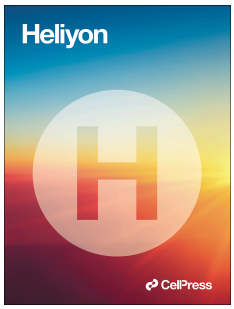
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.



Low-cost SYBR Green-based RT-qPCR assay for detecting SARS-CoV-2 in an Indonesian setting using WHO-recommended primers

Ratika Rahmasari, Muhareva Raekiansyah, Syifa Naura Azallea, Marvella Nethania, Navany Bilqisthy, Anna Rozaliyani, Anom Bowolaksono, Rani Sauriasari

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Authors: Ratika Rahmasari[1]*, Muhareva Raekiansyah[2*], Syifa Naura Azallea[1]*, Marvella Nethania[1], Navany Bilqisthy[1], Anna Rozaliyani[3], Anom Bowolaksono[4], Rani Sauriasari[5]**

[1] Microbiology and Biotechnology Laboratory, Faculty of Pharmacy, Universitas Indonesia, Depok 16424, West Java, Indonesia
[2] Helix Laboratory, Indonesia
[3] Department of Parasitology, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, West Java Indonesia
[4] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, West Java, Indonesia
[5] Clinical Pharmacy Laboratory, Faculty of Pharmacy, Universitas Indonesia, Depok 16424, West Java, Indonesia

*equal contribution

**Corresponding author: Rani Sauriasari

Address correspondence to Rani Sauriasari, Ph.D.
Clinical Pharmacy Laboratory
Faculty of Pharmacy, Universitas Indonesia
Depok 16424, West Java, Indonesia
rani@farmasi.ui.ac.id

Research article

Low-cost SYBR Green-based RT-qPCR assay for detecting SARS-CoV-2 in an Indonesian setting using WHO-recommended primers

Abstract. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent for the ongoing coronavirus disease 2019 (COVID-19) pandemic. For laboratory diagnosis, low-cost detection of SARS-CoV-2 is urgently needed, particularly in developing countries with limited resources. Probe- or TaqMan-based real-time reverse transcription polymerase chain reaction (RT-qPCR) is currently the gold standard for diagnosing infected individuals, as recommended by the World Health Organization (WHO). However, this assay is expensive, making it difficult to use for diagnosis on a large scale. Therefore, in this study, we develop and validate an alternative approach for RT-qPCR diagnosis by employing the DNA intercalating dye SYBR Green. We evaluate and use two WHO-recommended primers, namely CCDC-N and HKU-ORF1b-nsp14. The compatibility of the two primers was tested *in silico* with Indonesian SARS-CoV-2 genome sequences retrieved from the GISAID database and using bioinformatic tools. Using *in vitro*-transcribed RNA, optimization, sensitivity, and linearity of the two assays targeting the N and Nsp-14 genes were carried out. For further evaluation, we used clinical samples from patients and performed the SYBR Green-based RT-qPCR assay protocol in parallel with TaqMan-based commercial assay. Our results show that our methodology performs similarly to the broadly used TaqMan-based detection method in terms of specificity and sensitivity and thus offers an alternative assay for the detection of SARS-CoV-2 RNA for diagnostic purposes.

Keywords: SARS-CoV-2, one-step RT-qPCR, intercalating dye SYBR Green

1. Introduction

The emergence of viral diseases continues to represent a serious public health problem. At the end of 2019, a newly identified severe acute respiratory coronavirus 2 (SARS-CoV-2) caused a global and ongoing pandemic that triggered a humanitarian crisis. The novel SARS-CoV-2 virus was identified to be the causative agent of coronavirus disease 2019 (COVID-19) (Andersen et al., 2020). By the end of September 2021 the virus had infected more than 230 million people globally, with more than 4,700,000 associated deaths (Johns Hopkins Coronavirus Resource Center, 2021). As one of the most populous countries in the world, Indonesia has been severely affected by COVID-19. As at September 15, 2021, Indonesia had reported more than 4 million people infected and almost 140,000 deaths (WHO, 2021). In addition, between June and August 2021, the country experienced an escalating COVID-19 crisis caused by the Delta variant of SARS-CoV-2. Sharply increasing numbers of COVID-19 cases during that time made Indonesia Asia's new pandemic epicenter (Dyer, 2021).

The clinical picture of COVID-19 patients is similar to those suffering diseases caused by other coronaviruses, such as Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS) (Benvenuto et al., 2020). Although considerable knowledge has been acquired over time about the viral mechanism of infection and its mode of replication, at present, effective treatments for COVID-19 remain limited. Drug repurposing, which is an

effective approach for rapid drug discovery, could prove to be an advantageous tactic for identifying COVID-19 treatments (Rahmasari et al., 2020)

SARS-CoV-2 is an enveloped and non-segmented positive-stranded RNA virus belonging to the β-subgroup of the *Coronaviridae* (Zhou et al., 2020). The first complete genome of SARS-CoV-2 of 29.9 kb in length, was revealed from the Wuhan-Hu-1 coronavirus strain. Like other human coronaviruses, SARS-CoV-2 encodes a proofreading exoribonuclease nsp-14 for maintaining replication fidelity (Wu et al., 2020). However, as an RNA virus, SARS-CoV-2 has the potential to evolve rapidly through mutation, although its mutation rate is predicted to be slower than other RNA viruses (Smith et al., 2014). Following its initial outbreak in December 2019, the virus rapidly circulated throughout the world and accumulation of mutations has occurred in the genomes of circulating SARS-CoV-2 strains (Haddad et al., 2021; Khattak et al., 2021; Wang, Hozumi, Yin, & Wei, 2020)

Early diagnosis and isolation of suspected cases plays a key role in controlling the SARS-CoV-2 outbreak. Current diagnostic tests for SARS-CoV-2 use nucleic acid, antibody, and protein-based detections, but according to the World Health Organization (WHO), the gold standard method for detecting SARS-CoV-2 is reverse transcriptase polymerase chain reaction (RT-qPCR) using TaqMan probes for samples mainly taken from the nasopharynx and oropharynx of suspected individuals (WHO, 2020). Simplified RT-qPCR assays are now commercially available and target diverse regions of the SARS-CoV-2 genome, including N, E, S, ORF1ab and the RNA polymerase gene (RdRP).

As advised by the WHO, in addition to public health measures such as isolation of suspected individuals and social distancing, massive diagnostic testing must be implemented, particularly in affected areas, in order to contain the spread of the virus (WHO, 2020). However, SARS-CoV-2 testing capacity is a major issue worldwide. In addition, as the gold standard method, RT-qPCR protocol based on the use of fluorogenic probes can be expensive, making diagnosis on a large scale difficult, particularly in low- and middle-income countries.

The aim of this study was to develop and validate an alternative, lower-cost RT-qPCR method for detecting SARS-CoV-2. Instead of using fluorogenic probes (such as TaqMan), this assay employed intercalating dye SYBR Green as a less expensive alternative. We adapted two of the WHO TaqMan-based RT-qPCR protocols from the Chinese Center for Disease control (CDC) that target the N gene (China CDC, 2020) and one from the University of Hongkong that targets the Nsp-14 gene (Chu et al., 2020) to the SYBR Green-based assay. In our preliminary study, we performed *in-silico* analysis of the two primers against genomes derived from Indonesia. Since SARS-CoV-2 is constantly evolving, mutations between SARS-CoV-2 isolates may cause mismatched or imperfect binding of the primers and may impact the sensitivity of PCR reactions. Our results show that most CCDC-N and HKU-ORF1b-Nsp14 primer sequences largely match the currently reported Indonesian genomic sequences of SARS-CoV-2 and might therefore not affect the sensitivity of RT-qPCR detection. Furthermore, our developed SYBR Green-based RT-qPCR assay is as sensitive and reliable for SARS-CoV-2 detection as commercial TaqMan-based RT-qPCR, in particular for CCDC-N assay. Through dissociation curve analysis, the SYBR Green-based RT-qPCR assay allows for the verification of specific amplification of targeted genes, so as to avoid false-positive interpretation. Overall, our data indicates that relatively low-cost SYBR Green-based RT-qPCR assay is a reliable method for detecting SARS-CoV-2 from clinical samples.

2. Materials and Methods

2.1. *In-silico* primer analysis

Viral genome sequences of the SARS-CoV-2 virus from infected Indonesian individuals were downloaded from the Global Initiative on Sharing All Influenza Data (GISAID) EpicoV database (Shu & McCauley, 2017). The sequences were obtained upon free registration (https://www.gisaid.org/). All the genome sequence recorded in GISAID that contain the N gene or Nsp-14 gene were considered in the analysis. As of the end of May 2021, 1,938 sequences, including the Delta variant, were available for *in-silico* analysis. Two-pair primers recommended by the WHO were used in the present study, namely CCDC-N, developed by the Chinese CDC (China CDC, 2020), and HKU-Orf1b-Nsp14, developed by The University of Hong Kong (Chu et al., 2020). These primers target the conserved regions of the N and nsp14 genes, generating amplicons of 99 bp and 132 bp, respectively. The primer sequences are as follows: CCDC-N Fwd (5'-GGG GAA CTT CTC CTG CTA GAA-3'); CCDC-N-Rev (5'-CAG ACA TTT TGC TCT CAA GCT G-3'); HKU-ORF1b-nsp14-Fwd (5'-TGG GGY(C/T) TTT ACR(G/A) GGT AAC CT-3'); and HKU-ORF1b-nsp14-Rev (5'-AAC R(G/A) CG CTT AAC AAA GCA CTC-3'). Each primer was aligned with or compared to viral genome sequences using BioEdit Sequence Alignment Editor. Sequence variants or mismatches occurring in the primer binding region and their frequency were calculated and recorded.

2.2. Samples and ethical clearance

A total of 54 residual de-identified nasopharyngeal and oropharyngeal samples previously identified during routine diagnostic procedures, regardless of symptoms associated with COVID-19, were used. The study protocol was approved by the Research Ethics Committee of the Faculty of Medicine, University of Indonesia, with Approval number 21-05-0523.

2.3. Total nucleic acid extraction

Viral transport medium (VTM) samples from nasopharyngeal and oropharyngeal swabs were extracted for total nucleic acid content using the TANBead® Smart LabAssist-32 extraction system (Taiwan Advanced Nanotech Inc., Taiwan) according to the manufacturer's instructions. Nucleic acids were extracted from 300 µL of VTM samples with final elution volume of 100 µL for each sample. The nucleic acid was stored at −70°C until used.

2.4. Production of *in-vitro*-transcribed RNA for quantification standards

A positive control RNA standard was obtained by cloning a region of the SARS-CoV-2 genome in plasmid under the control of a T7 RNA polymerase promoter. To this end, synthetic fragments of 99 and 132 bps containing N- and ORF1b-nsp14 targets, respectively, were cloned into pBluescript II KS(-) (Genscript, USA) using SmaI and XhoI restriction sites and transformed in competent E.coli BL21 (D3) (Thermo Scientific, USA) by the heat shock method (42ºC, 30 s). Plating was carried out in LB medium containing 50 µg/mL ampicillin (Amp). After overnight incubation at 37ºC, three individual colonies were isolated and cultured overnight in LB containing 50 µg/mL ampicillin. Plasmid was isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) and quantified by spectrophotometric analysis (NanoDrop, Thermo Scientific, USA). For *in-vitro* transcription after plasmid confirmation, 1 ug of each plasmid was linearized with XhoI. *In-vitro* transcription was carried out using TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) following the

manufacturer's instructions. DNA in the reaction was degraded by incubation with RNAse-free DNase I. *In-vitro* transcript RNA was then purified with GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific, USA). Purified RNA was checked for size and integrity by gel electrophoresis, and absorbance-based measurement of the RNA yield was performed using NanoDrop (Thermo Scientific, USA). The number of copies/µl was calculated as (NA x C)/MW, where NA is the Avogadro constant expressed in mol-1, C is the concentration expressed in g/µl, and MW is the molecular weight expressed in g/mol.

2.5. SYBR Green-based and TaqMan-based RT-qPCR for SARS-CoV-2 detection

The SYBR Green-based RT-qPCR assays (one step) for monoplexes targeting N and Nsp-14 regions were performed using iTaq Universal SYBR Green Supermix (Biorad, USA), following the manufacturer's recommendations. In brief, each reaction consisted of a total volume of 10 µl containing 5 µl of iTaq Universal SYBR Green Supermix (2x), 0.125 µl of iScript reverse transcriptase, 1 µl of forward and reverse primers, 1.875 µl of nuclease-free water, and 2 µl of template RNA. In the initial experiment, final concentrations of primers were optimized using 0.4 uM, 0.3 uM, and 0.2 uM of each primer. Thermal cycling was run on a MA-6000 (Molarray, China) with the following cycle parameters: 50ºC for 10 min for reverse transcription, 95ºC for 1 min for initial denaturation, and then 40 cycles at 95ºC for 10 s and 60ºC for 30 s. The TaqMan-based RT-qPCR assays were performed using commercial 2019-nCoV Nucleic Acid Diagnostic Kit (Sansure Biotech, China). The Sansure kit detects the ORF1ab and N genes of SARS-CoV-2 virus and the human RNase P gene as an internal control to monitor the sample collection, sample handling and RT-qPCR process to avoid false-negative results. The result was considered positive when the Ct value of both the ORF1ab and N genes were ≤40; otherwise, when the Ct values of both the ORF1ab and N genes were >14, the results was considered negative. The kit were performed by following the manufacturer's recommendations with minor modification. In brief, each reaction consisted of a total volume of 25 µl containing 15 µl of PCR Master Mix and 10 µL of RNA extraction. Thermal cycling was run on an MA-6000 (Molarray, China) with the following cycle parameters: 50ºC for 30 min for reverse transcription, 95ºC for 1 min for initial denaturation and then 45 cycles of 95ºC for 15 s and 60ºC for 30 s, followed by a final cool-down to 25ºC for 10 s. A non-template control (nuclease-free water) was included in each RT-qPCR run. The PCR runs were analyzed with MA-6000 software, version 1.1 (Molarray, China). Auto threshold was set in all assays to determine the threshold cycle (Ct).

2.6. Melt curve analysis and gel electrophoresis

Specificities of SYBR Green-based RT-qPCR products were verified from melting curves recorded after each run. To this end, the fluorescence signal of each PCR product was monitored continuously as the temperature was increased from 65ºC to 95ºC, acquiring fluorescence data every 0.3ºC. In addition to melting curve analysis, amplicon sizes were confirmed by separating the RT-qPCR products under electrophoresis in a 2% agarose gel containing 0.01% v/v gel red (Biotium, USA) at 100 V for 40 min. The gels were then analyzed in a UV transilluminator. The expected amplicon sizes of N and Nsp-14 targeted genes were 99 bp and 132 bp, respectively.

2.7. Analytical sensitivity of SYBR Green-based assay

The analytical sensitivity of the SYBR Green-based assay was determined with seven serial 10-fold dilutions of *in-vitro*-transcribed RNA. All seven diluted RNA samples were subjected

to RT-qPCR. Each standard RNA was tested in duplicate. Standard or calibration curves were represented as Ct Vs RNA log copy number/reaction. Efficiency (E) was calculated as $E = 100 \times (10^{-1/s} - 1)$, where *s* is the slope of the calibration curve.

2.8. Intra-and inter-assays

To determine the reproducibility of the RT-qPCR assays, intra- and inter-assays were conducted. Six clinical samples of different Ct values were taken. The assays were performed using the conditions and systems described for primer concentration optimization mentioned above. The testing was conducted in three replicates on the same PCR plate for the intra-assay test. Under the same conditions and systems, three different PCR plates were repeated for evaluation of inter-assay performance. From both assays, the mean, SD and CV were acquired based on the Ct values obtained, in order to determine intra- and inter-assay reproducibility. A CV value of less than 10% (intra-assay CV) and 15% (inter-assay CV) was regarded as acceptable (Guidance for industry: bioanalytical method validation).

3. Results

3.1. Genetic diversity within primer binding regions

Genome variants in primer binding regions of CCDC-N and HKU-ORF1b-Nsp14 assays were analyzed using 1,938 viral sequences reported from Indonesia. Sequence variants or mismatches in primer binding regions of the two assays are presented in Figure 1. ~~More detailed information about variants and mutations is provided in Table S1-S4 of the supporting material.~~ More detailed information about sequence variants of the SARS-CoV-2 genomes within primer binding region of the primers are depicted in Table 1-4. Overall, sequence analysis showed that the genome variants in the primer binding regions presented in very low numbers, in particular for the HKU-Nsp-14 assay. Perfect match was demonstrated for the reverse primer of HKU-Nsp-14, indicating that no mutation had occurred in this target region. Meanwhile, for the forward primer region of HKU-Nsp-14, five variants were observed. All these variants mismatched with less than 0.2% of viral sequences. Furthermore, for the forward and reverse primers of the CCDC-N assays, 12 and five sequence variants existed, respectively. The most prevalent variant occurring for the CCDC-N forward primer was a substitution (at the seventh position of the binding region) that mismatched with 48.2% of the viral sequence. Meanwhile, the five variants in the reverse primer binding regions of the CCDC-N assay were found in less than 2% of the viral sequence. In addition, most of the mismatches observed for the CCDC-N forward primer and HKU-Nsp-14 forward primer were near the 5’ end of the primers or in the first half of the primer binding region. In contrast, all the mismatches observed for the CCDC-N reverse primer were present near the 3’ end.

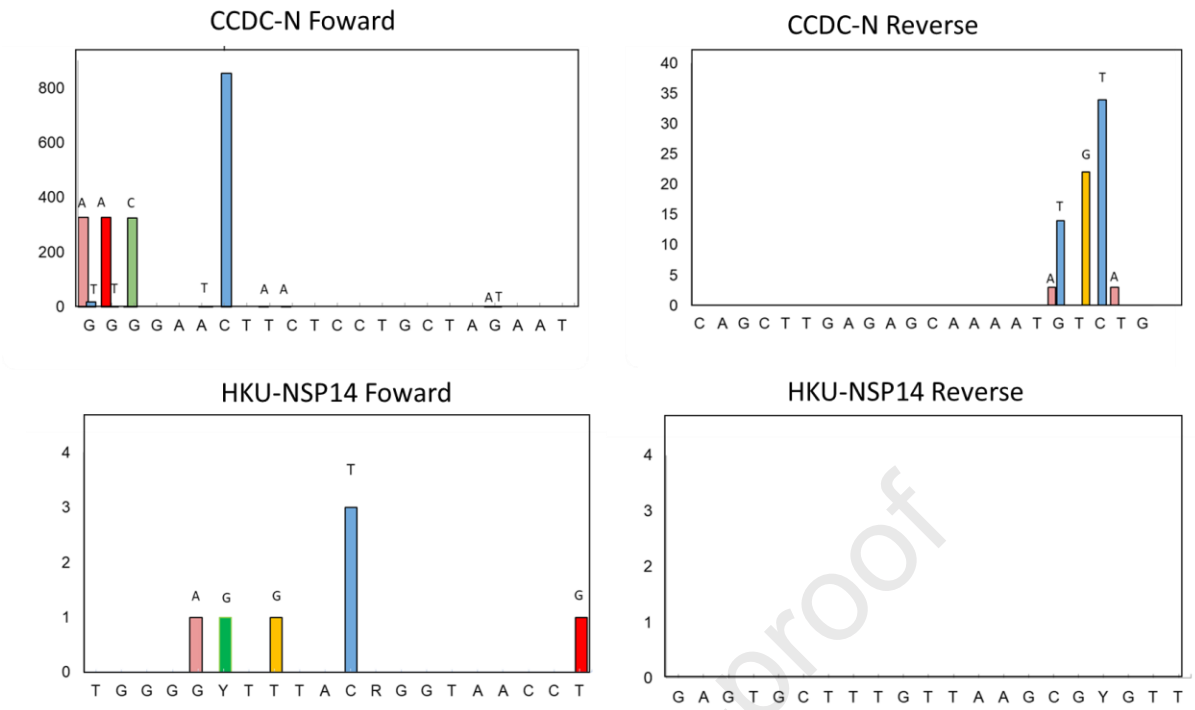


Figure 1. Sequence variants in primer binding regions for CCDC-N and HKU-Nsp-14 assay. SARS-CoV-2 genome sequences from 1,938 infected individuals of Indonesian origin submitted until the end of May 2021 were downloaded from the GISAID database (https://www.gisaid.org/). Each primer was aligned to the genome sequences using BioEdit Sequence Alignment Editor and genetic diversity in the primer binding regions was identified. The position and frequency of each variant/mismatch on the four primers was then plotted.

Table 1. Sequence variants of SARS-CoV-2 genome from Indonesia within primer binding region of HKU-ORF1b-nsp14 forward primer

No	HKU-ORF1b-nsp14 Forward Primer Sequence	Mutation	Percentage
1	T	-	-
2	G	-	-
3	G	-	-
4	G	-	-
5	G	G → A	0.06%
6	Y	Y → G	0.06%
7	T	-	-
8	T	T → G	0.06%
9	T	-	-
10	A	-	-
11	C	C → T	0.17%
12	R	-	-
13	G	-	-
14	G	-	-
15	T	-	-
16	A	-	-

17	A	-	-
18	C	-	-
19	C	-	-
20	T	T → G	0.06%

Table 2. Sequence variants of SARS-CoV-2 genome from Indonesia within primer binding region of HKU-ORF1b-nsp14 reverse primer

No	HKU-ORF1b-nsp14 Reverse Primer Sequence	Mutation	Percentage
1	G	-	-
2	A	-	-
3	G	-	-
4	T	-	-
5	G	-	-
6	C	-	-
7	T	-	-
8	T	-	-
9	T	-	-
10	G	-	-
11	T	-	-
12	T	-	-
13	A	-	-
14	A	-	-
15	G	-	-
16	C	-	-
17	G	-	-
18	Y	-	-
19	G	-	-
20	T	-	-
21	T	-	-

Table 3. Sequence variants of SARS-CoV-2 genome from Indonesia within primer binding region of CCDC-N forward primer

No	CCDC-N Forward Primer Sequence	Mutation	Percentage
1	G	G → A	18.43%
		G → T	1.01%
2	G	G → A	18.38%
		G → T	0.11%
3	G	G → A	0.06%
		G → C	18.32%
4	G	-	-
5	A	-	-
6	A	A → T	0.11%
		A → G	0.06%
7	C	C → T	48.20%
8	T	-	-
9	T	T → A	0.06%

10	C	C → A	0.06%
11	T	-	-
12	C	-	-
13	C	-	-
14	T	-	-
15	G	-	-
16	C	-	-
17	T	-	-
18	A	-	-
19	G	G → A G → T	0.11% 0.11%
20	A	-	-
21	A	-	-
22	T	-	-

Table 4. Sequence variants of SARS-CoV-2 genome from Indonesia within primer binding region of CCDC-N reverse primer

No	CCDC-N Reverse Primer Sequence	Mutation	Percentage
1	C	-	-
2	A	-	-
3	G	-	-
4	C	-	-
5	T	-	-
6	T	-	-
7	G	-	-
8	A	-	-
9	G	-	-
10	A	-	-
11	G	-	-
12	C	-	-
13	A	-	-
14	A	-	-
15	A	-	-
16	A	-	-
17	T	-	-
18	G	G → A G → T	0.17% 0.79%
19	T	T → G	1.24%
20	C	C → T	1.92%
21	T	T → A	0.17%
22	G	-	-

3.2. SYBR Green-based RT-qPCR assay for SARS-CoV-2 and its analytical sensitivity

The SYBR Green-based RT-qPCR protocol was set up using *in vitro* transcript RNA containing the SARS-CoV-2 sequences for both N and ORF1b-Nsp-14 targets. In the initial experiment, primer concentrations of the two assays were optimized. Figures 2A and B show amplification curves of the targeted regions of N and Nsp-14, respectively. Different primer concentrations

of 0.2, 0.3, and 0.4 µM generated almost identical results. No amplification curve was observed for the no-template control in the two assays, indicating that no contamination or unspecific signal occurred during the RT-qPCR process. The SYBR Green-based RT-qPCR results of the two assays were then confirmed with melting curve analysis and agarose gel electrophoresis. The melt curve of all amplified positive samples produced a clear and distinct melt peak at Tm = 82.05 (Figure 2C) and 79.89 (Figure 2D) for primer sets of N and Nsp-14, respectively. These unique melting peaks corroborate the presence of single PCR amplicons produced by the two assays. In addition to the melting peak analysis, the specificity of the assays was confirmed by 2% agarose gel electrophoresis of amplified products. This revealed single bands of 99 bp and 132 bp amplicons corresponding to expected product size of N and Nsp-14 target genes, respectively. No amplification appears in any negative controls (Figure 2E). Since all primer concentrations rendered similar results in the initial experiments, for the rest of the study, we decided to use primer concentration of 0.3 µM.

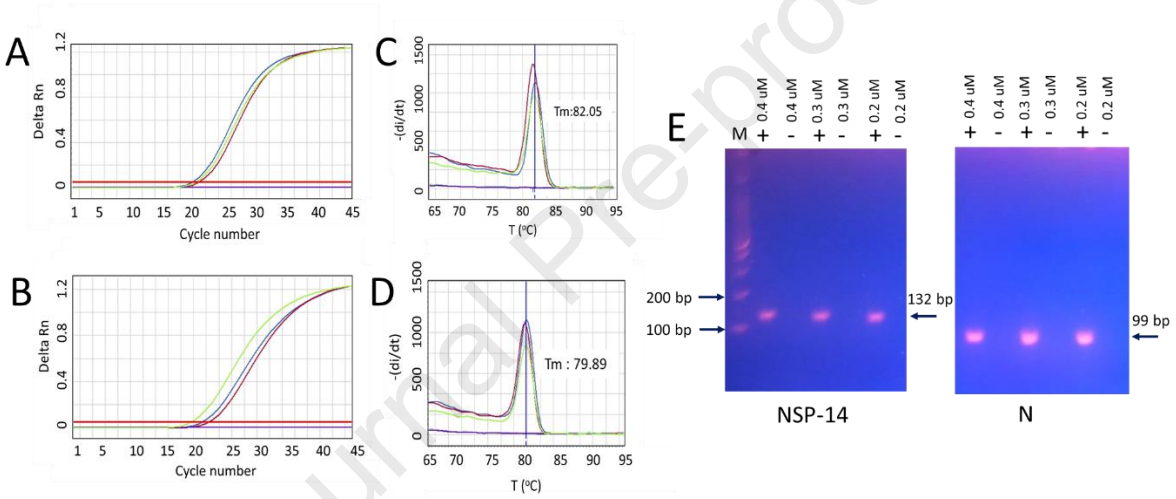


Figure 2. Characteristics of SYBR Green-based RT-qPCR. Amplification curves of (A) N- and (B) ORF1b-Nsp-14 assay of different primer concentrations (0.2, 0.3, and 0.4 µM) using *in-vitro*-transcribed RNA harboring both target sequences; melting curves for the products amplified with the N assay (C) and Nsp-14 assay (D); 2% agarose gel electrophoresis (E) of N (right panel) and Nsp-14 (left panel) PCR product amplified with SYBR Green-based RT-qPCR protocol. Notes: M:100 bp DNA molecular weight (Tiangen, China); (+) indicates positive template and (-) no template control; the arrows mark the amplified fragments of 99 bp and 132 bp and molecular weight of 100 bp and 200 bp. Green, blue, and red curves indicate different primer concentration of 0.2, 0,3, and 0,4 µM, respectively.

Next, sensitivity of SYBR Green-based RT-qPCR assay for both N- and Nsp-14 were determined. To this end *in-vitro* transcript RNA harboring N- or Nsp-14 gene targets were diluted serially in concentrations ranging from 10^7 to 10^1 copies/µL and used for RT-qPCR. The SYBR Green-based RT-qPCR was performed in duplicate and under optimal conditions. The results showed positive reactions with all serial dilutions of standard RNA except for Nsp-14 assay at the lowest RNA dilution (10^1) where only one amplification curve was generated (Figures 3A and B). Furthermore, it is shown that the Ct value linearly correlated with the logarithm of RNA copy number for N- and Nsp-14 assay with $R^2 = 0.995$ and $R^2 = 0.998$, respectively (Figures 3C and D). For N assay, the correlation standard curve

equation was Y = −3.587 × +41.377 and the amplification efficiency (E) was 90.01%. For Nsp-14 assay, the correlation standard curve equation was Y = −3.57 × +43.304 and the amplification efficiency (E) was 90.60%. The slope values of -3.59 and -3.57 for the CCDC-N and HKU-ORF1b-nsp14 assays, respectively, indicate that RT-qPCR amplifications are efficient (Svec et al., 2005). Verification of products of SYBR Green-based RT-qPCR with gel electrophoresis (Figures 3E and F) again shows correct amplicons with expected size of 99 bp and 131 bp for N and Nsp-14 assay, respectively. However, for Nsp-14 assay, no amplicon was detected at the lowest concentration of standard RNA (10^1 copy number), indicating that the SYBR Green-based RT-qPCR was more sensitive than conventional RT-PCR. Overall, the results obtained indicate that the performance of N and ORF1b-Nsp-14 SYBR Green-based RT-qPCR assay is sensitive for SARS-CoV-2 detection. From further experiments, the limit of detection (the lowest detectable concentration of *in-vitro* transcript RNA that returns a positive result in ≥95% of repeated measurements) of our SYBR Green-based protocols was 10 copies/µL for N assay (12/12 positives) and 1000 copies/ µL (7/7 positives) for Nsp-14 assay, as shown in Figure 4 and 5, respectively.

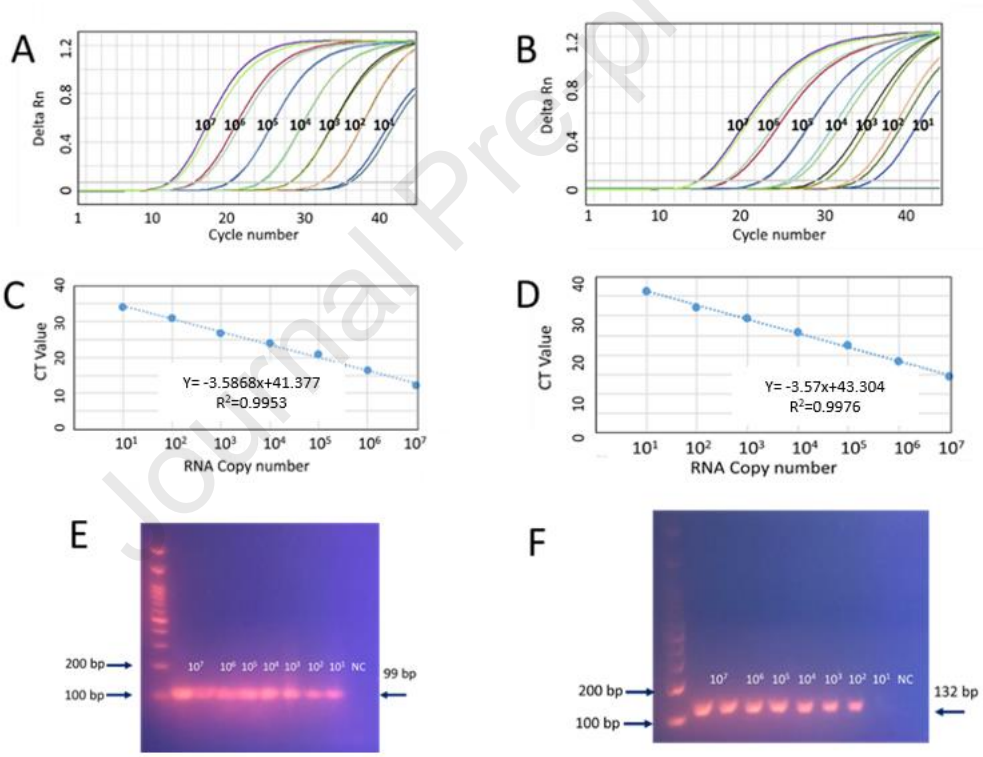


Figure 3. Analytical sensitivity of SYBR Green-based RT-qPCR for SARS-CoV-2 detection. Representative amplification curve of (A) N- and (B) Nsp-14 gene specific assay using RNA standard copy numbers. Amplification plots (cycle number versus fluorescence) of serially diluted *in-vitro*-transcribed RNA standards (copies/reaction); standard curves of (c) N- and (D) Nsp-14 gene specific assay. RNA copy number (log starting quantity) is indicated and plotted against the mean cycle threshold (Ct) value. The coefficient of determination (R2) and the equation of the regression curve (Y) were calculated. Representative (E) N- and (F) Nsp-14 RT-qPCR amplicons visualized in 2% agarose gel.

3.3. Validation of SYBR Green-based assay with clinical samples.

In order to validate our SYBR Green-based RT-qPCR protocols, we re-tested a set of 54 clinically relevant samples that had qualitatively tested positive and negative for SARS-CoV-2 during routine diagnosis (n = 34 positive and n = 20 negative). Performance of our SYBR Green-based methods was compared to a commercial TaqMan-based *in-vitro* diagnostic test kit. All results generated by RT-qPCR are shown in Table 5. In addition, representative SYBR Green-based RT-qPCR amplification curves, melt curves, and 2% agarose electrophoresis results of positive and negative results for the two assays are shown in Figure 6.

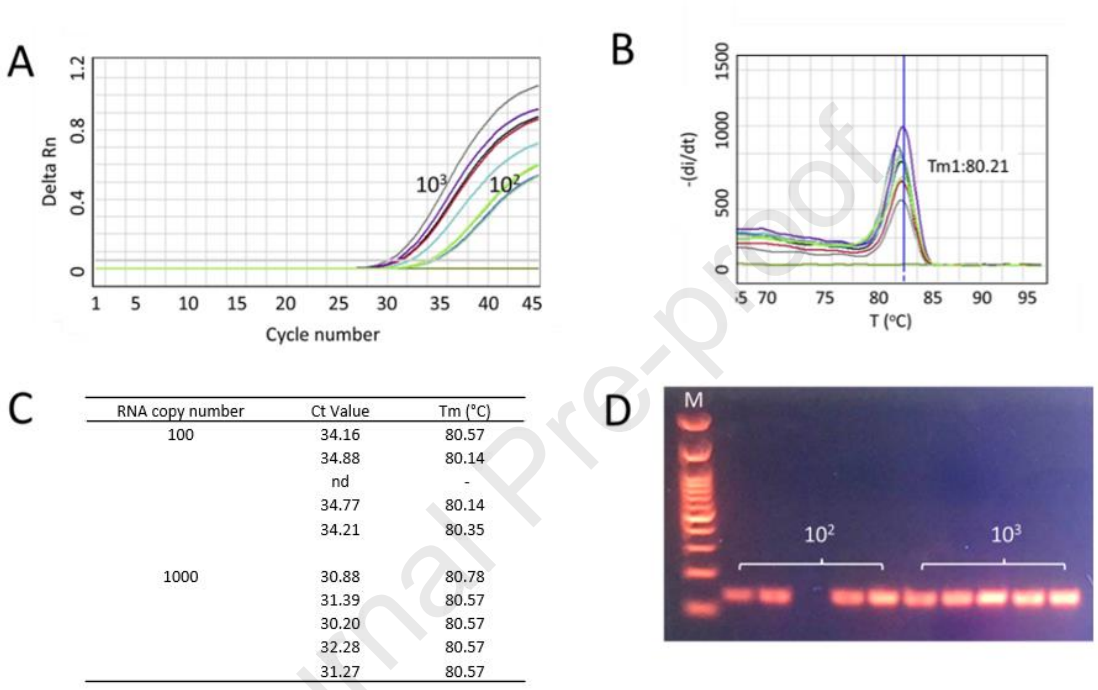
Table 5. Ct values and melting temperatures (Tm) of clinical samples tested by TaqMan-based and SYBR Green-based RT-qPCR.

Sample No.	Probe based assay (Ct value)		SYBR green-based assay (Ct value)			
	ORF1b	N	NSP-14	Tm (°C)	N	Tm (°C)
1	19.23	17.45	19.23	80.00	18.94	82.14
2	14.33	14.44	17.37	80.00	16.98	82.14
3	17.45	14.56	17.14	79.79	17.69	82.14
4	18.37	14.93	16.73	79.79	14.51	82.14
5	20.65	17.50	20.25	80.21	19.46	82.14
6	18.22	17.66	18.01	80.00	18.36	81.93
7	16.56	13.17	16.35	79.79	15.34	81.93
8	19.77	16.23	19.58	80.21	19.81	82.14
9	21.12	21.21	22.79	80.21	21.08	82.57
10	21.33	20.87	22.81	80.21	22.69	82.36
11	28.65	26.69	31.31	80.00	28.10	82.14
12	26.01	23.76	28.56	80.21	25.55	81.93
13	34.77	31.32	40.05	80.21	31.83	82.36
14	34.43	32.19	39.80	80.00	35.92	82.14
15	32.69	31.88	34.89	80.00	33.95	82.14
16	36.75	35.54	37.94	80.43	37.71	83.36
17	34.47	33.38	35.98	80.64	36.86	82.14
18	34.63	33.98	38.68	80.43	38.17	81.29
19	32.88	30.78	36.20	80.21	31.70	81.93
20	36.19	35.43	37.93	80.21	39.67	81.93
21	36.16	33.10	nd	-	36.76	82.40
22	39.18	37.41	nd	-	41.71	82.19
23	35.91	34.87	39.23	79.82	38.16	82.19
24	36.86	35.09	nd	-	36.94	82.19
25	38.61	35.07	nd	-	37.81	82.19
26	38.95	35.30	Nd	-	38.64	82.19
27	37.20	35.07	39.07	80.04	38.11	81.97
28	38.13	36.23	42.66	78.95	36.55	82.19
29	38.08	37.13	nd	-	36.90	82.19
30	37.45	34.81	nd	-	38.33	81.97
31	nd	nd	nd	-	nd	-
32	nd	nd	nd	-	nd	-
33	nd	nd	nd	-	41.37	75.93*
34	nd	nd	nd	-	44.09	75.71*
35	nd	nd	nd	-	nd	-
36	nd	nd	43.39	-	44.62	-
37	nd	nd	nd	-	nd	-
38	nd	nd	nd	-	44.30	-
39	nd	nd	nd	-	44.24	-
40	nd	nd	nd	-	nd	-
41	33.63	31.82	30.52	79.57	33.67	81.71
42	32.48	30.43	36.58	79.57	32.59	81.50
43	34.35	32.62	35.70	79.57	32.98	81.71
44	31.73	30.44	33.84	79.57	33.32	81.50
45	nd	nd	nd	-	nd	-
46	nd	nd	nd	-	nd	-
47	nd	nd	nd	-	nd	-

48	nd	nd	nd	-	nd	-
49	nd	nd	nd	-	nd	-
50	nd	nd	nd	-	nd	-
51	nd	nd	nd	-	nd	-
52	nd	nd	nd	-	nd	-
53	nd	nd	nd	-	nd	-
54	nd	nd	nd	-	nd	-

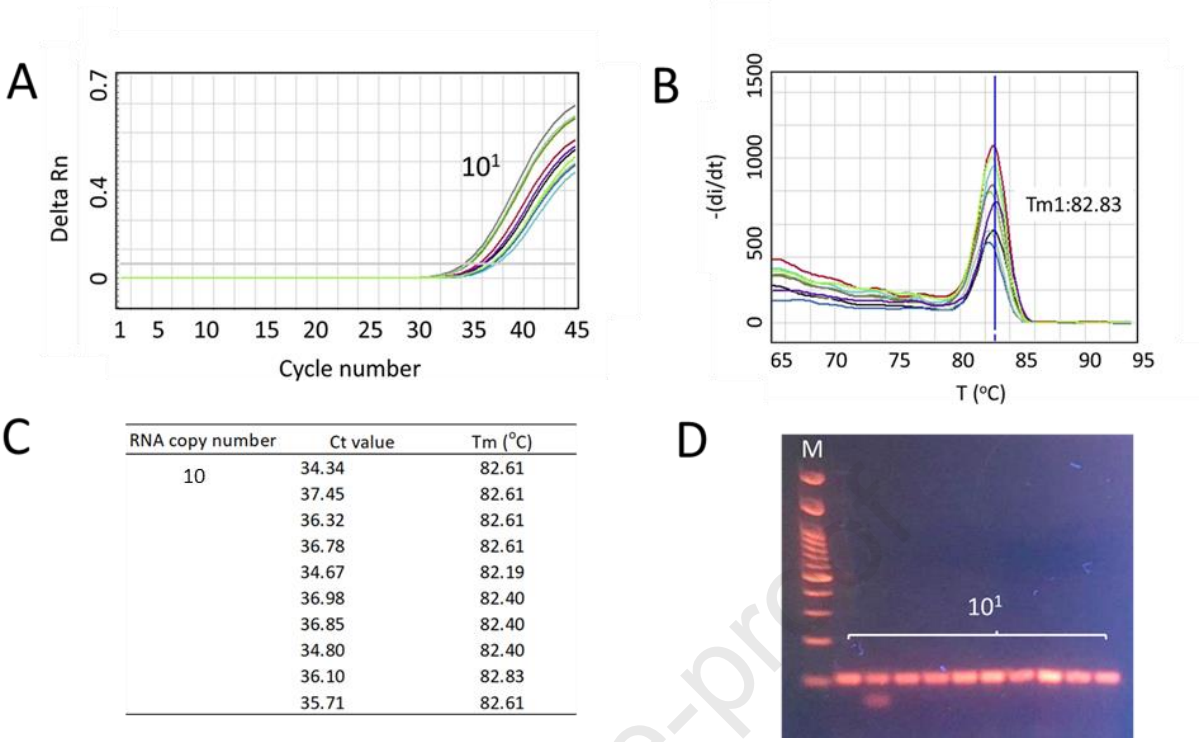
nd: not detected

* Non-specific signal



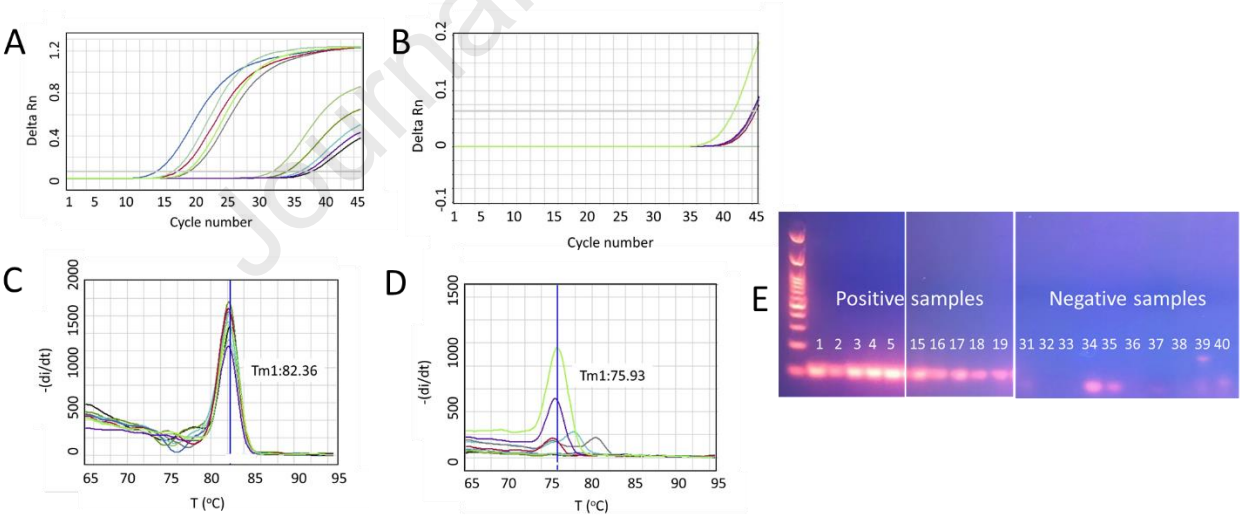
RNA copy number	Ct Value	Tm (°C)
100	34.16	80.57
	34.88	80.14
	nd	-
	34.77	80.14
	34.21	80.35
1000	30.88	80.78
	31.39	80.57
	30.20	80.57
	32.28	80.57
	31.27	80.57

Figure 4. Limit of detection of nsp-14 assay. Amplification curves of in-vitro transcribed RNA standard at concentration of 10^3 (n=5) and 10^2 copy/µL (n=5) (A); melting curves for the amplified products (B); Ct value and Tm and 2% agarose gel electrophoresis of the corresponding products (C and D).



RNA copy number	Ct value	Tm (°C)
10	34.34	82.61
	37.45	82.61
	36.32	82.61
	36.78	82.61
	34.67	82.19
	36.98	82.40
	36.85	82.40
	34.80	82.40
	36.10	82.83
	35.71	82.61

Figure 5. Limit of detection of N assay. Amplification curves of in-vitro transcribed RNA standard at concentration of 10^1 (n=10) (A); melting curves for the amplified products (B); Ct value and Tm and 2% agarose gel electrophoresis of the corresponding products (C and D).



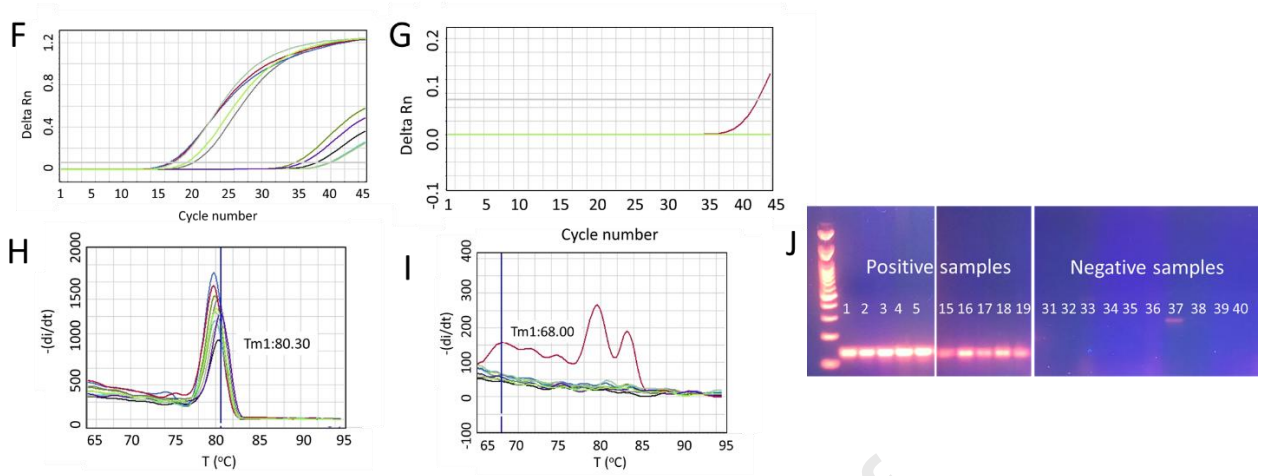


Figure 6. Validation of SYBR Green-based RT-qPCR protocol on clinical samples. Representative SYBR Green-based RT-qPCR amplification curve, melt curve, and 2% agarose electrophoresis for N target (the upper panel, Figures A to E) and Nsp-14 target (the lower panel, Figures F to J). (A and F) Amplification curves for 10 positive samples and (B and G)10 negative samples; nonspecific amplification generated in (B) five N assays and (H) one negative sample of Nsp-14 assay at the end of PCR cycles; corresponding melt curve dissociation for (C and H) positive and (D and I) negative samples; for N assay, unique single peaks with Tm of around 82^{o}C were generated in all positive samples. For Nsp-14 assay, single peaks were generated with Tm of around 80^{o}C in each positive sample. Nonspecific melt peaks were detected in some negative samples that correspond to the nonspecific amplification in both assays. (I and J) corresponding RT-qPCR amplicons for positive and negative samples are shown. For positive samples, amplicons with sizes of 99 bp and 132 bp were specifically amplified as expected in N and Nsp-14 assays, respectively. Nonspecific RT-qPCR amplicons with unexpected sizes were observed in some negative samples. The gels were cropped to present representative results.

Of these 54 samples, 34 were confirmed positive and 20 negative by both TaqMan and N assays, indicating that the performance of our SYBR Green-based assay targeting the N gene was comparable to the TaqMan assay. Of these 34 positive samples, one sample (sample no. 22) showed a late Ct value of 41.71. However, the melt curve analysis of this sample showed a Tm peak that matched the SARS-CoV-2 RNA positive control. For Nsp-14, seven borderline samples that tested positive using the TaqMan protocol (Ct ranged from 36 to 39 according to ORF1ab target) were shown as being negative. These results indicate that the SYBR Green assay targeting the Nsp-14 gene was less sensitive than that targeting the N gene, since it failed to detect the weak positive samples. For negative samples, both N and Nsp-14 assays were in 100% concordance with the TaqMan assays, indicating that our SYBR Green-based assays were specific. Although in N assay, for example, five samples generated amplification curves at very late cycles (Ct > 40), through melt curve analysis and gel visualization it was found that those amplification curves reflected nonspecific signals. This suggests that the outcome of tested samples obtained by SYBR Green protocols should be addressed taking into account the Tm peak.

Finally, using six clinical samples we performed intra- and inter-assays to determine the accuracy and reproducibility of our SYBR Green method (Table 6). The intra- and inter-assays were determined based on the Ct values obtained from triplicate samples within each run (intra-assay) and in three consecutive runs (inter-assay). For N assay, the intra- and inter-assay coefficients of variation (CV) ranged from 0.19% to 1.50% and 0.52% to 2.43%, respectively, whereas for Nsp-14 assay, the CV ranged from 0.36% to 2.73% (intra-assay) and from 1.19% to 2.16% (inter-assay). ~~Detailed information on the intra- and inter-assay results is provided in Table S5 (supplementary data).~~ The experimental variability values obtained are considered acceptable, indicating that our SYBR Green-based RT-qPCR is reproducible.

Table 6. Intra- and inter-reproducibility of SYBR green-based CCDC-N and HKU ORF1b-nsp14 assay

Primer	Sampel	Intra-assay reproducibility (n=3)		Inter-assay reproducibility (n=3)	
		Mean Ct ± SD	CV (%)	Mean Ct ± SD	CV (%)
N	Sample no. 4	13.86 ± 0.21	1.5	13.90 ±0.07	0.52
	Sample no. 10	22.43 ± 0.07	0.19	22.38 ± 0.12	0.53
	Sample no. 11	28.48 ± 0.15	0.54	27.74 ± 0.68	2.43
NSP-14	sample no. 1	19.16 ± 0.14	0.74	19.27 ± 0.42	2.16
	Sample no. 9	24.33 ± 0.09	0.36	24.81 ± 0.43	1.71
	Sample no. 12	25.74 ± 0.61	2.73	26.06 ± 0.31	1.19

4. Discussion

PCR has been used extensively for the diagnosis of viral infections because of its sensitivity, specificity, and reproducibility (Josko, 2010). For pathogenic RNA viruses such as SARS-CoV-2, RT-qPCR is regarded as the gold standard for detection of these viruses (Espy et al., 2006; WHO, 2020). The majority of RT-qPCR assays are probe-based, a method that is extremely effective but not accessible to all laboratories. Here, we describe a RT-qPCR methodology based on SYBR Green as an alternative molecular protocol for detecting SARS-CoV-2 from clinical samples without the need for TaqMan probes. For these purposes, we adapted two of the WHO-recommended TaqMan-based RT-qPCR protocols to SYBR Green-based assay.

In the preliminary study, genetic analysis within target regions of the two primers was performed. The aim was to investigate whether and how frequently mutation had already occurred within the primer binding region of the SARS-CoV-2 genome. As viruses evolve during an outbreak, genetic variants or mutations can emerge that could compromise the sensitivity and specificity of RT-PCR assay (Lippi, Simundic, & Plebani, 2020). To this end, using 1,938 SARS-CoV-2 genomes from Indonesia, the presence of mutations or mismatches in primer binding regions of CCDC-N and HKU-Nsp-14 assays was analyzed. Our observations show that there is only a low prevalence of genome variation in the primer binding region of the two assays. In particular, the reverse primer of HKU-Nsp-14 shows a perfect

match with 100% of the viral sequence, indicating that this region is entirely conserved. Our observation is in line with the estimates of moderate mutation rate in SARS-CoV-2 genome being similar to the SARS-CoV genome (Shen et al., 2020). SARS-CoV-2 has a higher fidelity in its transcription and replication process than other single-stranded RNA viruses because it has a proofreading mechanism regulated by NSP14 (Sevajol et al., 2014).

It worth noting that the presence of mismatch does not necessarily result in false negative results. It is well known that the sensitivity of diagnostic primers depends on their target position. In particular, the 5 prime half of a primer is not as important as its 3 prime half. In this study, the few mutations on the primer binding regions of CCDC-N and HKU-Nsp-14 primers that occurred were mostly located near the 3' end. Thus, this might not pose a major concern in relation to assay sensitivity (increasing the limit of detection). Decrease in primer sensitivity may occur if genetic variants increase in frequency. Specifically, if most of the variants present on the right end of a primer (the critical position), this might produce more false negatives in diagnostics. Given the fact that genetic variability in the SARS-CoV-2 genome is expected to increase through natural viral mutation, continuous monitoring of SARS-CoV-2 genomic variations is essential to providing rapid responses when assay re-design is needed in the future.

In this study we have successfully adapted the TaqMan-based RT-qPCR method recommended by the WHO that targets the nucleocapsid protein (N) gene and Nsp-14 gene of SARS-CoV-2 to an SYBR Green-based protocol. From analytical sensitivity results obtained using *in-vitro* transcript RNA, our SYBR Green-based assays were capable of detecting up to 10 copies of the *in-vitro* transcript RNA (Figure 5). Furthermore, in the studied clinical samples, our N assay, in particular, was comparable to the TaqMan-based assay (as our reference). Of 34 samples confirmed positive by the Taqman method, the N assay detected SARS-CoV-2 in the all samples, with no false positives. All negative samples tested by the TaqMan assay were also found to be negative in the N assay. In addition, our results were also reproducible, and thus we believe that our SYBR Green-based assay protocol could represent a valuable alternative for the molecular detection of SARS-CoV-2.

In our experiments we observed that the N gene assays were superior to the Nsp-14 assays in terms of sensitivity. When using *in-vitro* transcript RNA, the Ct values of the N gene assays were slightly lower than those of the Nsp-14-gene assays (Figures 3-5). Furthermore, the Nsp-14 gene assay failed to detect some borderline or weak positive samples (Table 5). Our results are in line with a recent study that showed that N gene–based RT-qPCR assay was more sensitive than open reading frame (ORF) 1 assay for detection of SARS-CoV-2 in clinical specimens (Chu et al., 2020; Etievant et al., 2020; Vogels et al., 2020)

In addition to primer sets, the sensitivity of RT-PCR is affected by many other factors, including cycling condition (annealing temperature). Thus at this point, we cannot rule out that better SYBR Green RT-qPCR assay results could be achieved by further optimization of the Nsp-14 assay process. Indeed, the superiority of N gene assay over NSp-14 gene assay in detecting SARS-CoV-2 RNA in the studied clinical samples was expected. This could be explained by the relative amount of N gene present in the samples. The N gene is the most abundant mRNA in coronaviruses and subgenomic mRNA of the N gene is produced during virus replication (Moreno et al., 2008). It is possible that the clinical samples might contain infected cells expressing subgenomic mRNA (Simons et al., 2005), resulting in more N gene copies in the samples.

RT-qPCR test for SARS-CoV-2 commonly targets multiple genes of the virus genome through multiplexing strategy (Corman et al., 2020). The justification is the high frequency of mutation in the viral genome that can make the primer less capable of viral detection. In the present study, our in-silico analysis shows that primer binding sequence of the N assay appear to be highly conserved. Our results and a previous study (MH et al., 2020) suggest that only a single primer set to the N gene could be used to test patients with this infection and thus could reduce testing cost.

Theoretically, probe-based methods such as TaqMan RT-qPCR is superior to SYBR Green-based assay. The use of an additional labeled probe significantly increases the sensitivity and specificity of the assay. Fluorescence emits from reporter dyes conjugated to the specific oligonucleotide sequence of the probes. In contrast, SYBR Green dye intercalates into any double-stranded DNA and emits a positive signal. Therefore, one drawback of the SYBR Green is that the dye is non-specific. Consequently, non-specific amplifications such as primer dimers may be captured as false positives. Primer dimers could present in negative samples or when the amount of target RNA is low. This phenomenon is likely related to the low annealing temperature and the high degeneracy and concentration of the primers. However, to ensure the accuracy of the SYBR Green-based methodology in which only specific amplification was obtained, melting curve analysis must be performed at the end of each PCR run. In this study, the optimal melting peak of N and Nsp-14 gene amplicons using RNA-transcribed RNA as a template is 82.05°C and 79.89^{o}C, respectively. We observed in clinical samples, that amplification signals were generated at a very late cycle in both the N and NSp-14 assays for some negative samples (Figure 4). However, from melting curve analysis we identified that the peaks were nonspecific, confirming that those signals were generated from nonspecific amplification. This suggests that in the SYBR Green-based methodology, melting curve analysis is an important step for correct interpretation of outcomes. One advantage of SYBR Green-based assay compared with TaqMan probes is that it is not sensitive to mutations in the amplified sequence. This chemistry is of particular interest when targeting an evolving RNA virus such SARS-CoV-2.

Finally, in order to rapidly detect and contain a SARS-CoV-2 outbreak, early detection of the virus and expanding testing capacity are critical. Low cost for early detection of the virus is needed, especially in resource-limited countries. The SYBR Green-based RT-qPCR assay as described in the present study and in previous reports (Wightman et al., 2021; Won et al., 2020; Marinowic et al., 2021; Pearson et al., 2021; and Pereira-Gómez et al., 2021) could be used as a cost-effective alternative to TaqMan-based assay for SARS-CoV-2 detection.

The sample size is a limitation of our current study although our methods had a great clinical performance compared with the TaqMan-based RT-PCR kit. Further evaluation of our SYBR Green-based RT-qPCR assay using higher number of clinical samples and performing comparison of population-relevant SARS-CoV-2 variants would be part of our future studies. In addition, ~~pe~~ cross validation of our assays using other human pathogenic coronaviruses including 229E, SARS, and MERS or its synthetic genes would be also important in the next studies.

5. Conclusion

We established and evaluated a relatively low-cost RT-qPCR method based on SYBR Green dye for SARS-CoV-2 detection. Our method is reliable for detecting SARS-CoV-2 from clinical samples and could be applied as an alternative method to TaqMan-based assay.

Declarations

Author contribution statement

Ratika Rahmasari, Muhareva Raekiansyah, and Syifa Naura Azallea, designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the paper.
Marvella Nethania performed the experiments, analyzed, interpreted the data and wrote the paper.
Navany Bilqisthy performed the experiments, analyzed and interpreted the data.
Anna Rozaliyani performed the experiments, analyzed and interpreted the data
Anom Bowolaksono designed experiments, analyzed and interpreted the data.
Rani Sauriasari conceived and designed the experiments, analyzed and interpreted the data, and wrote the paper.

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Declaration of interest statement

The authors declare no conflicts of interest.

Ethics approval

Our institutional review board approved this study and all biological samples were collected under the approval of the Ethics Committee of Faculty of Medicine, University of Indonesia.

Additional information

No additional information is available for this paper.

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