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RESEARCH ARTICLE

REVISED Denaturing and dNTPs reagents improve SARS-CoV-2 detection *via* single and multiplex RT-qPCR

[version 3; peer review: 2 approved with reservations]

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Abstract

Background

The COVID-19 pandemic, caused by SARS-CoV-2, highlighted the need for accurate and scalable diagnostic tools such as RT-qPCR. However, false-negative results may occur due to viral mutations and RNA secondary structures within target regions.



Methods

High-performance computing (HPC) was used to compile SARS-CoV-2 genomic sequences from GenBank and GISAID and generate consensus sequences for primer and probe design. A region within the *ORF8* gene was selected and evaluated alongside targets from the *E* and *N* genes and the *RNase P* control. Nasopharyngeal swab samples were collected from patients with a prior clinical diagnosis consistent with SARS-CoV-2 infection, as well as from volunteers, and total RNA was extracted using the MagMAX kit. RT-qPCR assays were performed

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2. **Juan Sebastian Quintero Barbosa** , University of Virginia, Charlottesville, USA

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in both single and multiplex formats. Denaturing solutions composed of tetraethylammonium chloride and dimethyl sulfoxide, as well as adjusted dNTP proportions based on viral nucleotide composition, were evaluated. Exploratory Ct-based performance metrics were estimated using predefined threshold criteria with the Caret package in R.

Results

A total of 126,576 SARS-CoV-2 genomes collected between January and December 2020 were used to construct a reference dataset. A target region within the *ORF8* gene exhibiting predicted secondary structures was selected for primer and probe design. Forty-nine clinical samples were analyzed, of which 22 tested positive across the evaluated gene targets. Variability in detection patterns was observed across sampling periods. The evaluated formulations were associated with changes in Ct values in both single and multiplex RT-qPCR assays, depending on the conditions and sample set.

Conclusions

The incorporation of denaturing solutions and the adjustment of nucleotide proportions were associated with changes in RT-qPCR performance under the evaluated experimental conditions. These findings suggest that RNA secondary structure and nucleotide composition may influence assay behavior; however, further studies are required to assess the broader applicability of this approach.

Keywords

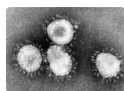
SARS-CoV-2, Diagnosis RT-qPCR, Adjuvant formulation, Primer and probe design, High performance computing



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REVISED Amendments from Version 2

This version has been substantially revised in response to the reviewer's comments to improve clarity, methodological transparency, and consistency between the reported results and their interpretation. The manuscript now more explicitly presents the study as an exploratory and methodological contribution rather than as a clinical diagnostic validation study. The Abstract, Introduction, Results, Discussion, and Conclusions have been revised to moderate statements that could be interpreted as overstating the findings. In particular, claims regarding assay performance have been reformulated to more accurately reflect the scope of the dataset and to avoid implying broad clinical diagnostic superiority. The Methods section has been clarified, particularly with respect to the Ct-based performance analysis. This analysis is now described as an exploratory internal comparison based on predefined operational Ct criteria, and its limitations are stated more explicitly. The corresponding Results section has also been revised so that these metrics are presented as comparative descriptors of assay behaviour under the evaluated experimental conditions rather than as measures of absolute diagnostic accuracy. In addition, a dedicated Limitations section has been included to acknowledge the restricted sample size, the constraints associated with sample availability during the COVID-19 pandemic, the absence of independent external clinical validation, and the ethical and legal restrictions that limited access to additional clinical information. Finally, the Data Availability section has been expanded to improve transparency and reproducibility by more clearly describing the genomic datasets, sequence alignments, and analysis scripts deposited in public repositories.

Any further responses from the reviewers can be found at the end of the article

Introduction

On January 23, 2020, the SARS-CoV-2 virus (*Coronaviridae: Betacoronavirus: Severe acute respiratory syndrome-related coronavirus*) was declared a public health emergency by the World Health Organization (WHO) International Health Regulations (IHR) Emergency Committee. At the time, the global public health authorities established that the spread of SARS-CoV-2 could be prevented if every nation adopted solid strategies for rapid and accurate disease detection (WHO, 2020).

Diagnostic methods based on gene-specific primers and probes for the detection of viruses *via* gene amplification include quantitative real-time polymerase chain reaction (RT-qPCR) or reverse transcription loop-mediated isothermal amplification (RT-LAMP), both of which can be conducted using oropharyngeal and nasopharyngeal swab samples from patients (Kevadiya *et al.*, 2021). Among these, the former method is considered the most sensitive and accurate for the detection of SARS-CoV-2 and other viruses (Martín *et al.*, 2021). This procedure could be effectively implemented due to the characterization of the viral genome, which encompasses approximately 10 genes (Zhu *et al.*, 2020). Therefore, WHO authorized the Berlin protocol which relies on the following genes: *ORF1ab*, which encodes proteins that enable viral replication (Nur *et al.*, 2015); *Spike (S)*, which interacts with the receptor of the host's angiotensin-converting enzyme 2 (ACE2) (Wan *et al.*, 2020); and *E*, which encodes the structural envelop protein (Tahan *et al.*, 2021). Other protocols such as the 2019-nCoV TaqMan RT-qPCR Kit authorized by the United States Centers for Disease Control and Prevention (CDC) utilize two regions of the *N* gene (N1-N2), which encode the nucleocapsid phosphoprotein (Navarathna *et al.*, 2021). Moreover, both protocols use the *RNase P* gene as a control to assess the efficiency of the RT-qPCR protocol (WHO, 2009).

Nevertheless, RT-qPCR-based diagnosis is conducted on a per-gene basis and therefore its widespread implementation would be both impractical and costly. Therefore, multiplex RT-qPCR (*e.g.*, the CDC kit or combined quantification of the *ORF1ab* and *S* genes) could be implemented as a promising approach to meet the current demand for accurate and cost-efficient diagnosis (Kudo *et al.*, 2020). Sample pooling is another approach that could increase population-wide SARS-CoV-2 diagnosis rates and has therefore been approved and implemented in several countries (Grobe *et al.*, 2021). However, sample numbers and sampling structure may limit the implementation of this strategy (Grobe *et al.*, 2021).

Although RT-qPCR-based SARS-CoV-2 diagnostic tools are generally considered the gold standard for disease detection, several studies have determined that this approach is prone to return false-negative results due to gene mutations (which is often the case for the *E* and *N* genes) (Hasan *et al.*, 2021; Tahan *et al.*, 2021) or primer dimer formation (Jaeger *et al.*, 2021). Another factor that affects RT-qPCR efficiency is the secondary structure of the RNA to be characterized/quantified (Hammerling *et al.*, 2020).

Previous studies have demonstrated the role of RNA secondary structure in viral evolution and transcript regulation (Andrews *et al.*, 2021; Huston *et al.*, 2021; Rangan *et al.*, 2020; Wacker *et al.*, 2020). However, their potential impact on RT-qPCR performance in clinical samples remains insufficiently characterized. Furthermore, the effects of adjuvants such as dimethyl sulfoxide (DMSO) or ammonium salts on RT-qPCR efficiency are not fully understood (Kovarova & Draber, 2000).

In this context, we used High-Performance Computing (HPC) to compile a global dataset of SARS-CoV-2 genomes available up to December 2020 and selected a region of the *ORF8* gene for evaluation in both single and multiplex RT-qPCR assays, alongside targets from the *E* and *N* genes. Additionally, we assessed the potential impact of adjusting denaturing solution composition and nucleotide proportions on RT-qPCR performance.

This study was designed as an exploratory and methodological approach to evaluate how RNA secondary structure and nucleotide composition may influence RT-qPCR assay performance under defined experimental conditions. The implications of these findings are discussed in the following sections.

Methods

Ethics statement

This study was approved by the Research Ethics Committees of the Industrial University of Santander, the Chicamocha Clinic, and the Chicamocha Clinical Laboratory (L3C) (Bucaramanga, Santander, Colombia). The study participants were hospitalized patients diagnosed with SARS-CoV-2, individuals presenting to the emergency room, or volunteers. All participants provided written informed consent and voluntarily participated in our study. Further, all participants were kept anonymous and were informed that the present study was conducted strictly for research purposes and that its outcomes were not intended to serve as treatment or diagnosis.

SARS-CoV-2 sample collection and storage

Nasopharyngeal swab samples were acquired by L3C personnel. Two samples were obtained per study participant, one for clinical diagnosis, as authorized by the Ministry of Health and Social Protection of Colombia, and the other for our study. The samples were placed in Universal Transport Medium (UTM) and stored at -80°C until required for downstream analyses (Rogers *et al.*, 2020). Samples were collected over a one-week period and packaged in accordance with applicable Colombian health regulations (Ministry of Health and Social Protection, 2020) prior to transport and processing. All samples were transferred to the Central Research Laboratory of the Faculty of Health Sciences at the Industrial University of Santander (LCI-FS-UIS) and maintained at 4°C during handling and processing. In all cases, written informed consent was obtained prior to sample collection, and documentation was properly verified and archived in accordance with institutional and national ethical guidelines.

Because sample collection was not conducted under a predefined experimental sampling framework but was instead constrained by voluntary participation and ethical requirements during the COVID-19 pandemic, an anonymized coding system was implemented to ensure participant confidentiality, in accordance with Resolution 8430 of 1993 and the applicable Colombian ethical framework (Ministry of Health and Social Protection, 1993). The alphanumeric code consisted of two digits corresponding to the participant, followed by “CO” (Colombia) and a consecutive number assigned to each sample.

Subsequently, samples were aliquoted in a Class II, Type A2 biosafety cabinet (Thermo Fisher Scientific). A total of three aliquots were obtained per sample, including one 200 μL aliquot used immediately for total RNA extraction and two 650 μL backup aliquots stored at -80°C .

SARS-CoV-2 genome database consolidation

To account for reported mutations in SARS-CoV-2 genomes that could affect primer and probe binding and potentially generate false-negative results, a region encoding the accessory protein ORF8 was selected for analysis. This region was chosen based on its predicted mutation profile, and monthly consensus sequences were generated to assess its identity pattern across newly available genomes. Publicly available SARS-CoV-2 genome sequences were obtained from the GenBank and GISAID databases (RRID:SCR_002760; RRID:SCR_018251) (Sayers *et al.*, 2021; Shu & McCauley, 2017). To gather data from the GenBank database, a Python script (RRID:SCR_008394) was executed in the GUANE-1 High Performance and Scientific Computing Center of the Industrial University of Santander (SC3UIS) through the Entrez Programming Utilities interface using the Biopython package (RRID:SCR_013249; RRID:SCR_007173) (Cock *et al.*, 2009; Geer *et al.*, 2010), whereas all GISAID data were manually downloaded. Genomes with uncharacterized regions/nucleotides were discarded using another Python script (RRID:SCR_008394), which was used to conduct monthly FASTA sequence alignments using the MAFFT software (RRID:SCR_011811) (Katoh & Standley, 2013) coupled with previously described DNA loss model parameters (Martínez-Pérez *et al.*, 2002, 2007). The consensus sequences were generated using BioEdit version 7.2 (RRID:SCR_007361) with a 100% threshold frequency (Hall, 1999).

Design and synthesis of *ORF8*-specific primers, probes, and substrate oligos

Using the SARS-CoV-2 (NC_045512) reference genome and consensus sequences from January to April 2020 (Zhu *et al.*, 2020), an approximately 150-base pair (bp) region of the *ORF8* gene was selected based on the secondary

structure of its RNA transcript, which in turn was predicted using the algorithms proposed by Zuker (Zuker & Jacobson, 1998) via the Mfold software (RRID:SCR_001360) (Zuker, 2003). Thus, the last set containing the codons of the central region of the *ORF8* gene, which encodes the secretion protein ORF8, was chosen because it allows proper viral adhesion to the host cell (Chan *et al.*, 2020). The obtained sequence was used as a template to create primers, a TaqMan FAM-BBQ probe, and substrate oligos for RT-qPCR. The specificity of the aforementioned molecules was confirmed via GenBank BLAST analyses (RRID:SCR_004870) (Ye *et al.*, 2006). Genes *E* and *N* from the Berlin and CDC protocols were used as controls (Biotek, 2020; Corman *et al.*, 2020).

All molecules were synthesized by Bioneer (Korea). The primers were purified *via* separation on a reverse-phase cartridge, whereas the probe and substrate oligos were purified *via* high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE), respectively. The *ORF8* RT-qPCR conditions were implemented as described by the Berlin protocol (Corman *et al.*, 2020) using the aforementioned molecules coupled with the 2019-nCoV TaqMan RT-qPCR Kit (Norgen Biotek Corp) developed by the CDC (Biotek, 2020).

Preparation of denaturing solutions and adjustment of dNTP concentrations

A:T and G:C ratios were calculated based on the monthly SARS-CoV-2 consensus sequences to obtain an average for each nucleotide. These averages were then used to determine the minimum concentrations of TEA (ABCAM-USA), DMSO (Scharlab-Spain), and dNTPs (100 mM each, Promega-USA) in molecular-grade ultrapure water (Promega-USA), in addition to the MgSO₄ concentration recommended by the Berlin protocol.

RNA extraction

Total RNA extraction was conducted using the MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (2000 RXNs) (Applied Biosystems-USA) using a KingFisher Duo Prime (5400110) DNA/RNA extraction system according to the manufacturer's instructions (Thermo Fisher Scientific-USA) (Fang *et al.*, 2007).

SARS-CoV-2 single and multiplex RT-qPCR

RT-qPCR was conducted using the *ORF8*-specific primers and probe designed herein, in addition to the *E* (Berlin protocol) and *N* genes (N1-N2; CDC protocol). The *RNase P* gene was used as an external control, as proposed by both of the aforementioned protocols. The reactions were conducted using the SuperScript III One-Step RT-qPCR System with Platinum Taq DNA Polymerase (Ref. 11732088; Invitrogen-USA) and the 2019-nCoV TaqMan RT-qPCR Kit (Ref. TM67100; Norgen-Biotek-Canada). Each reaction for each diagnostic system was conducted in either 15- or 25- μ L reaction volumes consisting of 2 μ L of patient-derived purified RNA, 2 \times One-Step RT-qPCR Master Mix, 2 \times nuclease-free buffer, and the respective primers/probe at the concentrations recommended by the CDC. Moreover, a denaturing stock solution was added to obtain a final concentration of 0.7% TEA, 0.2% DMSO, and 0.8 mMol MgSO₄. The dNTP reagent had a final concentration of 12 mMol dATP-dTTP, 10 mMol dCTP-dGTP, and 0.8 mMol MgSO₄. The reaction conditions were the following: 55°C for 15 minutes, 95°C for 3 minutes followed by 45 cycles of 95°C for 15 s and 58°C for 30 s for the SuperScript™ III One-Step RT-qPCR kit; and 95°C for 3 s followed by 55°C for 20 s for the 2019-nCoV TaqMan RT-qPCR kit. Fluorescence signals were quantified using a QuantStudio 1 Real-Time PCR System (No. A40427) in a 96-well 0.2 μ L block (Thermo Fisher Scientific-USA).

The above-described procedure was conducted using two different Multiplex One-Step RT-qPCR protocols. In the first instance, the primers and probes for the *E*, *ORF8*, and *N* (N1) genes were mixed, whereas the other reaction was performed by mixing the N1 and N2 sets of the *N* gene. The *RNase P* gene was independently assessed in both cases. All reactions were performed as described above.

Exploratory Ct-based performance analysis of SARS-CoV-2 RT-qPCR assays

Single and multiplex RT-qPCR assays were compared based on Ct values obtained under identical experimental conditions. Comparative analyses were performed at both the individual primer/probe level and at the sample level, allowing evaluation of performance differences between assay formats.

To estimate sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for the single and multiplex RT-qPCR assays, a custom script was implemented in R using the Caret package (<https://github.com/GenomicUIS/Sensitivity-specificity-PPV-and-NPV-for-SARS-CoV-2.git>) (Kuhn, 2008; R Core Team, 2020). All scripts and processed datasets used in these analyses are publicly available in the referenced repository.

Because this study was designed as an exploratory methodological study and not as a clinical diagnostic validation, performance estimates were derived from predefined Ct-based operational criteria applied uniformly across assays. For individual reactions, Ct values between 18 and 35 were considered within the operational positive range. Ct values

outside this interval, including values >35 or <18, were conservatively classified as non-positive or indeterminate for this exploratory internal analysis.

Ct values were subsequently pooled and evaluated at the sample level. A sample was considered positive when at least one primer/probe set met the predefined operational Ct criterion. For multiplex RT-qPCR assays, the same Ct-based criteria were applied, but results were evaluated collectively at the sample level.

These metrics were used to compare assay behavior under the evaluated experimental conditions. Because no independent external clinical validation was available, and because access to additional clinical information was restricted by ethical and legal considerations, these estimates should not be interpreted as measures of absolute diagnostic accuracy.

Results

SARS-CoV-2 GenBank and GISAID databases

A total of 19,317 genomes were retrieved from the GenBank database from January to October 2020 based on our search criteria, whereas 107,259 complete genomes were obtained from the GISAID database between January and December 2020. In both datasets, the number of available sequences increased progressively over time. However, the frequency of base substitutions in the monthly consensus sequences for the *E*, *ORF8*, and *N* genes was higher in the GISAID dataset compared to GenBank (Figure 1).

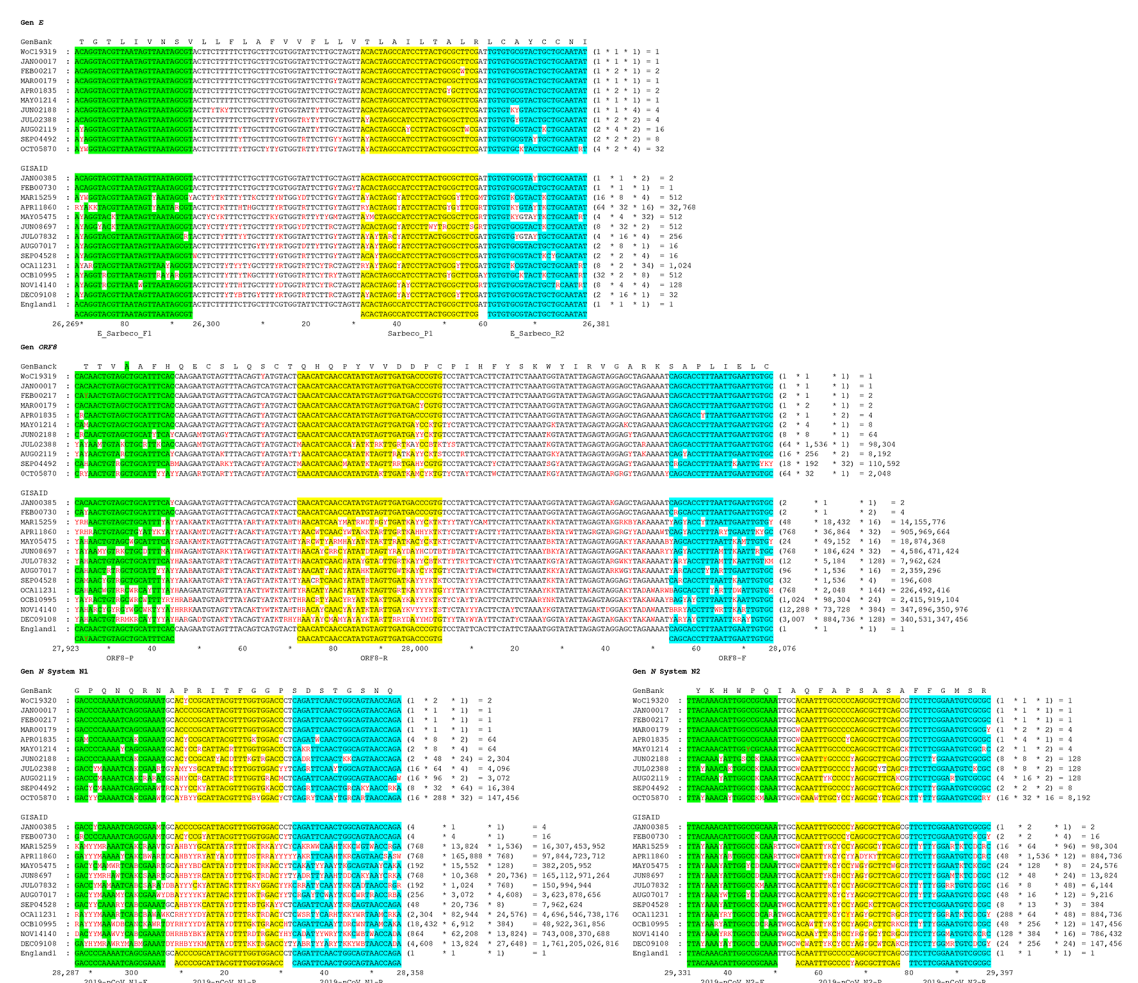


Figure 1. Primers and probes used for RT-qPCR detection of SARS-CoV-2. Nucleotide sequences are indicated as follows: forward primers are shown in green boxes, reverse primers in blue boxes, and probes in yellow boxes. Conceptual translation is displayed above each alignment. Numbers indicate nucleotide position within the sequence, and nucleotide combinations at each position follow international nomenclature. The nucleotide positions relative to the SARS-CoV-2 reference genome are shown in the lower consensus alignment.

Primer, probe, and substrate oligonucleotides design for SARS-CoV-2 detection based on RNA secondary structure

The region of the *E* gene employed herein exhibited a 113 bp length, whereas the amplicons of the N1-N2 system of the *N* gene were 72 and 67 bp in length, respectively. All regions exhibited loop-bubble structures. A 154 bp region within the first half of the *ORF8* gene also presented a loop-bubble structure similar to those observed in the *E* and *N* genes. The third loop of the N1 system and the second loop of the *ORF8* gene were formed by four and seven canonical base pairings, respectively. These structures are comparable to scorpion-type primer or probe configurations used in RT-qPCR; however, such loop-bubble structures are typically formed by 2–6 canonical base pairings (Figure 2). Table 1 summarizes the primer sequences used in this study.

SARS-CoV-2 detection via *E*, *ORF8*, and *N* RT-qPCR

A total of 49 samples were collected between October 25, 2020, and January 21, 2021, of which 22 tested positive and 27 tested negative. Samples collected in October were used to evaluate the detection of SARS-CoV-2 using the *E*, *ORF8*, and *N* gene targets by RT-qPCR (Table 2, Figure 3). Based on the secondary structure and nucleotide composition analyses, which indicated an A:T ratio between 63% and 70% across consensus sequences, denaturing and dNTP solutions were incorporated into the reactions (see Methods for details). Under these conditions, changes in Ct values were observed compared to the commercial procedures, although variability was detected among samples (Table 2, Figure 3).

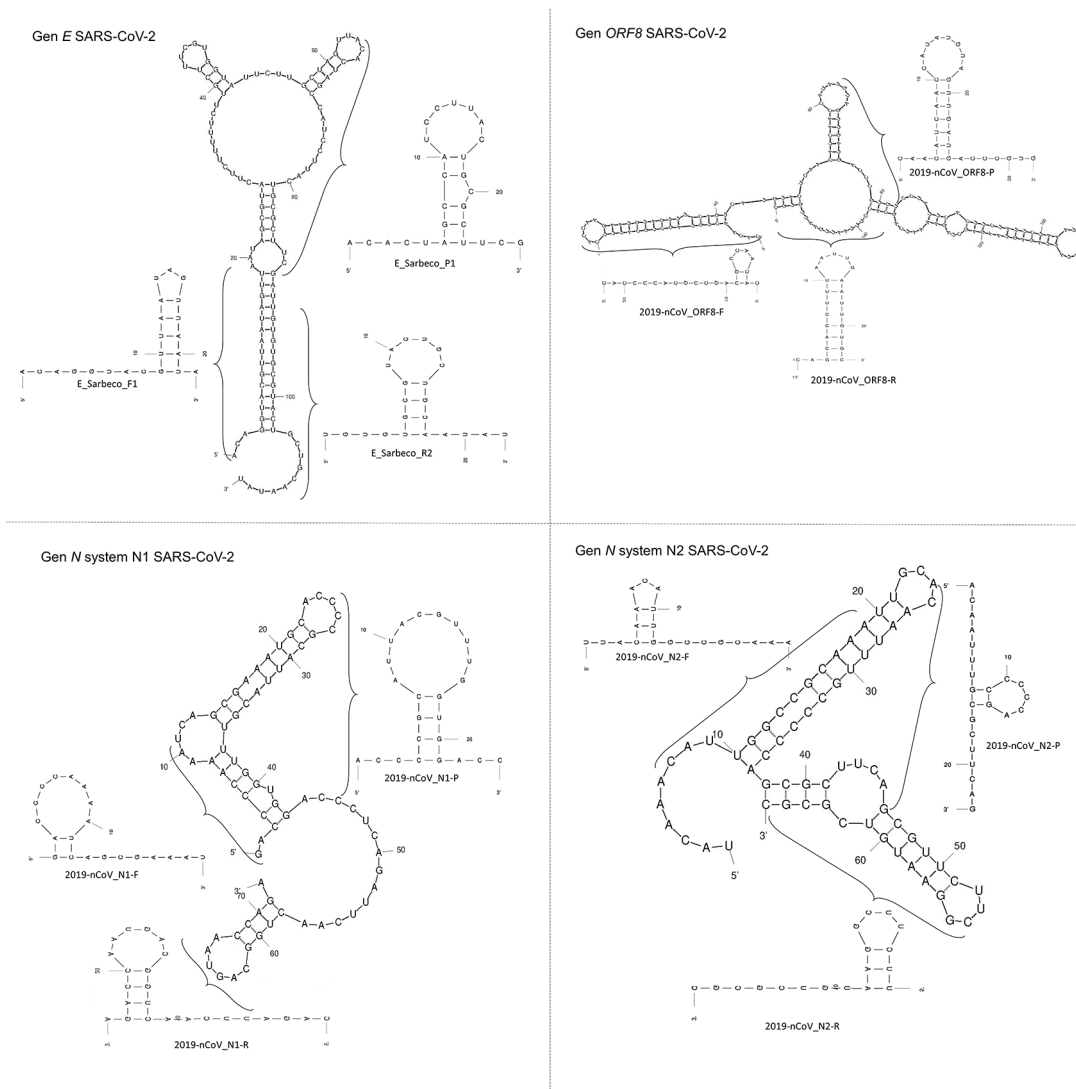


Figure 2. Predicted secondary structures of primers and probes used for the *E*, *ORF8*, and *N* (N1–N2) genes. Stem and stem-loop structures are observed in both the 5' and 3' orientations. Numbers indicate the number of nucleotides in each structure, and parentheses delineate individual primers and probes within each segment.

Table 1. Primers, probes, and substrate oligonucleotides used for RT-qPCR in this study.

Gen	Type	Code	Sequence	Total nmole	Reference
ORF8	Primer Forward	FwO820CoV	CAYAACTGTAGCTGCATTTCAC	24.3	This work
	Primer Reverse	RvO820CoV	GCACAATTCAATTAAAGGTGCTG	21.9	
	Probe	TqMO820CoV	FAM-CAACATCAACCATATGTAGTTGATGACCCGTG-BBQ	8.7	
	Substrate Oligonucleotide	TrGtO8	CAYAACTGTAGTGCAATTCACCAAGAATGTAGTTACAG TCATGTACTCAACATCAACCATATGTAGTTGATGACCCGT GTCCATTTCACCTTCTATTCTAAATGGTAATTAGAGTAGG AGCTAGAAAATCAGCACCTTTAAATTGAATTGTGC	0.3	
E	Primer Forward	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	34.0	(Corman et al., 2020)
	Primer Reverse	E_Sarbeco_R1	ATATTGCAGCAGTACGCACACA	49.8	
	Probe	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	22.5	
N	Primer Forward	2019-nCoV_N1-F	GACCCAAAATCAGCGAAAT	22.5	(Biotek, 2020)
	Primer Reverse	2019-nCoV_N1-R	TCTGGTACTGCCAGTTGAATCTG	22.5	
	Probe	2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ	22.5	
	Primer Forward	2019-nCoV_N2-F	TTACAAAACATTGGCCGCAAA	22.5	
	Primer Reverse	2019-nCoV_N2-R	GCGCGACATCCGAAGAA	22.5	
	Probe	2019-nCoV_N2-P	FAM-ACAATTTGCCCCAGCGCTTCAG-BHQ	22.5	
RNase P	Primer Forward	RP-F	AGATTTGGACCTGCGAGCG	54.3	(Corman et al., 2020)
	Primer Reverse	RP-R	GAGCGGTGTCTCCACAAGT	53.2	
	Probe	RP-P	FAM-TTCTGACCTGAAGGCTCTGGCGG-BHQ	12.8	

Table 2. Single-target RT-qPCR results for SARS-CoV-2 detection.

Sample	Collection/ Process Date	Reagent	E	ORF8	N1	N2	RNAse P	Sample	Collection/ Process Date	Reagent	E	ORF8	N1	N2	RNAse P
02	25/10/2020	Comm	41.884	44.458	27.527	27.471	28.500	03	27/10/2020	Comm	23.300	24.131	27.904	28.130	28.316
	18/11/2020	Desn	39.498	59.736	28.837	28.598	28.763		19/11/2020	Desn	24.518	24.539	27.489	28.017	28.900
07	03/12/2020	dNTPs	35.338	16.999	28.278	31.674	27.675	10	04/12/2020	dNTPs	25.556	25.851	27.616	29.531	28.090
	04/12/2020	Comm	---	20.662	Not determined	24.383	28.827		17/12/2020	Comm	14.417	---	Not determined	Not determined	27.623
11	05/12/2020	Desn	36.554	23.382	24.195	24.195	30.331	12	06/12/2020	Desn	21.025	---	Not determined	---	27.188
	11/12/2020	Comm	20.806	19.893	Not determined	---	32.268		17/12/2020	Comm	29.291	26.454	Not determined	---	27.776
14	14/12/2020	Desn	35.808	33.855	---	---	27.518	15	14/12/2020	Desn	25.257	---	---	---	28.202
	19/12/2020	Comm	7.970	10.416	Not determined	Not determined	30.167		20/12/2020	Comm	44.182	---	Not determined	Not determined	27.994
16-21	14-18/12/2020	Desn	21.082	4.873	---	---	31.051	22	18/12/2020	Desn	---	---	---	---	25.933
	16-18/12/2020	Comm	Similar results to sample 15	---	---	---	+++		27/12/2020	Comm	20.559	---	Not determined	32.003	26.813
23	18/12/2020	Desn	24.533	---	Not determined	33.795	29.757	24	21/12/2020	Desn	---	26.017	Not determined	---	31.614
	27/12/2020	Comm	---	25.500	---	34.371	34.371		27/12/2020	Comm	17.016	---	Not determined	22.325	26.014
		Desn	---	---	---	---	---			Desn	---	17.192	31.312	31.392	

E, ORF8, N1-N2 and RNAse P = Primers and probes to WHO, this work, N gen from CDC and Universal control, respectively.

Values correspond to Ct result by RT-qPCR.

Dot line = Result not generated.

Comm = Commercial reagent.

Desn = Denaturalization reagent.

dNTPs = dNTPs reagent.

+++ = Positive result.

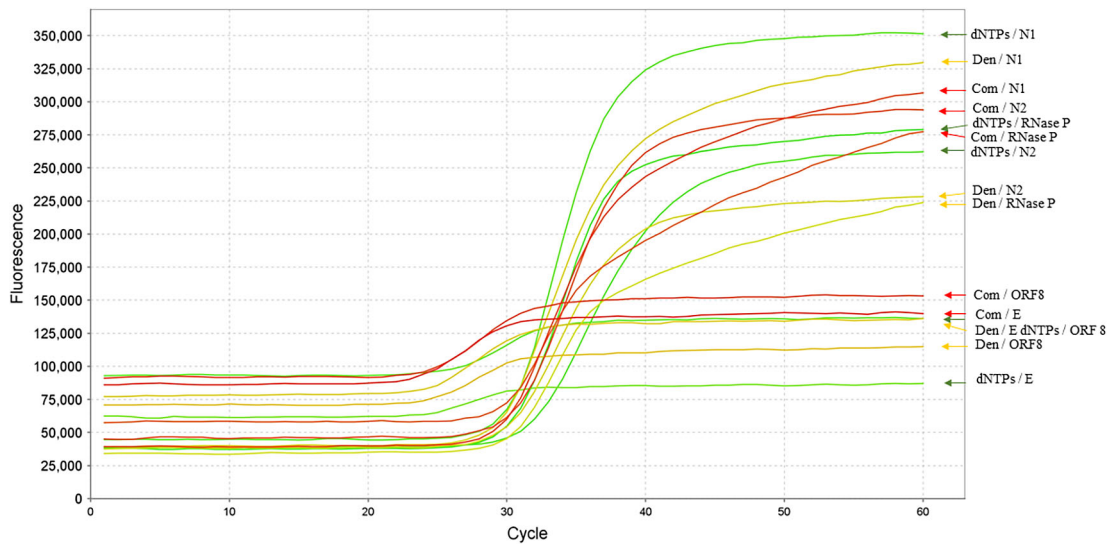


Figure 3. RT-qPCR amplification curves for SARS-CoV-2 detection in patient-derived samples. RNA extracted from patient samples was analyzed using primers and probes targeting the *E* gene (WHO protocol), *ORF8* (this study), and the *N* gene (N1–N2; CDC protocol). Green, yellow, and red arrows indicate amplification curves obtained using denaturing solution (Den), dNTP-adjusted conditions, and commercial kits, respectively. The *RNase P* gene was used as an internal control.

A decrease in amplification performance was observed in samples collected from December onward. This effect was first detected in the *E* gene system, followed by the N2 system and subsequently *ORF8*. This pattern was evaluated using ten samples collected between December 14 and 21, 2020. The observed results were classified into three outcomes depending on the detection system: (1) positive results were obtained following the manufacturer's protocol with the addition of the denaturing solution; (2) positive results were obtained only in the presence of the denaturing solution; and (3) positive results were obtained only in the absence of the denaturing solution (Table 2).

In silico estimation of *E*, *ORF8*, and *N* RT-qPCR primer and probe combinations

Analysis of global SARS-CoV-2 mutation patterns using GenBank and GISAID databases showed that the number of mutations increased from March 2020 onward relative to the reference genome (GenBank accession: NC_045512.2). This trend was particularly evident in the quencher and forward primer regions of the *ORF8* and *N* genes. The number of potential primer and probe combinations also increased over time, with a marked rise observed from November onward in the GISAID dataset. Higher values were observed in December. For example, the *ORF8* system and the N1 set exhibited 3.4×10^{11} and 1.7×10^{12} possible combinations, respectively, whereas the N2 set and the *E* gene exhibited 1.4×10^5 and 32 combinations (Figure 1).

Multiplex RT-qPCR

Multiplex RT-qPCR controls using the *E*, *ORF8*, and *N* (N1) systems with positive samples 02 and 03 produced amplification profiles consistent with detection. Similar patterns were observed in samples 05 and 06 collected in October 2020. Under the same reaction conditions, the addition of the denaturing solution was associated with decreased Ct values in samples collected in October, whereas increased Ct values were observed in samples collected in November. In contrast, the dNTP solution exhibited an opposite trend (Table 3, Figure 4).

The influence of the denaturing and dNTP solutions was further evaluated using nine samples collected between December 4 and 7, 2020, and January 14 and 15, 2021, using the N1–N2 primers and probe described by the CDC. Six samples exhibited the same pattern described above, whereas three samples tested negative based on both amplification curves and Ct values (Table 3, Figure 5). The multiplex RT-qPCR assay detected SARS-CoV-2 in samples collected between January 19 and 29, 2021 (Table 3).

Sensitivity, specificity, and predictive values of SARS-CoV-2 detection

Under the predefined Ct-based classification framework, the denaturing solution was associated with higher apparent sensitivity values than the commercial solution in single RT-qPCR assays, whereas the commercial solution showed higher apparent specificity values. In multiplex RT-qPCR assays, no major differences in apparent sensitivity were observed between commercial and denaturing conditions.

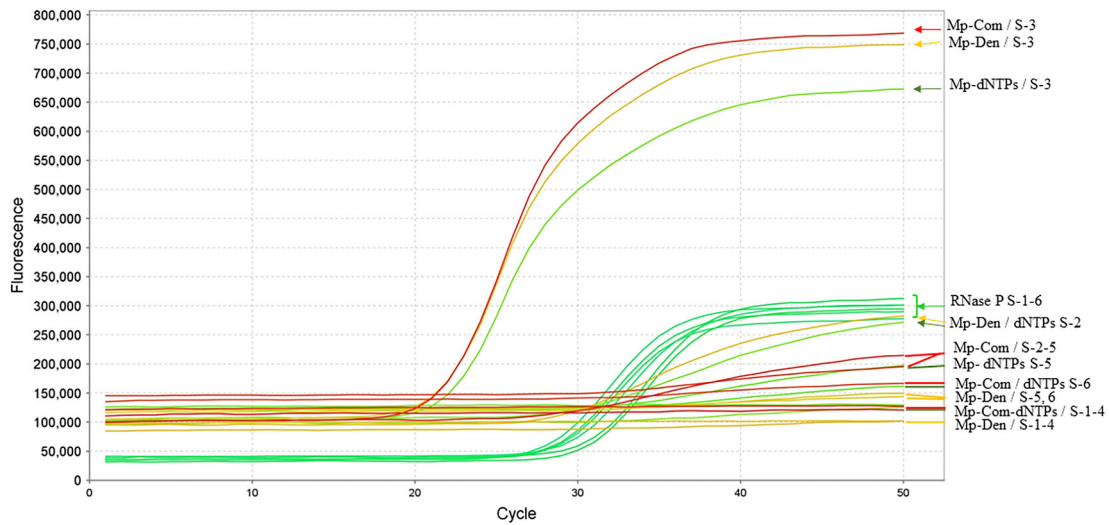


Figure 4. Multiplex RT-qPCR (Mp) amplification curves for SARS-CoV-2 detection using *E*, *ORF8*, and *N* (N1) targets. RNA samples from six patients are indicated by their corresponding identifiers. For each curve, the first number in the nomenclature corresponds to the upper panel, and the second to the lower panel. The *RNase P* gene was used as an internal control. Arrows and nomenclature are consistent with those described in Figure 3.

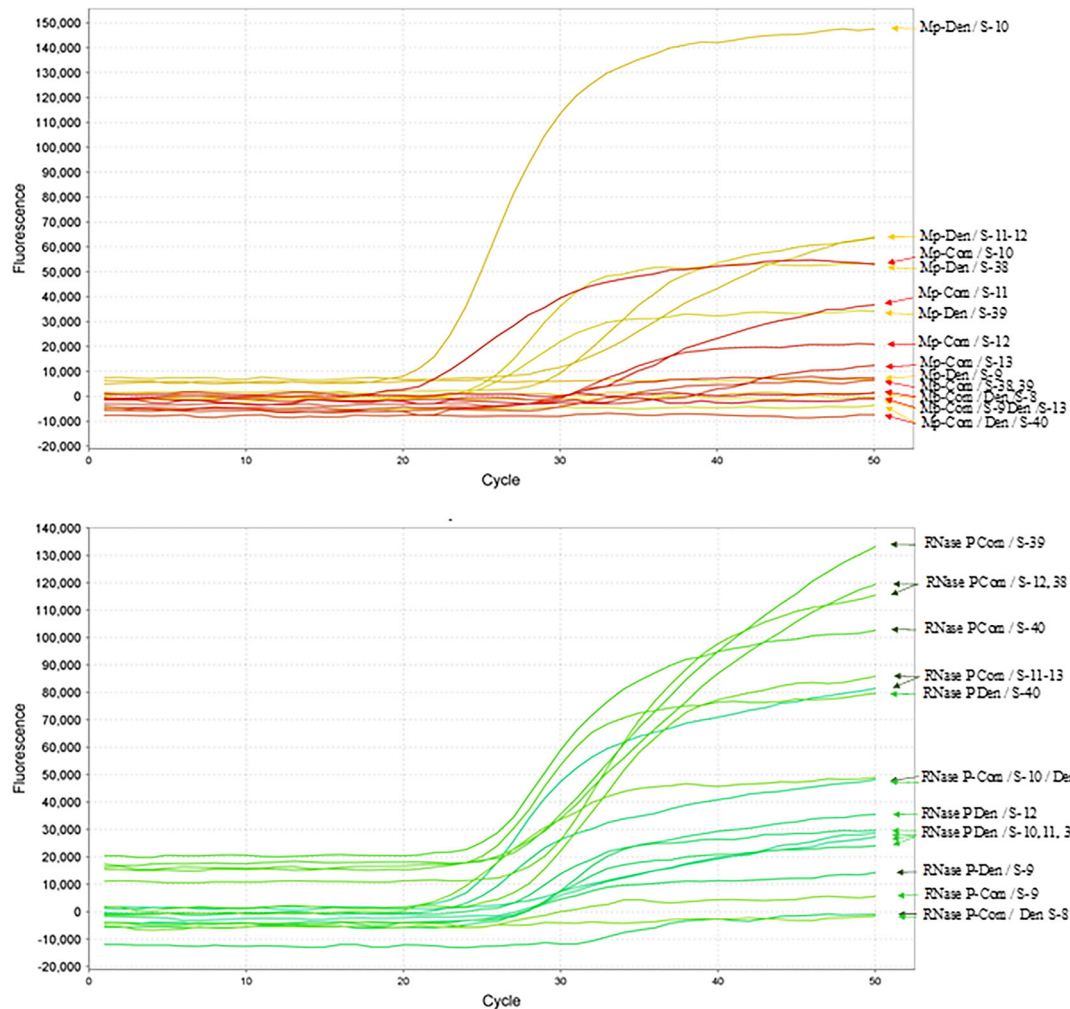


Figure 5. RT-qPCR Multiplex (Mp) curves for the detection of SARS-CoV-2 using the *N* gene (N1-N2). CDC-described primers and probes were used in combination with the denaturing reagent. The RNA samples from 9 patients are indicated with the respective numbers. The multiplex curves corresponding to N1-N2 are indicated at the top, whereas the results corresponding to the *RNase P* primers and probe used as controls are indicated below. The abbreviations and nomenclature are the same as in Figure 3.

Table 4. Exploratory Ct-based performance metrics for single-target and multiplex RT-qPCR assays under different reagent conditions.

Calculation of sensitivity and specificity for single RT-qPCR					
Comm			Desn		
	Event	No Event		Event	No Event
Event	6	2	Event	2	8
No Event	8	1	No Event	0	0
Kappa:	-0.1333		Kappa:	0	
McNemar's Test P-Value:	0.1138		McNemar's Test P-Value:	0.01333	
Sensitivity:	0.4286		Sensitivity:	1.0	
Specificity:	0.3333		Specificity:	0.0	
Pos Pred Value (PPV) :	0.7500		Pos Pred Value (PPV) :	0.2	
Neg Pred Value (NPV) :	0.1111		Neg Pred Value (NPV) :	NaN	
Prevalence:	0.8235		Prevalence:	0.2	
Calculation of sensitivity and specificity for multiplex RT-qPCR					
Comm			Desn		
	Event	No Event		Event	No Event
Event	16	4	Event	15	2
No Event	2	0	No Event	5	0
Kappa:	-0.1379		Kappa:	-0.1493	
McNemar's Test P-Value:	0.6831		McNemar's Test P-Value:	0.4497	
Sensitivity:	0.8889		Sensitivity:	0.7500	
Specificity:	0.0000		Specificity:	0.0000	
Pos Pred Value (PPV) :	0.8000		Pos Pred Value (PPV) :	0.8824	
Neg Pred Value (NPV) :	0.0000		Neg Pred Value (NPV) :	0.0000	
Prevalence:	0.8182		Prevalence:	0.9091	

In multiplex reactions, specificity values were 0.0 under both evaluated conditions in this dataset. Therefore, these metrics should be interpreted as exploratory descriptors of assay behavior under the defined experimental conditions rather than as evidence of clinical diagnostic performance. The dNTP conditions were not included in this analysis because the number of evaluable positive results was limited (Table 4).

Performance metrics were calculated using predefined Ct-based operational criteria for internal comparative analysis and should not be interpreted as externally validated diagnostic accuracy estimates.

Discussion

Since the first report of the SARS-CoV-2 and the onset of the pandemic, public health strategies have emphasized the need for rapid, cost-effective, and accurate diagnostic tools capable of processing large numbers of samples. Numerous RT-qPCR-based diagnostic systems have been developed targeting SARS-CoV-2 marker genes (Ruhan *et al.*, 2020; Nalla *et al.*, 2020). However, similar to the Berlin protocol (Corman *et al.*, 2020), several of these approaches require sequential reactions, which may prolong diagnostic workflows and limit efficiency.

To our knowledge, this study represents one of the first approaches to evaluate the use of three gene targets both independently and in multiplex RT-qPCR reactions for SARS-CoV-2 detection. This strategy combined primers and probes from the *E* gene (Berlin protocol), the *N* gene (CDC protocol), and the *ORF8* gene, together with reagents designed to reduce RNA secondary structures and account for nucleotide composition variability. These modifications were associated with changes in Ct values under the evaluated experimental conditions.

High-Performance Computing (HPC) analyses enabled large-scale SARS-CoV-2 genome alignment and database curation using publicly available sequences from GISAID and GenBank up to December 31, 2020 (Zhu *et al.*, 2020). This approach supported primer and probe design as well as *in silico* evaluation. However, the compiled database may not fully represent the global distribution of viral variants, as sequencing capacity varies across regions.

The use of a global genomic dataset also enabled the prediction of RNA and cDNA secondary structures associated with amplified regions, informing primer and probe design. Additionally, this approach supported the formulation of denaturing solutions and nucleotide compositions that were associated with changes in Ct values under the evaluated conditions (Kovarova & Draber, 2000). These factors may contribute to differences in RT-qPCR assay performance within the experimental framework of this study.

The reagents and methods evaluated showed Ct values comparable to those reported in previous studies (Arakawa *et al.*, 2024; Aranha *et al.*, 2021; Chen *et al.*, 2022). However, the proposed approach relies on the use of multiple gene targets and internal controls to ensure detection accuracy. Furthermore, the high mutation rate of SARS-CoV-2 may affect assay performance over time, highlighting the need for continuous updates of genomic databases.

Based on the results obtained, multiplex RT-qPCR represents a potential approach to address some of the limitations described. However, no consistent differences in Ct values were observed across all samples when compared with commercial methods. Additionally, the implementation of multiplex assays requires multiple fluorescent dyes and specialized equipment, which may increase operational costs. Despite these considerations, combining multiple gene targets may increase the likelihood of detecting a signal under specific conditions. The modular nature of the proposed approach allows flexibility in selecting gene targets according to diagnostic requirements.

Another advantage of multiplex RT-qPCR is the possibility of analyzing multiple samples from a single patient (Grobe *et al.*, 2021). Although preliminary studies suggest that sampling at different time points may improve detection consistency, this approach requires additional clinical monitoring to optimize its implementation.

Taken together, these findings suggest that integrating HPC-based design, primer and probe optimization, and RT-qPCR protocols may support improvements in SARS-CoV-2 detection workflows under the evaluated conditions.

Limitations

This study has several limitations. The sample size was constrained by logistical factors and low participation rates during the pandemic, including reluctance to undergo additional sampling procedures (McElfish *et al.*, 2021), as well as compliance with legal and ethical requirements. Consequently, only participants who provided informed consent were included.

Additionally, sample classification was based on Ct threshold criteria without independent external validation. Therefore, the reported performance metrics reflect comparative assay behavior under the evaluated conditions rather than absolute diagnostic accuracy.

These limitations should be considered when interpreting the results. Further studies incorporating larger datasets and additional validation approaches are required to assess the broader applicability of the proposed methodology. In addition, clinical characterization of participants, including symptom severity and extent of infection, was not available for analysis because this information was protected under the informed consent framework and applicable legal restrictions.

Conclusions

The results of this study suggest that the incorporation of denaturing solutions and the adjustment of nucleotide proportions may influence RT-qPCR performance under the evaluated experimental conditions. These effects were observed in both single and multiplex assays, although variability was detected across samples.

The integration of computational approaches, including HPC-based genome analysis, with primer and probe design provided a framework to evaluate the potential impact of RNA secondary structure and nucleotide composition on assay performance.

Given the exploratory nature of this study and the limited sample size, these findings should be interpreted within the context of the experimental design. Additional studies incorporating larger datasets and independent validation strategies will be necessary to determine the broader applicability of the proposed approach.

Data availability

Underlying data

This genomic sequence is available in GenBank:

GenBank: Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome. Accession number NC_045512; https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.

Extended data

All genomic data used in this study were obtained from publicly available databases, including GenBank and GISAID. The sequences analyzed, along with their corresponding alignments, are available in the associated Zenodo repository:

Zenodo: Denaturing and dNTPs reagents improve SARS-CoV-2 detection *via* single and multiplex RT-qPCR. <https://doi.org/10.5281/zenodo.6337537> (Cadena-Caballero *et al.*, 2022).

This repository contains:

- SARS-CoV-2 genomic sequences obtained from GenBank and GISAID databases.
- Multiple sequence alignments used for primer and probe design.

Additionally, all scripts used for sequence processing and analysis are publicly available on GitHub:

- <https://github.com/drueaplata/bio>.
- <https://github.com/GenomicUIS/Sensitivity-specificity-PPV-and-NPV-for-SARS-CoV-2>

These scripts were used for downloading, filtering, and aligning genomic sequences, including the removal of undetermined nucleotides (“N”) from the datasets, as well as for statistical analysis.

All data and resources are openly accessible and available under the terms of the [Creative Commons Zero](https://creativecommons.org/licenses/by/4.0/) (CC0 1.0) Public Domain Dedication, ensuring full transparency and reproducibility of the analyses performed in this study.

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? **Juan Sebastian Quintero Barbosa** 

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This manuscript presents an interesting exploratory study on the optimization of SARS-CoV-2 RT-qPCR detection through in silico design of an ORF8-targeted system and the evaluation of modified reaction conditions in both single and multiplex assays. The topic is relevant, and the integration of computational design with experimental testing in clinical samples is a clear strength of the work. Overall, I believe the study has value as a methodological contribution, but several aspects would benefit from clarification and a more cautious interpretation. For this reason, I responded “Partly” to all six evaluation questions.

For the question of whether the work is clearly and accurately presented and cites the current literature, I selected Partly because the manuscript is generally understandable and addresses a relevant body of literature, but some statements are stronger than the data support and some sections would benefit from clearer wording and greater precision.

For the question of whether the study design is appropriate and the work technically sound, I selected Partly because the overall concept is appropriate and the study includes both in silico and experimental components, but the validation is relatively limited and does not fully support the strength of some of the claims made by the authors.

For the question of whether sufficient details of methods and analysis are provided to allow replication, I selected Partly because the manuscript includes useful methodological information, but some aspects still require clarification, particularly how sample-level classifications were assigned and how the different assay formats were compared.

For the question of whether the statistical analysis and its interpretation are appropriate, I selected Partly because the authors provide performance metrics and comparative analyses, but the interpretation should be more cautious. In particular, the basis for sensitivity, specificity, and related measures should be explained more clearly, and their limitations should be acknowledged

more explicitly.

For the question of whether all source data underlying the results are available to ensure full reproducibility, I selected Partly because the manuscript makes an effort toward transparency, but I was not fully convinced that all underlying information needed for complete reproducibility is presented in a sufficiently clear and accessible manner.

For the question of whether the conclusions are adequately supported by the results, I selected Partly because the study provides promising findings, but the conclusions regarding improved diagnostic performance, especially in multiplex RT-qPCR, seem somewhat broader than what the current dataset can firmly support.

In summary, I consider this a useful exploratory study with methodological interest, but I recommend clearer presentation of the methods, more cautious interpretation of the performance analyses, and moderation of the conclusions.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology, immunology, vaccine research, molecular biology, and experimental assay evaluation. My assessment is primarily focused on study design, methodological clarity, data interpretation, and whether the conclusions are adequately supported by the results.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 31 Mar 2026

Francisco Martinez-Perez

Dear Reviewer 2,

We sincerely thank you for your careful and thoughtful evaluation of our manuscript. We appreciate your recognition that the study has methodological value and that the integration of *in silico* design with experimental testing in patient-derived samples represents a strength of the work. We have carefully revised the manuscript in response to your comments and observations.

Below we summarize the principal revisions made to the manuscript in relation to your concerns.

1. Clarity of presentation and wording

Comment: You indicated that the manuscript addresses a relevant topic, but that some statements were stronger than the data support and that several sections would benefit from clearer wording and greater precision.

Response: We revised the Abstract, Introduction, Results, Discussion, and Conclusions to improve clarity, precision, and overall consistency throughout the manuscript. We also moderated statements that could be interpreted as overstating the findings. In particular, the study is now explicitly framed as an exploratory and methodological contribution, and the wording of the conclusions has been revised to avoid implying clinical validation or broad diagnostic superiority.

2. Study design and technical soundness

Comment: You considered the overall concept appropriate, but noted that the validation was limited and did not fully support some of the stronger claims made in the manuscript.

Response: We agree with this observation and revised the manuscript accordingly. The study is now more clearly described as exploratory in nature. In addition, we added a dedicated Limitations section to explicitly acknowledge the restricted sample size, the context of sample acquisition during the COVID-19 pandemic, and the absence of independent external validation. These changes were introduced to better align the interpretation of the results with the actual scope of the study.

3. Methodological clarity and reproducibility

Comment: You requested clarification regarding sample-level classification and the comparison of different assay formats, and also noted that the information required for reproducibility was not sufficiently clear and accessible.

Response: We substantially revised the Methods section to clarify these points. In particular, we added and reformulated the subsection now entitled "Exploratory Ct-based performance analysis of SARS-CoV-2 RT-qPCR assays." In this section, we explain that the

performance estimates were derived from predefined Ct-based operational criteria applied uniformly across assays, and that these criteria were used solely for internal exploratory comparison. We also clarified how Ct values were pooled and evaluated at the sample level for both single and multiplex assays.

We additionally revised the Data Availability section to more clearly describe the genomic datasets, sequence alignments, and scripts used in the analyses, and to improve transparency regarding the public repositories associated with these resources. These revisions were intended to facilitate reproducibility within the scope of the study.

4. Statistical analysis and interpretation

Comment: You noted that the interpretation of sensitivity, specificity, and related metrics should be more cautious, and that their basis and limitations should be explained more explicitly.

Response: We carefully revised both the Methods and Results sections in response to this concern. The Ct-based performance analysis is now explicitly presented as exploratory and internal, rather than as a measure of absolute diagnostic accuracy. In the Results section, these values are now described as apparent comparative metrics under a predefined Ct-based classification framework. We also added explicit language stating that, because no independent external clinical validation was available, these metrics should not be interpreted as evidence of clinical diagnostic performance.

5. Conclusions

Comment: You considered that the conclusions, particularly those related to improved diagnostic performance in multiplex RT-qPCR, were broader than what the dataset could firmly support.

Response: We revised the Discussion and Conclusions to moderate these statements. The manuscript no longer presents the findings as demonstrating clinical diagnostic superiority. Instead, the conclusions now emphasize that the evaluated denaturing and dNTP-based conditions were associated with changes in RT-qPCR behaviour under the experimental conditions tested, and that additional studies with larger datasets and independent validation would be required to assess broader applicability.

6. Additional clarification regarding study scope and clinical information

Response: To further clarify the context of the study, we now explicitly state in the manuscript that this was not a clinical validation study and that access to participant clinical characterization, including symptom severity and extent of infection, was restricted by the informed consent framework and applicable legal requirements. This clarification was added to the Limitations section to explain why these data were not available for further analysis.

We are grateful for your constructive comments, which helped us improve the manuscript

substantially. We believe that the revised version is clearer, more methodologically transparent, more cautious in its interpretation, and better aligned with the exploratory scope of the work.

Sincerely,

Francisco Martinez-Perez
On behalf of all authors
Industrial University of Santander

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 16 May 2022

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Andrew D. Beggs

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The authors have set out to design a new primer set to target the ORF8 gene region of the SARS-CoV-2 genome, as well as understand the effect of denaturing agents on the efficiency of detection by reducing secondary structures and increasing RT-QPCR. It is certainly an interesting approach. However I have a few comments and suggestions to further improve the manuscript

1. The abstract is confusing. They state that they design ORF8 primers using E and N as targets but what they actually mean is that they use these as references. Also they state that the denaturing agents reduce secondary structure and increase efficiency but don't really get the results of this across in the abstract
2. They talk in detail about the characterisation in the GISAID/GenBank databases, but don't explain well that the reason they are doing this is to help design the ORF8 gene probes. This should be clarified.
3. The primer/probe concentrations are not given but should be to increase the reproducibility of the experiments
4. A 2xn table of the primer sets with a set threshold in positive/negative and quoted

sensitivity, specificity etc would greatly aid interpretation of the data

5. The authors state in the discussion that "our study is the first to demonstrate the use of three genes both independently or in multiplex reactions to detect the SARS-CoV-2 virus" - the Thermo Taqpath assay uses ORF, N, and S genes for detection and so is multiplex.
6. In the discussion of their analysis of global SARS-CoV-2 mutations they discuss "thousands of millions" of mutations. This makes it seem like there have been this number of mutations rather than reports of mutations, as there will be genomes submitted that have overlapping mutations between genomes. Some clarification on language would be useful.
7. In addition a discussion of why they chose to target the ORF8 gene would be useful. I assume it is because of the relatively low mutational rate here, but it would be nice to see this explained.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: SARS-CoV-2 genetics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Mar 2024

Francisco Martinez-Perez

Reviewer 1.

Andrew D. Beggs
Institute of Cancer and Genomic Sciences,
University of Birmingham, Birmingham, UK.

The authors have set out to design a new primer set to target the *ORF8* gene region of the SARS-CoV-2 genome, as well as understand the effect of denaturing agents on the efficiency of detection by reducing secondary structures and increasing RT-qPCR. It is certainly an interesting approach. However, I have a few comments and suggestions to further improve the manuscript.

Response: We greatly appreciate the comments, suggestions and corrections made by reviewer 1, which greatly enriched the work.

1. The abstract is confusing. They state that they design *ORF8* primers using *E* and *N* as targets but what they actually mean is that they use these as references. Also, they state that the denaturing agents reduce secondary structure and increase efficiency but don't really get the results of this across in the abstract.

Response: The correction is accepted. The abstract of the manuscript is modified and expanded, providing clarity in the methods and results sections.

Abstract

Background: The COVID-19 pandemic, caused by the SARS-CoV-2, can be effectively managed with diagnostic tools such as RT-qPCR. However, it can produce false-negative results due to viral mutations and RNA secondary structures from the target gene sequence.

Methods: With High Performance Computing, the complete SARS-CoV-2 genome was obtained from the GenBank/GISAID to generate consensus sequences to design primers/probes for RT-qPCR. *ORF8* gene was selected, meanwhile, *E* and *N* and *RNAse P* were according to CDC protocol. Nasopharyngeal swab samples were collected from patients diagnosed with SARS-CoV-2. Total RNA was purified according MagMAX kit, it was used in single, and multiplex RT-qPCR. To avoid templated secondary structures, compensate nucleotide proportions and improve Ct values, a solution composed of tetraethylammonium chloride and dimethyl sulfoxide and other with corresponding to dNTPs proportions in accordance SARS-CoV-2 genome were included. Sensitivity and specificity according to Ct values were determined with the Caret package in R software.

Results: 126,576 SARS-CoV-2 genomes from January to December 2020 comprised a database. From this, a region near of 5' *ORF8* gene showed three stem-loops was used for primers/FAM-probe. 49 samples were obtained, from them, 22 were positive to gene selected regions. Interestingly, samples from October to November 2020 were positive for all regions, however, in January 2021 different results were observed in *ORF8*. An improvement in Ct with the adjuvant solutions was determined in all samples with others SARS-CoV-2 primers/probes, for both single and multiplex RT-qPCR. The inclusion of the denaturing solution in single RT-qPCR increased its sensitivity with respect to the commercial method, while in multiplex the opposite was generated.

Conclusions: Including adjuvant solutions to prevent the formation of RNA secondary structures and the adjustment of the nucleotide ratios of SARS-CoV-2 improved single and

multiplex RT-qPCR for viral identification, demonstrating its potential application in health public.

2. They talk in detail about the characterization in the GISAID/GenBank databases, but don't explain well that the reason they are doing this is to help design the ORF8 gene probes. This should be clarified.

Response: Clarity is provided in the methods to use of databases for ORF8 design.

Due to the appearance of mutations in several genes of the SARS-CoV-2 reference genome that could generate false negatives with the primers and probes authorized for identification by RT-qPCR. A region coding for the accessory protein ORF8 was selected, hypothesizing that it did not have a high mutation frequency. Therefore, monthly consensus sequences were generated to determine its identity pattern with respect to new genomes.

3. The primer/probe concentrations are not given but should be to increase the reproducibility of the experiments.

Response: The concentration in nmole of the primers/probes is reported below. It could also be included in Table 1.

4. A 2xn table of the primer sets with a set threshold in positive/negative and quoted sensitivity, specificity etc. would greatly aid interpretation of the data.

Response: The calculation of the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the viral identification tests of the participants was included in the manuscript.

Methodology

Calculation of sensitivity, specificity and predictive values of SARS-CoV-2.

To calculate the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of each of the reactions with the single and multiplex primers used, a script was implemented in R using the Caret package to calculate the values generated by each RT-qPCR with their respective primers/probes (<https://github.com/GenomicUIS/Sensitivity-specificity-PPV-and-NPV-for-SARS-CoV-2.git>) (Kuhn, 2008; R Core Team 2020). Cycle threshold (Ct) was defined for each of the primers in the single reactions of each of the processed samples, where, the value of 18-35 Ct was considered true positive, Ct 35 > false positive, Ct 18 < true negative. Subsequently, the Cts values obtained were pooled and evaluated per sample. Therefore, if the result generated by a set of primers/probes was at the above-mentioned threshold, it was considered a true positive identification. Whereas, results outside the threshold were considered false negative and indeterminate. Cts were identical for multiplex RT-qPCR reactions, but the results were evaluated as a whole.

R Core Team. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. 2020; Vienna, Austria. URL <https://www.R-project.org/>.

Kuhn M: Building Predictive Models in R Using the caret Package. *J Stat Softw.* 2008; 28(5): 1–26. Doi: 10.18637/jss.v028.i05

Results

Sensitivity, specificity and predictive values of SARS-CoV-2

The performance of single and multiplex RT-qPCRs with the solutions used showed that single denaturing was more sensitive than the commercial solution, but the latter is 33% more specific than the former. As for the multiplex reactions, the commercial solution showed non-significant difference in sensitivity with respect to the denaturing solution. Similarly, the specificity of the single reactions was better for the commercial solution with respect to the denaturing solution, but in the multiplex reactions this difference was not evident in the detection of true negatives as both solutions yielded results of 0. dNTPs were not evaluated due to the number of positive results (Table 4).

Discussion

The reagents and methods described herein allow for an immediate, facile, and cost-effective detection of the SARS-CoV-2 virus; which was demonstrated by comparing the Cts of the positive results used to determine sensitivity and specificity with those published for other countries (Arakawa *et al.*, 2024; Aranha *et al.*, 2021; Chen *et al.*, 2022).

Arakawa Y, Nishida Y, Sakanashi D, *et al.*: Clinical evaluation of a modified SARS-CoV-2 rapid molecular assay, ID NOW™ COVID-19 2.0. *J. Infect. Chemother.* 2024; S1341-321X(24): 00073-4. Doi: 10.1016/j.jiac.2024.02.032.

Aranha C, Patel V, Bhor V, *et al.*: Cycle threshold values in RT-PCR to determine dynamics of SARS-CoV-2 viral load: An approach to reduce the isolation period for COVID-19 patients. *J. Med. Virol.* 2021; 93(12): 6794-6797. Doi: 10.1002/jmv.27206.

Chen YY, Shen X, Wang YJ, *et al.*: Evaluation of the cycle threshold values of RT-PCR for SARS-CoV-2 in COVID-19 patients in predicting epidemic dynamics and monitoring surface contamination. *J. Infect. Public. Health.* 2022; 15(12): 1494-1496. Doi: 10.1016/j.jiph.2022.11.012.

Based on our findings, we concluded that multiplex RT-qPCR is an optimal solution for the aforementioned limitations, even though the Cts used to establish sensitivity and specificity did not show a difference in some samples between commercial methods compared to the one proposed here.

5. The authors state in the discussion that "our study is the first to demonstrate the use of three genes both independently or in multiplex reactions to detect the SARS-CoV-2 virus" - the Thermo Taqpath assay uses ORF, N, and S genes for detection and so is multiplex.

Response: To our knowledge, at the time the experiments for this study were conducted (19 September 2020 to 30 January 2021), there was no kit that performed viral identification with three genes.

According to the official website of Thermo Fisher Scientific, the Taqpath kit is based on sequences obtained from GenBank and GISAID as of 7 May 2021 (<https://www.thermofisher.com/co/en/home/clinical/clinical-genomics/pathogen-detection-solutions/covid-19-sars-cov-2/multiplex.html>), and subsequently obtained a letter of approval for use by the Food and Drug Administration (FDA) on 18 May 2022 (<https://www.fda.gov/media/136113/download>). Our public database was generated by HPC between January and December 2020 (<https://zenodo.org/records/6337537>).

6. In the discussion of their analysis of global SARS-CoV-2 mutations they discuss "thousands of millions" of mutations. This makes it seem like there have been this number of mutations rather than reports of mutations, as there will be genomes submitted that have overlapping mutations between genomes. Some clarification on language would be useful.

Response: The correction is accepted, and clarity is given in the manuscript.

Our analysis of the global SARS-CoV-2 mutation patterns based on the GenBank and GISAID databases indicated that most mutations began in March 2020, and subsequently increased significantly, relative to the SARS-CoV-2 reference genome (GenBank access: NC_045512.2).

7. In addition, a discussion of why they chose to target the *ORF8* gene would be useful. I assume it is because of the relatively low mutational rate here, but it would be nice to see this explained.

Response: Correction accepted.

Using the SARS-CoV-2 (NC_045512) reference genome and consensus sequences from January to April 2020 (Zhu et al., 2020), an approximately 150-base pair (bp) region of the *ORF8* gene was selected based on the secondary structure of its RNA transcript, which in turn was predicted using the algorithms proposed by Zuker (Zuker & Jacobson, 1998) via the Mfold software (RRID:SCR_001360) (Zuker, 2003). Thus, the last set containing the codons of the central region of the *ORF8* gene, which encodes the secretion protein *ORF8*, was chosen because it allows proper viral adhesion to the host cell (Chan et al., 2020).

Chan JF, Kok KH, Zhu Z, *et al.*: Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg. Microbes. Infect.* 2020; 9(1): 221-236. DOI: 10.1080/22221751.2020.1719902.

Competing Interests: No competing interests were disclosed.

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