Diagnostic Performance and Clinical Feasibility of a Novel One-Step RT-qPCR for Detecting Multiple Severe Acute Respiratory Syndrome Coronavirus

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Abstract

The pandemic coronavirus disease 2019 (COVID-19) is characterized as an acute respiratory infection in the majority of cases and is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, other coronaviruses (CoVs) can infect humans, although the majority only cause mild respiratory symptoms. As early diagnosis of SARS-CoV-2 is critical to prevent further transmission events and to improve clinical outcomes, it is important to be able to distinguish SARS-CoV-2 from other CoVs in respiratory samples. Therefore, we developed and evaluated a novel quantitative reverse transcription PCR (RT-qPCR) assay, targeting the spike (S) and membrane (M) genes, to enable the rapid identification of SARS-CoV-2 including several new circulating variants, and other emerging pan-SARS-like CoVs. Using RNA extracted from cell culture supernatants, our multiple simultaneous SARS-CoV-2 assays confirmed a limit of detection of 1×10^0 TCID₅₀/ml and no cross-reaction with human coronaviruses or other respiratory viruses. We also validated our method using human clinical samples from COVID-19 patients and healthy individuals, including nasal swab and sputum samples. This novel one-step multiplex RT-qPCR assay can be used to improve the laboratory diagnosis of human-pathogenic coronaviruses, including SARS-CoV-2, and may be useful for the identification of other pan-SARS-like CoVs of zoonotic origin.

Introduction

Coronaviruses (CoVs) are single-stranded and positive-sense RNA [10] viruses that belong to the *Sarbecovirus* subgenus of the *Coronaviridae* family, which can infect both animals and humans [7, 8]. The CoVs are further classified into four distinct genera: α -, β -, γ -, and δ -coronavirus. α -, and β -CoVs have been associated with human infection, with seven CoVs known to cause respiratory diseases in humans, including SARS-CoV and MERS-CoV, and SARS-CoV-2, the causative agent of the ongoing pandemic [14, 35]. *Sarbecovirus*, a subgenus of the β -CoVs genus was distinguished from four other subgenera (*Hibe*-, *Nobe*-, *Merbe*- and *Embecovirus*) following the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) human outbreak in southern China in 2002 [35].

In December 2019, cases of pneumonia of unknown origin were reported in Wuhan city, Hubei, China [35]. A novel β -CoV and later, officially named as SARS-CoV-2, was discovered to be the causative of coronavirus disease 2019 (COVID-19) [1]. Next generation sequencing (NGS) analysis of the whole SARS-CoV-2 genome revealed that the virus shares 80–89% identity with SARS-CoV. However, the genome sequence homology of SARS-CoV-2 showed approximately 96% identity to bat-derived SARS-related CoVs, such as BetaCoV/bat/Yunnan/RaTG13/2013, suggesting that SARS-CoV-2 may have originated in a bat species. Therefore, pan-SARS CoV molecular diagnostics are no longer used to detect SARS-CoV-2 and international research groups have been designing specific primers and probes for the detection of SARS-CoV-2 based on the most conserved genes, such as RNA-dependent RNA polymerase (RdRp), envelope (E), or nucleocapsid (N) genes [9]. Initial RT-qPCR assays were based on single target genes, however, assays for detecting SARS-CoV-2 RNA are now available targeting single, double, and/or triple target genes, resulting in complicated diagnostic criteria for positive and negative results [5, 6, 18, 23]. For example, verification of the SARS-CoV-2 positive detection assay requires the detection of all target genes to be positive, including RdRp, N, and E genes [3, 31]. However, primers targeting the RdRp gene for detection of SARS-CoV-2 were found to cross-react with SARS-CoV RNA[3] and some researchers found the sensitivity of RT-qPCR to be approximately 70% for SARS-CoV-2 diagnosis[12], which may be attributed to false-negative results of SARS-CoV-2 infection. These results suggested that although a protocol using RT-qPCR assay for SARS-CoV-2 detection using RdRp, E, and N genes has been established, alternative molecular diagnostic tools have utility for the detection of SARS-CoV-2 with improved sensitivity.

Our goal was to develop a novel RT-qPCR assay to rapidly detect SARS-CoV-2 by targeting the spike (S) and membrane (M) genes, to effectively distinguish this SARS-CoV-2 from other bat-related SARS-like CoVs. The rationale behind this approach was not only to design a highly sensitive and specific primer and probe set for the detection of SARS-CoV-2 but to also produce a tool that would be able to distinguish this virus from other similar zoonotic CoVs, such as those found in the bat reservoir, which would have utility for use in resource-poor regions which may have limited laboratory capacities. The S encoded to a trimeric protein, with each monomer is comprised of an S1 (receptor-binding) and an S2 (membrane-fusion) subunit [15], whilst several amino acid substitutions [29] and deletions [32] have been reported in the S1 subunit of SARS-CoVs, the S2 subunit is conserved as per the other CoVs genes including RdRp, E, and N. As such, the S gene represents a molecular diagnostics target for SARS-CoV-2 detection in nasopharyngeal or throat swabs, as shown [4, 33]. The M is a conservative gene and encode to one of important glycoprotein in maintaining virion size and shape, and assistant of other structure proteins assembly [20]. The M gene is well set up for molecular diagnostics in viral pathogens detection [25, 28], however it not be screening for SARS-CoV-2 detection.

Herein, we developed and assessed the sensitivity and specificity of a RT-qPCR assay targeting the S2 subunits of the S gene and M gene for the simultaneous detection of SARS-CoV-2 in, and Pan-SARS-like viruses and to protect against false negative results. We developed this assay using human and bat-derived CoVs and other respiratory viruses. Notably, we evaluated our one-step multiplex RT-qPCR method to simultaneously detect viruses in human clinical samples including nasal swabs and sputums, thus evaluating its utility in the study of zoonotic CoVs. Overall our novel one-step multiplex RT-qPCR assay was sensitive and specific and showed utility in the specific detection of CoVs across the broad *Sarbecovirus* subgenera and human-pathogenic coronaviruses, including SARS-CoV and SARS-CoV-2.

Materials And Methods

Primer and probe design for RT-qPCR.

To detect multiple CoVs including SARS-CoV-2 using a one-step multiplex RT-qPCR, primers and probes were designed based on the 265 reference of published coronavirus sequences (isolated from 2005 to 2020) in GenBank and Global Initiative on Sharing All Influenza Data (GISAID) database. The sequences were aligned using ClustalW in BioEdit software (version 6.0) and used as a template for the design of the multiplex RT-qPCR. The probes were labelled with FAM (5-Carboxyfluorescein) and HEX (5-hexachloro-fluorescein) for detection of the SARS-CoV-2 and pan-SARS-like CoVs on M gene, respectively. And Cy5 (Cyanine Dye 5) and Texas Red for detection of the SARS-CoV-2 and Pan-SARS-CoVs on S2 domain of S gene, respectively. All primers

and probes were synthesized by Cosmogenetech Inc. (Seoul, Republic of Korea) and detailed information regarding all the designed primers and probes including WHO guidelines [2] are presented in Table 1.

Viruses and viral RNA samples

To evaluate the sensitivity and specificity of our multiplex RT-qPCR, cell culture supernatants from cells infected with coronaviruses and other respiratory viruses were used. In total eighteen different cultured viruses were used. Korean isolated four of SARS-CoV-2 (wild type, alpha variant, beta variant, and delta variant), human coronavirus-NL63 (Korea/CN0601/14), human coronavirus-229E (Korea/KUMC-9), and MERS-CoV (MERS-CoV/KOR/KNIH/002_05_2015) were obtained from the Korea Centers for Disease Control and Prevention (KCDC, Republic of Korea), and other viruses were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) or purchased from the Korea Bank for Pathogenic Viruses (KBPV, Republic of Korea) [16]. The RNA of SARS-CoV (HKU-39849) was provided by Dr. Seungtaek Kim[22] and viral RNA samples of bat-derived SARS-related CoV (GenBank accession number: MK991935, MK991936) from bat fecal samples that were used in the specificity assays described in table 2 were provided by Prof. Hye Kwon Kim[21].

SARS-CoV-2 virus propagation

VERO cells were purchased from the American Type Culture Collection (CRL-1586) and were maintained in Dulbecco's Modified Eagle Medium (DMEM, Corning, NY, USA) containing 5% Fetal Bovine Serum (FBS, Thermo Fisher Scientific, MA, USA) and Penicillin (100 IU/ml, Thermo Fisher Scientific), and incubated at 37 °C in 5% CO₂. For the titration of the SARS-CoV-2 virus, VERO cells were seeded at 1×10^5 cells per well in 96-well-plates for 24 h before inoculation with serial, ten-fold dilutions of SARS-CoV-2. After 72 h incubation, TCID₅₀/ml was determined using the method described by Reed and Muench [24]. All SARS-CoV-2 experiments were conducted at KRIBB (Daejeon, Republic of Korea) under the approval and in accordance with the guidelines of the Institutional Biosafety Committee (IBC, approval number KRIBB-IBC-20200208) of KRIBB. Experimental work with SARS-CoV-2 was conducted in a biosafety level-3 (BL-3) facility at KRIBB (permission number KCDC-HP-19-3-01).

One-step multiplex RT-qPCR analysis

Viral RNA was purified using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, and samples were stored at -80°C until further analysis. Viral RNA of SARS-CoV-2 was extracted in a BL-3 containment facility in KRIBB and all RT-qPCR were conducted in BL-2 facilities at the KRIBB. The RT-qPCR assays were performed using the LightCycler 96 instrument and analysis software (Roche, Switzerland). The one-step RT-qPCR reaction mixture included 5 µl of RNA template, 10 µl of 2X reaction buffer supplemented with the SensiFAST Probe No-ROX One-Step Mix (Bioline, UK), 0.2 µl of reverse transcriptase (Bioline, UK), 0.4 µl of RNase inhibitor (Bioline), 1 µl of each primer (10 pmol), 1 µl of each probe (5 µM), and RNase-free water to add up to a final volume of 20 µl. PCR cycling conditions used were as follows; initial at 45°C for 15 mins and 95°C for 10 mins for the reverse transcription step, followed by 40 cycles at 95°C for 5 s and 58°C for 30 s.

Determination of sensitivity and specificity of the RT-qPCR analysis

To assess the sensitivity of the one-step multiplex RT-qPCR assay, SARS-CoV-2 was serially diluted 10-fold from 10^0 to 10^6 TCID₅₀/ml and then viral RNA was extracted from these samples using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen). The intact viral RNA was then amplified by RT-qPCR. After RT-qPCR, the presence of amplicons was confirmed using 2% agarose gel electrophoresis and Sanger sequencing. To optimize the specificity of one-step multiplex RT-qPCR for SARS-CoV-2 detection, different intact viral RNA templates were used from human coronaviruses (SARS-CoV, wildtype of SARS-CoV-2, three of SARS-CoV-2 variants, MERS-CoV, hCoV-229E and hCoV-NL63), two bat-derived SARS-related coronaviruses, influenza A viruses (A/California/04/09, A/Korea/37/2012) and B virus (B/Brisbane/60/2008); and five other swine/human pathogens, including Porcine epidemic diarrhea virus (PEDV/Korea/SM98), Dengue type 2 (Korea/DENV-2/KBPV-VR-29); Parainfluenza virus type 1 (Korea/KUMC-44); Human Respiratory Syncytial virus (HRSV-A/IC688/12); and Adenovirus type 3 (KUMC-62).

Collection of human clinical samples

Virus-negative nasal swabs and sputum samples were acquired from healthy donors and clinical samples were obtained from COVID-19 patients in the Chungnam National University School of Medicine (Daejeon, South Korea). A total of 67 clinical samples (40 males and 27 females) were used in this study, including 29 cases of laboratory-confirmed COVID-19 that tested positive for SARS-CoV-2 RNA by the RdRp2 assay and 38 cases with healthy donors (ranging in age from 18 to 64 years old). Written informed consent was obtained from all human subjects who participated in this investigation. All samples were collected under the approval of the Institutional Review Board of the National Medical Center, Republic of Korea (IRB number: 2020-03-057). All experiments using human clinical samples were performed following approved guidelines.

Results

Primer and probe design for RT-qPCR

To develop the RT-qPCR assay for detecting various types of coronaviruses, four specific probes targeting the S (S2 domain) and M genes of SARS-CoV-2, and pan-SARS-like viruses were developed. To inform the design, we obtained 265 reference sequences from GenBank and GISAID, which included 200 reference sequences of SARS-CoV-2 worldwide (up to April 5 2021), 15 reference isolates of SARS-CoV and 40 reference sequences of non-human SARS-related viruses isolated in bat and pangolin species (isolated between 2005 to 2019). The SARS-CoV-2, S2 subunit of S gene and M gene may be used for a wider screening to detect other SARS coronaviruses, including SARS-CoV, SARS-CoV-2, and pan-SARS-like viruses from humans and bats. The selection of targeted sequence regions was graphic in Figure 1 using 20 representative strains for SARS-CoV, SARS-CoV-2, and Bat-SARS-related coronaviruses. The most conservative sequences for primers and probes designation of each targets detection were selected (Table 1).

Specificity of the multiplex RT-qPCR

To determine the specificity of the RT-qPCR assay for distinguishing between SARS-CoV-2, SARS-CoV, other human pathogenic coronaviruses and respiratory viruses, we tested the specificity of the assay using 19 viral RNA samples extracted from cultured viruses. These included human and swine coronaviruses, human influenza A (subtype H1N1 and H3N2) and B virus, Dengue virus type 2, parainfluenza virus type 1, human respiratory syncytial virus, and adenovirus type 3. The multiplex RT-qPCR assay showed no cross-reactivity with different viruses in each assay (Table 2). In particular, the SARS coronaviruses and bat-associated SARS-like viruses were successfully detected by the pan-SARS-like CoV probe, whereas the MERS-CoV and other viruses were not detected by our multiplex RT-qPCR assay. Moreover, we confirmed the performance of the RT-qPCR using the viral samples with reference primers and probes targeting the E, and RdRp genes published in the WHO guidelines [2]. These primer/probe sets could also detect four different of SARS-CoV-2 as well as SARS-related coronaviruses from bats (Table 2).

Sensitivity of multiplex RT-qPCR using intact viral RNA

To confirm the sensitivity of the multiplex RT-qPCR assay for SARS-CoV-2, the four types of SARS-CoV-2 virus (wild type, alpha-, beta- and delta-variant) were used for viral genomic RNA extracted from cultured with known titers virus. To compare the sensitivity of each singleplex and multiplex assay, both assays were performed in triplicate in three independent experiments. The efficiency of the singleplex assay was 102.65 % to 105.68% for the individual assays and the limits of detection for all assays was viral RNA sample from 1×10^0 the 50% of tissue culture infective dose (TCID₅₀)/ml of culture supernatants (Figure 2). The performance of the multiplex assay was 97.91 % to 101.95 % for the multiple assays, and the limits of detection for all assays, determined using a standard curve of the Cq values derived from RNA extracted from serially diluted original SARS-CoV-2 viruses [11] was 1×10^0 TCID₅₀/ml (Figure 2). The sensitivity of our multiplex RT-qPCR assay for detecting SARS-CoV-2 (Cq values between 34.15 and 35.32) was similar to that of the WHO reference protocol (Cq values between 34.41 and 36.42) (Table 3). These results suggest that the multiplex RT-qPCR assays were capable of screening for a broader range of coronaviruses while maintaining an analytical sensitivity comparable to that of singleplex assays.

Multiplex RT-qPCR of human clinical samples

To evaluate diagnostic performance of the multiplex assay, we tested 29 clinical samples, including nasal swabs and sputum from previously confirmed COVID-19 patients with respiratory disease exhibiting symptoms including fever, cough and/or respiratory failure, obtained from Chungnam National University Hospital (Daejeon, South Korea) and 38 negative specimens from healthy donors. Asymptomatic patients were not included in this study. As shown in Table 4, all of the specimens from COVID-19 patients tested positive for SARS-CoV-2 (100% sensitivity) and there was no cross-reactivity with any of the other target viruses in our one-step multiplex RT-qPCR assay. We also tested the same samples with the WHO-recommended protocol that uses the RdRp and E gene-specific primer/probe sets. The resulting Cq-value detected with our multiplex assay was comparable to E gene target and approximate 2 log₂ Cq values earlier than RdRp gene target with the WHO recommended RT-qPCR assays. These results suggest that the sensitivity and specificity of the multiplex RT-qPCR assay for SARS-CoV-2 RNA in swabs and sputum was similar to the WHO-recommended protocol.

Discussion

Here, we developed a novel multiplex molecular diagnostic assay based on RT-qPCR targeting the S- and M-gene to distinguish SARS-CoV-2 from other CoVs. The assay was highly sensitive and specific for the detection of SARS-CoV-2 RNA in cultured viruses and clinical samples obtained from COVID-19 patients. Notably, our assay was able to detect other CoVs, including human-pathogenic coronaviruses, SARS-CoV and those from the *Sarbecovirus* subgenus. Overall, our multiplex RT-qPCR assay showed detection of sensitivity and specificity for SARS-CoV-2 RNA with the added advantage of simultaneous detection of emerging coronaviruses in the *Sarbecovirus* subgenus. This gives our assay utility as not only a highly sensitive and specific method with which to detect SARS-CoV-2 but to also detect other potentially zoonotic coronaviruses.

Our approach showed that the CoVs, S and gene M can be a useful target for molecular diagnostics. The S glycoprotein comprises two functional subunits responsible for binding to the host cell receptor (S1 domain), and fusion of the viral and cellular membranes (S2 domain). Therefore, spike glycoproteins mediate the entry of the coronavirus into host cells and regulate the pathogenicity and human-to-human transmission of SARS-CoV [17, 27]. Based on the sequence analysis, S glycoproteins from SARS-CoV-2 have 76% amino acid sequence similarity with SARS-CoV and approximately 80% identity with bat-derived coronaviruses [13, 27, 30], indicating a utility for S glycoproteins as targets for molecular diagnostics that can discriminate between human pathogenic-CoVs and related zoonotic viruses. The M glycoprotein from SARS-CoV-2 is high conservative and shared approximately 90% identity with SARS-CoV and bat-derived coronaviruses. Herein, we designed a novel primer/probe set targeting S and M genes for the detection of SARS-CoV-2, and pan-CoVs, and successfully confirmed its function using human clinical samples from COVID-19 patients as well as several different cultured viruses. Currently, the WHO has announced a list of several RT-qPCR assays to detect SARS-CoV-2 using RNA transcript standards, including ORF1/nsp14, N, E, and RdRp genes. Here we developed and evaluated a one-step multiplex assay for the simultaneous detection by RT-qPCR of human-pathogenic coronaviruses, targeting to the S and M genes. Our goal was to enhance the sensitivity and specificity of detection for the wider screening of *Sarbecoviruses*, including SARS-CoV, SARS-CoV-2, and pan-SARS-like viruses from humans and bats. Compared with the previously established S gene target assays for detecting SARS-CoV-2, our multiplex RT-qPCR assay showed a difference in specificity between positive and negative of 1 to 2 log Cq units using cultured viruses (Supplementary Table 1). In particular, our multiplex RT-qPCR assay was comparable to E gene target and approximate 2 log₂ Cq values earlier than RdRp gene target for the detection of SARS-CoV-2 in respiratory tract clinical specimens and cultured viruses (Figure 3). Overall, our one-step multiplex molecular diagnostic assay provided consistent and reliable results that were comparable with the WHO-guideline based on RdRp and E gene targets diagnostics.

In late 2002, SARS-CoV was discovered in China before the SARS-CoV-2 pandemic. SARS-CoV is an emerging virus that is transmitted rapidly among humans and cross-species transmission between bat and Palm civets [10, 34]. SARS-CoV has been previously assigned to the *Sarbecovirus* subgenus, and it originates

from bat SARS-related coronaviruses (SARSr-CoV) [19, 26]. These reports suggested the possibility of human-to-human transmission and/or multiple spillover events, probably due to multiple events of viral recombination from several SARSr-CoVs in bat species. Therefore, longitudinal surveillance of SARS-CoV in bat species is needed for the prevention of novel coronavirus outbreaks in humans. Our multiplex RT-qPCR assay includes *Sarbecovirus* detection and confirms the applicability of the assay for viral detection in the bat fecal samples.

In summary, we developed a novel one-step multiplex RT-qPCR assay that can be used in clinical laboratories to support the detection of human-pathogenic coronaviruses, including SARS-CoV and SARS-CoV-2 and other potentially related CoVs of the *Sarbecovirus* genus. Thus, our assay would contribute to the public health responses and disease control strategies of countries by providing efficient detection measures, especially to those with limited laboratory capacities.

Declarations

Author Contributions: Designed research and preparation of the manuscript: T.B.L., H.K.K., M.Z., S.S.W., J.A.K., D.G.J., S.W.Y. Performed experiments: T.B.L., M.J.A., V.T.L., S.L., Z.J. Contributed preparation of clinical reagents: H.K.K., P.K.B., S.K., Y.S.K. Drafted the paper: all authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Tables

Table 1. Novel primers and probes for detection of coronaviruses

Primers/Probes		Sequence (5'– 3')	Target	Position	Amplicon size (bp)	Reference
primer/probe set A	Pan-SARS-Forward	GTG CTT GCT GCT GTY TAC AG	Membrane gene	26697	152	EPI_ISL_413017
	SARS-Cov-2-Probe	FAM – CAC CGG TGG AAT TGC TAT CGC – BHQ1		26729		
	Pan-SARS-Probe	HEX – GAA GTA GCT RAG CCA CAT CAA GCC – BHQ1		-26789		
	Pan-SARS-Reverse	GTT TCT GGR TTG AAT GAC CAC A		-26848		
primer/probe set B	Pan-SARS-Forward	ATY AGR GCT GCW GAA ATC AG	Spike gene	24578	218	
	Pan-SARS-Probe	Texas red-GCT TCT GCY AAY CTT GCT GC-BHQ2		24599		
	SARS-Cov-2-Probe	Cy5-ATG AGG TGC TGA CTG AGG GA-BHQ2		-24715		
	Pan-SARS-Reverse	CCW TCA TGA CAA ATD GCW GG		-24795		

Table 2. Specificity analysis of multiplex RT-qPCR assays using known cell culture supernatants.

Virus	Virus Family	Host	Accession Number	Mean <i>ct</i> value (Standard deviation)					
				Multiplex assay				WHO reference	
				Spike target		Membrane target		RdRp	E
				SARS-CoV-2 (Cy5 probe)	Pan-SARS like-CoVs (Texas-red probe)	SARS-CoV-2 (FAM probe)	Pan-SARS like-CoVs (Hex probe)		
Wildtype SARS-CoV -2 (hCoV-19/South Korea/KUMC01/2020)	<i>Coronaviridae</i>	Human	EPI_ISL_413017	15.35 (0.21)	14.03 (0.07)	14.15 (0.13)	14.22 (0.16)	17.31 (0.29)	15.1 (0.45)
Alpha variant SARS-CoV -2 (hCoV-19/South Korea/KDCA0001/2020)	<i>Coronaviridae</i>	Human	EPI_ISL_738139	14.23 (0.19)	14.13 (0.12)	13.36 (0.09)	14 (0.11)	17.42 (0.24)	14.0 (0.21)
Beta variant SARS-CoV -2 (hCoV-19/South Korea/KDCA0463/2020)	<i>Coronaviridae</i>	Human	EPI_ISL_762992	14.49 (0.09)	14.58 (0.14)	14.37 (0.1)	14.88(0.15)	18.57 (0.13)	15.2 (0.22)
Delta variant SARS-CoV -2 (hCoV-19/South Korea/KDCA0464/2021)	<i>Coronaviridae</i>	Human	EPI_ISL_833249	14.64 (0.18)	15.07(0.01)	13.96(0.02)	14.4(0.02)	18.04(0.01)	15.1
SARS-CoV (HKU-39849)	<i>Coronaviridae</i>	Human	AY278491	N.D	14.75 (0.31)	N.D	14.52 (0.16)	17.2 (0.33)	15.3 (0.26)
Bat SARS-related coronavirus (B18-83)	<i>Coronaviridae</i>	Bat	MK991935	N.D	21.92 (0.13)	N.D	22.19 (0.23)	24.88 (0.27)	22.5 (0.17)
Bat SARS-related coronavirus (B18-92)	<i>Coronaviridae</i>	Bat	MK991936	N.D	22.18 (0.38)	N.D	22.32 (0.15)	25.05 (0.18)	21.9 (0.35)
Coronavirus-NL63 (Korea/CN0601/14)	<i>Coronaviridae</i>	Human	MG772808	N.D	N.D	N.D	N.D	N.D	N.D
Coronavirus-229E (Korea/KUMC-9)	<i>Coronaviridae</i>	Human	AY386391	N.D	N.D	N.D	N.D	N.D	N.D
MERS-CoV (Korea/KNIH/002_5_2015)	<i>Coronaviridae</i>	Human	KT029139	N.D	N.D	N.D	N.D	N.D	N.D
PEDV (Korea/SM98)	<i>Coronaviridae</i>	Swine	GU937797	N.D	N.D	N.D	N.D	N.D	N.D
Dengue type 2 (Korea/DENV-2/KBPV-VR-29)	<i>Flaviviridae</i>	Human	KP406804	N.D	N.D	N.D	N.D	N.D	N.D
Human H3N2 Influenza A (Korea/37/2012)	<i>Orthomyxoviridae</i>	Human	KT889146	N.D	N.D	N.D	N.D	N.D	N.D
Human H1N1 Influenza A (California/04/09)	<i>Orthomyxoviridae</i>	Human	GQ117044	N.D	N.D	N.D	N.D	N.D	N.D
Influenza B (B/Brisbane/60/2008)	<i>Orthomyxoviridae</i>	Human	FJ766842	N.D	N.D	N.D	N.D	N.D	N.D
Parainfluenza virus 1 (Korea/KUMC-44)	<i>Paramyxoviridae</i>	Human	MG255129	N.D	N.D	N.D	N.D	N.D	N.D
Human respiratory syncytial virus (HRSV-A/IC688/12)	<i>Pneumoviridae</i>	Human	KP663728	N.D	N.D	N.D	N.D	N.D	N.D
Adenovirus type 3 (KUMC-62)	<i>Adenoviridae</i>	Human	KY320276	N.D	N.D	N.D	N.D	N.D	N.D
H ₂ O (Double Distilled water)				N.D	N.D	N.D	N.D	N.D	N.D

Table 3. Limits of detection of the multiplex RT-qPCR assays

Sensitivity		Target gene	Limit of detection (TCID ₅₀ /ml)	Wildtype (KUMC01/2020)	Alpha Variant (KDCA0001/2020)	Beta Variant (KDCA0463/2020)	Delta Variant (KDCA0464/2021)
One-step multiplex assay	SARS-CoV-2 (FAM)	Membrane	1 x 10 ⁰	35.14 (0.32)	35.1 (0.29)	35.32 (0.1)	35.18 (0.45)
	Pan-SARS-like (HEX)		1 x 10 ⁰	34.62 (0.35)	34.83 (0.21)	34.92 (0.12)	34.79 (0.31)
	SARS-CoV-2 (Cy5)	Spike (S2 domain)	1 x 10 ⁰	34.68 (0.3)	34.56 (0.22)	34.82 (0.15)	34.68 (0.21)
	Pan-SARS-like (Texas red)		1 x 10 ⁰	34.42 (0.46)	34.15 (0.24)	34.48 (0.3)	34.35 (0.22)
WHO reference	SARS-CoV-2 (FAM)	RdRp	1 x 10 ⁰	36.1 (0.11)	36.35 (0.13)	36.42 (0.36)	36.29 (0.26)
		Envelop	1 x 10 ⁰	34.42 (0.1)	34.41 (0.33)	34.61 (0.13)	34.48 (0.17)

Table 4. Diagnostics validation of SARS-CoV-2 RT-qPCR multiplex assay using human clinical samples

Swab (Sw#) sample	Our detection system				WHO reference		Sputum (Sp#) sample	Our detection system				WHO reference	
	SARS- CoV- 2_M (FAM)	Pan- SARS_M (Hex)	Pan- SARS_S2 (Tex-RD)	SARS- CoV- 2_S2 (Cy5)	E	RdRp		SARS- CoV- 2_M (FAM)	Pan- SARS_M (Hex)	Pan- SARS_S2 (Tex-RD)	SARS- CoV- 2_S2 (Cy5)	E	RdRp
Sw#1	N.D	N.D	N.D	N.D	N.D	N.D	Sp#1	N.D	N.D	N.D	N.D	N.D	N.D
Sw#2	N.D	N.D	N.D	N.D	N.D	N.D	Sp#2	N.D	N.D	N.D	N.D	N.D	N.D
Sw#3	N.D	N.D	N.D	N.D	N.D	N.D	Sp#3	N.D	N.D	N.D	N.D	N.D	N.D
Sw#4	N.D	N.D	N.D	N.D	N.D	N.D	Sp#4	N.D	N.D	N.D	N.D	N.D	N.D
Sw#5	N.D	N.D	N.D	N.D	N.D	N.D	Sp#5	N.D	N.D	N.D	N.D	N.D	N.D
Sw#6	N.D	N.D	N.D	N.D	N.D	N.D	Sp#6	N.D	N.D	N.D	N.D	N.D	N.D
Sw#7	N.D	N.D	N.D	N.D	N.D	N.D	Sp#7	N.D	N.D	N.D	N.D	N.D	N.D
Sw#8	N.D	N.D	N.D	N.D	N.D	N.D	Sp#8	N.D	N.D	N.D	N.D	N.D	N.D
Sw#9	N.D	N.D	N.D	N.D	N.D	N.D	Sp#9	N.D	N.D	N.D	N.D	N.D	N.D
Sw#10	N.D	N.D	N.D	N.D	N.D	N.D	Sp#10	N.D	N.D	N.D	N.D	N.D	N.D
Sw#11	N.D	N.D	N.D	N.D	N.D	N.D	Sp#11	N.D	N.D	N.D	N.D	N.D	N.D
Sw#12	N.D	N.D	N.D	N.D	N.D	N.D	Sp#12	N.D	N.D	N.D	N.D	N.D	N.D
Sw#13	N.D	N.D	N.D	N.D	N.D	N.D	Sp#13	N.D	N.D	N.D	N.D	N.D	N.D
Sw#14	N.D	N.D	N.D	N.D	N.D	N.D	Sp#14	N.D	N.D	N.D	N.D	N.D	N.D
Sw#15	N.D	N.D	N.D	N.D	N.D	N.D	Sp#15	N.D	N.D	N.D	N.D	N.D	N.D
Sw#16	N.D	N.D	N.D	N.D	N.D	N.D	Sp#16	N.D	N.D	N.D	N.D	N.D	N.D
Sw#17	N.D	N.D	N.D	N.D	N.D	N.D	Sp#17	23.48	24.04	23.21	24.32	24.34	26.49
Sw#18	N.D	N.D	N.D	N.D	N.D	N.D	Sp#18	24.73	25.38	24.41	25.71	25.52	28.92
Sw#19	N.D	N.D	N.D	N.D	N.D	N.D	Sp#19	23.91	24.36	23.69	24.59	25.09	27.99
Sw#20	N.D	N.D	N.D	N.D	N.D	N.D	Sp#20	17.09	17.73	16.78	18.05	18.5	21.23
Sw#21	N.D	N.D	N.D	N.D	N.D	N.D	Sp#21	19.69	20.29	19.4	20.59	21	23.17
Sw#22	N.D	N.D	N.D	N.D	N.D	N.D	Sp#22	26.14	26.62	25.9	26.87	26.75	29.27
Sw#23	22.88	23.37	22.64	23.62	23.88	27.33	Sp#23	15.5	16.16	15.17	16.5	16.52	19.63
Sw#24	23.24	23.75	22.99	24.01	24.17	26.53	Sp#24	21.61	22.13	21.36	22.39	22.72	24.8
Sw#25	15.78	16.4	15.47	16.71	17.25	19.93	Sp#25	32.21	32.95	31.84	33.32	32.32	34.71
Sw#26	28.1	28.56	27.87	28.8	28.61	32.37	Sp#26	20.16	20.78	19.85	21.09	21.18	24.86
Sw#27	15.53	16.17	15.22	16.49	16.74	19.95	Sp#27	19.39	19.97	19.1	20.26	20.61	23.51
Sw#28	16.8	17.44	16.48	17.77	18.17	19.65	Sp#28	15.98	16.73	15.61	17.11	17.08	19.78
Sw#29	33.35	34.16	32.95	34.57	33.81	35.17	Sp#29	27.14	27.68	26.87	27.96	27.96	30.28
Sw#30	32.35	32.67	32.2	32.83	33.14	34.44	Sp#30	32	32.82	31.59	33.24	32.46	34.7
Sw#31	22.95	23.47	22.69	23.73	23.87	26.03							
Sw#32	16.02	16.72	15.67	17.08	16.96	19.92							
Sw#33	29.67	29.97	29.52	30.12	29.99	34.23							
Sw#34	25.67	26.36	25.33	26.71	24.01	27.03							
Sw#35	23.44	23.95	23.19	24.21	24.27	26.53							
Sw#36	25.41	26.01	25.11	26.31	24.22	26.82							
Sw#37	31.08	32.17	30.54	32.72	32.33	34.67							

Supplementary Table

Figures

Figure 1

Positions of amplicon targets and oligonucleotide binding regions on Membrane (A) and Spike (B) genes. The panels show oligonucleotide binding regions in rectangle boxes for primers/probes of set A and B selection (n=20). Dots represent identical nucleotides and dashes indicated to nucleotides deletion compared with hCoV-19/Spain/Valencia50/2020 sequence, which was the most recently released sequence at the time (Accession GISAID number EPI_ISL_420131). The full structure of Spike gene is annotated, SP: signal peptide; NTD: N-terminal domain; RBD: receptor-binding domain; RBM: receptor (ACE2)-binding motif; SD: subdomain; MCS: multiple cleavage site; FP: fusion peptide; HR: heptad repeat; TM: transmembrane domain; CP: Cytoplasmic.

Figure 2

Amplification plots and standard curves of the singleplex and multiplex RT-qPCR assays. The RT-qPCR assay used FAM-, Hex-, Cy5- and Texas Red-dye for each probe (showed in Table 1). The assays were performed using intact viral RNA corresponding to SARS-CoV, SARS-CoV-2, and Pan-SARS-like virus; the range of serial virus dilution from 4 to 0 log₁₀TCID₅₀/ml. The cycle threshold in amplification plots, correlation coefficient (R²), slope of the standard curve for the assays and efficiency were constructed automatically by Lightcycler 96 software. Each concentration had triple replicates.