




ORIGINAL RESEARCH



## Developing multiplex ddPCR assays for SARS-CoV-2 detection based on probe mix and amplitude based multiplexing

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### ABSTRACT

**Introduction:** With the ongoing SARS-CoV-2 pandemic, different articles have been published highlighting the superiority of droplet digital PCR (ddPCR) over the gold-standard reverse transcription PCR (RT-PCR) in SARS-CoV-2 detection. However, few studies have been reported on developing multiplex ddPCR assays for SARS-CoV-2 detection and their performance. This study shows steps on how to develop different ddPCR SARS-CoV-2 assays including higher order multiplex assays for SARS-CoV-2 detection and antiviral screening.

**Methods:** Using multiple primer/probe sets, we developed, optimized, and analyzed the performance of simplex (1 target), duplex (2 targets), triplex probe mix (3 targets), and quadruplex (4 targets) SARS-CoV-2 ddPCR assays based on a two-color ddPCR detection system.

**Results:** Results showed that the quadruplex assay had similar limits of detection and accuracy to the lower multiplex assays. Analyzing 94 clinical samples demonstrated that the ddPCR triplex probe mix assay had better sensitivity than the RT-qPCR assay. Additionally, the ddPCR multiplex assay showed that remdesivir could inhibit the growth of SARS-CoV-2 *in vitro* while another testing drug could not.

**Conclusion:** Our research shows that developing multiplex ddPCR assays is possible by combining probe mix and amplitude-based multiplexing, which will help in developing multiplexed ddPCR assays for different SARS-CoV-2 applications.

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## 1. Introduction

Since the discovery and isolation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Wuhan, China [1], the virus has spread to multiple countries across the globe causing a global public health crisis. Many health systems have been crippled by this pandemic and all efforts are geared toward prevention, diagnosis, and treatment of the virus and its associated disease coronavirus disease 2019 (COVID-19) [2,3].

As much as many companies and research institutes have come up with drugs, vaccines, and other potent solutions to stop this virus, a permanent solution to stop this pandemic still does not exist [4]. This has left many countries to rely on diagnostics to detect, isolate, and treat infected patients. The most commonly used method and gold-standard for the detection of infection cases in this pandemic is reverse transcription quantification real-time PCR (RT-PCR). PCR is not a new technique as it has grown since its discovery in the 1990s [5] to become a well-established and commonly used technique to diagnose infectious diseases.

PCR has seen a transformation over the past decades since its discovery. The analogous qualitative conventional PCR that relies on gel electrophoresis was improved to quantitative real-time PCR

(qPCR) for detection and relative quantification. However, due to the need for a standard curve to perform relative quantification using qPCR, the third generation of PCR, digital PCR (dPCR), was developed to absolutely quantify pathogens directly [6]. Since its advent, dPCR has undergone lots of technological improvements and applications that are slowly overshadowing qPCR [7,8]. Technological refinements in dPCR have led to the development of better platforms like droplet digital PCR (ddPCR) capable of portioning samples to thousands of droplets for direct detection and quantification. Recent publications have highlighted the superiority of dPCR [6] in detection and quantification of targets including SARS-CoV-2 [9–16] when compared to RT-PCR. In their publication [15], Suo et al. showed that dPCR could detect SARS-CoV-2 reliably in clinical samples that tested negative by RT-PCR. This meant that the assay detection capabilities were superior to that of RT-PCR and it could avoid false-negative results that were recorded by RT-PCR.

Similar to RT-PCR, multiplexing is possible by digital PCR [17]. Using probe-based or Evagreen assays, one can design multiple assays to quantify more than one target in a single well. This has been highlighted to save on costs when running simplex assays and also increase the chances of target

detection [18]. So far, no publication has been made publicly available to explain the different approaches and steps on how to develop multiplex ddPCR assays that can detect SARS-CoV-2. The available multiplexed ddPCR assays have been commercialized and do not explain steps on how to develop similar assays. This poses a challenge to prospective researchers and clinicians who want to develop in-house multiplex assays for SARS-CoV-2 detection, diagnosis, and/or research.

Therefore, in this study, using already RT-PCR tested [10–13,15] China CDC SARS-CoV-2 primers/probe sets [19] and additional in-house primers, we aim to show steps on how to develop simplex, duplex, triplex probe mix, and quadruplex ddPCR assays that can reliably detect and also quantify SARS-CoV-2 from clinical and research samples.

## 2. Materials and methods

### 2.1. Ethical considerations

Wuhan Institute of Virology (WHIOV) is among the labs and institutes approved by China CDC of Wuhan city to conduct research on SARS-CoV-2 and detect COVID-19 from clinical samples. Research on developing new diagnostic techniques for COVID-19 using clinical samples has also been approved by the ethical committee of Wuhan Institute of Virology (2020FCA001).

During the outbreak in Wuhan, numerous clinical isolates from different hospitals were transported to WHIOV for detection. To ensure biosafety, all samples were received and inactivated first in the biosafety level 2 laboratory (BSL 2) with personal protection equipment for biosafety level 3 (BSL 3) laboratory.

### 2.2. Sample categories for assay development

Since the assays may be used for research or diagnosis, three sample categories were used to develop the various ddPCR assays. Sample 1 was a sample containing only the SARS-CoV-2 genome (SARS-CoV-2 only) obtained from the cultured virus in Vero E6 cells to represent research work; sample 2 was the human gene sample (IC only) pooled from oral swabs of healthy volunteers to represent negative clinical samples during diagnosis; and sample 3 was the SARS-CoV-2 virus from Vero E6 cells spiked with the human gene (SARS-CoV-2+ IC) to represent a positive clinical sample during diagnosis.

### 2.3. Sample processing

All the samples used in this work were firstly inactivated in the BSL 2 laboratory by heating at 56°C for 30 min. Inactivated samples were then extracted (bead extraction) using the Purifier™ Modesty automated RNA extraction machine. Post extraction, 4 µL RNA was converted to cDNA using the TakaRa PrimeScript™ RT Master Mix according to the manufacturer's instructions for subsequent experiments (RT-qPCR and/or ddPCR).

### 2.4. Primers and probes

China CDC Primers and probes (Table 1) targeting the open reading frame 1ab (ORF1ab) and nucleoprotein (N) gene regions [19] of SARS-CoV-2 were used to develop the various assays. Two additional primer sets targeting the receptor-binding domain 2 (RBD2) [20] of SARS-CoV-2 and an endogenous internal control Ribonuclease P protein subunit p30 (RPP30) [12] targeting the human gene were also synthesized and used to develop the various assays. All primers and probes were synthesized by the Sangon Biotech Company, Beijing, China.

### 2.5. Real-Time PCR Assay Composition

The Luna® Universal probe qPCR Master Mix (New England BioLabs) was used for developing the qPCR assay according to the manufacturer's instructions. The qPCR assay was composed of 0.4 µM and 0.2 µM primer and probe (Table 1) concentrations, respectively, 10 µL Luna Universal qPCR Probe Master Mix, 2 µL cDNA sample, and nuclease free water to a final volume of 20 µL. All qPCR experiments were performed in a CFX96 Touch™ real-time PCR (BioRad) instrument under the reaction conditions initial denaturation at 95°C for 5 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing (reading step) at 64°C for 1 minute.

### 2.6. Droplet digital PCR workflow and assay composition

The ddPCR workflow begins with making the detection assay mix. The ddPCR™ Supermix for Probes (No dUTP) was used to develop all the assays after generating cDNA. Different primer and probe concentrations were used to develop the simplex, duplex, triplex probe mix, and quadruplex assays. The 1× concentration of the various assays included:

**Table 1.** Primer and probe sequences for SARS-CoV-2 and an endogenous human control gene.

Target		Sequence 5' to 3'	Probe dye(s)	Ref
ORF1ab	Forward	CCCTGTGGGTTTTACACTTAA	5'- FAM and BHQ1-3'	[19]
	Reverse	ACGATTGTGCATCAGCTGA		
N	Probe	CCGCTGCGGTATGTGGAAAGGTTATGG	5'- FAM and BHQ1-3'	[19]
	Forward	GGGGAACTTCTCCTGCTAGAAT		
RPP30	Reverse	CAGACATTTTGTCTCAAGCTG	5'- HEX and BHQ1-3'	[12]
	Probe	TTGCTGCTGCTTGACAGATT		
RBD2	Forward	AGTGCATGCTTATCTCTGACAG	5'- HEX and BHQ1-3'	[20]
	Reverse	GCAGGGCTATAGACAAGTTCA		
RBD2	Probe	TTTCTGTGAAGGCG ATTGACCGA	5'- FAM and BHQ1-3'	[20]
	Forward	CTCAAGTGTCTGTGGATCACG		
	Reverse	CCTGTGCTGTAAACCATTG		
	Probe	ACAGCATCAGTAGTGCAGCAATGTCTC		

### 2.6.1. Simplex assays

Each assay here was composed of a primer and probe pair for a particular target. The general 1× assay was composed of 11 μL 2× ddPCR Supermix for probes (No dUTP), 900 and 250 nM primer and probe concentrations, respectively, 2.2 μL cDNA, and nuclease free water to a final volume of 22 μL.

### 2.6.2. Duplex assays

The duplex assay was composed of two sets of primers and probes. Each duplex assay was set to detect a target (N/ORF1ab) in the FAM channel and the human gene (IC) RPP30 in the HEX channel. The 1× assay was similar to that of the simplex assay with only a variation in the volume of nuclease free water due to the addition of a new primer set.

### 2.6.3. Triplex probe mix assay

The triplex probe mix assay was composed of three sets of primers and probes to detect two targets (ORF1ab and N) and the IC (RPP30). The general 1× assay was composed of 11 μL 2× ddPCR Supermix for probes (No dUTP), 900 and 250 nM primer and probe concentrations, respectively, 2.2 μL cDNA, and nuclease free water to a final volume of 22 μL. However, for the probes, the targets were labeled with different dyes (FAM and/or HEX) at different ratios. Target 1 was labeled with FAM (1:0), target 2 was labeled with 50% FAM and 50% HEX (1:1), and target 3 was labeled with HEX (0:1). Targets 1 and 2 were ORF1ab and N gene used interchangeably while target 3 was RPP30.

### 2.6.4. Quadruplex amplitude-based assay

The quadruplex assay was composed of four sets of primers and probes to detect three SARS-CoV-2 targets (ORF1ab, N, and RBD2) and the IC (RPP30). The 1× quadruplex assay was composed of 11 μL 2× ddPCR Supermix for probes (No dUTP), primers at 900 nM (1×) and 450 nM (0.5×) concentrations, probes at 250 nM (1×) and 125 nM (0.5×) concentrations, 2.2 μL cDNA, and nuclease free water to a final volume of 22 μL. Each channel was used to detect two targets at 0.5× and 1× primer and probe concentrations. For FAM targets, RBD2 primers and probe were added at a concentration of 0.5× while N primers and probe were added at 1× concentrations. For the HEX channel, RPP30 primers and probe were added at 0.5× concentrations while ORF1ab primers and probe were added at 1× concentrations. Since this assay included an in-house primer set (RBD2), it was excluded in subsequent optimization experiments and only used for demonstration purposes.

After distributing the different assays in a ddPCR plate, approximately 20,000 nanoliter-sized droplets per well were generated using an automated droplet generator (QX200™ AutoDG ddPCR system (Bio-Rad, US)). Resultant droplets were then heat-sealed with a pierceable aluminum foil using a PX1 PCR plate sealer (Bio-Rad) set to run at 180°C for 5 sec before being loaded into a C100 Touch™ Thermal Cycler (Bio-Rad, US) for amplification. Thermal cycling with a ramp rate of 2°C/sec at every step was set to run for 10 min enzyme activation at 95°C; followed by 40 cycles of denaturation at 94°C for 30 sec and 1 min annealing/extension at 57°C; enzyme deactivation at 98°C for 10 min; and a final hold step at 4°C

for at least 30 minutes to stabilize the droplets. Post amplification, droplets were read in a QX200™ Droplet Reader.

## 2.7. Annealing temperature optimization

During thermal cycling of the droplets, a temperature gradient between 65°C and 55°C was inserted at the annealing temperature step to determine the best temperature for which droplet separation will be achieved in the simplex, duplex, and triplex probe mix assays.

## 2.8. Inter and intra-assay variability

Eight replicates per assay of a positive sample spiked with a human gene were used to test inter and intra-assay variability. The sample was tested on simplex (ORF1ab, N, and RPP30 (IC)), duplex (N and IC, and OFR1ab and IC), and triplex probe mix assays. For the triplex assays, two assays with interchanging N or ORF1ab targets to 50% (FAM and HEX) probe concentrations were used.

## 2.9. Determining LoB/LoD

The triplex probe mix assay was used to determine SARS-CoV-2 detection limits. For Limit of Blank (LoB), 21 SARS-CoV-2 negative human samples and 3 nontemplate controls (NTC) were used. However, due to the absence of a SARS-CoV-2 standard, SARS-CoV-2 cDNA from Vero E6 cells was serially diluted in a background of already pooled human oral swab matrix to determine the assay Limit of Detection (LoD). A total of 16 two-fold serial dilutions were done and detected in triplicates using the triplex probe mix assay. The LoD was set to be the lowest concentration at which two of the three (2/3) triplicate samples could be detected in the dilution series. The LoD for individual targets (ORF1ab and N) were determined in copies/reaction.

### 2.9.1. Application of the multiplex assay

The triplex probe mix assay approach was used to demonstrate the applicability of multiplex assays in detecting clinical samples and performing research. For the diagnostic performance, 94 clinical samples that were negative or with low virus concentrations were used to test the triplex probe mix ddPCR assay (N (1:0), ORF (1:1), and RPP30 (0:1)). The same ddPCR primers and probes were also used to perform qPCR. For qPCR, no optimization was done as the Luna® Universal probe qPCR Master Mix was used according to the manufacturer's instructions. A one-step commercial RT-qPCR kit approved by China FDA (DaAn Gene Co., Shenzhen) targeting the ORF1ab (VIC), N (FAM), and an endogenous internal control (Cy5) was used according to the manufacturer's instructions to validate the qPCR and ddPCR detection results.

For the research application, we tested the applicability of the triplex probe mix assay (RBD2 (1:0), N (1:1), and ORF1ab (0:1)) in determining the inhibition efficiency of remdesivir and a drug we named Code 30 (due to ongoing research) against SARS-CoV-2 *in vitro*. Vero E6 cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.001. One set was used as a control while the other two sets were treated with remdesivir and Code 30 to a final concentration of 10 μM. Using the MTT assay, both drugs (remdesivir and code 30) were found having no

toxicity to VERO E6 cells at a concentration of 10  $\mu$ M. Twenty-four hours post infection (hpi), 200  $\mu$ L of the cell supernatant from all sets was extracted in triplicate and the RNA quantified using the ddPCR triplex probe mix assay.

### 2.9.2. Data analysis

A droplet count of  $\geq 10,000$  droplets was set as the cutoff when analyzing all ddPCR experiments [15]. If the number of droplets were lower, the results were discarded or the experiment repeated. All ddPCR data were generated using BioRad's QuantaSoft™ software version 1.7.4.0197. Simplex and duplex assay experiments were analyzed directly using the same software. However, for the triplex probe mix and quadruplex assays, the QuantaSoft™ Analysis Pro software version 1.0.596 was used for analysis. Since the QX200 ddPCR system has a narrow dynamic range from 1 to 120,000 copies/20  $\mu$ L reaction, it is recommended that when quantifying samples with extremely high amounts of target molecules (like cell culture samples) plan to reduce the starting sample accordingly. In the advent, the target copy number/genome is unknown; it is recommended that one determines the optimal starting amount by performing four tenfold serial dilutions of each sample at the expected digital range. Further statistical analysis was done using the GraphPad Prism Version 6.01 software.

## 3. Results

### 3.1. Droplet separation

The three sample categories highlighted in the methodology section 3.2 were used to determine how droplets separated in the simplex, duplex, triplex probe mix, and quadruplex assays. Since it is important to have a control during diagnosis, the RPP30 gene was included in all multiplex assays. The results were as follows:

#### 3.1.1. Simplex assay

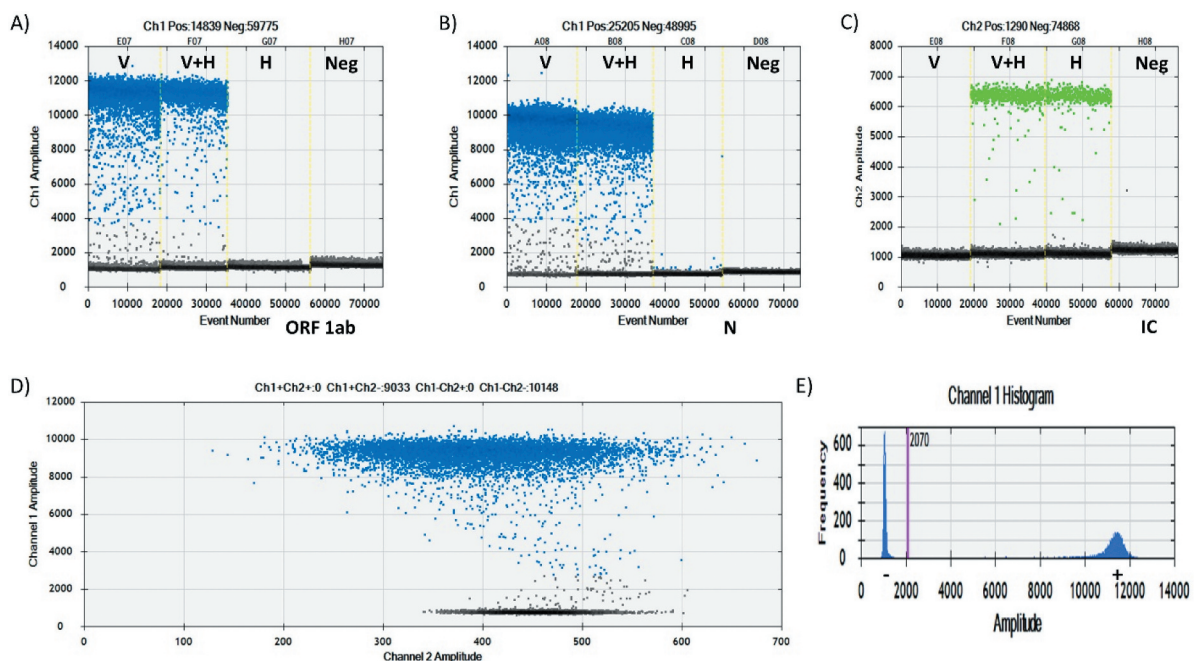
Droplets could be detected in all the simplex assays as seen in Figure 1. Both the ORF1ab and N gene targets were labeled with FAM hence read in Channel 1. From the results, the virus could be detected in the sample categories with only the virus (V) and the virus spiked in a background of the pooled human gene (V + H). No droplets were observed for both the ORF1ab and N genes when only the healthy human sample (H) and negative (Neg) control samples were detected as seen in Figure 1A and B. The RPP30 was labeled with HEX hence read in Channel 2. The human gene could be detected in only the H, and V + H samples as seen in Figure 1C. The human gene could not be detected in the V and Neg samples as they did not contain the human gene.

#### 3.1.2. Duplex assay

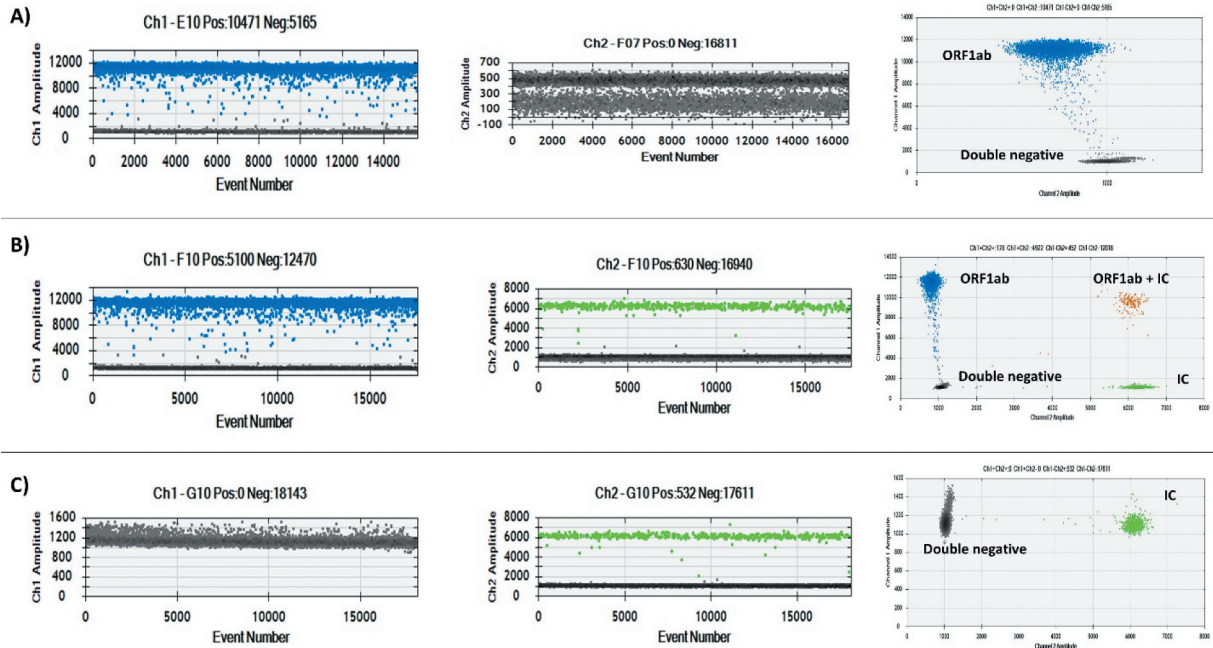
For the duplex assays, one target was FAM labeled (ORF1ab) and the other HEX labeled (RPP30). This meant that droplets were to be observed in both Channel 1 and Channel 2 as seen in Figure 2. As expected, four droplet clusters were observed in the 2D channel and two in the 1D channel (for each target) when a sample containing the virus spiked in a background of the pooled human gene was detected (Figure 2B). However, when a sample contained only the virus was read (Figure 2A), we observed a positive signal in the FAM channel and no signal in the HEX channel with a consequent 2 droplet amplitude partition in the 2D channel. The reverse was true when only a sample containing only the human gene was detected (Figure 2C).

#### 3.1.3. Triplex probe mix assay

Eight droplet clusters were observed (Figure 3A) when a triplex probe mix assay was run to detect SARS-CoV-2 spiked in a background of the pooled human sample. The normal QuantaSoft™ software used for reading droplets



**Figure 1.** Simplex assay droplet separation results. A, B, and C are 1D amplitude results of the ORF1ab, N, and RPP30 targets respectively using various sample categories. D is a representative 2D amplitude result. E is a representative histogram result of a simplex assay.

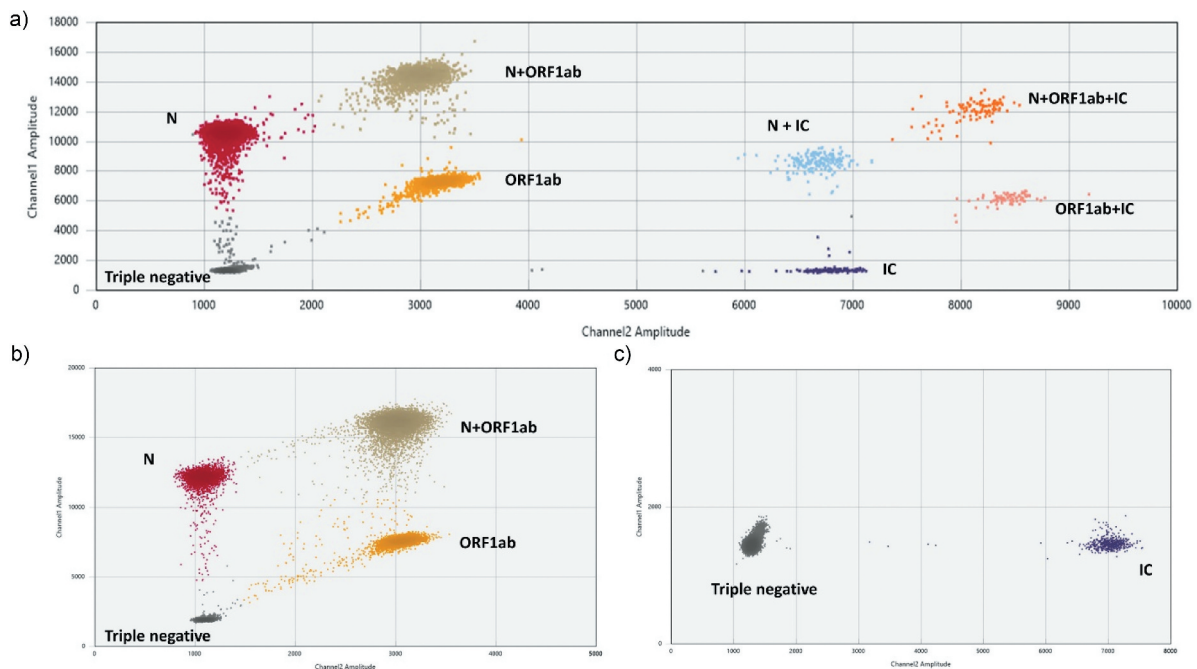


**Figure 2.** Duplex assay droplet separation results in the 1D and 2D amplitudes. A) SARS-CoV-2 only sample. B) SARS-CoV-2+ IC sample. C) IC only sample.

could not classify which targets the droplets belonged to as seen in Supplementary Figure S1. To analyze the data, we used the QuantaSoft™ Analysis Pro software as it was designed to curb this inefficiency. Using the circular threshold tool in the software, the droplet clusters were designated as follows in the 2D amplitude: triple negative (bottom left gray cluster); N (single positive (FAM channel) directly above the triple negative); ORF1ab (single positive (FAM and HEX channels) at a  $\sim 45^\circ$  angle from the triple negative); IC (single positive (HEX channel) immediately to

the right of triple negative); N+ ORF1ab (double positive above N and ORF1ab); N+ IC (double positive above IC); ORF1ab+IC (double positive at a  $45^\circ$  angle from IC); and N + ORF1ab+IC (triple positive and right above ORF1ab+IC).

From the different sample categories in the triplex probe mix assay, the clusters of double positives and triple positive reduce when only the virus (Figure 3B) or the human gene (Figure 3C) are detected. Additionally, the target cluster positions are maintained with different samples.



**Figure 3.** Droplet separation in a triplex probe mix assay results. A) SARS-CoV-2+ IC sample. B) SARS-CoV-2 only sample. C) IC only sample.

### 3.1.4. Quadruplex amplitude-based assay

Sixteen droplet clusters were observed (Figure 4A) in the quadruplex assay using a sample containing SARS-CoV-2 spiked in a background of the pooled human sample. Similar to the triplex assay, the QuantaSoft™ software used for reading the droplets could not directly classify the clusters as seen in Figure S2; hence, the QuantaSoft™ Analysis Pro software was used. The droplet clusters were assigned using the circular threshold tool available in the QuantaSoft™ Analysis Pro software. The droplets were clustered as: quadruple negative (bottom left gray cluster); RBD2 (single positive (FAM channel) directly above the quadruple negative); N (single positive (FAM channel) directly above RBD2); RBD2 + N (double positive (FAM channel) directly above N), IC (single positive (HEX channel) immediately to the right of quadruple negative); ORF1ab (single positive (HEX channel) immediately to the right of IC); IC+ORF1ab (double positive (HEX channel) immediately to the right of ORF1ab); and quadruple positive (highest droplet cluster above IC+ORF1ab)). Resultant double-positive and triple-positive droplets clusters of different targets could be located at the intersections of the arrows. The different sample types also helped in the location of different clusters. From the amplitude result of the 2D channel, the droplet clusters of the SARS-CoV-2 targets were maintained at the same positions even when different sample categories were used (Figure 4).

### 3.2. Temperature gradient analysis

As seen in Figure 5, there was a general increase in the positive droplet amplitude with a decrease in annealing temperature from 65°C to 55°C. In the 2D channel of both the duplex and triplex probe mix assays, there was no clear separation of droplet clusters till the temperatures dropped

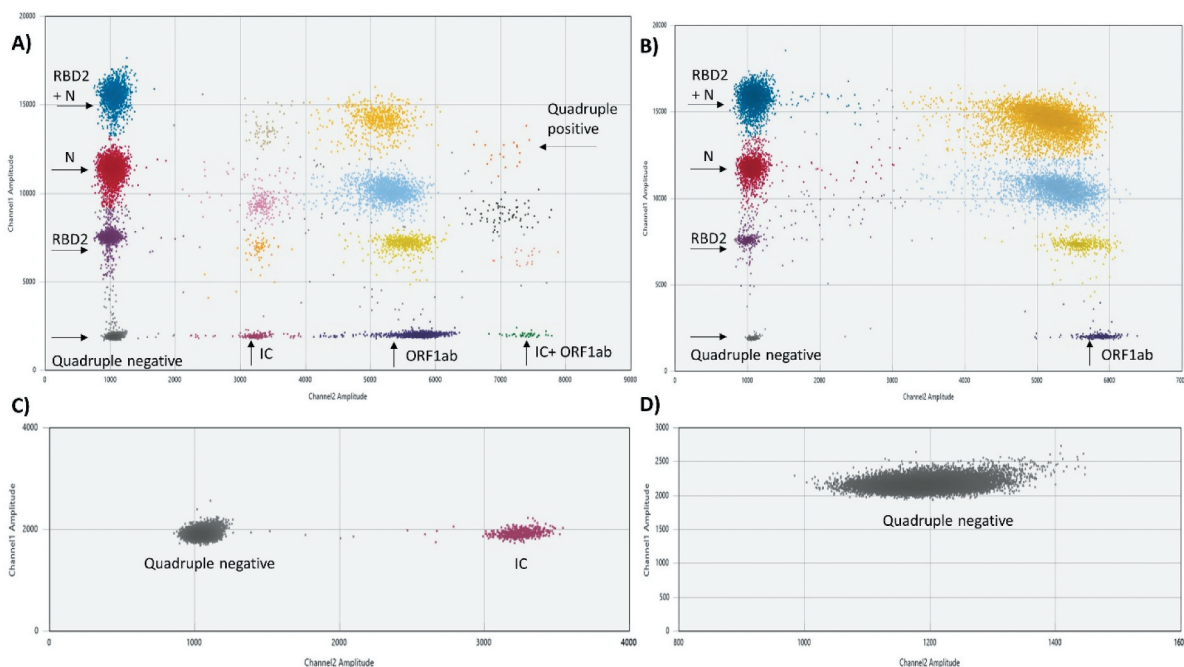
from 65°C to 63°C. Even though clusters could be assigned at 63°C, the better separation was yet to be achieved. For the duplex assay, the 1D plot results (data not shown) were similar to those of the simplex assays. An annealing temperature of 57°C was found optimum for subsequent experiments as optimum separation was achieved at 56.9°C. This temperature was suitable for all the assays.

### 3.3. Assay variability

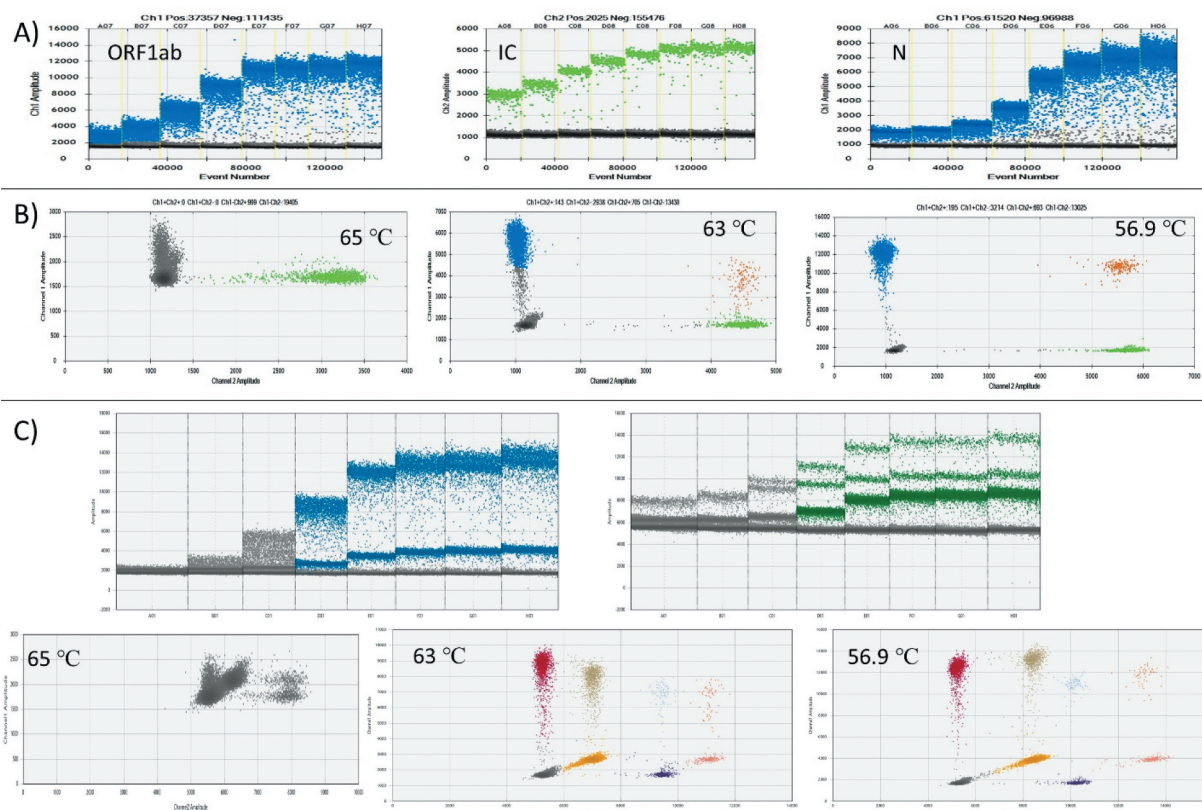
Intra- and inter-assay variability of replicate wells was used to determine the reproducibility of results using different assays. The percentage co-efficient of variation (%CV) of all tested assays was determined. As seen in Table 2, there was little intra- and inter-assay variability (%CV < 6) between replicate wells of simplex, duplex, and triplex probe mix assays post quantification. All the assays also had high reproducibility as target copies were almost at par as seen in Figure 6. This meant that the triplex probe mix assay performed similarly to the simplex and duplex assay hence found fit for further experiments.

### 3.4. Assay LoB and LoD

From the 24 samples used to determine the triplex probe mix assays LoB, 4/24 samples had one positive droplet in the N target with 3/4 arising from human samples, and 1/4 from NTC samples as seen in Table S1. Hence, the LoB for the N target and ORF1ab target was determined to be 1 and 0 positive droplets, respectively. After detection of the serially diluted cDNA (Table S2), the LoD for N and ORF1ab target was determined to be 1.42 copies/reaction and 2.75 copies/reaction, respectively. Consequently, to classify a sample as SARS-CoV-2 triplex assay ddPCR positive; a) the number of copies per reaction was either >1.42 copies/reaction



**Figure 4.** Droplet separation in a quadruplex amplitude-based assay. A) SARS-CoV-2 spiked in a background of pooled human sample. B) SARS-CoV-2 sample. C) Pooled human sample. D) Negative sample.



**Figure 5.** Temperature gradient analysis of simplex (A), duplex (B), and triplex probe mix (C) assays from 65°C to 55°C. There was a general increase in droplet amplitude with a decrease in annealing temperature. Optimum separation was achieved at an annealing temperature of 56.9°C in all the assays. Well: A-65°C; B-64.3°C; C-63°C; D-61.1°C; E-58.8°C; F-56.9°C; G-55.7°C; H-55°C.

**Table 2.** Inter- and intra-assay variability of replicate wells.

Intra-assay	N			ORF1ab			IC		
	Mean	%CV	N	Mean	%CV	N	Mean	%CV	N
Simplex	853.625	1.765	8	383.250	1.649	8	36.325	3.687	8
Duplex (N/ORF1ab IC) <sup>a</sup>	833.625	2.276	8	386.250	1.636	8	35.638	3.797	8
Triplex (N 50%) <sup>b</sup>	846.125	1.915	8	344.125	2.057	8	34.088	4.414	8
Triplex (ORF1ab 50%) <sup>b</sup>	866.500	1.778	8	362.500	3.606	8	35.013	5.392	8
<b>Inter-assay</b>	<b>849.969</b>	<b>2.330</b>	<b>32</b>	<b>369.031</b>	<b>5.196</b>	<b>32</b>	<b>35.266</b>	<b>4.774</b>	<b>32</b>

<sup>a</sup>Two duplex assays were used. One with N plus the IC and another with ORF1ab plus IC.

<sup>b</sup>The bracket represents which target had its probe conjugated i.e. 50% FAM and 50% HEX.

and >2.75 copies/reaction when droplets are only observed in either N or ORF1ab channels, respectively, or b) when one or more droplets are observed in both N and ORF1ab channels.

### 3.5. Fitness for purpose

The triplex probe mix assay was used to investigate the fitness of the multiplex assay in diagnosis and research. Considering that different labs may use different sample processing procedures including sample inactivation and ddPCR droplet preparation, the turn-around-time (TAT) for the triplex probe mix SARS-CoV-2 ddPCR assay may be different. In our current setup using the QX200 AutoDG system, the TAT was estimated to be ~6 hours.

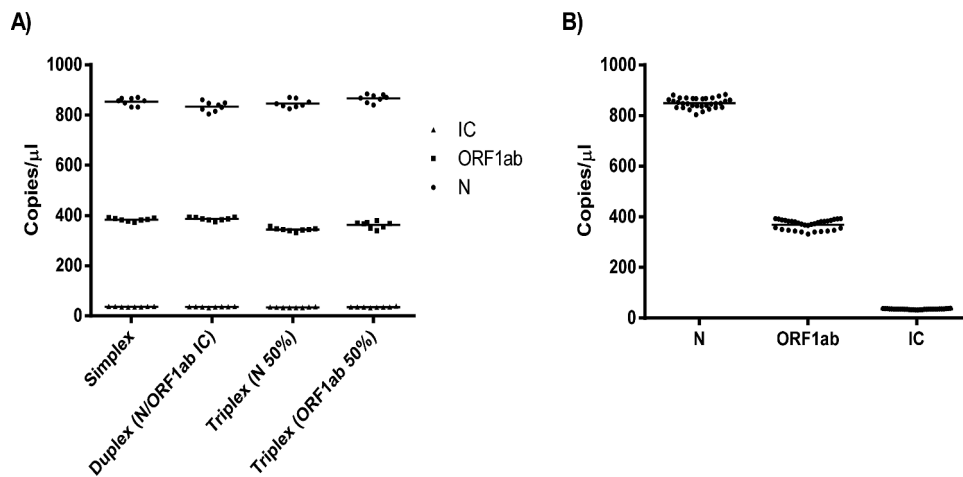
#### 3.5.1. Diagnosis

Ninety-four clinical samples were used to test the performance of the triplex probe mix assay in the diagnosis of COVID-19. The same targets used in the triplex probe mix assay were also detected

using qPCR. Both the two assays were validated using the DaAn commercial RT-qPCR kit for SARS-CoV-2 detection in clinical samples. The viral load data of the clinical samples tested and the cycle threshold (Ct) values from qPCR are shown in Table S3. As summarized in Table 3, the ddPCR triplex probe mix assay performed better than the qPCR assay. Notably, the sensitivity of the ddPCR assay (96.3%) was slightly higher than that of the qPCR assay (92.59%) as two samples that failed to be detected by qPCR were detected by both ddPCR and the commercial kit. This consequently improved the accuracy of ddPCR in detecting SARS-CoV-2 from COVID-19 samples when compared to qPCR.

#### 3.5.2. Research

Two drugs, remdsevir and Code 30, were used to demonstrate the fitness of the triplex probe mix assay (N (1:0), ORF1ab (1:1) and RBD2 (0:1)) in performing research. Of note, the RPP30 gene here was replaced with RBD2 as this research was done on Vero E6 cells. As seen in Figure 7A, no growth occurred in Vero E6 cells infected



**Figure 6.** Reproducibility of replicate wells. A) Intra-assay reproducibility of eight replicate wells using different assays. B) General reproducibility after the replicate wells data of each target in all assays are merged together.

**Table 3.** Diagnostic performance of the triplex probe mix and qPCR assays in detecting clinical samples.

N = 94	Commercial qPCR kit		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	NLR (95% CI)	Accuracy (95% CI)	
	Positive	Negative							
ddPCR assay	Positive	52	0	96.3%	100%	100%	95.24%	0.04	97.87%
	Negative	2	40	(87.25 – 99.55%)	(91.19–100%)	(N/A)	(83.7–98.73%)	(0.01–0.14)	(92.52–99.74%)
qPCR assay	Positive	50	0	92.59%	100%	100%	90.91%	0.07	95.74%
	Negative	4	40	(82.11–97.94%)	(91.19–100%)	(N/A)	(79.57–96.25%)	(0.03–0.19)	(89.46–98.83%)

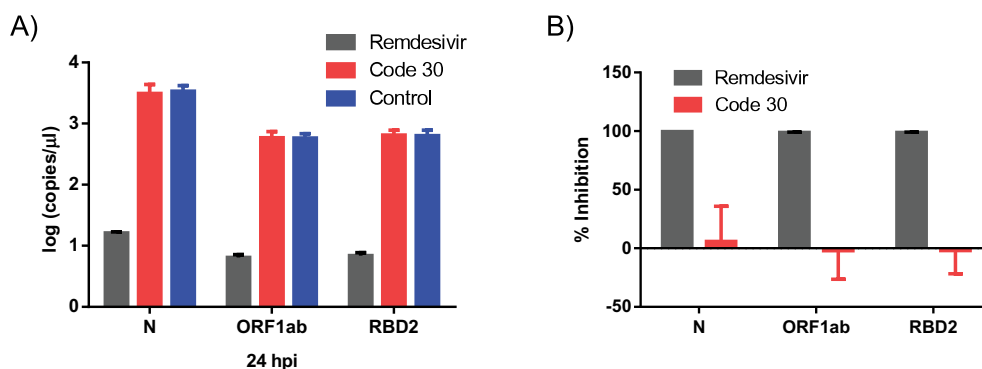
N-total samples; CI-confidence interval; PPV-positive predictive value; NPV-negative predictive value; NLR-negative likelihood ratio  
Calculated online by MEDCALC ([https://www.medcalc.org/calc/diagnostic\\_test.php](https://www.medcalc.org/calc/diagnostic_test.php))

with remdesivir 24 hours post infection (hpi). However, Code 30 grew equally as the control 24 hpi. This meant that Code 30 had no inhibitory effect against SARS-CoV-2 while remdesivir had the ability to inhibit SARS-CoV-2 *in vitro* as seen in Figure 7B. At a concentration of 10  $\mu$ M, remdesivir's percentage inhibition efficiency against SARS-CoV-2 *in vitro* was calculated to be 99.53%, 98.90%, and 98.92% for targets N, ORF1ab, and RBD2, respectively.

#### 4. Discussion

Multiplexing has been used widely to save on costs, increase sample throughput, and maximize the number of targets that can be sensitively detected within a small sample. When developing multiplexed qPCR assays, each target has to be labeled with

a unique probe to differentiate the targets. In ddPCR, few systems have this ability to detect targets using more than two probes [17]. Detection in these systems is done in two discrete optical channels, e.g. FAM and HEX/VIC as seen in Bio-Rads' QX200 system that was used in this work. Despite this, ddPCR systems have a unique advantage that unlike qPCR, more than two targets can still be detected in a single reaction using higher order multiplexing. When developing higher order multiplex ddPCR assays, two main options exist: probe mix multiplexing and amplitude-based multiplexing. In this article, we use already established primers that target different regions of the SARS-CoV-2 sequence to explain the two. To make it easy and help in the discussion, we developed a flowchart illustration (Figure 8) for reference on steps taken toward developing the multiplex assays for SARS-CoV-2 detection.



**Figure 7.** The antiviral activity of remdesivir and Code 30 against SARS-CoV-2 in cell culture. A) Quantified log copies/ $\mu$ l of different targets by ddPCR triplex probe mix assay 24 hpi. B) Inhibition efficiency of remdesivir and Code 30 against SARS-CoV-2 growth in Vero E6 cells.



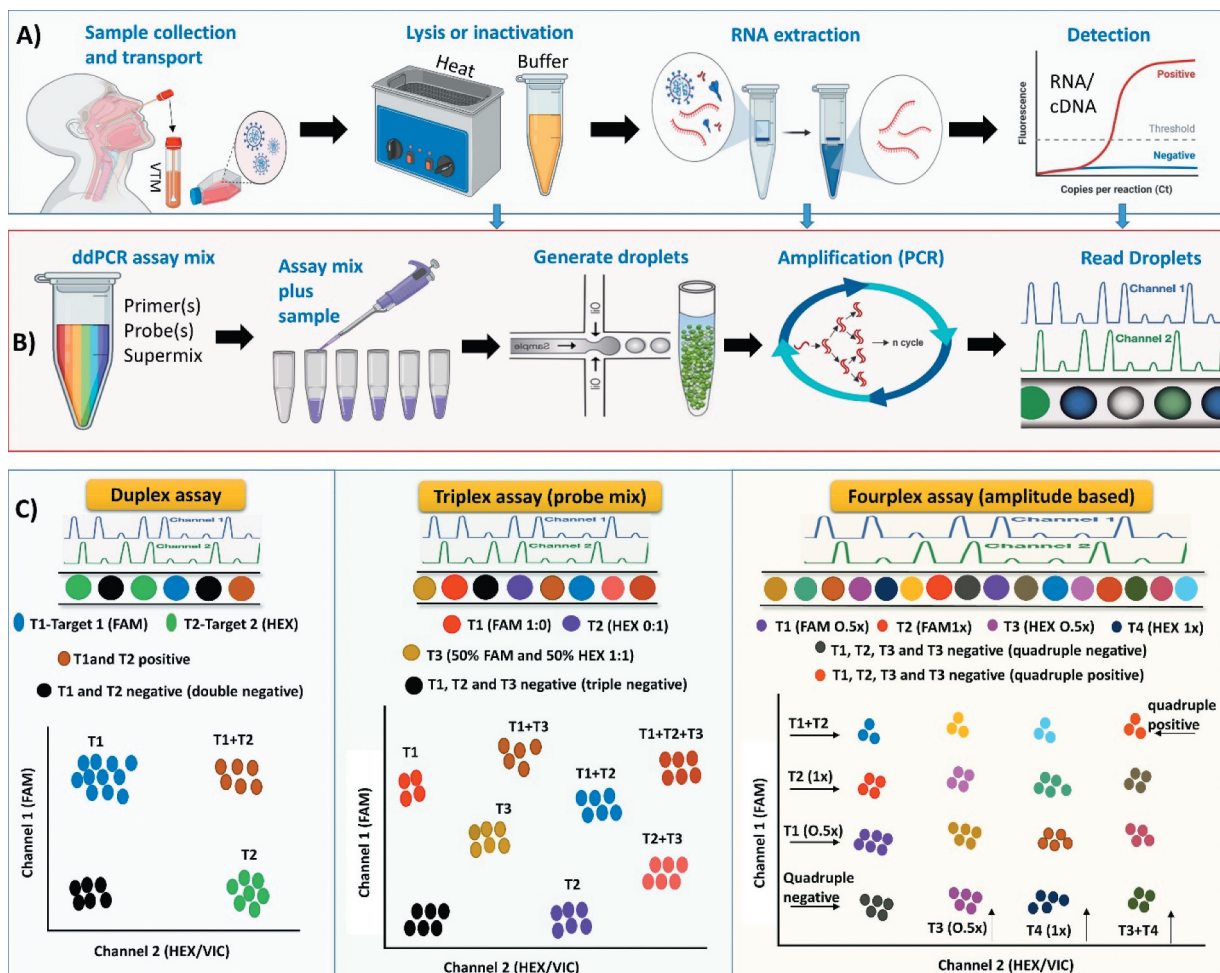
Sample handling and preparation workflow: So far, clinical [9,10,12,14–16] and environmental [11,13,21] samples have been used for SARS-CoV-2 detection using ddPCR. Due to these advancements, we designed our workflow to not only aim at developing the multiplex assay for clinical application but also for research as seen in Figure 8A. To actualize this, we developed three sample matrix containing only the virus (SARS-CoV-2) to represent laboratory research work, virus in a background of pooled human oral swabs (SARS-CoV-2 + IC) to represent clinical work, and a sample with only pooled human oral swabs (IC) negative for SARS-CoV-2 as a control representing clinical patients negative for SARS-CoV-2. Additionally, the three sample categories were used to help locate targets and assign droplet clusters when developing the assays.

Using ddPCR, it has been shown that SARS-CoV-2 could reliably be detected from nasopharyngeal samples without RNA extraction [22,23]. In our flowchart (Figure 8A), we also highlight this as a possibility even though we did not explore it. However, majority of the research done on SARS-CoV-2 using ddPCR have used samples that have undergone RNA extraction [9–16,19]. This is similar to our work. Due to the high costs incurred when performing ddPCR, we also recommended that SARS-CoV-2 samples can be detected by RT-

qPCR first before ddPCR (last step in Figure 8A). As highlighted by Liu et.al. in their article [10], RT-qPCR can be used in the mass diagnosis of SARS-CoV-2 due to low costs; however, in cases where sensitivity and precision are low, ddPCR would be the ideal method for quantification.

ddPCR workflow: The general ddPCR workflow is summarized in Figure 8B. The workflow begins with preparing your assay mix (primers/probe(s) mixed with the ddPCR supermix at different concentrations to make simplex or multiplex assays). The assay mix and sample(s) are then added to PCR strips or 96-well PCR plates dependent on the ddPCR system. Post sample addition, droplets are generated using a droplet generator. Partitioning samples to generate thousands of droplets makes multiplexing by ddPCR easy [17]. The droplets are then thermocycled to end-point. End-point detection post PCR helps ddPCR to become resistant to a number of efficiency differences between targets, improving accuracy and precision [24]. Post amplification the droplets are read using a droplet reader to generate data for analysis.

Data analysis and droplet separation: As seen in Figure 8C, different approaches can be made to develop multiplex SARS-CoV-2 ddPCR assays. Dependent on the assay, droplets will be separated differently according to the fluorescence amplitude exhibited by the droplets. The most basic in this work was the



**Figure 8.** Flowchart for this research showing steps toward developing multiplex ddPCR assays (including higher order multiplex assays) for SARS-CoV-2. A) Sample handling and preparation workflow. B) ddPCR workflow. C) Data analysis and droplet separation.

development of the duplex assays. Since two channels existed, we used non-competing duplex reactions (two primer and probe pairs) to detect targets. However, there also exists other strategies to develop duplex assays including [17]; competing duplex reactions (one primer pair with two probes binding the same region); non-competing (hybrid) duplex reactions; and duplexing using nonspecific double-stranded DNA binding dyes/non-competing hybrid probe assays [18]. For the higher order multiplex assays, we developed the triplex probe mix assay (probe mix multiplexing) and the quadruplex assay (amplitude-based multiplexing).

Amplitude-based multiplex assay design is quite similar to that of duplex double-stranded DNA binding dye but with more targets ( $\geq 3$ ). Essentially, since there are two channels, each channel can be used to detect two targets (T1-T4) at 0.5 $\times$  and 1 $\times$  concentrations of primers and probes as seen in Figure 8C. T1 and T2 are detected in Channel 1 (FAM) at 0.5 $\times$  and 1 $\times$  concentrations of primers and probes, respectively, while T3 and T4 are detected in Channel 2 (HEX/VIC) at 0.5 $\times$  and 1 $\times$  concentrations of primers and probes, respectively, giving rise to 16 ( $2^4$ ) droplet clusters in the 2D amplitude. In our work, we used four SARS-CoV-2 primer and probe pairs targeting RBD2, N, ORF1ab, and RPP30 to demonstrate this phenomenon. T1 and T2 were correspondent to RBD2 and N targets, respectively, while T3 and T4 were corresponding to RPP30 and ORF1ab, respectively. The separation of 16 clusters was achieved and additionally, the exclusion of different targets using our sample matrix yielded expected results. Currently, no SARS-CoV-2 assay has been published or commercialized that demonstrates this approach.

Triplex SARS-CoV-2 ddPCR assays including two that use the ddPCR triplex probe mix assay approach have been developed by different companies for commercial purposes. Notably, these assays can be used in specific instruments and have different targets hence pose a challenge when one wants to change targets for research or clinical applications. To curb this, we used primers targeting ORF1ab, N, and RPP30 to demonstrate how to develop similar assays. Using the triplex probe mix approach, eight droplet clusters are expected to arise from three targets (T1-T3) as seen in Figure 8C. In the eight clusters, two targets T1 and T2 are detected in a conventional manner (100% probe concentration) in channel 1 (FAM) and Channel 2 (HEX/VIC) respectively. However, the third target (T3) is conjugated with half probe concentrations, i.e. 50% FAM and 50% HEX/VIC to constitute the final 100% probe concentration hence located between T1 and T2 in the 2D amplitude with a consequent lowered amplitude in both Channels 1 and 2 amplitudes. Since the triplex probe mix assay was able to accommodate the recommended China CDC primers, it was used to show steps on optimization of multiplex assays.

After optimization tests, we analyzed the fitness of the triplex probe mix assay in detecting clinical samples and performing research. From our results, the assay was fit for SARS-CoV-2 diagnosis and research. Similar to the other ddPCR results [9,12,14–16], we also found that the N gene was more sensitive in the detection of COVID-19 from low-concentrated samples than the ORF1ab gene. This improved diagnostic sensitivity has been previously

speculated to be due to the relative abundance of the N gene subgenomic mRNA produced during viral replication [25–27]. Since the triplex probe mix assay was fit for diagnosis, it could be used to greatly cut on costs when performing simplex assays for each target. Despite these advantages, there is still more to be done to further prove the diagnostic performance of the triplex probe mix assay using more swab samples and different types of clinical samples by comparison with the widely used and gold-standard RT-PCR test.

Remdesivir has been extensively used in clinical applications after the drug was found to inhibit SARS-CoV-2 *in vitro* [28]. We used this drug and another drug we termed Code 30 to show the applicability of multiplex assays in performing research. As expected, the multiplex assay could quantify the activity of the two drugs against SARS-CoV-2. This may support the suggestion by Yu et.al to use ddPCR in quantifying antiviral efficacy against SARS-CoV-2 [16]. Only two non-diagnostic research applications of ddPCR against SARS-CoV-2 exist [11,13]. We believe that multiplex assays can also help in testing how different targets respond when performing research like gene expression, copy number variations, and rare mutation detections.

## 5. Conclusion

This study shows steps on how to develop different ddPCR SARS-CoV-2 assays based on a two-color ddPCR system including higher order multiplex assays for SARS-CoV-2 detection and research like antiviral screening. It will not only aid in developing better diagnostic assays for SARS-CoV-2 through reducing false positive and false negative results during detection but also help laboratory research by maximizing the number of targets that can be sensitively detected within a small volume of sample.

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## Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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