Robust and sensitive detection of SARS-CoV-2 using PCR based methods

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32 **Abstract**

33 The World Health Organization (WHO) has declared the Coronavirus disease 2019 (COVID-19) as an
 34 international health emergency. Current diagnostic tests are based on the reverse transcription-
 35 quantitative polymerase chain reaction (RT-qPCR) method, the gold standard test that involves the
 36 amplification of viral RNA. However, the RT-qPCR assay has limitations in terms of sensitivity and
 37 quantification. In this study, we tested both qPCR and droplet digital PCR (ddPCR) to detect low
 38 amounts of viral RNA. The cycle threshold (CT) of viral RNA by RT-PCR significantly varied
 39 according to the sequence of primer and probe sets with in vitro transcript (IVT) RNA or viral RNA as
 40 templates, whereas the copy number of viral RNA by ddPCR was effectively quantified with IVT
 41 RNA, cultured viral RNA, and RNA from clinical samples. Furthermore, the clinical samples were
 42 assayed via both methods, and the sensitivity of the ddPCR was determined to be significantly higher
 43 than RT-qPCR. These findings suggest that ddPCR could be used as a highly sensitive and compatible
 44 diagnostic method for viral RNA detection.

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57 **Keywords:** COVID-19; SARS-CoV-2; Reverse transcription-quantitative polymerase chain reaction
 58 (RT-qPCR); Droplet digital PCR (ddPCR); Nucleocapsid protein gene; Envelope protein gene

59 **Introduction**

60 Coronaviruses have caused several notable respiratory disease outbreaks in humans, such as severe
 61 acute respiratory syndrome (SARS). The SARS coronavirus was the first fatal pathogenic human
 62 coronavirus to be identified and caused 8,096 infections and 774 deaths in 26 countries (“WHO |
 63 Summary of probable SARS cases with onset of illness from 1 November 2002 to 31 July 2003,”
 64 2015). Prior to the emergence of SARS-CoV, only two HCoV (HCoV-229E and HCoV-OC43) were
 65 known, both of which caused mild respiratory symptoms (Fung & Liu, 2019). Bats were the natural
 66 hosts of SARS-CoV and civet cats were regarded as the intermediate host (Li et al., 2006). Extensive
 67 studies on bat coronaviruses showed that such coronaviruses could be potential human pathogens (Ge
 68 et al., 2013; Wu, Peng, Wilken, Geraghty, & Li, 2012). MERS-CoV was first reported in 2012 and
 69 caused 2,494 infections and 858 deaths in 27 countries as of November 2019 (“WHO | Middle East
 70 respiratory syndrome coronavirus (MERS-CoV),” in press). MERS-CoV also originated from bat
 71 coronaviruses and camels were the intermediate host (Ashour, Elkhatib, Rahman, & Elshabrawy,
 72 2020).

73 According to the World Health Organization (WHO), the WHO China Country Office was informed
 74 of cases of pneumonia of unknown etiology in Wuhan City, Hubei Province, on December 31st, 2019
 75 (World Health Organization (WHO), 2020). The novel coronavirus, currently termed SARS-CoV-2,
 76 was officially announced as the causative agent by the International Committee on Taxonomy of
 77 Viruses (ICTV) (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses,
 78 2020; Gorbalenya, 2020). SARS-CoV-2, or severe acute respiratory syndrome coronavirus 2, has
 79 resulted in 2,114,269 laboratory-confirmed cases and 145,144 deaths as of April 17th, 2020 (da Costa,
 80 Moreli, & Saivish, 2020). A viral genome sequence was released for immediate public health support
 81 via the community online resource virological.org on January 10th (Wuhan-Hu-1, GenBank accession
 82 number MN908947) (Li et al., 2006), followed by four other genomes that were deposited on January
 83 12th in the viral sequence database curated by the Global Initiative on Sharing All Influenza Data
 84 (GISAID). The genome sequences suggest the virus is closely related to the members of a viral
 85 species termed severe acute respiratory syndrome (SARS)-related CoV, a species defined by the agent

of the 2002/03 outbreak of SARS in humans (Ge et al., 2013; Wu et al., 2012). The species also comprises of a large number of viruses mostly detected in rhinolophid bats in Asia and Europe. For the past several decades, quantitative polymerase chain reactions (qPCR) have become the gold standard for quantifying relative gene expressions. On the other hand, the recently developed digital polymerase chain reaction (dPCR) enables the highly sensitive measurement and absolute quantitation of nucleic acids (Hindson et al., 2011, 2013). dPCR does not require calibration with qualified standards for comparison. However, DNA or RNA quantities should be metrologically traceable to a reference (Bhat & Emslie, 2016; Whale et al., 2018). As of this writing, multiple nucleic acid quantitation methods have been developed, such as chemical analysis methods based on isotope-dilution mass spectrometry (IDMS), capillary electrophoresis (CE), and enumeration-based flow cytometric method (FCM) counting (Kwon, Jeong, Bae, Choi, & Yang, 2019; Yoo et al., 2016). These methods can be accurately calibrated with solutions of nucleic acids. In addition, an international comparison study was performed between national metrology institutes (NMI) using the dPCR method (Corbisier et al., 2012). Recently, the droplet digital PCR (ddPCR) method has emerged as a powerful analytical technique for clinical utility (Miotke, Lau, Rumma, & Ji, 2014; Pinheiro et al., 2012). For example, ddPCR can be used for the rapid enumeration of viral genomes and particles (Americo, Earl, & Moss, 2017; Hayden et al., 2013; Martinez-Hernandez et al., 2019). In the present case of SARS-CoV-2, virus isolates or samples from infected patients have yet to be made available to the international public health community. In this study, we conclude that optimized conditions are required to increase the precision of ddPCR to develop reference materials with matrix conditions.

Materials and Methods

Construction of RNA standards.

Full-length N and E genes of SARS-CoV-2 (GeneBank: MN908947) were synthesized and cloned in pET21a plasmid. The plasmids were amplified with the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3').

113 The amplicons were spectrophotometrically quantified at 260 nm. A total of 200 ng of the PCR
114 product was used for in vitro transcription (MEGAscript T7 Transcription kit; Thermo Fisher
115 Scientific, Waltham, MA, USA), which was performed at 37°C overnight in a 20 µl reaction mixture
116 containing 2 µl of reaction buffer, 2 µl of each nucleoside triphosphate, and 2 µl of enzyme mix. The
117 template DNAs were removed by digestion with 2 U of Turbo DNase I for 15 min at 37°C. The RNAs
118 were precipitated by adding 6 µl 3 M sodium acetate and 150 µl of 98% ethanol which was followed
119 by a subsequent incubation at -20°C for 30 min. After 15 min of centrifugation at 17,000 rpm, the
120 supernatant was removed and 200 µl of 70% ethanol was added. After another 10-min centrifugation
121 at 17,000 rpm, the supernatant was removed and the pellet was dissolved in 20 µl RNase-free H₂O
122 (Takara Bio Inc., Otsu, Shiga, Japan). Quantitation of the RNAs was performed
123 spectrophotometrically at 260 nm. The measurement of the RNA concentrations was performed in
124 duplicate, and the concentration was then converted to the molecule number (Fronhoffs et al., 2002).

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126 **Cell and virus RNA extraction**

127 The genomic RNA of clinical isolate SARS-CoV-2 (NCCP 43326) was obtained from the National
128 Culture Collection for Pathogens (NCCP). The obtained RNA genomes were maintained at -80 °C
129 and were used as a template for the assays. Vero E6 cells (ATCC CRL-1586) were purchased from the
130 American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle
131 medium (DMEM) supplemented with 2% (v/v) fetal bovine serum (FBS) and 1% antibiotic-
132 antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% humidified
133 CO₂ incubator. The SARS coronavirus HKU-39849 (GenBank: AY278491.2, provided by Dr. Malik
134 Peiris of the University of Hong Kong) was inoculated into Vero E6 cells. Cytopathic effects were
135 observed two days after inoculation and the viral titer was determined via a plaque assay. The viral
136 RNA was extracted from the culture medium using the MagMAX-96 viral RNA isolation kit (Thermo
137 Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Human hepatoma
138 Huh-7 were obtained from the Japan Cell Research Bank (National Institutes of Biomedical
139 Innovation, Health and Nutrition, Japan). The cells were maintained in Dulbecco's modified Eagle's

medium (DMEM, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) at 37 °C in a 5% CO₂ incubator. The patient-derived isolate MERS-CoV strain KNIH/002_05_2015 (Kim et al., 2016) was obtained from KNIH and inoculated into cultured Huh-7 Cells. After 3 days of incubation, the viral RNA was extracted from the culture medium using the QIAamp viral RNA extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Human lung fibroblast MRC-5 cells were obtained from the American Type Culture Collection (ATCC). The culture medium for the cell line was Gibco Minimum Essential Media (MEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 1% Sodium Pyruvate, 1% penicillin, and 1% non-essential amino acids. The cell lines were cultured in a T75 flask in a humidified atmosphere of 5% CO₂ at 37 °C. The subcultures were produced with a ratio of 1:2 when the cell confluency reached 80%–90% every 3 or 4 days. HCoV OC43 (ATCC VR1588) was obtained from ATCC and was inoculated into a virus growth medium (MEM with 2% heat-inactivated FBS, 1% penicillin, and 1% non-essential amino acids) and propagated to the MRC-5 cells with 90% confluence. The flasks were maintained at 33 °C in a humidified atmosphere of 5% CO₂. When the cells reached 50%-60% confluence, the supernatant was harvested, filtered, and stored at -80 °C until RNA extraction. Viral RNA was extracted using the viral RNA Minikit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

Clinical samples and RNA preparation

The clinical samples used in this study were collected from subjects according to registered protocols approved by the Institutional Review Board (IRB) of Jeonbuk National University Hospital, with all patients having signed written informed consent forms (IRB registration number: CUH 2020-02-050-008). The clinical characteristics of the patients are shown in Table 2. Upper respiratory tract specimens (naso- and oropharyngeal swabs) from COVID-19 patients were suspended in a transport medium (eNAT; COPAN, Murrieta, CA, USA) and stored at -80 °C until use. The RNA extraction from the clinical samples was performed using the viral RNA Minikit (QIAGEN, Hilden, Germany)

166 according to the manufacturer's protocol. The extracted viral RNA was used as a template for cDNA
167 synthesis.

168

169 Primer and probes

170 The primer-probe sets used in this study are listed in Table 1. For the detection of SARS-CoV-2,
171 primer-probe sets of the Centers for Disease Control and Prevention (CDC, USA), The University of
172 Hong Kong (HKU, Hong Kong), the National Institute of Infectious Disease Department of Virology
173 III (NIID, Japan), and the National Institute of Health (NIH, Thailand) were selected according to
174 laboratory guidance from WHO (Jung et al., 2020). The specific primer-probe sets for MERS-CoV
175 and OC43 were used for the quantification of the viral RNA genomes (Corman et al., 2012; Vijgen et
176 al., 2005). All primers and probes were synthesized by Neoprobe (Daejeon, Korea). All probes were
177 labeled with the reporter molecule 6-carboxyfluorescein (FAM) at the 5'-end and with the quencher
178 Black Hole Quencher 1 (BHQ-1) at the 3'-end.

179

180 cDNA synthesis of viral RNA genomes

181 The cDNAs of the clinical and cultured (SARS-CoV-2, SARS-CoV, MERS-CoV, and OC43) viral
182 RNA genomes were synthesized using the LunaScript RT SuperMix Kit (New England Biolabs,
183 Ipswich, MA, USA). The reaction mixture had a volume of 20 μ L and comprised of 4 μ L of 5X
184 LunaScript RT supermix, 1 μ L of cDNA template, and 15 μ L of distilled water. The reactions were
185 carried out according to the manufacturer's instructions. The synthesized cDNAs were serially diluted
186 with distilled water and used as templates for qPCR and ddPCR without purification.

187

188 qPCR and droplet digital PCR measurement

189 The templates were serially diluted to few copy numbers/ μ L. RT-qPCR analysis was performed on the
190 StepOne and StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA).
191 The reaction mixture had a volume of 25 μ L and comprised of 12.5 μ L of maxima Probe/ROX
192 qPCR master mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 1 μ L of cDNA template,

193 1 μ L of 10 μ M forward primer, 1 μ L of 10 μ M reverse primer, 1 μ L of 5 μ M probe labeled with
194 FAM, and 8.5 μ L of distilled water. The ddPCR analysis was performed using a QX200 system
195 (BioRad Laboratories, Hercules, CA, USA). The reaction mixture had a volume of 20 μ L and
196 comprised of 10 μ L of ddPCR supermix for probes (BioRad Laboratories, Hercules, CA, USA),
197 1 μ L of cDNA template, 1 μ L of 10 μ M forward primer, 1 μ L of 10 μ M reverse primer, 1 μ L
198 of 5 μ M probe labeled with FAM, and 6 μ L of distilled water. All valid copy numbers were selected
199 according to the manufacturer's instructions (BioRad, 2015).

200

201 **Data analysis**

202 The qPCR data were initially analyzed using the StepOne software (Thermo Fisher Scientific,
203 Waltham, MA, USA). Raw data (i.e., the fluorescence values for C_T) were exported from the StepOne
204 software to Microsoft Excel 2016. A series of diluted templates were used to determine the C_T value,
205 which can establish a standard curve for evaluating the reaction efficiency. Droplet fluorescence data
206 were initially analyzed using the QuantaSoft software (BioRad Laboratories, Hercules, CA, USA).
207 Raw data (i.e., the fluorescence values for the droplets) were exported from the QuantaSoft software
208 to Microsoft Excel 2016. Depending on the separation of positive and negative droplets, an objective
209 separation value k was automatically calculated.

210

211 **Results**

212 **Quantification of viral and IVT RNAs**

213 In this study, the primer-probe sets of each E gene were used to quantify the viral genomic RNA of
214 SARS-CoV-2 and related coronaviruses (Table 1) (Corman et al., 2012, 2020a; Jung et al., 2020;
215 Vijgen et al., 2005). The clinical characteristics of confirmed COVID-19 patients with symptoms are
216 summarized in Table 2.

217 The copy numbers of SARS-CoV-2, SARS-CoV, MERS, and OC43 viral RNA were measured using
218 ddPCR, and the measured values of viral genomic RNA were 8.04×10^5 , 6.52×10^5 , 9.37×10^4 and
219 9.57×10^4 copies/ μ L, respectively (Table 3). To assess the quantification of viral genomic RNA and

220 partial synthetic viral RNA, *in vitro* transcript (IVT) N and E gene RNA were measured via qPCR and
221 ddPCR. The copy numbers of N IVT RNA and E IVT RNA were measured as 1.8×10^9 and 3.47×10^9 ,
222 respectively. The amplification efficiencies were calculated from standard curves of SARS-CoV-2
223 viral RNA and IVT RNAs.

224

225 **IVT RNAs as a standard for RT-qPCR**

226 The R^2 values of viral RNA and IVT N IVT RNA were calculated as 0.9973 and 0.9987, respectively,
227 whereas the R^2 values of viral RNA and IVT E IVT RNA were calculated as 0.9956 and 0.9993,
228 respectively (Tables 3 and 4). These values indicate that the standard curves of both viral and IVT
229 RNAs are linear and accurate. The amplification efficiency of both viral and IVT RNAs were
230 calculated from the slopes of the standard curves. The efficiencies of both viral and IVT RNAs
231 according to the target genes were similar (Tables 3 and 4). The similar amplification efficiencies
232 show that the quantified IVT RNA can be used as a standard for RT-qPCR measurement of viral RNA.

233

234 **Comparison of qPCR and ddPCR assays for COVID-19 clinical samples**

235 According to the qPCR and ddPCR results, all patient samples were positive when we used 0.1 ng of
236 extracted RNA for the template. The non-template control did not produce any signals from both the
237 qPCR and ddPCR assays. The result of the serially diluted sample showed that the C_T value was
238 undetermined. On the other hand, all ddPCR signals were detected in all of the diluted samples (Table
239 5). Furthermore, the positive copies (P2 for 0.67 and P3 for 0.60) from the two samples were
240 exceptionally low compared to the other specimens, but still only produced positive signals in the
241 ddPCR results. This suggests that the ddPCR method can be applied to detect ultra-low amounts of
242 sample with high sensitivity.

243

244 **Discussion**

245 The outbreak of COVID-19 is a pandemic threat caused by the emergence of SARS-CoV-2, a newly
246 discovered human coronavirus (Wu et al., 2020; Zhou et al., 2020). SARS-CoV-2 has four structural

247 proteins, known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins (Walls
248 et al., 2020). The SARS-CoV-2 S protein binds with angiotensin-converting enzyme 2 (ACE2) with
249 high-affinity, which leads to clinical features of pneumonia in many patients (Wrapp et al., 2020; Zhu
250 et al., 2020). SARS-CoV-2 is highly contagious and has quickly spread across the world, which
251 further emphasizes the essential role of diagnostics in the control of communicable diseases (Winter
252 & Hegde, 2020).

253 Currently, a reliable test (also the most widely used) for detecting acute infections of SARS-CoV-2 is
254 the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method (Lu et al., 2020).
255 Multiple national laboratories have developed assays targeting conserved regions that could
256 potentially detect pan-sarbecovirus (Chu et al., 2020; Corman et al., 2020b). A previous study
257 provided information such as the specific primer and probe sequence, thermal profile, and reagents for
258 RT-qPCR to optimize the reaction conditions for the detection of SARS-CoV-2 RNA (Bustin, 2020).
259 ddPCR is a method that improves upon conventional PCR methods that can individually amplify and
260 directly quantify pathogen RNA or DNA (Li et al., 2018). Several studies have compared RT-qPCR
261 and ddPCR for the quantification of viral genomes (Abachin et al., 2018; Dobnik et al., 2019;
262 Kiselina et al., 2014).

263 In the present study, SARS-CoV-2 RNA concentrations were measured using the qPCR and ddPCR
264 methods (Table 3). The qPCR results showed that the C_T value of a given template was greatly
265 dependent on the individual primer-probe sets. However, the copy numbers determined via ddPCR
266 were stable and reliable in the quantification of low-viral RNA samples, regardless of the primer-
267 probe sets. A previous study reported that qPCR and ddPCR results were consistent for 95 positive
268 samples and that there was a high correlation between the C_T values of qPCR and the copy number
269 values of ddPCR among patient samples (Yu et al., 2020). Although the C_T values of each primer-
270 probe set exhibited variance, the copy numbers from ddPCR showed that N genes are more abundant
271 than E genes. These findings are also consistent with another previous study, indicating N genes are
272 good targets for diagnostics (Kim et al., 2020).

273 The qPCR method requires the use of a standard curve to obtain quantitative measurements of an

unknown sample (Adamski, Gumann, & Baird, 2014). However, in the case of ddPCR, the distribution of target-specific amplification into partitions is calculated using the Poisson distribution, enabling the absolute quantification of the target gene based on the ratio of positive against all partitions at the end of the reaction without dependence on an external standard (Gutiérrez-Aguirre, Rački, Dreó, & Ravnikar, 2015). In addition to these advantages, our results revealed that ddPCR is a more sensitive and accurate method for SARS-CoV-2 detection in low viral copies (Table 5). Further experiments should be performed even if patients are cured of COVID-19, and such tests should be conducted using ddPCR to lower the limit of detection (LOD) to be able to detect low amounts of viral RNA.

The Coronavirus Standards Working Group led by the Joint Initiative for Metrology in Biology (JIMB) is conducting international collaborative experiments to develop guidelines for the purpose of evaluating and establishing the availability of common and appropriate standards, diverse reference materials, validation tests, and reference measurement protocols that can enable reliable COVID-19 testing (“Coronavirus Standards Working Group — The Joint Initiative for Metrology in Biology,” in press; “ISO - COVID-19 response: freely available ISO standards,” in press). Our results serve as a guide for the standardization of analytical methods and the development of reference materials that are required for accurate COVID-19 diagnosis based on both RT-qPCR and ddPCR. Consequently, ddPCR-based diagnostics can serve as a companion method to the current standard RT-qPCR to provide sensitive and accurate quantitative results for emergency testing that can be adopted for clinical use.

Conclusion

Our study demonstrated that CT values of samples from qPCR assays were significantly variable depending on the sequence of primer and probe sets, whereas copy numbers of samples from ddPCR assays were hardly affected by the sequence of primer and probe sets. These results indicate that ddPCR can be performed with sub-optimal primer-probe sets without the loss of sensitivity. A comparison of the standard curves of viral RNA and IVT RNA showed that IVT RNA quantified via

ddPCR could be used as a standard for the absolute quantification of qPCR assays. The results from the clinical samples showed that the ddPCR method could result in highly sensitive and quantitative diagnostics. Although qPCR produced different CT values using different primer-probes sets, the ddPCR results obtained using different primer-probe sets were compatible with each other. Therefore, the deployment of ddPCR-based diagnostics could produce highly sensitive and quantitative results that are compatible between different laboratories.

Author Contributions

Conceptualization, S. K. and H. M. Y.; methodology, S. K. and H. M. Y.; validation, S. K. and H. M. Y.; formal analysis, C. P.; investigation, C. P., J. L., Z. U. H., K. B. K., S. J. K., H. G. K., E. C. P., G.-S. P., D. P., S.-H. B., D. K., D. P., J. L., S. J., S. K., and C.-S. L.; resources, C. P., J. L., Z. U. H., K. B. K., E. C. P., S.-H. B., D. P., J. L., S. J., S. K., and C.-S. L.; data curation, S. K. and H. M. Y.; writing—original draft preparation, C. P., Z. U. H., K. B. K., E. C. P., S. K., C.-S. L., S. K. and H. M. Y.; writing—review and editing, S. K. and H. M. Y.; visualization, C. P., S. K. and H. M. Y.; supervision, S. K. and H. M. Y.; project administration, S. K.; funding acquisition, S. K.

Acknowledgements

This work was supported by National Research Council of Science and Technology grant (Grant No. CRC-16-01-KRICT). This research was also supported by the “Development of recombinant coronavirus reference materials”, grant number KRISS-2020-GP2020-0003-08, and “Development of Measurement Standards and Technology for Biomaterials and Medical Convergence”, grant number KRISS-2020-GP2020-0004 programs, funded by the Korea Research Institute of Standards and Science. ORCID ID (Hee Min Yoo: 0000-0002-5951-2137; Seil Kim: 0000-0003-3465-7118; Changwoo Park: 0000-0001-7083-9037; Jina Lee: 0000-0002-3661-3701; Zohaib Ul Hassan: 0000-0002-4982-2262; Dongju Park: 0000-0001-6516-6755)

Competing interest

328 The authors declare no conflict of interest.

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486
487 **Table legends**

488 **Table 1.** Information regarding the primers and probes used in this study.

489 **Table 2.** Clinical characteristics of laboratory-confirmed patients with COVID-19.

490 **Table 3.** R^2 values calculated with mean C_T and copy number quantified via qPCR and ddPCR.

491 **Table 4.** Comparative analysis of C_T values and copy numbers obtained via qPCR and ddPCR.

492 **Table 5.** C_T values and copy numbers of patient samples via qPCR and ddPCR.

493 **Table 1.** Information of primers and probes used in the study

Institute	Gene	Name	Type	Sequence (5' → 3')	Target	Ref.	
CDC (USA)	N	2019-nCoV_N1-F	F	GACCCCAAAATCAGCGAAAT	SARS-CoV-2	(Jung et al., 2020)	
		2019-nCoV_N1-R	R	TCTGGTTACTGCCAGTTGAATCTG			
		2019-nCoV_N1-P	P	ACCCCGCATTACGTTTGGTGGACC			
		2019-nCoV_N2-F	F	TTACAAACATTGGCCGCAAA	SARS-CoV-2		
		2019-nCoV_N2-R	R	GCGCGACATTCCGAAGAA			
		2019-nCoV_N2-P	P	ACAATTTGCCCCAGCGCTTCAG			
NIID (Japan)		NIID_2019-nCOV_N_F2	F	AAATTTTGGGGACCAGGAAC	SARS-CoV-2		
		NIID_2019-nCOV_N_R2	R	TGGCAGCTGTGTAGGTCAAC			
		NIID_2019-nCOV_N_P2	P	ATGTCGCGCATTGGCATGGA			
NIH (Thailand)		WH-NIC N-F	F	CGTTTGGTGGACCCTCAGAT	SARS-CoV-2		
		WH-NIC N-R	R	CCCCACTGCGTTCTCCATT			
		WH-NIC N-P	P	CAACTGGCAGTAACCA			
HKU (Hong Kong)		HKU-NF	F	TAATCAGACAAGGAACTGATTA	Sarbeco		
		HKU-NR	R	CGAAGGTGTGACTTCCATG			
		HKU-NP	P	GCAAATTGTGCAATTTGCGG			
Charité (Germany)		E_Sarbeco_F1	F	ACAGGTACGTTAATAGTTAATAGCGT	Sarbeco		(Corman et al., 2020a)
		E_Sarbeco_R2	R	ATATTGCAGCAGTACGCACACA			
		E_Sarbeco_P1	P	ACACTAGCCATCCTTACTGCGCTTCG			
University of Bonn Medical Centre (Germany)	upE-F	F	GCAACGCGCGATTTCAGTT	MERS	(Corman et al., 2012)		
	upE-R	R	GCCTCTACACGGGACCCATA				
	upE-P	P	CTCTTCACATAATCGCCCCGAGCTCG				
University of Leuven (Belgium)	OC43-FP	F	ATGTTAGGCCGATAATTGAGGACTAT	OC43	(Vijgen et al., 2005)		
	OC43-RP	R	AATGTAAAGATGGCCGCGTATT				
	OC43-TP	P	CATACTCTGACGGTCACAAT				

Table 2. Clinical characteristics of laboratory-confirmed patients with COVID-19.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (years)/Sex	72/M	57/M	72/F	48/M	51/F
Fever	-	+	+	+	+
Chills	-	+	-	-	-
Cough	+	+	+	-	+
Dyspnea	-	+	+	-	+
Diarrhea	-	-	-	+	-
Laboratory findings					
WBC	3,570	3,710	6,720	4,730	5,820
Hemoglobin	10.3	12.9	16.1	12.9	10.8
Platelet	157,000	42,000	190,000	316,000	175,000
AST	23	57	32	34	43
ALT	22	55	13	38	18
Creatinine	0.83	0.77	0.55	0.31	0.38
Used drug					
Lopinavir/ritonavir	+	+	+	+	+
Hydroxychloroquine	+	+	+	+	+
Outcome	Cured	Cured	Cured	Cured	Cured

499

Table 3. R² values calculated with mean C_T and copy number quantified via qPCR and ddPCR.

Target	E_Sarbeco			
	SARS-CoV-2		SARS-CoV	
Dilution factor	qPCR (C _T)	ddPCR (copy/μl)	qPCR (C _T)	ddPCR (copy/μl)
10 ⁻¹	19.81	98533	19.96	90000
10 ⁻²	23.09	9780	23.34	8747
10 ⁻³	26.79	804	27.30	652
10 ⁻⁴	31.20	46	30.77	57
Efficiency (%)	83.65		88.30	
R ²	0.9956		0.9991	
Target	E_upE		E_OC43	
	MERS		OC43	
Dilution factor	qPCR (C _T)	ddPCR (copy/μl)	qPCR (C _T)	ddPCR (copy/μl)
1	24.46	86400	24.69	99800
10 ⁻¹	27.73	8980	27.93	10887
10 ⁻²	31.06	937	31.46	957
10 ⁻³	35.11	74	35.16	93
Efficiency (%)	92.01		93.24	
R ²	0.9972		0.9991	
Target	2019-nCoV_N2		E_Sarbeco	

Dilution factor	IVT N gene		IVT E gene	
	qPCR (C _T)	ddPCR (copy/μl)	qPCR (C _T)	ddPCR (copy/μl)
10 ⁻⁴	19.61	6794000	20.94	20000000
10 ⁻⁵	24.03	22280	24.97	43490
10 ⁻⁶	28.09	1803	28.90	3470
10 ⁻⁷	31.85	154	32.49	318
Efficiency (%)	75.88		81.71	
R ²	0.9987		0.9993	

* The initial concentrations of SARS-CoV-2, SARS-CoV, MERS, OC43 viral RNA, and IVT N gene and E gene RNAs were measured using a Quantus fluorometer and the measured values of each RNA were 78.3, 35.7, 30.8, 66.2, 91.4, and 50.9 ng/μL, respectively.

Table 4. Comparative analysis of C_T values and copy numbers obtained via qPCR and ddPCR.

Dilution factor	2019-nCoV_N1		2019-nCoV_N2		NIID_2019-nCoV_N		WH-NIC_N		HKU_N	
	qPCR	ddPCR	qPCR	ddPCR	qPCR	ddPCR	qPCR	ddPCR	qPCR	ddPCR
10 ⁻²	18.89	6806000	18.87	175333	18.14	163000	21.40	6797333	20.79	165400
10 ⁻³	22.16	21173	22.19	20340	21.63	15173	24.65	19993	24.31	18473
10 ⁻⁴	25.99	1907	26.06	1659	25.40	1177	28.52	1599	28.09	1553
10 ⁻⁵	30.66	135	30.60	132	29.93	60	33.11	105	32.68	105
10 ⁻⁶	34.12	11	34.57	8	34.91	7	36.71	5	36.71	12
Efficiency (%)	80.58		78.28		73.40		80.26		77.28	
R ²	0.9968		0.9973		0.9943		0.9971		0.9978	

* A copy number quantified as a target of Germany E gene using ddPCR

524 **Table 5.** C_T values and copy numbers of patient samples via qPCR and ddPCR.

Dilution factor	Template	2019-nCoV_N2 primer-probe set									
		P1 [*]		P2 [*]		P3 [*]		P4 [*]		P5 [*]	
		qPCR (Ct)	ddPCR (copy/μl)	qPCR (Ct)	ddPCR (copy/μl)	qPCR (Ct)	ddPCR (copy/μl)	qPCR (Ct)	ddPCR (copy/μl)	qPCR (Ct)	ddPCR (copy/μl)
10 ⁻¹		26.87	1051	35.03	7.20	35.15	2.40	Undetermined	0	35.05	7.53
10 ⁻²		30.52	82	37.03	1.33	Undetermined	0.73	Undetermined	0.53 ^{**}	Undetermined	1.20
10 ⁻³		33.81	5.93	Undetermined	0.67	Undetermined	0.60	Undetermined	0	Undetermined	1.07

525 ^{*} The initial concentrations of the viral RNA templates were 46.9, 21.1, 27.5, 17.2, and 15.9 ng/μL, respectively.

526 ^{**} Only one of the triplicate was positive (single droplet).