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Methods Article

Optimization and application of digital droplet PCR for the detection of SARS-CoV-2 in saliva specimen using commercially available kit

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Abstract

The coronavirus disease-19 pandemic has resulted in a significant global health crisis, causing hundreds of millions of cases and millions of deaths. Despite being declared endemic, SARS-CoV-2 infection continues to pose a significant risk, particularly for immunocompromised individuals, highlighting the need for a more sensitive and specific detection. Reverse transcription digital droplet polymerase chain reaction (RT-ddPCR) possesses a sensitive and absolute quantification compared to the gold standard. This study is the first to optimize RT-ddPCR for detecting SARS-CoV-2 in saliva specimens using a commercially available RT-qPCR kit. Optimization involved the assessment of the RT-ddPCR reaction mixture, annealing temperature adjustments, and validation using 40 stored saliva specimens. RT-qPCR was used as a reference method in this study. Compatibility assessment revealed that ddPCR Supermix for Probes (no dUTP) was preferable with an optimal annealing temperature of 57.6°C. Although a 25% higher primer/probe concentration provides a higher amplitude in droplet separation of positive control, the number of copy numbers decreased. An inverse correlation between Ct value and copy number concentration was displayed, presenting that the lower the Ct value, the higher the concentration, for the N and E genes with r^2 values of 0.98 and 0.85, respectively. However, ORF1ab was poorly correlated (r^2 of 0.34). The sensitivity of targeted and E genes was 100% and 93.3%, respectively; as for the specificity, the percentage ranged from 80.8% to 91.3%. This study implicates the applicability of a modified method in the ddPCR platform for similar types of pathogens using saliva specimens.

Keywords: COVID-19; SARS-CoV-2; digital droplet PCR, RT-ddPCR, RT-qPCR, saliva

Introduction

Coronavirus disease-19 (COVID-19) is a disease caused by SARS-CoV-2 that has been a pandemic and has caused more than 687 million cases until the end of the pandemic period in May 2023 [1]. SARS-CoV-2 has been classified as endemic in recent days, co-existing as a common virus similar to other respiratory symptoms [2]. However, the severity of the infection varies among individuals, particularly those with immunocompromised conditions. Transmission of COVID-19 can cause mild to serious symptoms in immunocompromised individuals, which may result in a more severe and higher mortality risk [3]. Thus, a more sensitive and specific detection of SARS-CoV-2 still highly relevant to be developed in order to improve the accuracy, time efficiency, and convenience for diagnostic methods. The development may be further implemented in the diagnosis of other diseases with similar modes of action.

Based on the recommendation of the World Health Organization (WHO), the nucleic acid amplification test (NAAT) is used for the detection of COVID-19, where reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is mainly used as the gold standard for COVID-19 diagnosis [4]. RT-qPCR has good sensitivity and specificity to diagnose COVID-19, but on a low viral count specimen, this testing method usually results in a false negative specimen [5–7]. In addition, RT-qPCR relies on a standard curve produced during the amplification process to relatively quantify nucleic acid targets [8]. Other than RT-qPCR, one of the other NAAT methods, reverse transcription digital droplet polymerase chain reaction (RT-ddPCR), has been known for its sensitive and absolute quantification results using the Poisson statistic principle.

In RT-ddPCR, the specimen will be distributed into thousands of uniform-sized droplets with water-oil emulsion before being amplified in a smaller segment, causing fewer biases to occur by the amplification of other targets or inhibitors that come along with the specimen. The need for calibration can be dismissed, as ddPCR does not required a standard curve for analysis.

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Furthermore, the principles of limited dilution and end-point PCR that are used in ddPCR further increase its accuracy. Several studies have revealed that ddPCR is more sensitive and accurate compared to RT-qPCR in both singleplex or multiplex targeted detection and in the implementation of pooling [9–11]. However, ddPCR is yet to be used routinely for diagnosis, which could be due to its relatively high cost of reagents worldwide compared to qPCR reagents [12]. One strategy to overcome this challenge is through technological advancement by harmonizing reagents that can be used across different platforms, as in RT-qPCR. Consequently, this approach could become readily available to numerous testing laboratories, which may increase competition that will gradually lower costs. In addition, the modified method would potentially establish itself as the gold standard for future diagnostic applications.

On the other hand, saliva, a clinically approved specimen for emergency use for SARS-CoV-2 diagnosis according to the Food and Drug Administration (FDA), has a promising role in molecular diagnostics [13, 14]. Aside from a non-invasive sampling technique, saliva collection is simple to be performed individually, which eventually reduces the risk of infecting healthcare workers. The usage of saliva in the detection of SARS-CoV-2 has proven to be reliable on both RT-PCR [15] and ddPCR platforms [9, 16, 17]. Minimally processed saliva using a multiplexed paired-pooled assay was revealed to have high throughput and sensitivity in SARS-CoV-2 screening [16, 18]. Similarly, detection using RT-ddPCR demonstrated an accuracy similar to that of nasal swabs in detecting infected cases, with a concordance of 93.75% compared to RT-qPCR [8]. Absolute SARS-CoV-2 RNA viral load rapid increment is able to be observed through RT-ddPCR in presymptomatic individuals, supporting its reliability for viral detection [17].

Taking into account the strategy of harmonizing RT-qPCR reagents on the RT-ddPCR platform and utilizing minimally treated saliva specimens, this study will be the first to optimize the application of RT-ddPCR in detecting SARS-CoV-2 in saliva specimens using the commercially available RT-qPCR kit. Furthermore, the modified method will be validated across 40 saliva specimens, with the results being compared to the RT-qPCR gold standard for SARS-CoV-2 detection. The RT-ddPCR optimization procedure that our study has implemented may serve as a potential strategy to increase the detection and diagnosis accuracy of other viral and respiratory infections or even other pathogens with similar modes of infection.

Materials and methods

Specimen collection

A total of 40 saliva specimens from the COVID-19 Laboratory Center, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia (SMHS-AJCUI) collection, which was kept at −80°C, were utilized to optimize and validate the performance of RT-qPCR commercial kit on ddPCR. Those specimens consisted of 20 positive and 20 negative SARS-CoV-2 saliva specimens. The saliva specimens were collected and tested for COVID-19 from November 2022 - November 2023. This study has been approved by the Institutional Review Board of SMHS-AJCUI (06/05/KEP-FKIKUAJ/2022, 19 May 2022).

Viability test using RT-qPCR

Prior to optimization and validation, viability tests were performed on the 40 selected specimens using RT-qPCR to ensure the quality of the specimens' post −80°C storage. The saliva specimen (90 μL) was heated at 95°C for 10 minutes [18] before the addition of internal control (10 μL) and centrifuged at 8000 rpm for 1 minute. The supernatant of the saliva specimen was used as a viral RNA template. The xABT Multiple Real-Time PCR Kit for Detection of 2019-CoV (Beijing Applied Biological Technologies Co., Ltd, Beijing, China, #CT8223-48T) was used as a reference kit in this study. The RT-qPCR master mixture was prepared in a 20 μL reaction volume according to the manufacturer's instructions, consisting of 10 μL of the nucleic acid amplification reaction solution, 2 μL of nuclease-free water, 1 μL of xABT reverse transcriptase (RT), 2 μL reaction of either xABT solution A (ORF1ab and N gene) or B (E gene), and 5 μL of heat-treated saliva specimen. Upon the addition of the viral RNA template, the plate was briefly spun down to ensure that all solution was positioned at the bottom of the well. The amplification process was performed on the CFX96 Touch Real-Time PCR detection platform (Bio-Rad Laboratories, Hercules, CA, USA) under the following cycling conditions: 45°C for 10 minutes (RT), 95°C for 5 minutes (DNA polymerase activation), 45 cycles of 95°C for 15 seconds (denaturation), and 60°C for 45 seconds (annealing). Fluorescence was measured at 60°C. Each RT-qPCR run included negative and positive controls (SARS-CoV-2 pseudovirus (RNA) containing ORF1ab, N, and E gene fragments). Ct value analysis was conducted using CFX Maestro 1.0 software version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA). Amplification of internal control gene (detected by Cy5 channel) in specimens with no amplification (N/A) or Ct values greater than 40 on any of the target genes (ORF1ab and E gene detected by FAM channel; N gene detected by VIC channel) was interpreted as a negative result. Specimens were regarded as viable when exhibiting concordance results with pre-storage testing. Specimens with a detectable cycle threshold (Ct) value after long-term storage are considered positive. Negative specimens are specimens that remained undetected above Ct cut-off values. Viable specimens were selected for further assessment.

Assessment of RT-ddPCR reaction mixture for RT-qPCR

There were two available mixture types offered by ddPCR that could facilitate the assay. The compatibility of two different RT-ddPCR reaction mixtures, namely ddPCR Supermix for Probes (no dUTP) (Bio-Rad Laboratories, Hercules, CA, USA, Cat#1863023) and One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, Cat#1864021), was assessed three times in the detection of ORF1ab, N, and E genes toward the reference kit. As for the detection of internal control (phocine herpesvirus 1—PhHV-1) used in this study was assessed using EvaGreen Supermix (Bio-Rad, Cat#1864034). PhHV-1 is a universal internal amplification control, which consisted of a complete DNA virus of the non-human seal herpes virus type 1 [19, 20]. Two viable SARS-CoV-2 saliva specimens, consisting of both positive and negative specimens, were selected as representative and utilized in this step.

The RT-qPCR mixture for probes was prepared according to the reference kit instructions with the substitution of the nucleic acid amplification reaction solution with either ddPCR Supermix for Probes (no dUTP) (Bio-Rad, Cat#1863023) or One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, Cat#1864021). The amplification process and analysis were performed in a similar setting as mentioned above. The compatible RT-ddPCR reaction mixture was further evaluated using the RT-ddPCR platform.

Annealing temperature and primer/probe optimization in RT-ddPCR

Nuclease-free water (NFW), xABT positive control (PC), and SARS-CoV-2 negative and positive saliva specimens were used as templates to assess the optimal annealing temperature. The standard RT-ddPCR cycling program was modified by replacing the annealing temperature step with a thermal gradient between 57°C and 65°C for a 45-second extension time. An increased concentration by the addition of 25% primer–probe concentrations was compared with the reference condition using NFW as no template control (NTC) and PC.

RT-ddPCR

A two-color ddPCR detection system, the QX200 droplet reader (Bio-Rad), which is able to perform two-plex target detection in channel 1 (FAM/EvaGreen) and channel 2 (VIC/HEX) was used in this study. The RT-ddPCR was run twice. First to detect double targets in xABT solution A of the ORF1ab gene using channel 1 to measure FAM fluorophore and the N gene using channel 2 to measure VIC fluorophore, and single target detection in xABT solution B of the E gene using channel 1 to measure FAM fluorophore. Second, the detection of PhHV-1 as an internal control using channel 1, which measured EvaGreen dye in the QX200 droplet reader.

For ORF1ab, N, and E gene detection, 24 µL of each reaction mixture was prepared to reach similar end concentrations according to the reference kit by substituting nucleic acid amplification reaction solution with the compatible ddPCR reaction mixture for probes obtained in the previous step (Supplementary Table 1). The volume per reaction of the master mixture is solely calculated to achieve the same end concentration as the commercial kit, as the manufacturer has not disclosed information regarding the specification of the targeted genes, which includes the primer-probe set and concentration. The reaction mixture was inserted into a ddPCR 96-well semi-skirted plate (Bio-Rad Cat#12001925) on an ice block, followed by the addition of 6 µL of heat-treated saliva specimens, totaling a volume of 24 µL. The plate containing the mixture was covered with a pierceable foil heat seal (Bio-Rad Cat#1814040) and sealed using a thermal PX1 PCR Plate Sealer (Bio-Rad) at 180°C for 5 seconds. The mixture was thoroughly homogenized and spun down shortly to remove visible bubbles which can cause failure during the generation of droplets and PCR [21, 22]. The plate was conditioned to reach room temperature, transferred into the Automated Droplet Generator—AutoDG (Bio-Rad) machine along with the DG32 Automated Droplet Generator Cartridge (Bio-Rad, Cat#1864108) and a new ddPCR 96-well semi-skirted plate. The master mixture and specimen solution were converted into droplets in the AutoDG machine. The plate was gently removed from the droplet generator machine and briefly sealed, as previously stated. Within a maximum of 30 minutes post-droplet generation, plate-containing partitioned specimens were cycled in a C1000 Touch Thermal Cycler (Bio-Rad) under a similar thermocycling protocol as the reference RT-qPCR, with an additional post-cycling step of 98°C for 10 minutes (enzyme inactivation) and an infinite 4°C hold. A ramp rate of 2°C/second was added to each cycling step. The cycled plate was then gently transferred and read in the FAM and VIC channels to detect the ORF1ab/E gene and N gene, respectively, using the QX200 droplet reader (Bio-Rad). Figure 1 depicts the general workflow of RT-ddPCR procedure.

For PhHV-1 detection, 18 µL of reaction mixture was prepared consisting of 12 µL of the QX200 ddPCR EvaGreen Supermix (Bio-Rad, Cat#1864034), 5.64 µL of nuclease-free water, and 0.18 µL of both forward and reverse primers. Conversion of the mixture into droplets and amplification were performed as previously stated in the RT-ddPCR procedure. The cycled plate was further read in the EvaGreen channel.

Protocol validation

The validation of viable specimens was performed on both RT-qPCR and RT-ddPCR systems, as illustrated in Fig. 2.

Data analysis

A threshold was manually adjusted in regard to the NTC and PC or as close as reasonably possible to the amplitude value of the cloud corresponding to the negative droplets, which is also considered the background, and in accordance with the corresponding NTC results on the ORF1ab, N and E genes in each RT-ddPCR run [23–26]. The interpretation of positive SARS-CoV-2 specimens was in consideration of the positive droplet detection across all targeted genes [26]. Specimens with a droplet value above 10 000 were considered viable [24] and thus subjected to further statistical analysis. According to the ddPCR guideline, the specimen is considered positive if 1–3 positive droplets are present, taking into account (i) the recommendation of having more than one positive droplet to reduce false positives caused by noise and (ii) the possible impact of the saliva specimen matrix on fluorescent readings in the RT-ddPCR platform due to the direct heating method as a substitute for RNA extraction, thus showing the possibility of impurities that persist in the viral RNA template; consequently, specimens with two positive droplets are considered positive in this study [24, 27]. In accordance with the commercially available RT-qPCR kit manufacturer's instructions, additional conditions were considered for interpreting the RT-ddPCR detection results. The exemption was made for ORF1ab due to noisy data. Therefore, a specimen is deemed positive if one or two target genes (N or/and E) exhibit positive droplets with a copy number concentration (copies/µL). Conversely, if both N and E genes are not detected, the specimen is classified as negative (Tabel 1). Sensitivity and specificity were calculated for RT-ddPCR, considering the RT-qPCR results as references. Analysis of copy number was performed using QX Manager 1.2 Analysis Software (Bio-Rad) and analyzed using simple linear regression. GraphPad Prism 9.1.1 (GraphPad Software, La Jolla, CA, USA) was used to visualize the results.

Results and discussion

In this study, we conducted viability tests on 40 stored saliva specimens to ensure the quality of the specimens' post-storage. Current and previous detections displayed consistent results. All negative SARS-CoV-2 specimens with no detected Ct values remained undetected, and two of the positive SARS-CoV-2 specimens with previously detected Ct values (Sample ID 35—ORF1ab: 33.45, N: 34.36, E: 34.3; Sample ID 39—ORF1ab: 37.87, N: 36.35, E: N/A) were detected as negative (Table 1). Consequently, 95% of stored specimens (n = 40) were considered viable for SARS-CoV-2 detection, implicating the applicability of stored specimens for further studies that utilize similar types of viruses. A total of 18 positive and 22 negative SARS-CoV-2 saliva specimens determined by current RT-qPCR were further included in the detection using RT-ddPCR.

The compatibility assessment was conducted using two different types of commercially available ddPCR reaction mixtures, namely: ddPCR Supermix for Probes (no dUTP) and the One-Step RT-ddPCR Advanced Kit for Probes. The digital droplet PCR

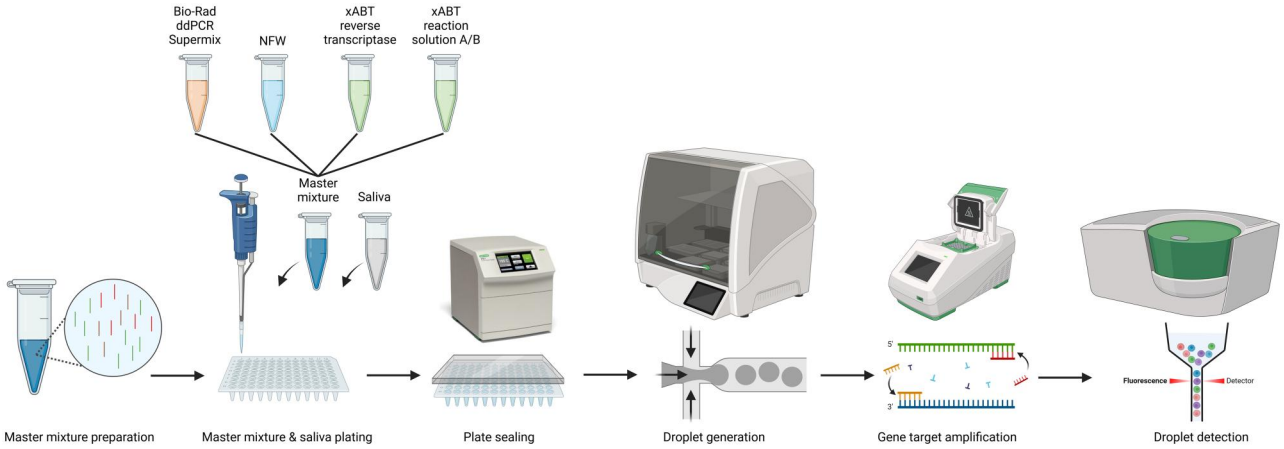

Figure 1. The workflow of the RT-ddPCR procedure. The RT-ddPCR workflow consists of six essential stages: master mix preparation (combined reagents of RT-ddPCR and RT-qPCR–xABT), pipetting into the plate, thorough sealing using a thermal sealer, loading into the ddPCR droplet generator, amplification, and final processing by the ddPCR droplet detection. The droplet generator instrument automatically partitions samples and mixtures into smaller droplets, while the droplet reader quantifies droplets using a two-color detection system (created with BioRender.com).

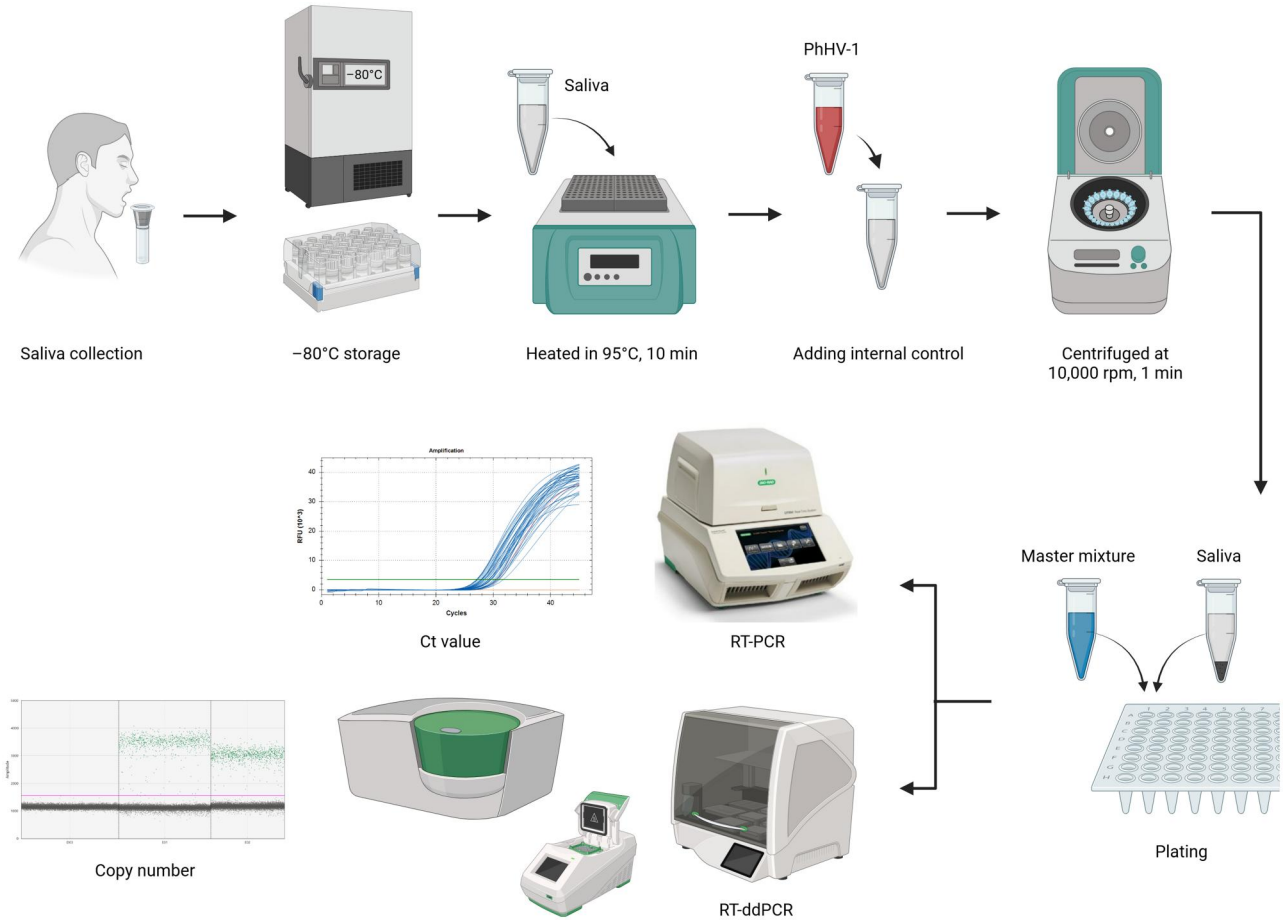

Figure 2. Overview of RT-ddPCR SARS-CoV-2 detection using saliva specimens. Saliva specimens were collected in cryotubes and stored at −80°C. Upon usage, the specimens were heated to 95°C for 10 minutes, followed by the addition of an internal control. The mixture was thoroughly homogenized and subsequently centrifuged at 10 000 rpm for 1 minute. The PCR master mixture and the supernatant of the heat-treated specimens were pipetted into a 96-well plate. RT-PCR and RT-ddPCR amplification were performed according to the established procedure.

supermix has a hot start feature at the polymerase stage, allowing the sample to partition into droplets while keeping the enzyme inactive at room temperature. Supermix has been designed to support the amplification and detection of target genes using commercially available probe-based assays [28, 29]. The ddPCR Supermix for Probes (no dUTP) mixture was revealed to be compatible with the reference kit. The assessment has been conducted three times, showing consistent results (Fig. 3d–f) when compared to the reference mixture (Fig. 3a–c). The ddPCR Advanced Kit for Probes mixture comprises of One-Step

Table 1. Ct value, copy number concentration, and interpretation result of positive SARS-CoV-2 saliva specimen collection.

Sample code	Ct value			RT-qPCR SARS-CoV-2 intrepretation result	Copy number concentration (copies/μL)			RT-ddPCR SARS-CoV-2 intrepretation result
	ORF1ab	N	E		ORF1ab	N**	E**	
21	35.13	35.59	36.20	Positive	1.97	0.26 (P)	0.62 (P)	Positive
22	29.16	29.69	29.67	Positive	8.2	4.1 (P)	1.24 (P)	Positive
23	29.34	28.78	29.12	Positive	0.79	10.74 (P)	7.3 (P)	Positive
24	35.65	37.06	35.74	Positive	0.61	0.09 (N)	0.09 (N)	Negative
25	35.88	34.94	35.82	Positive	N/A*	N/A*	N/A*	No amplification
26	30.44	31.35	30.72	Positive	0.88	4.27 (P)	3.54 (P)	Positive
27	33.30	34.55	34.06	Positive	1.36	0.06 (N)	0.16 (P)	Positive
28	34.56	37.19	35.05	Positive	3.81	0.08 (N)	0.19 (P)	Positive
29	35.76	35.55	35.72	Positive	1.17	0.15 (P)	0.28 (P)	Positive
30	33.01	34.23	33.47	Positive	1.08	0.59 (P)	0.21 (P)	Positive
31	37.25	36.12	38.19	Positive	0	0 (N)	0 (N)	Negative
32	29.20	29.14	29.10	Positive	0.2	5.54 (P)	6.15 (P)	Positive
33	38.42	39.09	–	Positive	3.91	0 (N)	2.01 (P)	Positive
34	22.30	22.86	22.48	Positive	240.61	220.95 (P)	125.77 (P)	Positive
35	–	–	–	Negative	0	0 (N)	0 (N)	Negative
36	30.13	30.51	30.64	Positive	3.9	3.32 (P)	0.9 (P)	Positive
37	32.53	32.59	32.69	Positive	0.27	1.25 (P)	0.69 (P)	Positive
38	32.46	33.08	32.03	Positive	2.43	1.56 (P)	4.29 (P)	Positive
39	–	–	–	Negative	0	0 (N)	0.7 (P)	Negative
40	24.43	24.67	24.62	Positive	39.44	89.96 (P)	54.57 (P)	Positive

* N/A: No amplification, droplet counts below 10 000.
** Individual interpretations of positive (P) and negative (N) for the N and E genes are displayed alongside the copy number concentration values.

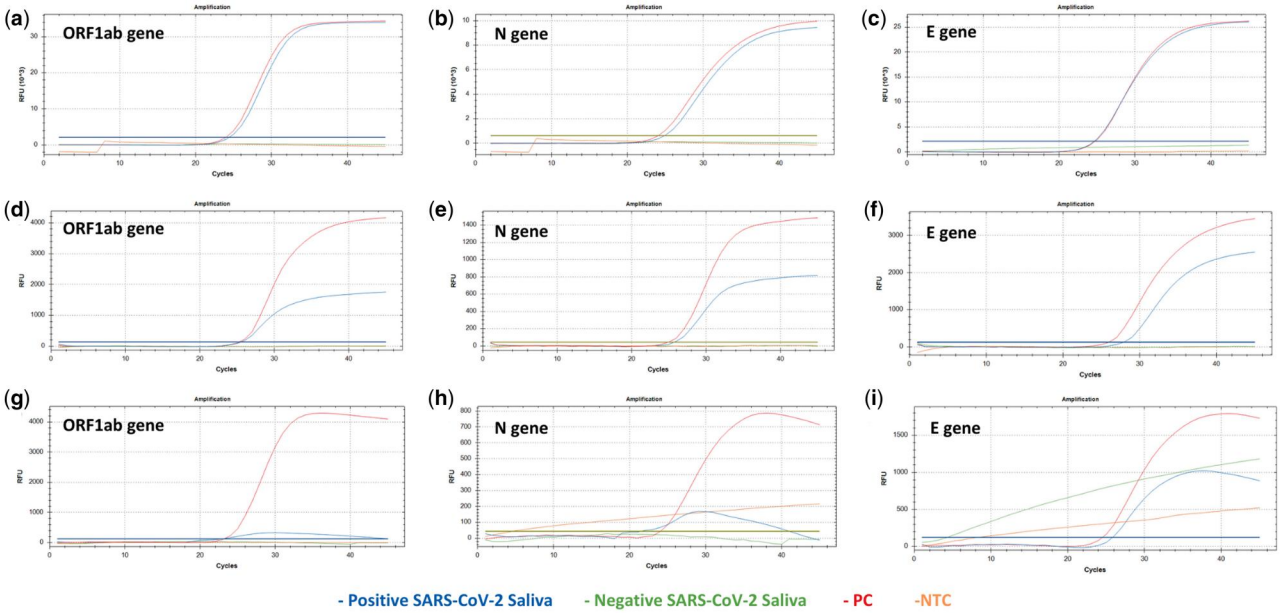

Figure 3. Compatibility of ddPCR mixtures tested using RT-qPCR. (a)–(c): xABT reference running condition of ORF1ab, N and E gene; (d)–(f): modified condition using ddPCR Supermix mixture for Probes (no dUTP) of ORF1ab, N and E gene; (g)–(i): modified condition using One-Step RT-ddPCR Advanced Kit mixture of ORF1ab, N and E gene. The compatibility test was done on positive SARS-CoV-2 saliva, negative SARS-CoV2 saliva, PC, and NTC, which are traced in blue, green, red, and orange lines, respectively.

RT-ddPCR supermix solution, RT enzyme, and dithiothreitol (DTT), which claimed increased efficiency, specificity, and sensitivity for quantification of RNA targets in the ddPCR process. Apart from that, the supermix contains RNase inhibitors, which function to protect RNA during the test process [28, 30]. However, our test has proven that the One-Step RT-ddPCR Advanced Kit for Probes was not compatible with our experiment setting (Fig. 3g–i). It might be due to the existence of DTT and incompatibility between the Bio-Rad RT enzyme and the commercial kit primer/probe set. Dithiothreitol (DTT) is a strong reductor agent that is used as a reaction stabilizing agent and to accelerate the degradation of black hole quenchers (BHQ), which can imitate positive signals, thus decreasing test sensitivity. It was observed that DTT disrupted real-time PCR by causing the passive reference signal to be quenched, leading to an overestimation of DNA concentrations. This compound was discovered to yield fluctuating signals from negative droplets in control reactions [26, 31–33]. Therefore, it could be possible that the One-Step RT-ddPCR Advanced Kit for Probes is less stable and can produce different numbers of detectable droplets with each test repetition. It is

also possible that the RT enzyme produced by Bio-Rad is incompatible with the primer/probe set of the xABT commercial kit. However, the compatibility comparison between the two components cannot be studied further in our study because the detailed base pair sequences of the commercial kit belong to company confidentiality (trade properties). Following the viability test, our assessment of RT-ddPCR reaction mixtures in RT-qPCR was revealed to be compatible.ddPCR Supermix for Probes (no dUTP) was further utilized in the RT-ddPCR platform, which showed a non-optimal quantification result, displaying unclear visible separation between positive and negative droplets for each gene target (Fig. 4). Further optimization, which includes annealing temperature adjustment, could potentially yield an efficient amplification result. Typically, the efficiency disparity between the intended target and co-amplified target can be widened by increasing the annealing temperature, which consequently enhances the specificity of the primer binding. This can frequently occur to the extent that the co-amplified population fuses with the negative population. Additionally, adjusting the annealing temperature to lower values may improve digital resolution and droplet separation clarity by minimizing the presence of stragglers or "rain" [26]. Therefore, in this study, we further conducted annealing temperature modifications. Changes in temperature at the annealing stage gave positive results regarding the amplitude separation between droplets with SARS-CoV-2 genetic material (positive droplets) and droplets without amplification of SARS-CoV-2 genetic material (negative droplets) with minimal background signal. Reducing the reaction temperature at the annealing stage causes the primer to bind to the template; however, if the reaction temperature is too low, it can allow binding to other non-specific genes, which can reduce reaction efficiency [31]. In this study, we observed droplet separation on a thermal gradient ranging from 57°C to 65°C, specifically 57°C, 57.6°C, 58.7°C, 60.2°C, 62°C, 63.5°C, 64.5°C, and 65°C which were automatically set by the C1000 Touch Thermal Cycler. Droplet separations were clearly visible using 57.6°C as the annealing temperature for the N and E genes. For both targets, there was no significant difference in the amplitude of this temperature (Fig. 5b–c, e–f). The annealing temperature is usually 3–5°C lower than the primary melting temperature [31], which in the xABT kit occurs at a temperature of 60°C. During the PCR step of the RT-ddPCR, a total volume of 40 µL consisting of 20 µL of master mixture and specimen and 20 µL of droplet generation oil was subjected to amplification, implying the need for adjustments in annealing temperature according to the total volume. These results are higher than a study by Zhang et al. (2018), who stated that the optimal temperature at the annealing stage for ddPCR detection of SARS-CoV-2 was 53.6°C, and in this study, most of the primer sets and probes for SARS-CoV-2 virus detection had tolerance to a wide temperature range and a very low background signal at an annealing temperature range of 50–60.5°C [32]. In addition, a study revealed that lower temperatures lead to better amplitude separation, while the distance between amplitudes decreases and becomes less distinct for temperatures above 60°C [25]. The applied temperatures ranged from 50°C to 64°C and were tested on a primary probe set with three replicates per temperature. Our experiment concluded that 57.6°C was the optimal annealing temperature for ddPCR, which is the highest temperature that still produces clear amplitude separation [34]. However, in the amplification of the ORF1ab target gene (Fig. 5a, d), the difference

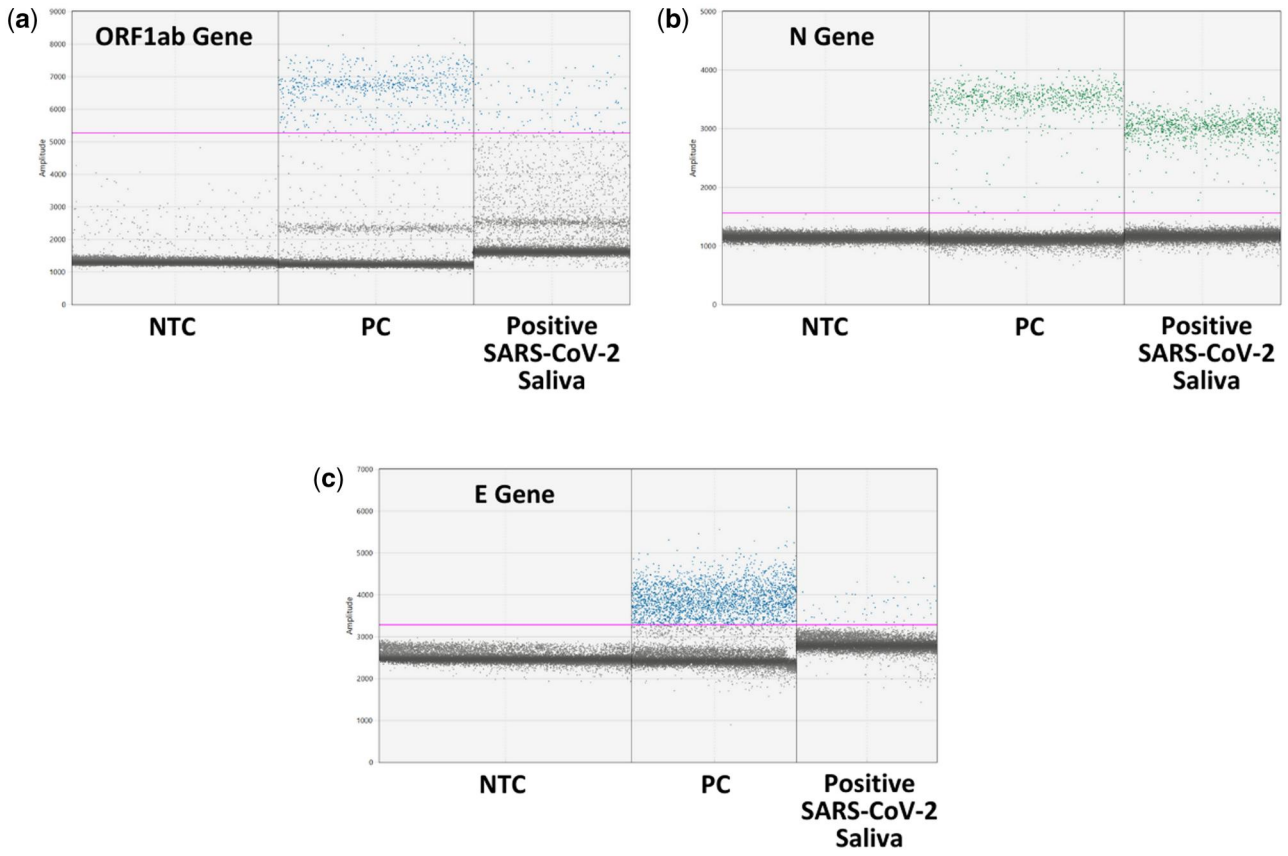

Figure 4. Droplet separation using ddPCR Supermix for probes (no dUTP) on targeted genes. (a) ORF1ab, (b) N gene, and (c) E gene. NTC, PC, and positive SARS-CoV-2 saliva are utilized in chronological order display

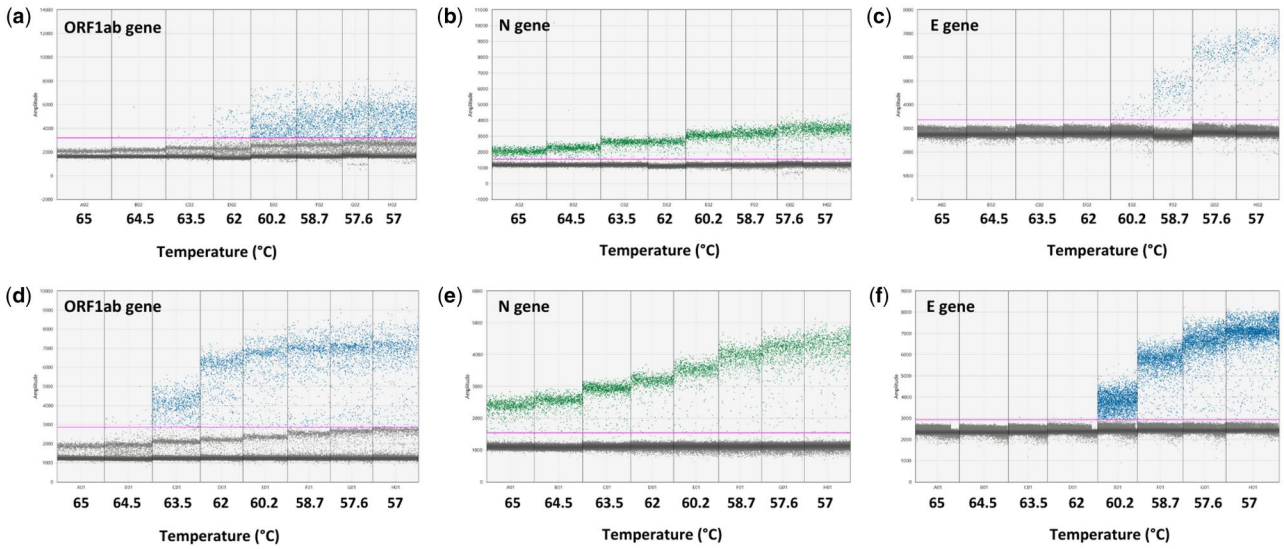

Figure 5. Droplet separation of the thermal annealing gradient ranges from 57°C to 65°C. (a)–(c): ORF1ab, N, and E genes of the positive SARS-CoV-2 saliva. (d)–(f): ORF1ab, N, and E genes of PC

in the amplitude of positive and negative droplets in the specimens was not clearly visible with changes in annealing temperature. Therefore, the threshold was adjusted manually using the PC and NTC as a reference, as has been done by several previous studies [9, 34–37]. This is suspected to occur due to incompatibility, the presence of certain inhibitors that only affect the ORF1ab gene, or it might be due to the unspecific target of ORF1ab in this commercial kit causing high cross-reactivity, although further study into this cause still needs to be done.

An additional optimization was performed by increasing the concentrations of the primer and probe by 25%. This optimization provides supporting data that may be utilized, as it was conducted solely with a PC and was limited to the testing of SARS-CoV-2 saliva specimens (Fig. 6). In the case of the ORF1ab target gene (Fig. 6a), the PC with reference concentration presented a higher copy number of 79.41% (82.26 copies/μL) compared to the additional 25% primer/probe concentration (16.93 copies/μL). A similar result was demonstrated on the N and E genes at the reference concentration (Fig. 6b–c), with a percentage of 79.01% (reference: 79.39 copies/μL, higher concentration: 16.66 copies/μL) and 10.41% (reference: 257.82 copies/μL, higher concentration: 230.98 copies/μL) higher compared to the additional 25% primer/probe condition, respectively. Although a higher primer/probe concentration provides a higher amplitude in droplet separation, the reference concentration scheme still presented a clear separation. Therefore, during the specimens' validation, we only used the result of the optimized annealing temperature as the reference concentration deemed sufficient. Even though the research did not use additional higher concentrations, it can be concluded that increasing the concentration of the primer and probe can be one way to obtain significant droplet separation. This is because by increasing the concentration of primer and probe, more binding of the target gene will occur, so the amount of amplification will increase. Further increasing the amount of amplification can cause separation of positive and negative droplet amplitudes [38, 39].

The detection of our custom internal control, phocine herpesvirus 1 (PhHV-1), which has commonly served as an amplification control [20], also demonstrated a concordance result on both PCR methods. PhHV Ct value and concentration in the form of copy number were detected during the RT-qPCR and RT-ddPCR, respectively, ensuring the accuracy and reliability of the result, including the efficiency of the process [40]. The detection of IC was further validated on forty saliva specimens, displaying corresponding results with a Ct value ranging from 27.09 to 31.78 and a copy number concentration ranging from 8.24 to 109.83 copies/μL (Supplementary Fig. 1). As our internal control has proven to be valid, we conducted further validation of 40 samples for each target of the ORF1ab, N, and E genes. Thirty-eight out of 40 specimens are valid for this detection since the droplet numbers are all above 10 000 and displayed clear separation. Fig. 7 depicts the droplets separation of targeted genes in representative saliva specimens. Both N and E genes presented high concordance of copy number (range 0.06-220.95 copies/μL, 0.09-125.77 copies/μL) and Ct value (range 22.86-39.09, 22.48-38.19) with an r^2 value of 0.98 and 0.85, respectively (Fig. 8). An inverse correlation between Ct value and copy number concentration was displayed: the lower the Ct value, the higher the copy number concentration. Our analysis revealed that a copy number concentration below 0.1 copies/μL indicates the presence of only one positive droplet, which is therefore considered a negative for the detection of the targeted gene (Table 1). We encountered challenges in establishing an appropriate threshold for ORF1ab gene, potentially stemming from the nature of this targeted gene, and thus the ORF1ab target was poorly detected, r^2 of 0.34 (Ct value range: 22.3-38.42, copy number: 0.2-240.61 copies/μL). A similar result was reported in studies that the N gene was reported to have less nucleotide variation than ORF1ab which may cause a more stable detection of the N gene compared to ORF1ab [41, 42]. Our study suggests that an approach to finding a better target for the detection of SARS-CoV-2, in particular, to modify the ORF1ab target, is an option to avoid unspecific detection.

The RT-qPCR analysis revealed 18 positive and 22 negative SARS-CoV-2 saliva specimens, whereas the RT-ddPCR analysis identified 15 positive and 23 negative specimens. One out of each positive and negative SARS-CoV-2 saliva specimen was categorized as unviable in the RT-ddPCR detection of all targeted genes with droplets below 10 000 (Table 1). Despite the fact that some saliva specimens were interpreted as positive for SARS-CoV-2 in the RT-qPCR setting, a positive at a low level (reaching $Ct > 35$) was observed and might be a false positive [43]. On the other

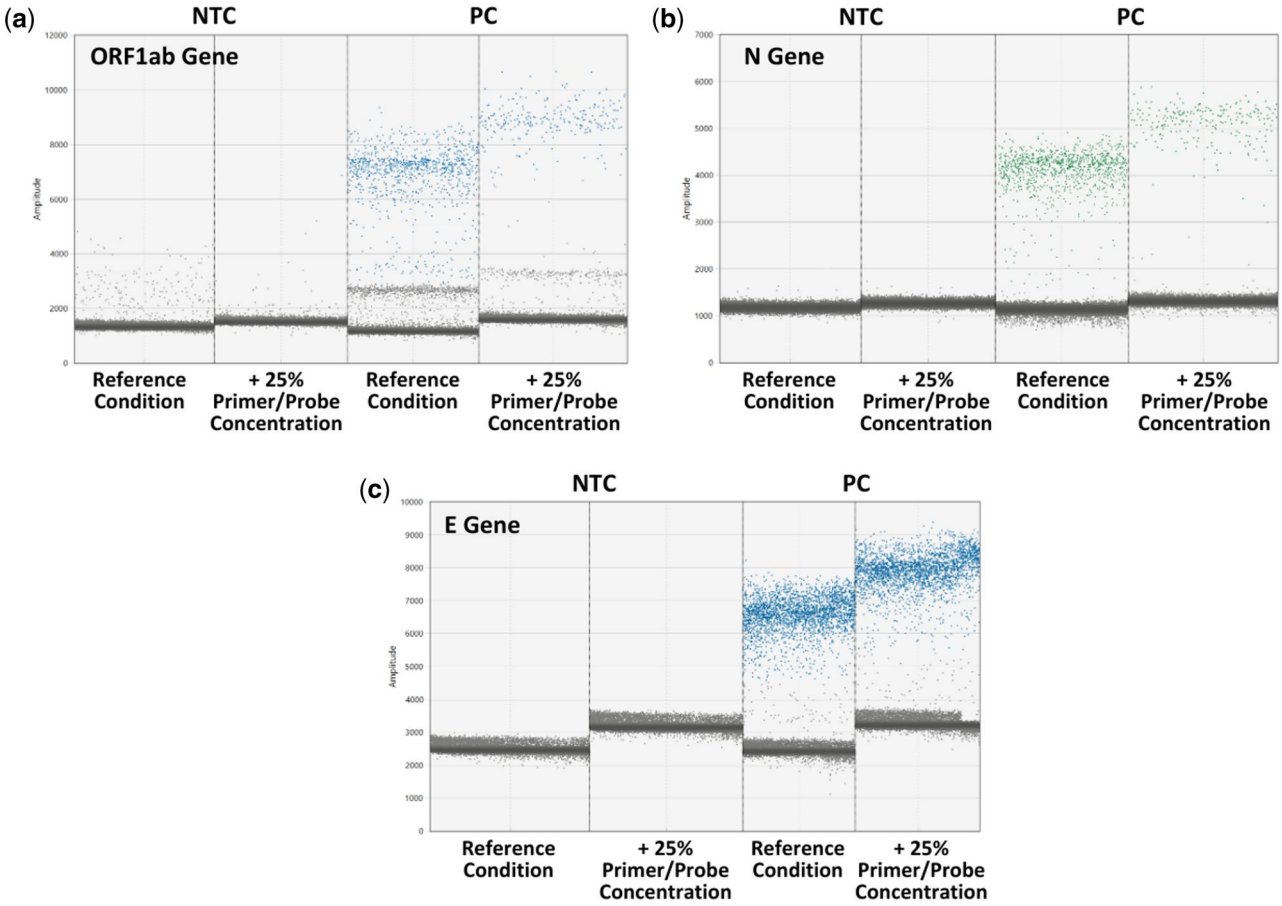

Figure 6. Comparison of droplet separation between reference and the additional 25% primer–probe concentrations. (a)–(c): ORF1ab, N, and E genes of NTC and PC in reference and modified conditions

hand, the RT-ddPCR platform has the capacity to detect the smallest quantity of viral particles [44, 45] and has been able to identify positive cases with low viral load that were initially tested negative with RT-qPCR; the condition may occur in Sample ID-29 [7, 11, 46, 47]. This interpretation could be due to the increased tolerance of RT-ddPCR toward PCR inhibitors compared to RT-qPCR [48]. After conducting an additional overall interpretation by excluding the ORF1ab due to threshold-setting challenges, we observed that samples ID-27 and ID-29 might be negative for SARS-CoV-2 because of ambiguous threshold lines for the E and N genes, respectively. However, this does not alter the conclusion of our study, which aims to demonstrate the feasibility of optimizing the commercial RT-qPCR kit on the RT-ddPCR platform.

Based on the aforementioned reason, the sensitivity values were calculated for N and E targeted genes, which were shown to be 100% and 93.3%, as for the specificity, the percentage was 80.8% and 91.3% for N and E genes, respectively. However, despite exhibiting a high degree of specificity, the ORF1ab demonstrated the presence of "rain" droplets in the 1D analysis, indicating that it may not be specific (Supplementary Fig. 2). During the validation of both ORF1ab and N genes, which were the targets of solution A, we found a percentage of double-positive droplets to the total positive droplets of 21% and 22% for ORF1ab and N genes, respectively. This result presented a highly double-positive indicating that in one droplet of the reaction mixture containing both the ORF1ab and N genes, thus detected in FAM and VIC ddPCR channels. Meanwhile, a low double-positive is also present in this study, with a percentage of 4 and 6 for the ORF1ab and N genes, respectively. Our study indicated that the lower Ct value or higher copy number concentration resulted in a stronger double-positive correlation, providing a clear and clustered separation in the 2D analysis. However, further research should be conducted to comprehend this result. Supplementary Fig. 3 displays the 2D plot of two double-positive detected specimens.

Our method optimizes existing molecular detection kits for coherent integration with the RT-ddPCR platform. Combining components from the current kit with those of ddPCR (such as ddPCR Supermix and oil) enables direct conversion. The optimization process involves assessing the compatibility of the RT-ddPCR master mixture, optimizing primers/probes concentrations, and annealing temperature adjustment, thus facilitating the detection of targets not limited to SARS-CoV-2. This approach might contribute to the enhanced precision in pathogen detection, fostering progress in molecular diagnostics. Generally, cost is still a drawback of implementing advanced technologies. However, the cost will align with the demand for technology by means of integrating the economy of scale. According to our study, the cost of RT-ddPCR is twice as high as that of RT-PCR. Nonetheless, the situation might differ among the countries/areas. In many developing countries, including Indonesia, most equipment, reagents, and consumables are still imported, thus scaling up the cost of the experiment itself. Despite its drawback, RT-ddPCR generates an absolute quantification of genetic material without the necessity of running standard curves, serving as a potential strategy to increase the detection and diagnosis

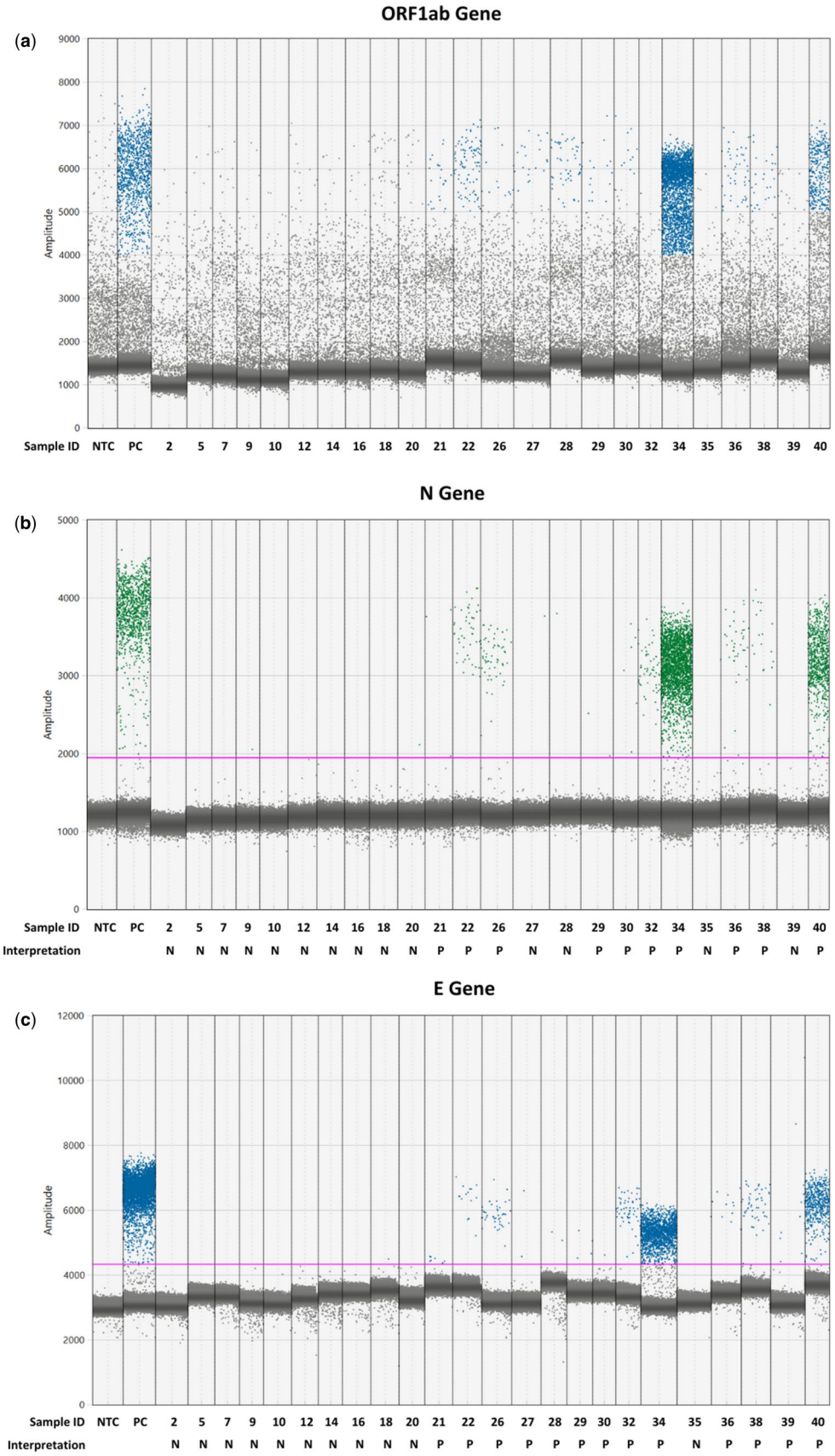

Figure 7. Droplet separation of targeted genes in representative saliva specimens. (a)–(c): ORF1ab, N, and E genes, respectively, of NTC, PC, and representative saliva specimen in chronological order. Blue or green dots represent positive droplets, and samples are intrepreted as positive (P) and negative (N) on each gene of N and E. Sample IDs 2–20 and 21–40 have an overall interpretation as negative and positive SARS-CoV-2 saliva specimens, respectively, except for Sample IDs 35 and 39, which are both interpreted as negative in the RT-ddPCR platform

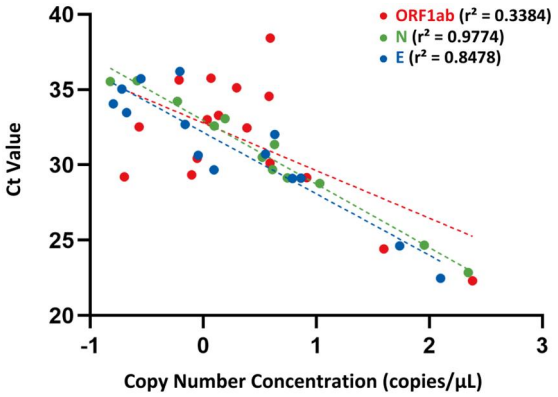

Figure 8. Correlation of Ct value and copy number concentration. ORF1ab, N, and E genes are plotted in red, green, and blue dots, respectively. The slope of linear regression is presented as a traced line

accuracy of the targeted pathogen(s). Moreover, absolute copies are needed to investigate the tested method's sensitivity and provide a diagnostic test's LoD (Limit of Detection).

In conclusion, our study revealed that utilizing the commercially available RT-qPCR kit was proven possible and recommended as an option for optimization on the RT-ddPCR platform, especially when the primer/probe sets produce a high yield in the RT-qPCR setting. Adjustment of annealing temperature presented an optimized result, although an increment in primer/probe concentration is not adapted as the reference condition is deemed sufficient. In addition, we recommend conducting a complementary experiment. For instance, designing primer–probe sets similar to those employed in commercial RT-qPCR assays, which could provide comparable method in terms of accuracy and performance. This approach may advance molecular diagnostics, regardless of the pathogen types. Considering our ORF1ab detection challenges in establishing an appropriate threshold for analysis, our study may also serve as a suggestion for industries and researchers for further development in selecting optimal primer–probe designs for the targeted gene of commercial or in-use kits. Post-validation revealed an inverse correlation between copy number concentration and Ct value. A strong double positive was observed in a low Ct value SARS-CoV-2 saliva specimen. However, additional research is required to achieve a deeper understanding. Implementation of this established procedure may be employed to evaluate a suboptimal amplification in a targeted gene of RT-ddPCR setting.

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Author contributions

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Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

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Data availability

The data that support the findings of this study are made available from the corresponding author upon request.

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