and 110% are considered optimal [21,22].

Ethical Approval

The patient's samples are obtained from the Parasitology laboratory-Advanced Biomedical Laboratory of Universitas Padjadjaran, with no association to identifiable individual patients, and with approval from the Research Ethics Commission of Padjadjaran University, Bandung (Number: 139/UN6.KEP/EC/2022).

Clinical Sample Testing with Multiplex rRT-PCR

According to the protocol, the rRT-PCR reaction mixture containing the designed primers and probes at optimized concentrations was tested on clinical samples and one negative control under PCR cycle conditions.

Multiplex rRT-PCR Kit Stability Test

A master mix for multiplex PCR was prepared for 20x reaction and divided into two tubes. The first tube was immediately tested on seven clinical samples and one negative control, while the second tube was stored in a -20°C freezer for seven days. On the seventh day, testing was conducted on the same seven clinical samples and one negative control.

Results and Disscusion

Primer and Probe Design

The most crucial step in primer and probe design is the selection of the nucleotide sequence of the target gene [23]. The template sequence for primer and probe design is mutation-free and conserved. The designed primers, their parameters, and the probes can be observed in Table 1. The nucleotides of the designed primers and probes range between 15 to 30 nucleotides, with a GC content of 45-55% and no more than two C or G bases at the fifth position from the 3′ end of the primer [24]. Additionally, the primer pairs do not contain CG dinucleotides to avoid the formation of highly stable secondary structures. The designed primers also contain G or C nucleotide bases at the 3′ end to enhance primer-template binding strength. In contrast, the probes do not contain G bases at the 5′ end to prevent continuous quenching that may reduce fluorescence values [21].

The primer and probe sequences were validated by aligning them with the reference genomes of Wuhan, Indonesian SARS-CoV-2, and closely related SARS-CoV-1 sequences. Alignment was performed using the Multiple Sequence Alignment NCBI tool (Figure 1).

Table 1. Primers and probes were used in this study.

Primer	Sequence (5'-3')	%GC	Length	Tm (˚C)	Product length	Fluorescent
NSP14_f	TGCCTGTTGGCATCATTCTA	45.0	20 bp	60.2	197 bp	Cy5
NSP14_P	CCATGGTAATGCACATGTAGCT	45.5	22 bp	59.9		
NSP14_r	CACGCTTAACAAAGCACTCG	50.0	20 bp	59.7		
RdRp_f	GGCCTCACTTGTTCTTGCTC	55.0	20 bp	55.0	112 bp	HEX
RdRp_P	CAACGTGTTGTAGCTTGTCACA	45.5	22 bp	45.5		
RdRp_r	GCCACACATGACCATTTCAC	50.0	20 bp	59.8		
RPP30_f	CAATTTCCAGTGCCCTCAAT	45.0	20 bp	59.9	116 bp	FAM
RPP30_P	TAGTGCTGCAGAAAGGCCTT	50.0	20 bp	60.2		
RPP30_r	GCCTAGATTTGCCACGTCAT	50.0	20 bp	60.1		

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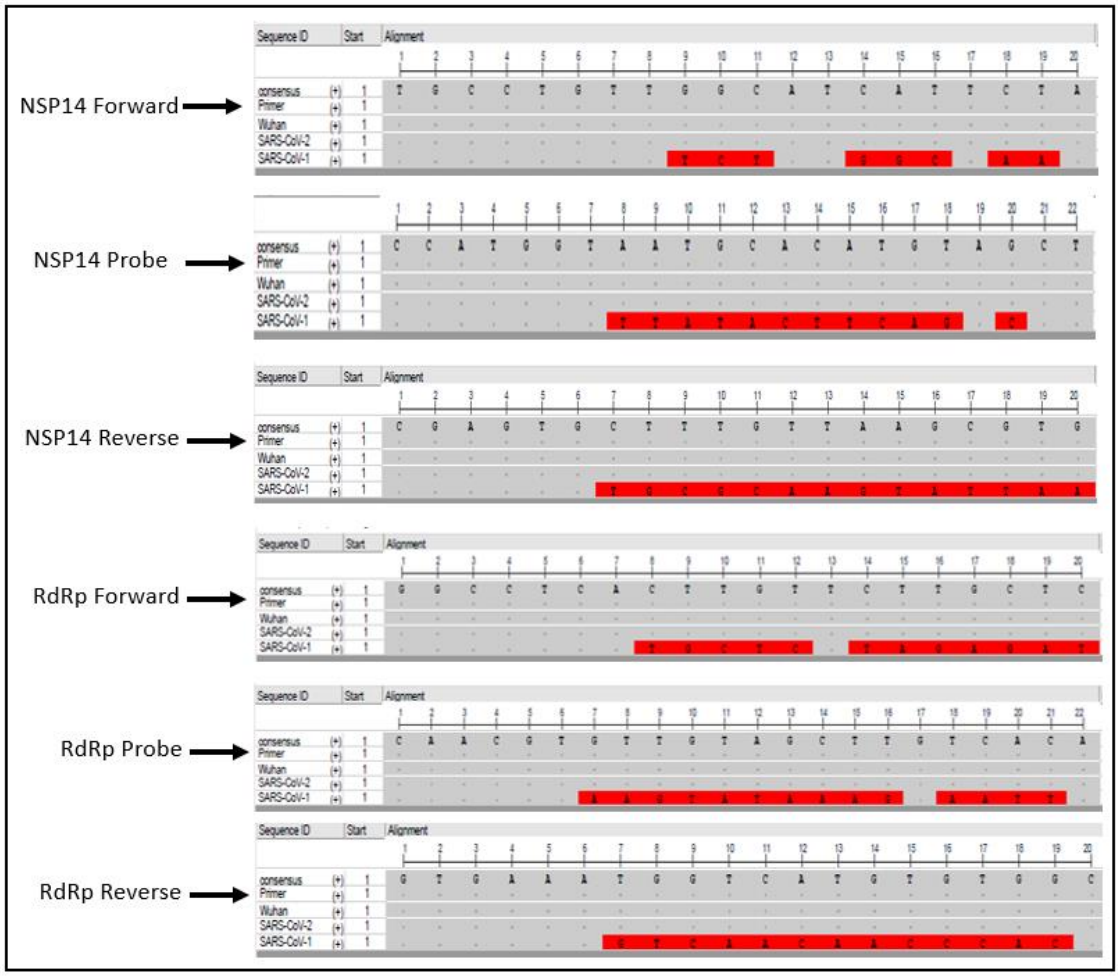


Figure 1. Alignment of primers and probes for NSP14 and RdRp targets against the Wuhan, Indonesian SARS-CoV2, and SARS-CoV1 consensus sequences.

The melting temperature (Tm) of primers and probes ranged between 55-60˚C, with a difference in Tm between the primers not exceeding 5˚C. The Tm value represents the temperature required for primer dissociation/binding release. A difference in Tm exceeding 5˚C should be avoided as it might hinder the amplification process or even prevent amplification [23,25].

The length of the PCR product should ideally be less than 150 bp to enhance PCR efficiency. Shorter PCR products have a reduced likelihood of forming secondary structures. This is because primers and probes are more likely to denature during the extension process at temperatures of 92-95˚C, allowing them to bind more effectively to the complementary target gene sequence [23]. Among the three primer-probe pairs for the target gene, only the NSP14 gene resulted in an amplification product longer than 150 bp. However, this did not increase the formation of secondary structures based on the analysis obtained via OligoAnalyzer.

The fluorescent dyes used in this study are FAM, HEX, and Cy5. Probes must be labeled with reporter dyes with different fluorescence spectra and minimal overlap. The combination of reporter and quencher fluorescent dyes should align with the detection capabilities of the rRT-PCR machine and involve non-fluorescent quenchers [23]. The overlap of the wavelengths of the selected fluorescent dyes is a little small, and the fluorescence intensity used is also suitable for detecting samples in small quantities.

The specificity of the primer probe towards the target gene was analyzed using NCBI BLAST. The BLAST results indicated that the selected primer and probe were specific for SARS-CoV-2 and the human RNase P gene, with a 100% homology. Based on this alignment result, it is estimated that the primer probe can detect the SARS-CoV-2 target gene in both sample and internal control.

The secondary structure analysis of the primer probe was conducted using the Oligoanalyzer program (https://sg.idtdna.com/pages/tools/oligoanalyzer). The free energy value (ΔG) determined the primer secondary structure's stability. Overall, the ΔG values of the designed primer probe were greater than -6.0 kcal/mol. A ΔG value greater than -6.0 kcal/mol is necessary for a primer to disrupt dimer structures. If the ΔG value of the secondary structure is -9 kcal/mol or more negative, it can potentially affect the PCR reaction [23,26].

Characterization of RT-PCR result

The PCR products of the NSP14, RdRp, and RPP30 target genes showed specific bands, respectively sized at 197 bp, 112 bp, and 116 bp (**Figure 2**).

Optimization of Probe Concentration using Singleplex rRT-PCR

The optimum probe concentration for each target gene from the five tested concentration variations based on the highest fluorescence intensity generated in consecutive amplification plots was NSP14 (1 μM), RdRp (1.5 μM), and RPP30 (1.5 μM) (**Figure 3**). The probe concentration with the highest fluorescence intensity exhibited the smallest Ct value among all probe concentration variations. The Ct value is inversely related to the amount of target nucleic acid in the initial sample; the lower the Ct value, the more significant the amount.

Multiplex rRT-PCR.

Multiplex rRT-PCR using primers and probes for the NSP14, RdRp, and RPP30 target genes at their optimum concentrations yielded Ct values for NSP14, RdRp, and RPP30 targets as follows: 27.76, 27.57, and 35.33, respectively (Figure 4). The RNA samples used in this study were isolated from nasopharyngeal swab solutions of COVID-19-positive patients, and their concentrations were predetermined. Amplification using the RPP30 primer-probe (internal control) showed a positive Ct, where the RPP30 target functions as the RNase P gene serving as an internal control. The specificity of the multiplex rRT-PCR was observed in the amplification plot, which showed a rise in the negative controls. All negative controls should not yield a Ct value, indicating the specificity of all primer pairs.

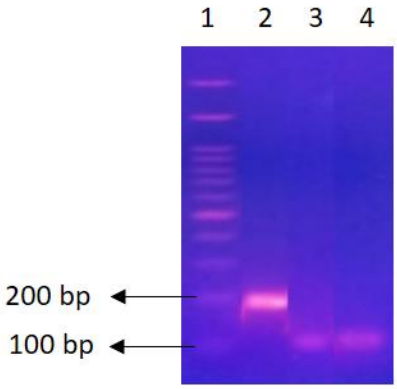
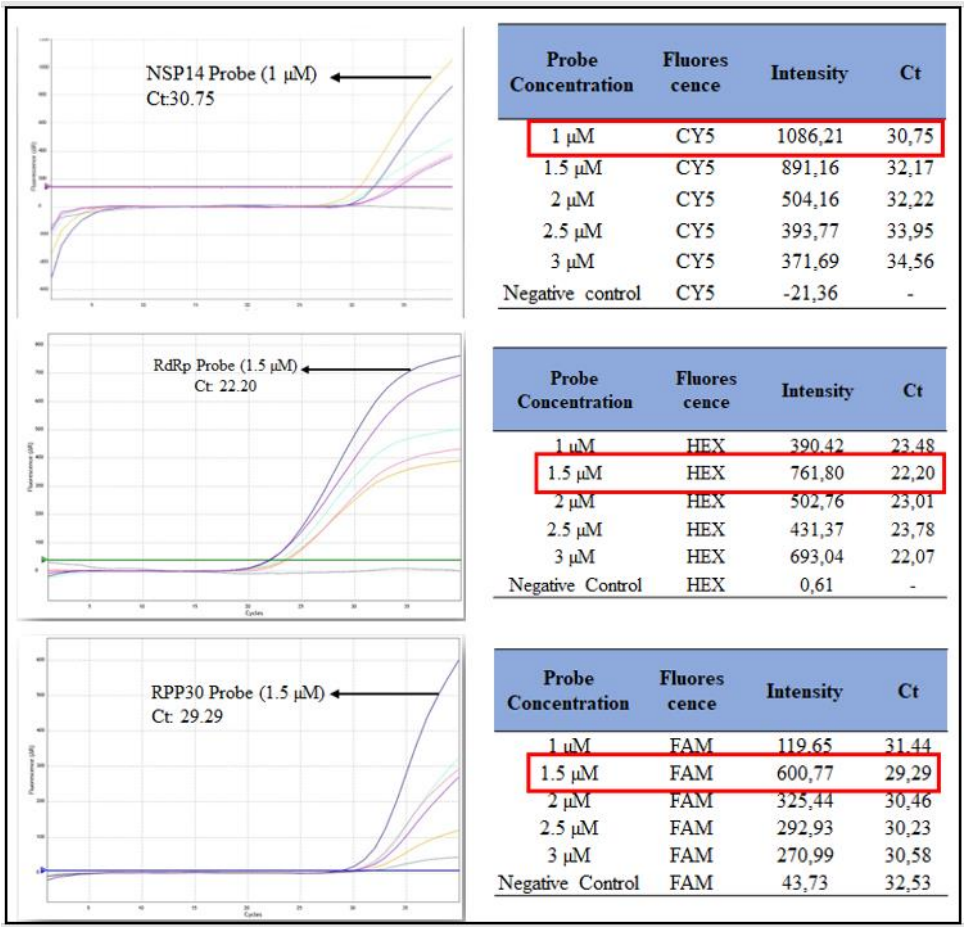


Figure 2. Visualization results of 2% agarose gel electrophoresis. (1) NSP14 primer amplicon (197 bp); (2) RdRp primer amplicon (112 bp); and (3) RPP30 primer amplicon (116 bp).

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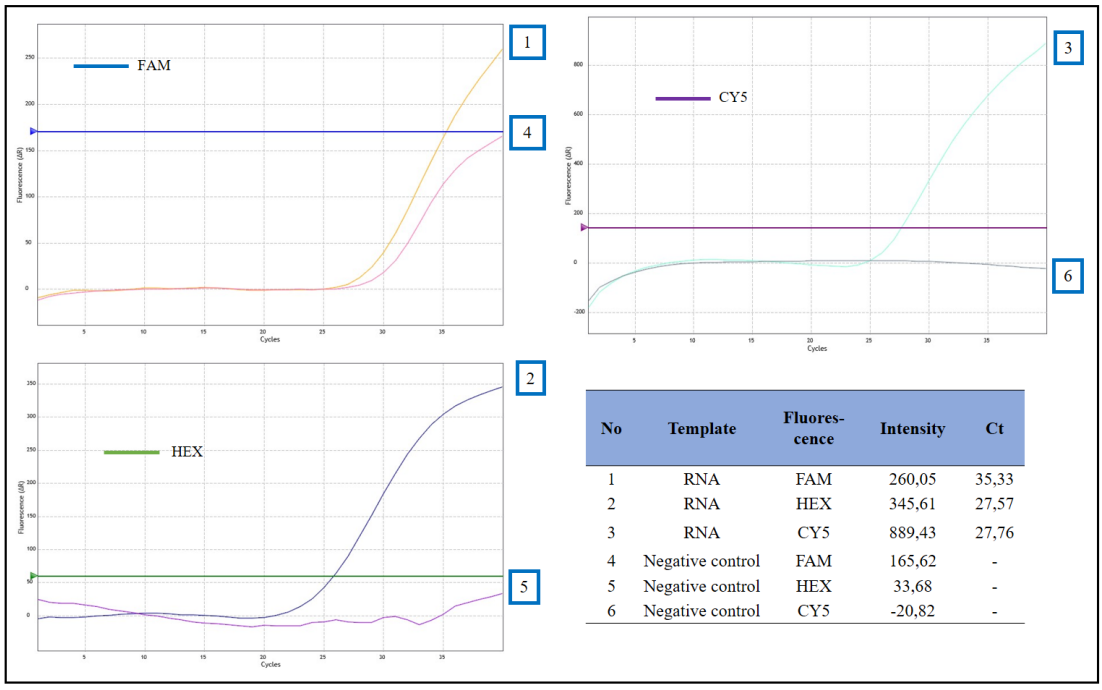


Probe Concentration	Fluorescence	Intensity	Ct
1 µM	CY5	1086,21	30,75
1.5 µM	CY5	891,16	32,17
2 µM	CY5	504,16	32,22
2.5 µM	CY5	393,77	33,95
3 µM	CY5	371,69	34,56
Negative control	CY5	-21,36	-

Probe Concentration	Fluorescence	Intensity	Ct
1 µM	HEX	390,42	23,48
1.5 µM	HEX	761,80	22,20
2 µM	HEX	502,76	23,01
2.5 µM	HEX	431,37	23,78
3 µM	HEX	693,04	22,07
Negative Control	HEX	0,61	-

Probe Concentration	Fluorescence	Intensity	Ct
1 µM	FAM	119,65	31,44
1.5 µM	FAM	600,77	29,29
2 µM	FAM	325,44	30,46
2.5 µM	FAM	292,93	30,23
3 µM	FAM	270,99	30,58
Negative Control	FAM	43,73	32,53

Figure 3. Amplification plot optimizing probe concentration with singleplex rRT-PCR..



No	Template	Fluorescence	Intensity	Ct
1	RNA	FAM	260,05	35,33
2	RNA	HEX	345,61	27,57
3	RNA	CY5	889,43	27,76
4	Negative control	FAM	165,62	-
5	Negative control	HEX	33,68	-
6	Negative control	CY5	-20,82	-

Figure 4. Multiplex rRT-PCR plot using optimized probe concentration

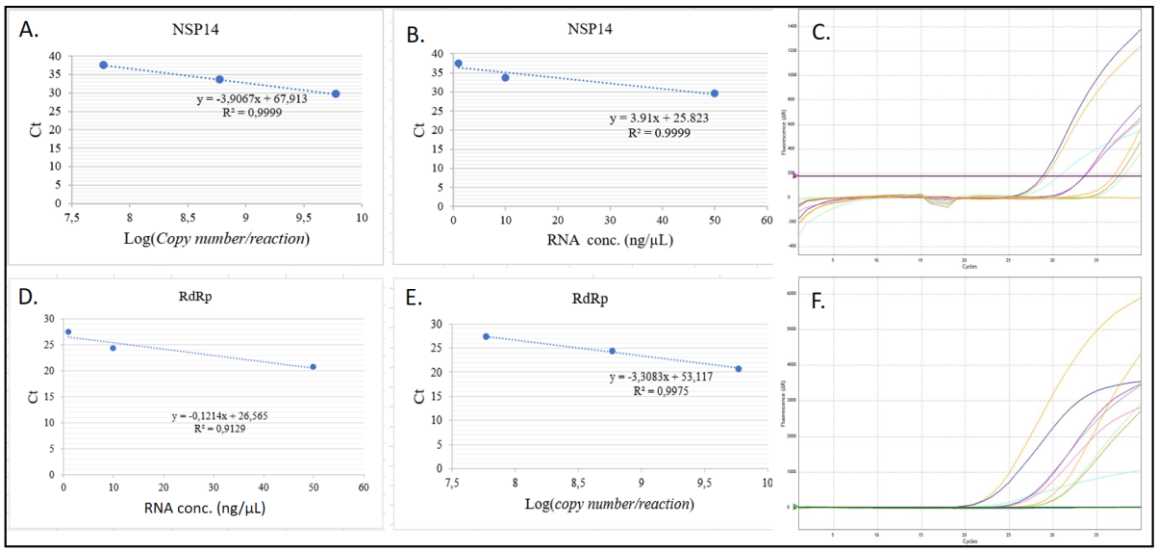


Figure 5. Standard curve and amplification plot for LoD and LoQ determination. NSP14 target genes (A, B, C); RdRp target genes (D, E, F).

Determination of LoD, LoQ, and PCR Efficiency.

LoD and LoQ were obtained from the RNA concentration vs. Ct value plot on the standard curve, while the PCR efficiency value was obtained from the Log (copy number/reaction) vs. Ct value plot on the standard curve. Figure 5 shows the standard curve for LoD and LoQ calculations. We could only test three concentrations due to sample limitations, namely 1, 10, and 50 ng/µL, yet the obtained curves were sufficiently robust with an R-value of 0.99. The calculation results indicated that the LoD and LoQ for the NSP14 target gene were 0.22 and 0.67 ng/µL, respectively, and for the RdRp target gene, they were 1.08 and 3.28 ng/µL, respectively. Furthermore, the PCR efficiencies for the NSP14 and RdRp genes were 80.3% and 100.6%, respectively.

The PCR efficiency should ideally range between 90-110% with a slope value between -3.6 and -3.3. An efficiency of 100% or E=1 signifies that each sample dilution will have a Ct value difference of 3.32, indicating that the amount of newly formed DNA amplicon doubles with each cycle [23]. The PCR efficiency value for the RPP30 gene is also below 110% (data not shown). PCR efficiencies above 110% can be caused by several factors, including contamination during sample preparation or extraction stages, or the presence of polymerase enzyme activators, causing the PCR efficiency to exceed 100%. Meanwhile, PCR efficiency below 100% may result from suboptimal reagent concentrations and PCR conditions; Ct value differences below 3.3 between repetitions of each sample concentration indicate contamination, and significant differences in Ct values between repetitions can also affect the slope value of the standard curve, leading to lower PCR efficiency [27,28].

We also analyzed the data with a Whisker plot, as shown in Figure 6, with 3x repetition of the experiment. The whisker plot was used to visually represent the distribution of data obtained from multiple repetitions of the experiment, providing insights into the variability and consistency of the results. Each whisker plot likely represents the Ct values obtained for each target gene (NSP14, RdRp, and RPP30) across multiple repetitions of the experiment, helping to assess the robustness and reproducibility of the assay.

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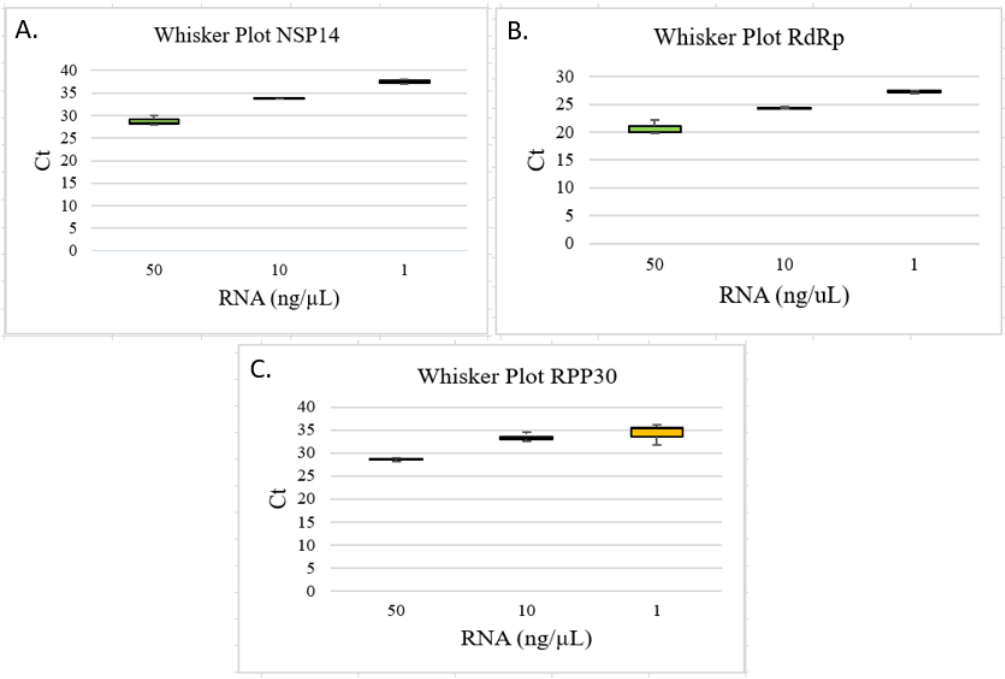


Figure 6. Whisker Plot A. NSP14, B. RdRp, C. RPP30

Table 2. Results of multiplex rRT-PCR testing with clinical samples

Samples		Ct-RPP30	Ct-RdRp	Ct-NSP14
Patient	Positive 1	28.87	23.64	32.23
	Positive 2	29.24	24.15	34.08
	Positive 3	30.96	21.83	30.76
	Positive 4	29.78	23.61	31.10
	Positive 5	29.40	22.88	30.22
	Positive 6	30.73	27.94	33.05
	Positive 7	31.50	24.66	31.36
	Negative 1	31.98	-	-
	Negative 2	31.83	-	-
	Negative 3	31.36	-	-

Multiplex rRT-PCR Testing on Clinical Samples

We tested this multiplex PCR kit against ten clinical samples, with seven accurate positives and three actual negative samples. The results show that for the positive samples, the Ct values for the NSP14 gene ranged from 30-34, the RdRp gene ranged from 21-27, and the RPP30 gene ranged from 28-31 (Table 2). Using this kit, the RdRp gene target provides better Ct values than the NSP14 gene target, aligning with the gene

targets recommended by the WHO. The difference in Ct value between one target and another can be caused by the secondary structure of the RNA and the potential reasons for Ct range variation among the samples depends on viral load, sample quality, sample type. Importantly, all samples analyzed in this study had been pre-confirmed as positive or negative by a commercial PCR kit, and our in-house assay consistently reproduced these results, validating its diagnostic concordance.

Stability test of Multiplex rRT-PCR Kit

We tested the storage stability of this in-house qPCR kit with ten additional samples, consisting of 5 negative samples, two positive samples, and one control. The results indicate that this multiplex rRT-PCR kit remains stable for up to one month of storage. The Ct values between the kit without storage and the kit stored for one month at -20°C show almost no difference (Table 3). The two negative samples using the RdRp primer probe had Ct values of 37-37.92. These Ct values are relatively high, likely due to background signals since Ct values exceed 35 and result in non-sigmoidal amplification plots. Thus, these negative samples are still categorized as unfavorable. These results suggest that this kit can be used due to its good stability after one month of storage at -20°C. However, further stability testing might be needed, such as extending the storage duration or varying thawing frequencies. However, we cannot establish the cut-off value for this kit as the sample size used was limited.

In 2023, the WHO announced COVID-19 as an endemic disease, indicating the continued need to develop rRT-PCR kits for COVID-19 detection. Indonesia, with a population exceeding 200 million people, holds the potential for a higher number of cases, hence the continued necessity for the development of in-house rRT-PCR kits for production and local use. We hope that this kit will be beneficial.

The study presents the development of an in-house multiplex rRT-PCR kit for detecting SARS-CoV-2, aiming to contribute to COVID-19 diagnostics. The kit demonstrated satisfactory results through meticulous primer and probe design, optimization of probe concentrations, and extensive clinical sample testing. The specificity and sensitivity of the kit were confirmed, with optimal probe concentrations identified for each target gene. Moreover, stability testing indicated that the kit remains viable after one month of storage at -20°C. These findings suggest that the developed kit could be a reliable and cost-effective diagnostic tool, particularly in regions facing challenges accessing commercial PCR kits. It also highlights the importance of continued efforts in developing diagnostic tools to combat the COVID-19 pandemic, particularly in countries with high population densities like Indonesia.

Table 3. Stability Test Results of the Multiplex rRT-PCR Kit on Clinical Samples

Samples		Cy5		HEX		FAM	
		H-0	H+7	H-0	H+7	H-0	H+7
Patient	Positive 1	24.46	24.83	20.47	20.11	29.32	28.56
	Positive 2	28.03	28.85	24.13	24.91	29.32	29.54
	Positive 3	30.96	29.88	25.54	25.42	30.97	29.60
	Positive 4	28.88	29.16	25.31	25.15	29.73	29.93
	Positive 5	27.68	28.85	24.76	25.19	28.51	29.10
	Negative 1	-	-	37.02	37.00	29.22	29.45
	Negative 2	-	-	37.92	-	29.81	30.04

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Negative control	-	-	-	-	35.67	37.78

Our in-house PCR kit targets NSP14, RdRp, and RPP30 genes, which differ from several commercial kits. Although we have not yet directly evaluated the kit's performance against specific variants of concern (VOCs), we selected NSP14 as one of our target genes based on its highly conserved nature across SARS-CoV-2 isolates. Previous studies have shown that NSP14 encodes a bifunctional enzyme with 3′-to-5′ exoribonuclease (ExoN) and guanine-N7 methyltransferase (N7-MTase) activities, playing a crucial role in viral replication fidelity and RNA proofreading [17,18]. Because of its essential role in maintaining replication accuracy, NSP14 is relatively conserved and less prone to mutations compared to other viral structural proteins (such as Spike or Nucleocapsid), even across emerging variants. Moreover, previous reported the successfully developed and utilized RT-qPCR assays targeting the NSP14 region for SARS-CoV-2 detection in nasopharyngeal swabs and complex matrices like wastewater, highlighting its stability and reliability as a diagnostic target[19,20]. Given that mutations in the ExoN domain of NSP14 significantly impair coronaviruses' replication fidelity and viability, the evolutionary pressure maintains NSP14 sequence conservation, minimizing the risk of diagnostic failure due to new variant emergence[29]. Thus, although we have not yet validated our kit against specific variants, the biological characteristics of NSP14 strongly support its robustness as a target, even in the presence of SARS-CoV-2 genetic evolution. Future research should include validation against VOCs.

Conclusions

The developed in-house multiplex rRT-PCR kit can be used for detecting SARS-CoV-2, with the limit of detection and quantification for each target gene being 0.22 and 0.67 ng/μL for the NSP14 gene, and 1.08 and 3.28 ng/μL for the RdRp gene. Testing on clinical samples showed that this kit is usable and remains stable for up to one month of storage. This in-house multiplex rRT-PCR kit is expected to enhance both the efficiency and accuracy of detection compared to commercially available options.

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Declaration of Generative AI and AI-Assisted Technologies

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

Author Contributions

S.G.: Conceptualization, supervision, and corresponding author responsibilities. R.N.P.: Methodology, investigation, and data collection. H.S.: Formal analysis and data curation. I.Y.S.: Methodology and validation. H.L.W.: Visualization and writing —original draft preparation. S.E.: Data collection and formal analysis. S.I.: Validation and writing —review and editing. Y.W.H.: Formal analysis and data curation. L.F.: Visualization and writing — review and editing.

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Declaration of Conflicting Interests

The authors declare no conflict of interest.

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