Chimica et Natura Acta
p-ISSN: 2355-0864 e-ISSN: 2541-2574
Homepage: http://jurnal.unpad.ac.id/jcena

New Custom Primers for the Detection of SARS-CoV-2 using the Singleplex rRT–PCR SYBR Green-Based Method with the NSP10 and N genes as Targets

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DOI: https://doi.org/10.24198/cna.v13.n1.53493

Abstract: Although COVID-19 is no longer a global health emergency, rapid, sensitive, and specific detection tests are still needed. In this study, we developed a cost-effective test, the SYBR Green-based rRT–PCR kit, using new custom primers targeting the N and NSP10 genes of the SARS-CoV-2 virus. The specificity of the designed primers was determined through agarose gel electrophoresis. A standard curve generated from a ten-fold dilution of SARS-CoV-2 RNA was used to determine the efficiency and sensitivity of the kit. Validation of this protocol was carried out on ten clinical specimens. As expected, the results showed that the N and NSP10 gene primers produced 134 and 161 bp products, respectively. The limits of detection and limit of quantification with N gene primers were 7.74 and 23.46 copies/µL, respectively, and those with the NSP10 gene primers were 4.69 and 14.21 copies/µL, with a PCR efficiency of 102.5% and 110.6%, respectively. The validation results with clinical specimens revealed that seven samples were true-positive for COVID-19 (Ct range 15.09–21.33), and three were confirmed to be true-negative. Costs associated with COVID-19 patient testing can be anticipated to decrease with the use of custom primers for the detection of SARS-CoV-2 via the use of the singleplex rRT–PCR mix SYBR Green.

Keywords: custom primers, singleplex *r*RT–PCR kit, SARS-CoV-2, SYBR Green.

***Abstrak*:** *Meskipun COVID-19 bukan lagi darurat kesehatan global, tes deteksi yang cepat, sensitif, dan spesifik tetap diperlukan. Pada penelitian ini, kami mengembangkan tes yang hemat biaya, yaitu kit rRT–PCR berbasis SYBR Green, dengan menggunakan primer khusus baru yang menargetkan gen N dan NSP10 dari virus SARS-CoV-2. Spesifisitas primer yang dirancang ditentukan melalui elektroforesis gel agarosa. Kurva standar yang dihasilkan dari pengenceran sepuluh kali lipat RNA SARS-CoV-2 digunakan untuk menentukan efisiensi dan sensitivitas kit. Validasi protokol ini dilakukan terhadap sepuluh spesimen klinis. Sesuai dengan yang diharapkan, hasil penelitian menunjukkan bahwa primer gen N dan NSP10 menghasilkan produk masing-masing sebesar 134 dan 161 bp. Batas deteksi dan batas kuantifikasi dengan primer gen N berturut-turut adalah 7,74 dan 23,46 salinan/µL, dan primer gen NSP10 adalah 4,69 dan 14,21 salinan/µL, dengan efisiensi PCR masing-masing sebesar 102,5% dan 110,6%. Hasil validasi dengan spesimen klinis menunjukkan bahwa tujuh sampel benar-benar positif COVID-19 (kisaran Ct 15,09–21,33), dan tiga sampel dipastikan negatif nyata. Biaya yang terkait dengan pengujian pasien COVID-19 diperkirakan akan berkurang dengan penggunaan primer khusus untuk mendeteksi SARS-CoV-2 melalui penggunaan singleplex rRT–PCR SYBR Green.*

Kata kunci: *primer baru, kit singleplex rRT–PCR, SARS-CoV-2, SYBR Green.*

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, which emerged from December 2019 to early 2023, has infected more than 623 million people and caused the deaths of 16.6 million people worldwide. The significant decrease in cases caused the COVID-19 pandemic to become endemic, which means that COVID-19 still existed, but the number of cases decreased. This means that the development of a COVID-19 detection method is still needed. The real-time reverse transcription-polymerase chain reaction (rRT-PCR) detection method is still the "gold standard" for accurately detecting SARS-CoV-2. Although the antigen rapid test method yields good

results, it cannot accurately determine the patient's virus titer in real-time (Lu *et al*. 2020; Udugama *et al*. 2020).

rRT–PCR is a method for detecting RNA targets that consists of two steps: reverse transcription, in which RNA is converted to cDNA; and cDNA amplification. The rRT–PCR method requires a pair of primers that bind to specific oligonucleotides that target the SARS-CoV-2 gene and fluorescent dyes that detect amplification in real-time (Mo *et al*. 2012). The SARS-CoV-2 rRT-PCR detection kits are generally multiplexed and use two or more pairs of primers to amplify DNA fragments simultaneously.

The target genes for the detection of SARS-CoV-2 are generally conserved genes in SARS-CoV-2, such as the nucleocapsid (N), envelope (E), RNA-dependent RNA polymerase (RdRp), ORF1a, and ORF1b genes (Afzal 2020; Brown *et al*. 2021; Mathuria *et al*. 2020; Tombuloglu *et al*. 2022). The protocol from the United States CDC targets two regions in the N gene (N1 and N2), and the human RNase P gene was used as an internal control (Lu *et al*. 2020). In addition, there are several published primers and probe sequences. The WHO recommends primer and probe sequences that target two fragments of the RdRp gene (IP2 and IP4) developed by the National Reference Center for Respiratory Viruses, Institut Pasteur, Paris, and the E gene (E-Sarbeco) developed by Corman *et al*. (2020).

Commercial rRT-PCR kits for detecting SARS-CoV-2 in Indonesia are still available imported from abroad, so the testing capacity of these kits in Indonesia is relatively low compared to that in other countries (Sucahya 2020). rRT–PCR kits generally use fluorescent probes that recognize specific sequences on the PCR amplicon. These fluorescent probes are generally much more expensive than PCR primers.

Several studies have developed a more cost-effective alternative method for detecting SARS-CoV-2, specifically the rRT-PCR test using SYBR Green dye (Dorlass *et al*. 2020; Pearson *et al*. 2021; Pereira-Gómez *et al*. 2021). SYBR Green is a nonspecific dye that costs less than probes (Houghton & Cockerill 2006) because it does not require a specially designed probe for the target gene. However, there is a limitation associated with using SYBR Green. It can interact with all dsDNA formed during PCRs, including nonspecific products and primer dimers (Arya *et al*. 2005). Therefore, a diagnosis can produce false positives if it only depends on the amplification plot. Therefore, it is necessary to carry out a melting curve analysis to determine the specificity of the fluorescence signal (Pereira-Gómez *et al*. 2021).

Pereira-Gómez *et al*. (2021) compared the TaqMan Rt-qPCR and SYBR Green-based rRT–PCR methods. The amplification efficiencies of the ORF1b-nsp14 and N regions for the TaqMan RT–qPCR reference assays were 103.67% and 100.99%, respectively. However, the SYBR Green-based qPCR products had an amplification efficiency of 99.77% for the ORF1b-nsp14 region and 102.56% for the N region. For ORF1b-nsp14 and its N targets, probe-based qPCR had a limit of detection of 10 copies/reaction (2.5 copies/L). The limit of detection for the ORF1b-nsp14 target in the SYBR Green-based assays was 50 copies/reaction (12.5 copies/L), and for the N target, it was 250 copies/reaction (62.5 copies/L). Therefore, the SYBR Green-based test showed lower sensitivity than the probe-based methods. The limits of detection (LODs) for the ORF1b-nsp14 and N target genes determined using the SYBR Green-based method were 12.5 copies/μL and 62.5 copies/μL, respectively (Pereira-Gómez *et al*. 2021).

In this study, to develop a low-cost in-house rRT-PCR kit, we used different primer sequences from the existing ones. We developed a singleplex rRT-PCR kit based on SYBR Green, which can be used as an alternative method for detecting the SARS-CoV-2 virus. Primer specificity was determined by characterization via agarose gel electrophoresis. Then, we measured the efficiency and analytical sensitivity of the kit using a standard curve containing various concentrations of SARS-CoV-2 RNA at tenfold dilution. Finally, we used clinical specimens to evaluate the ability of the SYBR Green-based method to detect seven positive and three negative COVID-19 samples, after that the results were confirmed via RT–PCR with a commercial kit.

MATERIALS AND METHODS

Materials

The materials used for this study were as follows: 2019-nCoV_Positive Control_v2 (MBS-4101, 1st BASE), agarose (Sigma–Aldrich), buffer TE (tris-Cl 1 M pH 7.4, EDTA 0.5 M pH 8.0), buffer TAE (tris base, acetate acid glacial, EDTA 0.5 M pH 8.0), loading dye buffer, 100 bp marker (Thermo Fisher), MyTaq™ One-Step RT–PCR Kit (Cat. No. BIO-65049, Bioline, Meridian Bioscience), nuclease-free water (Thermo Fisher), primer (Integrated DNA Technologies), positive and negative samples of COVID-19, and SensiFAST™ SYBR® No-ROX One-Step Kit (Cat. No. BIO-72001, Bioline, Meridian Bioscience).

Instrument

Real-time PCR equipment (AriaMx Real-Time PCR System), ectrophoresis gel agarose equipment (Biorad), an isofreeze PCR rack 96-well system, a micropipette (Thermo Scientific), a microplate spectrophotometer (Thermo Scientific™ Multiskan Sky), a microcentrifuge, an optical PCR 8-cap strip (GenFollower), a PCR plate 96-well plate (GenFollower), a PCR tube stripe (GenFollower), a microtube rack (Biologix), a sterile microtube 1.5 mL, and tips for micropipettes.

Design of primers

A total of 98 sequences of the whole genome of SARS-CoV-2 from Indonesian patients were retrieved from the Global Initiative on Sharing Avian Influenza Data database (GISAID; https://www.gisaid.org/). The Indonesian SARS-CoV-2 genome sequence was aligned using SeqMan Lasergene software to obtain a consensus sequence of the Indonesian SARS-CoV-2 genome. Furthermore, the nucleocapsid (N) gene sequence, nonstructural protein 10 (NSP10), and RNA-dependent RNA polymerase (RdRp; as positive control primers) were cut from the consensus sequences of the SARS-CoV-2 genome obtained using EditSeq Lasergene software, after which the region of the gene was selected for primer design. The primers used were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The specificity of the primers used in this study was analyzed in silico using nucleotide BLAST (blastn; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers were subsequently analyzed for their secondary structures, such as hairpin, homodimer, and heterodimer structures, by using OligoAnalyzer on the Integrated DNA Technologies website (IDT; https://www.idtdna.com/pages/tools/ oligoanalyzer). The melting curve of each primer was predicted in silico with uMelt Quartz (https://www.dna-utah.org/umelt/quartz/um.php). The predictions were made using the thermodynamic parameters of Blake & Delcourt (1998) with the assumption of a free [Mg^{2+}] of 3.0 mM and a [monovalent ion+] of 20.0 mM (Dwight *et al*. 2011). The designed primers were subsequently ordered to IDT for synthesis.

Determination of primer specificity via the conventional RT–PCR technique

PCRs were performed in a total volume of 50 µL. The mixture consisted of 25 µL of 2X MyTaq One-Step Mix, 2.0 µL of each of the 10 µM forward and reverse primers, 0.5 µL of reverse transcriptase enzyme, 1.0 µL of RiboSafe RNase inhibitor, 5 µL of RNA template and DEPC-$_{H2O}$. The thermal cycling procedure was set as follows: 45°C for 20 minutes (reverse transcription), 95°C for 1 minute (DNA polymerase activation), 40 cycles of 95°C for 10 seconds (denaturation), 60°C for 10 seconds (annealing) and 72°C for 30 seconds (elongation).

Amplicon Characterization Using Agarose Gel Electrophoresis

PCR products were characterized using 2% agarose gel electrophoresis and stained with red gel dye. Then, 3 µL of the amplicon was added to 1 µL of loading dye buffer for a total volume of 4 µL. The marker mixture consisted of 1 µL of 100 bp marker, 1 µL of loading dye buffer, and 4 µL of nuclease-free water; hence, the total volume was 6 µL. Later, the markers and PCR products were put into the wells of the agarose gel. The electrophoresis apparatus was set at 80 V for 45 minutes. The migration of the amplicon was observed through visualization with UV light.

Preparation of Standard Curves for Determination of Primer Efficiency and Sensitivity

The concentration of SARS-CoV-2 RNA was determined using a Multiskan Sky microplate spectrophotometer, and various RNA concentrations were diluted to 10x: 100, 10, 1, 0.1, and 0.01 ng/µL. Each sample was put into a tube containing a master mix of 10 µL of 2X SensiFASTTM SYBR® No-ROX One-Step Mix, a pair of forward primers, and 10 µM reverse primers (0.8 µL each), 0.2 µL of reverse transcriptase enzyme, 0.4 µL of RiboSafe RNase inhibitor, and DEPC-H2O for a total volume of 20 µL. The experiment was carried out in triplicate for each concentration of RNA. The thermal cycling procedure was set as follows: 45°C for 10 minutes (reverse transcription); 95°C for 2 minutes (denaturation); 40 cycles of 95°C for 5 seconds (denaturation) and 60°C for 20 seconds (annealing and elongation); and 65°C to 95°C in increments of 0.5°C for 5 seconds for melting curve analysis. Later, a standard curve for the primer qPCR results of the N and NSP10 genes was generated by plotting the Ct value on the Y axis and the log (copy number/reaction) on the X axis.

PCR Efficiency, Limit of Detection, and Limit of Quantification

The PCR efficiency, limit of detection, and limit of quantification of the kits were determined by the following formulas: %Efficiency = $(10(-1/S) - 1) \times 100$; Limit of detection (LOD) = $(3.3 \times \sigma)/S$; Limit of quantification (LOQ) = $(10 \times \sigma)/S$. Where σ is the standard deviation of the linear regression y-intercept and S is the slope of the standard curve.

Clinical Specimens Assay

Ten COVID-19 clinical specimens were used to validate the SYBR Green-based rRT-PCR method. Briefly, 10 µL of 2X SensiFAST™ SYBR® No-ROX One-Step Mix was added to a pair of forward primers and reverse primers (10 µM, each 0.8 µL), 0.2 µL of reverse transcriptase, 0.4 µL of RiboSafe RNase inhibitor, and DEPC-H_2O to a volume of 16 µL. Then, 4 µL of clinical specimen RNA was added, so the mixture had a total volume of 20 µL. Positive and negative controls were also added to the different tubes.

RESULTS AND DISCUSSION

Primer design

Primer design is the principal procedure, particularly in developing the rRT–PCR method based on SYBR Green. SYBR Green can bind with all dsDNA that may be generated during PCR,

including nonspecific products and primer dimers (Dorlass *et al*. 2020). Therefore, primers must be designed prudently to bind specifically to the targeted gene. In addition, primers are essential components that determine the specificity, sensitivity, and efficiency of PCR (Pereira-Gómez *et al*. 2021).

In the SARS-CoV-2 virus assay, the use of at least two target genes is recommended to avoid the possibility of genetic drift from SARS-CoV-2 (Tombuloglu *et al*. 2022). The targeted genes used in this study were the conserved genes of SARS-CoV-2 (the N and NSP10 genes). A total of 98 Indonesian SARS-CoV-2 genome sequences obtained from the Global Initiative on Sharing Avian Influenza Data database (GISAID; https://www.gisaid.org/) were aligned with SeqMan Lasergene software to obtain the Indonesian SARS-CoV-2 consensus genome sequence. The N, NSP10, and RdRp gene sequences (as positive control primers) were subsequently obtained by cutting the consensus sequences into N, NSP10, and RdRp gene sequences from the Wuhan-Hu-1 sequence (GenBank accession number: NC_045512) available from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) database.

The primers used for the N, NSP10, and RdRp target genes were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The parameters for primer design were set as follows: PCR product length range of 100-200 bp, primer length of 20 bp, Tm of 60ºC with a difference in Tm of a pair of primers (ΔTm) not exceeding 2ºC, % GC of primer range of 40-55%, and minimization of self-complementarity, especially at the 3' end of the primer, to avoid the formation of secondary structures such as primer dimers and hairpins. In addition, primers targeting RNase P or the ribonuclease P subunit P30 (RPP30) were also designed as internal controls. Internal controls are needed to control the quality of clinical specimens, nucleic acid extraction, and PCR amplification (Houghton & Cockerill 2006). The primer design results used in this study are shown in Table 1.

The prediction of the melting curve for each primer was carried out using uMelt Quartz (https://www.dna-utah.org/umelt/quartz/um.php) to determine the theoretical Tm product for each primer. The melting curves were obtained by increasing the temperature after the last PCR cycle; the fluorescence decreased as the temperature increased. An increase in temperature results in the denaturation of dsDNA such that SYBR Green is no longer bound to dsDNA, which is reflected by a decrease in fluorescence. To more efficiently observe the analyzed Tm product, a negative derivative of the melting curve (-dF/dT) is usually used until a melting peak is produced, indicating the analyzed Tm product (Arya *et al*. 2005; Dwight *et al*. 2011; Klymus *et al*. 2019). The designed primers for the N, NSP10, RdRp2, and RPP30 genes are predicted to contain Tm products of 85°C, 80.5°C, 80°C, and 79°C, respectively.

In silico analysis of the specificity of the primer was performed using BLAST (blastn; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the BLASTN results, primers were predicted to be able to specifically detect SARS-CoV-2. Further analysis was then carried out by conducting an alignment between the design primers and the consensus sequences for SARS-CoV-2 Indonesia, SARS-CoV-2 Wuhan-Hu-1 (GenBank accession number: NC_045512.2), and SARS-CoV (GenBank accession number: NC_004718.3) using the MSA Viewer on

Table 1. The sequence of designed primers and their characteristics.

Target gene	Primer Name	Sequences (5'-3')	Primer length (bp)	Tm (ºC)	%GC	Product length (bp)
N	GnNF	TTTGGTGGACCCTCAGATTC	20	59.9	50%	134
	GnNR	TTGCCATGTTGAGTGAGAGC	20	60.0	50%	
NSP10	NSP10F	CAGTTACACCGGAAGCCAAT	20	60.0	50%	161
	NSP10R	ACCCACAGGGTCATTAGCAC	20	59.9	55%	
RdRp2	RdRp2For	CATGTGTGGCGGTTCACTAT	20	59.4	50%	104
	RdRp2Rev	CCGTGACAGCTTGACAAATG	20	60.3	50%	
RPP30	RPP30For	CAATTTCCAGTGCCCTCAAT	20	59.9	45%	116
	RPP30Rev	GCCTAGATTTGCCACGTCAT	20	60.1	50%	

(https://www.ncbi.nlm.nih.gov/projects/msaviewer/) (Figure 1).

The alignment results showed that the N, NSP10, and RdRp2 gene primers were not complementary to the SARS-CoV-2 gene primers (Figure 1(a), (b), and (c)). Concerning the RdRp2 forward primer, there is a possibility of incompatibility with the Indonesian SARS-CoV-2 sequence, as indicated by the presence of R or purine bases (A or G) in the Indonesian SARS-CoV-2 consensus sequence (Figure 1 (C)). This mutation was due to a G-to-A mutation at position 15451 (based on the Wuhan-Hu-1 sequence; GenBank accession number: NC_045512.2) in the RdRp gene region of the delta variant (B.1.617.2). Thus, the use of the RdRp2 primer for diagnosis may produce inaccurate results, so the RdRp2 primer was used only as a primer for the positive control.

Furthermore, the secondary structure of the primers was analyzed using OligoAnalyzer, which is available on the Integrated DNA Technologies website (IDT; https://www.idtdna.com/pages/tools/oligoanalyzer), by creating predictions through the value of ΔG. ΔG, or Gibbs free energy, is the energy required to break the secondary structure and determine the stability of the DNA molecule. A more negative ΔG value indicates a more stable secondary structure (Bustin & Nolan 2020). Hence, a secondary structure with a very negative ΔG value must be avoided because primer attachment to the template is disrupted during the annealing phase.

We analyzed the formation of secondary structures such as hairpins, homodimers, and heterodimers. A hairpin structure the primer, so folding occurs within the primer itself. Intermolecular interactions can form homodimer or self-dimer structures due to complementarity between two of the same primer sequences (forward primers with forward primers or reverse primers with reverse primers). Moreover, heterodimer or cross-dimer structures are formed by intermolecular interactions due to complementarity between a pair of primers (forward primer and reverse primer) (Ishige *et al.* 2020).

The designed primer hairpin, homodimer, and heterodimer structural analysis results showed ΔG > -9.0 kcal/mol (Table 2). A primer specificity assay using agarose gel electrophoresis (based on

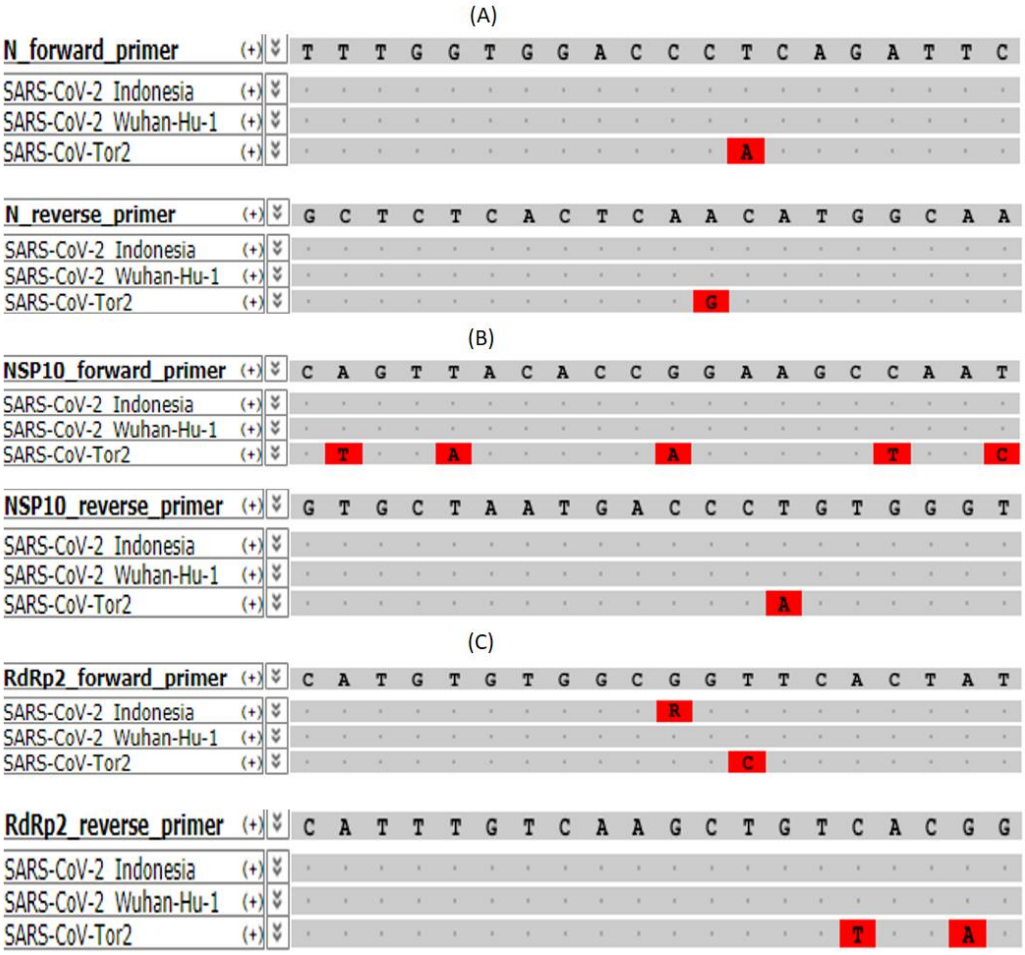

Figure 1. Primer alignment using MSA Viewer on NCBI. (a) N gene primer, (b) NSP10 gene primer, and (c) RdRp2 gene primer (used as positive control).

amplicon length) or melting curves (based on the Tm of the amplicon) can assess whether the predicted secondary structure is formed.

Primer specificity

The specificity of each primer was determined by using the N and NSP10 gene primers for SARS-CoV-2 RNA, with RdRp2 serving as the positive control and RPP30 serving as the internal control. Specificity testing is carried out to avoid false positive results caused by nonspecific primers amplifying the desired target gene or by primers forming secondary structures, giving rise to nonspecific results.

Each primer pair was tested, and a negative control was included. A negative control or non-template control was used to ensure that there was no contamination in the reaction by adding nuclease-free water (NFW) to the reaction mixture instead of the RNA template. The PCR products obtained using conventional RT–PCR techniques were further characterized using 2% agarose gel electrophoresis to determine the size of the product and the specificity of the designed primers. Agarose gel electrophoresis is a DNA characterization method based on differences in the movement of charged molecules in an electric field. The results of the characterization by agarose gel electrophoresis are shown in Figure 2. The electrophoresis results revealed that there was only one band for each primer pair, and the product lengths matched the theoretical length of each gene: 134 bp for the N gene, 161 bp for the NSP10 gene, 104 bp for the RdRp2 gene, and 116 bp for the RPP30 gene. These results showed that the designed primer produced the appropriate product and did not form secondary structures such as hairpins or primer dimers. The identity of the PCR products was further confirmed by sequencing and matching the results with the sequence database available at NCBI using the Basic Local Alignment Search Tools (BLAST) program.

Table 2 Results of secondary structure analysis using the OligoAnalyzer. The design resulted in a tolerable ΔG value (ΔG > -9.0 kcal/mol).

Target Gene	Primer name	ΔG Hairpin (kkal/mol)	ΔG Homo-dimer (kkal/mol)	ΔG Hetero-dimer (kkal/mol)
N	GnNF	-0.43	-4.41	-5.p13
	GnNR	0.29	-5.38	
NSP10	NSP10F	0.44	-9.75	-4.41
	NSP10R	-1.48	-7.48	
RdRp2	RdRp2For	-0.99	-5.38	-6.68
	RdRp2Rev	0.17	-6.34	
RPP30	RPP30For	1.10	-5.36	-3.90
	RPP30Rev	0.89	-6.30	

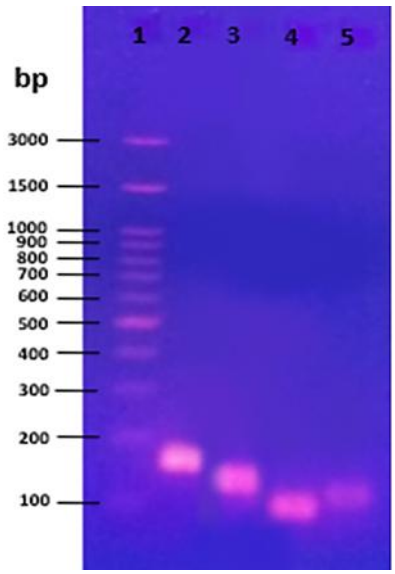

Figure 2. Electropherogram of PCR products (2% agarose gel). (1) DNA ladder 100 b p;. (2) The NSP10 primer product (161 bp); (3) N gene primer product (134 bp; (4) PC (positive control) with the primer RdRp2 (104 bp); (5) RPP30 gene primer was 116 bp (5).

Limitations of detection, Limit of quantification, and Efficiency of rRT–PCR

The limit of detection (LOD), limit of quantification (LOQ), and PCR efficiency were determined using a standard curve. The standard curve was generated by plotting the Ct values obtained from the amplification plot on the Y axis with the log number of copies of RNA at the start of the reaction (copy number/reaction) on the X axis. To obtain linear data to create a standard curve, the SARS-CoV-2 RNA template at a known concentration was diluted tenfold to obtain RNA concentrations of 100; 10; 1; 0.1; and 0.01 ng/µL.

The results of the amplification plot and the melting curves of the N and NSP10 gene primers are shown in Figure 3. Melting curve analysis revealed that the N gene primers produced products with a Tm of 83.50 - 84.50ºC, and the NSP10 gene primers had a Tm of 80.50 - 81.00ºC. The Tm product resulting from the melting curve analysis of each primer was close to the theoretical Tm product. The melting curve of the N gene primer has a difference range of 1.5 - 0.5°C compared to the theoretical product Tm, while the NSP10 gene primer has a maximum difference of 0.5°C compared to the theoretical product Tm. This finding showed that the NSP10 gene primer has better specificity than the N gene primer.

Based on the use of a tenfold diluted RNA template, the Ct value between the two concentrations was 3.3, so an efficiency of 100% was obtained (Tahmasebi *et al*. 2020). The results showed that the lower the concentration of RNA used was, the greater the Ct value was (Table 3). Each primer produced a different ΔCt, and the difference was not as large as 3.3, so the standard curve became less linear.

A standard curve was generated by plotting the Ct value on the Y-axis with the log number of RNA c opies at the start of the reaction (copy number/reaction) on the X-axis. The Y-intercept of the resulting linear regression provides information about the sensitivity (limit of detection) and the slope of the linear regression. The standard curves of the N and NSP10 gene primers are shown in Figure 4. The R2 values of the N and NSP10 gene primers were 0.9091 and 0.9645, respectively.

The limit of detection (LOD) and limit of quantification (LOQ) of the primers were subsequently determined using the slope and standard deviation based on the standard curve. The LODs for the N and NSP10 genes were 7.74 ng/µL and 4.69 ng/µL, respectively. Moreover, the primer LOQs for the N and NSP10 genes were 23.46 ng/µL and 14.21 ng/µL, respectively. Furthermore, the efficiency of each primary variable was determined based on the

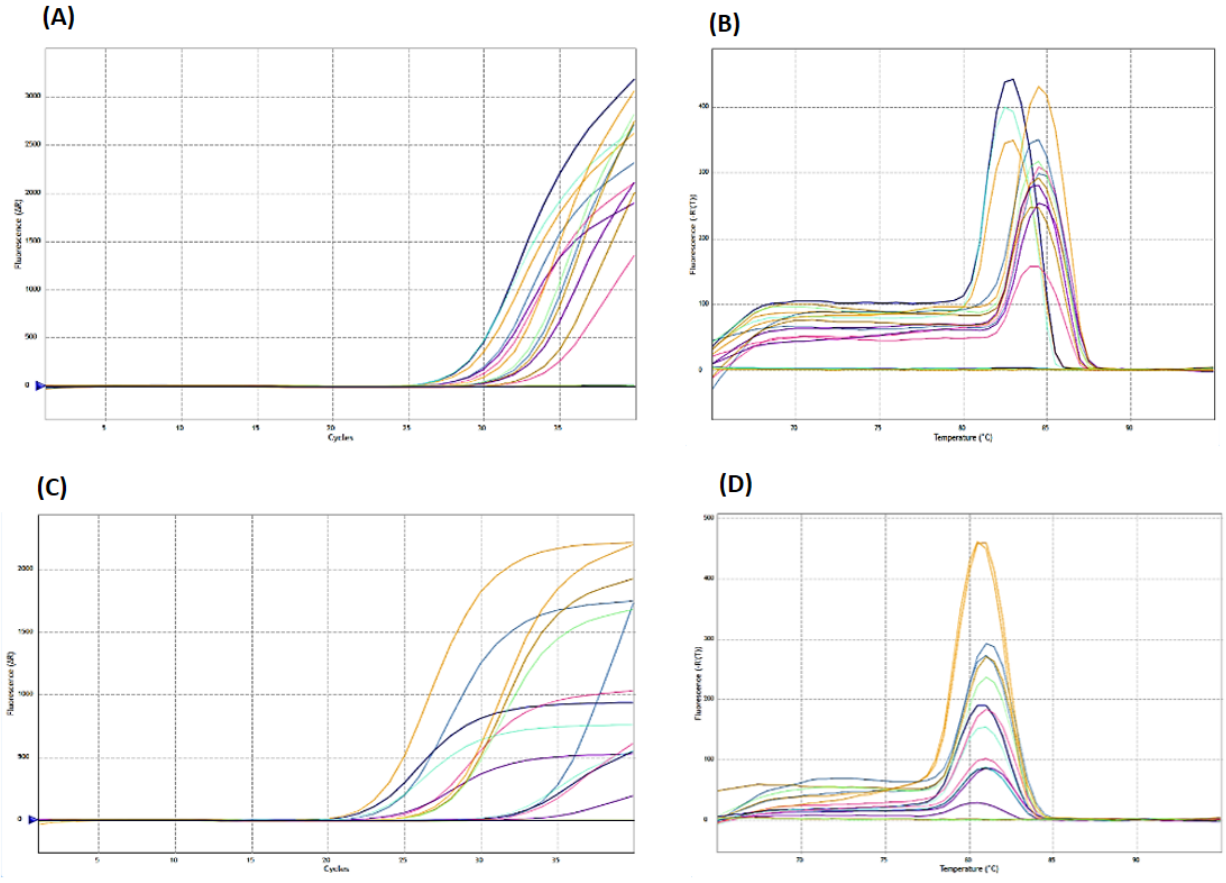

Figure 1. Amplification plot and melting curves for PCR sensitivity and efficiency determination. (A, B) N gene primer and (C,D) NSP10 gene primer.

Table 3. Ct value of PCR sensitivity and efficiency test results.

RNA Concentration (ng/µL)	Mean of Ct	
	N	NSP10
100	19.53	20.41
10	21.19	22.55
1	28.73	24.64
0.1	29.60	30.17
0.01	31.64	32.05
NC	-	-
	-	-

NC: *negative control*

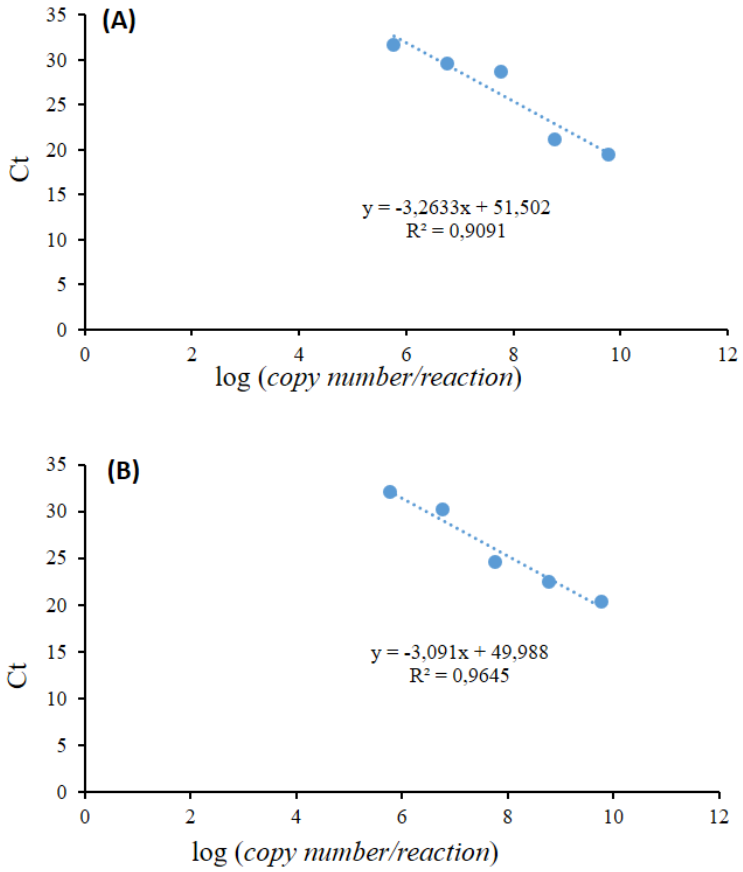

Figure 2. Standard curve for PCR sensitivity and efficiency determination. Standard curve using (a) N primer and (b) NSP10 primer.

slope of the linear regression equation. PCR efficiency shows how many templates are converted into amplification products in each cycle. At 100% PCR efficiency, each cycle had exactly double the number of amplification products, which indicated that each dsDNA molecule produced two dsDNA molecules. The efficiencies of the N and NSP10 gene primers were 102.5% and 110.6%, respectively, where both primers resulted in a PCR efficiency exceeding 100%. A percentage of PCR efficiency that exceeded 100% was also found for N target genes; Pereira-Gómez *et al*. (2020) used SYBR Green-based rRT-PCR, for which the PCR efficiency was 102.56% (Table 4). In addition, Table 4 shows that several target genes from commercial probe-based rRT-PCR kits resulted in PCR efficiencies exceeding 100%.

A PCR efficiency of 100% had a slope of -3.32, while the slopes obtained from the primers in this study were more positive than -3.32 for the N gene primers (-3.2633) and the NSP10 gene primer (-3.091). A good PCR mixture has an efficiency in the range of 90%-110% (slope between -3.58 and -3.10).

Table 4. The PCR efficiency and LOD of several studies were determined by the SYBR Green-based and probe-based rRT-PCR methods.

Method	Reference	Target Gene	PCR Efficiency	Limit of Detection
rRT-PCR based pn SYBR Green	This study	N	102.5%	7,74 ng/µL
		NSP10	110.6%	4,69 ng/µL
	(Sucahya, 2020)	N	102.56%	62,5 copies/µL
		NSP14	99.77%	12,5 copies/µL
rRT-PCR based on Probe (CDC China)	(Pratiwi, 2019)	N	95.2%	100 copies/µL
		NSP10	137.3%	100 copies/µL
rRT-PCR based on Probe (Charité)	(Pratiwi, 2019)	E	110.3%	100 copies/µL
		RdRp	121.5%	>1000 copies/µL
rRT-PCR based on Probe (*Hong Kong University*)	(Pratiwi, 2019)	N	93.4%	100 copies/µL
		NSP14	101.9%	100 copies/µL
rRT-PCR based on Probe (CDC Amerika)	(Pratiwi, 2019)	N1	124.1%	100 copies/µL
		N2	95.2%	100 copies/µL
		N3	128.1%	100 copies/µL

In the present study, the primers used for the N and NSP10 genes had relatively good efficacy, even though the percentage of these genes was greater than 100%. PCR efficiency exceeding 100% is generally caused by the inhibition of the polymerase enzyme. Contaminants that can become inhibitors can be generated from proteins such as hemoglobin and polysaccharides or from compounds used in nucleic acid extraction stages, such as ethanol, phenol, SDS, proteinase K, guanidinium, and sodium acetate (Tahmasebi *et al*. 2020).

Limit of detection, limit of quantification, and PCR efficiency based on the positive control linear regression curve

The sensitivity and efficiency of PCR based on the results of the positive control linear regression curve were compared with the results of the sensitivity and efficiency of the N and NSP10 gene primers. Linear regression curves were generated using positive control concentration variations (400,000; 200,000; 20,000; 2,000; and 200 copies/µL). The results of the amplification plot and the melting curve from this experiment are shown in Figure 5. Melting curve analysis revealed that the product formed from the positive control had a Tm ranging from 80.00 - 80.50ºC.

The Tm value from the analysis of the melting curve is appropriate for the prediction results and differs from the theoretical Tm product by only 0.5ºC. Furthermore, a linear regression curve was generated, as shown in Figure 6, so that the equation $y = -3.3256x + 35.853$ was obtained with $R^2 = 0.9951$. The R^2 value of the positive control linear regression curve indicates that the data obtained are more linear than the N and NSP10 gene primer data because the R^2 value of the positive control linear regression curve **approaches 1**.

The limit of detection and limit of quantification were 1.05 ng/µL and 3.17 ng/µL, respectively, with a PCR efficiency of 99.85%. PCR was more efficient than PCR with the N and NSP10 gene primers. A better percentage of PCR efficiency can be obtained when using a positive control as a template, which is different from the N and NSP10 gene primers, which use the extracted SARS-CoV-2 RNA as a template.

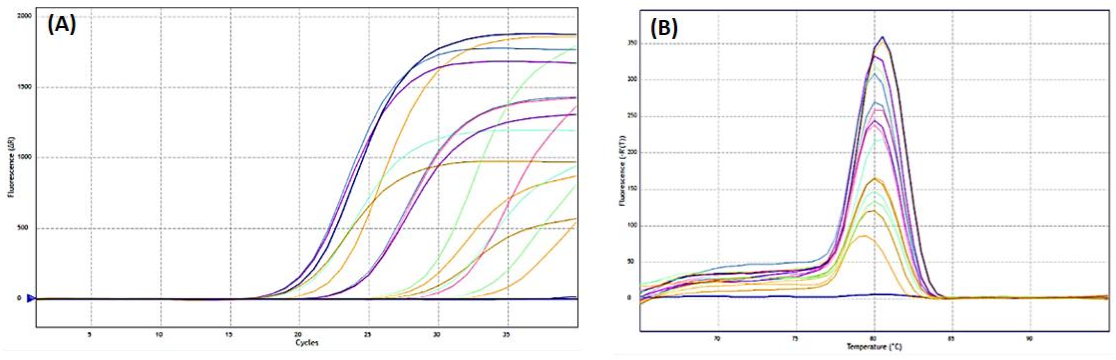

Figure 5. Amplification plot (A) and melting curve (B) for determination of sensitivity and efficiency of PCR using positive controls.

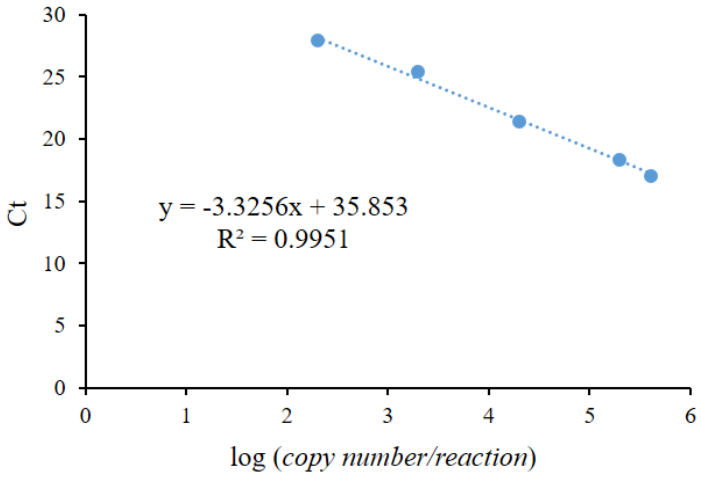

Figure 6. Positive control linear regression curve for determination of PCR sensitivity and efficiency.

Table 5 Ct values from clinical specimen testing using the rRT-PCR method based on SYBR Green and results from clinical laboratories using a probe-based method (target genes N1 and N2). The test was carried out targeting the N and NSP10 genes of SARS-CoV-2 and included internal controls (RPP30 target gene), negative control (H_2O as template), and positive control (RdRp target gene). The test results showed that seven samples were detected positive for COVID-19 and three samples were detected negative for COVID-19.

Sample		RT-PCR result with SYBR Green-based				RT-PCR result with commercial kit	
		Ct value				Ct value	
		N	NSP10	RPP30	RdRp2	N1	N2
Positive sample	1	19.91	18.76	26.48		15.87	17.18
	2	20.81	19.50	26.72		17.51	18.49
	3	16.69	15.09	27.03		11.74	12.31
	4	19.95	19.42	30.01		16.88	17.51
	5	19.01	19.00	30.36		15.65	16.19
	6	21.33	20.95	29.76		16.65	17.53
	7	17.97	16.81	30.23		13.58	13.61
Negative sample	1	-	-	35.70		-	-
	2	-	-	27.12		-	-
	3	-	-	29.00		-	-
Negative control		-	-	-			
Positive control*	1				22.63		
	2				23.43		

*Positive control from commercial kit *2019-nCoV_Positive Control*_v2 contain E *Sarbeco*, gen RdRp, and gen RNAse P sequence gene

Validation of the SYBR Green-based rRT–PCR method was carried out on ten clinical specimens, seven of which were confirmed to be positive for COVID-19 and three of which were confirmed to be negative for COVID-19 by a clinical laboratory; these results were tested using a probe-based method involving two target genes from nucleocapsid (N1 and N2). Tests on clinical specimens were carried out using two primer pairs with the N and NSP10 target genes of SARS-CoV-2 and the RPP30 primer as an internal control, including a negative control and a positive control (RdRp target gene) in duplicate. The Ct and Tm values of the test results for the clinical specimens are shown in Table 5 and Table 6, respectively.

The Ct values of the rRT–PCR based on SYBR Green in this study showed that of the ten clinical specimens tested, seven samples were true positive and three samples were true negative (Table 5). The Ct values of the positive samples tested by the

Table 6. The results of the melting curve of clinical specimens using the SYBR Green-based rRT-PCR method.

Sample		Tm (ºC)			
		N	NSP10	RPP30	RdRp2
Positive Patient	1	84.50	81.00	79.00	
	2	84.00	81.00	79.00	
	3	84.00	81.00	79.50	
	4	84.50	81.00	80.00	
	5	84.50	81.00	79.50	
	6	84.50	81.00	79.50	
	7	84.50	81.00	79.50	
Negative Patient	1	68.50	68.00	77.50	
	2	71.00	70.50	79.50	
	3	95.00	76.50	80.00	
Positive Control*	1				79.50
	2				79.50

rRT–PCR method based on SYBR Green using the N and NSP10 gene primers ranged from 15.09 to 21.33, while the Ct values tested from clinical laboratories had a lower range (11.74-18.49). The RPP30 primer, which gave positive results in all the samples, indicated that all the samples had been extracted properly.

The melting curve analysis showed that for the positive samples tested using the N and NSP10 gene primers, all had a Tm product that matched the prediction of the melting curve, with a Tm difference of 1.0 - 0.5°C (Table 6). Similarly, compared with those of the theoretical product, the Tm products of the internal control (using the RPP30 primer) and the positive control (using the RdRp2 primer) were suitable and had a Tm difference of 1.5 - 0.5°C.

CONCLUSION

The N and NSP10 gene primers used to target the SARS-CoV-2 gene complied with the primer design requirements and can be used to detect the SARS-CoV-2 virus via the SYBR Green-based rRT–PCR method. The limit of detection (LOD) and limit of quantification (LOQ) of the NSP10 gene primers were 4.69 and 14.21 ng/µL, respectively, which were better than those of the NSP gene primers, which were 7.74 and 23.46 ng/µL, respectively. Moreover, the NSP10 gene primer provided a better efficiency (102.5%) than the NSP10 gene primer (110.6%). The SYBR Green-based rRT–PCR kit was used to detect ten properly tested clinical specimens, with seven samples being positive for COVID-19 and three samples being negative for COVID-19. Costs associated with COVID-19 patient testing can be anticipated to decrease with the use of custom primers for the detection of SARS-CoV-2 via the use of the singleplex rRT–PCR mix SYBR Green, especially in Indonesia.

ACKNOWLEDGMENTS

We would like to acknowledge the financial support provided for this research by the Indonesia Endowment Funds for Education (67/FI/P-KCOVID-19.2B3/IX/2021) and Universitas Padjadjaran, Indonesia (2203/UN6.3.1/PT.00/2022).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The patient samples were obtained from the clinical laboratory of Universitas Padjadjaran with approval from the Research Ethics Commission of Padjadjaran University, Bandung (number: 139/UN6.KEP/EC/2022).

Consent for publication: Not applicable

Availability of data and materials:

No specific datasets or materials were used or generated for this study. The sequence GISAID ID of 98 whole genome of SARS-CoV-2 from Indonesian patients are available upon requested; All information and analyses presented in this publication are based on existing literature.

Declaration of competing interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding:

This research was funded by the Indonesia Endowment Funds for Education (67/FI/P-KCOVID-19.2B3/IX/2021) and Universitas Padjadjaran, Indonesia (2203/UN6.3.1/PT.00/2022), and the APC was funded by Padjadjaran University, Indonesia.

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