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Absolute quantification of SARS-CoV-2 with Clarity Plus™ digital PCR

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ABSTRACT

In recent years, the usage of digital polymerase chain reaction (dPCR) for various clinical applications has increased exponentially. In this study, a dPCR assay optimized on the Clarity Plus™ dPCR system was evaluated for the absolute quantification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the global coronavirus disease 2019 (COVID-19) outbreak. The assay demonstrated good inter- and intra- assay precision, accuracy, as well as excellent linearity across a range of over 6 orders of magnitude for target gene quantification. In addition, a comparison of the assay on both dPCR and qPCR platforms revealed that dPCR exhibited a slightly higher sensitivity compared to its qPCR counterpart when quantifying SARS-CoV-2 at a lower concentration. Overall, the results showed that the dPCR assay is a reliable and effective approach for the absolute quantification of SARS-CoV-2 and can be a valuable molecular tool in clinical applications such as detecting low viral loads in patients as well as in wastewater surveillance of COVID-19.

1. Introduction

Digital Polymerase Chain Reaction (dPCR) was first introduced by Volgelstein and Kinzler in a 1999 publication in which they utilized a series of four 96-well plates to physically partition samples obtained from colorectal cancer patients in order to detect mutations in the *ras* oncogene [1]. Being the third generation PCR platform, dPCR works by separating a PCR mixture into many independent nanoliter sub-reactions, leading to individual partitions having either a few or no target sequences. Upon completion of PCR, the proportion of positive and negative partitions for each sample is determined and used to compute the absolute nucleic acid copy number using Poisson statistics [2]. The nanoliter sub-reaction causes increased congregation of the PCR mix and reduces template competition as well as sample susceptibility to PCR inhibitors. Consequently, dPCR has shown to detect rare mutations in a background of wild-type sequences with greater relative sensitivity and precision [3]. For example, in the case of detecting rare circulating tumor DNA, dPCR was reported to provide a higher sensitivity of up to 0.001% without compromising on its accuracy [2,4]. In comparison to traditional PCR and qPCR, dPCR also obviates the need for a standard curve or calibrator during analysis. As such, robust and precise quantification of the desired nucleic acid targets is achievable [3,5].

Commercial dPCR platforms available in the market employ two main methods of sample partitioning. The first method involves the generation of water–oil emulsion droplets. This technology is employed by the QX100™/200™ droplet digital PCR systems (Bio-Rad Laboratories) and the Naica® System (Stilla Technologies). The second method is based on the chip/nanoplate technology currently adopted by BioMark™ HD (Fluidigm), QuantStudio® 3D (Thermo Fisher Scientific), QIAcuity (QIAGEN) and Clarity™/Clarity Plus™ (JN Medsys) [2,6]. Among these, the Clarity Plus™ dPCR system is one of the few that allows simultaneous detection and quantification of up to six target genes in a single reaction. This system also partitions each nucleic acid sample into 40,000 compartments using high-density chip which enables a wide dynamic range for detection with minimal sample loss. Since the mid-2000s, a plethora of new dPCR applications has been developed which led to an exponential increase in the number of scientific reports published on this topic [7]. A quick search on NCBI with "digital PCR" as the keyword returned a search result of close to 4,000 publications. These reports focused on a wide array of topics including rare mutation detection, copy number variation analysis, environmental DNA surveillance, and quantification of pathogens and genetically modified organisms [8–12].

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly transmissible virus and has caused a global pandemic of acute respiratory disease, which was subsequently named Coronavirus Disease 2019 (COVID-19). As of June 2021, over 180 million COVID-19 cases and nearly 4 million deaths have been reported worldwide [13]. This pandemic has posed a severe threat to global public health, which

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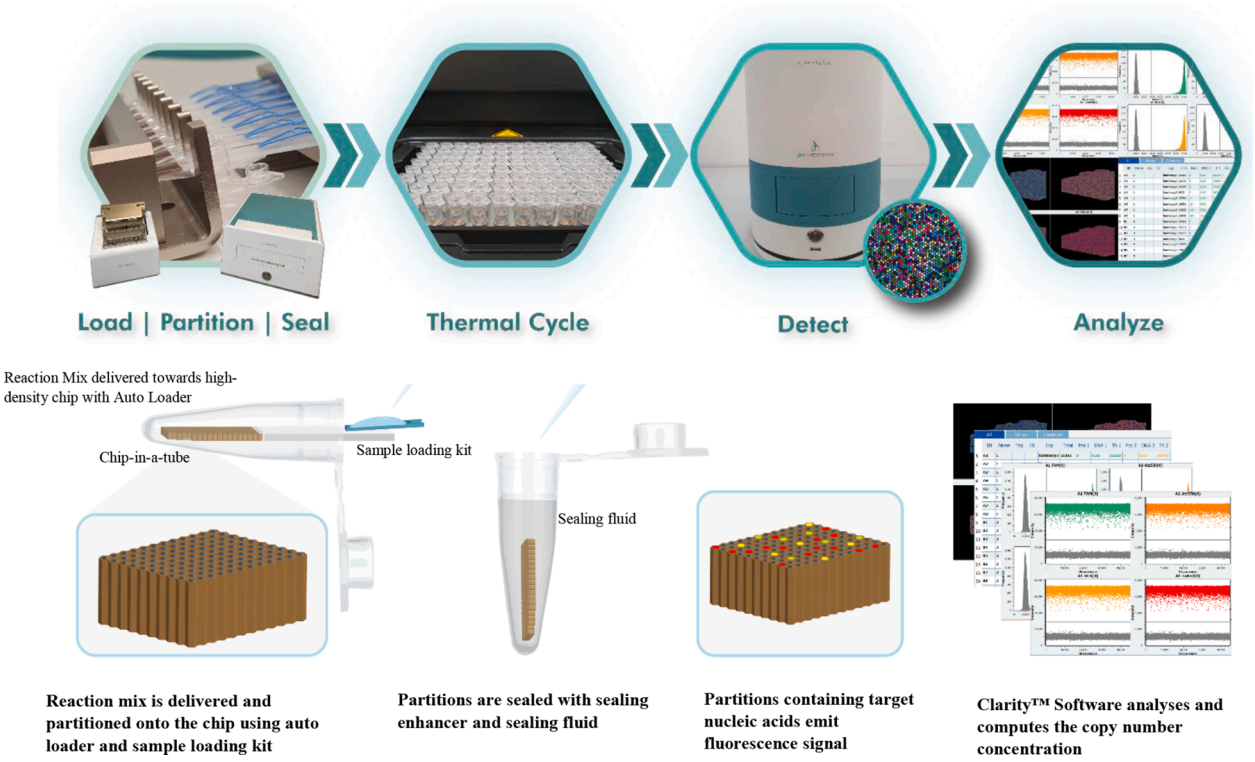

Fig. 1. Experimental workflow of Clarity Plus™ digital PCR. The Clarity Plus™ system adopts a unique chip-in-a-tube format which allows digital PCR to be performed with ease and speed via simultaneous loading and partitioning of eight reaction mixes using an auto loader. The reaction mixes are subsequently sealed in the partitions with the sealing enhancer and sealing fluid. Following which, the reactions are subjected to thermal cycling. After PCR, the tube-strips are transferred to the Clarity Plus™ reader where fluorescence signals in the partitions are detected. The proportion of positive to negative partitions is then analyzed by the Clarity™ software to compute the copy number concentration of each sample using Poisson statistics.

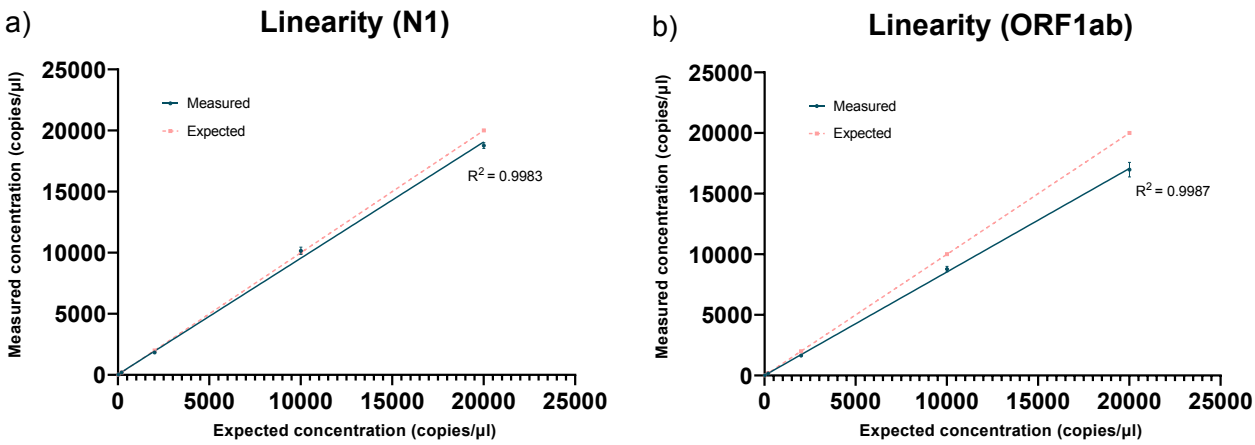

Fig. 2. Linearity of the SARS-CoV-2 RT-dPCR assay analyzed with serially diluted N1 (a) and ORF1ab (b) synthetic RNA from 0.2 to 20,000 copies/µl.

necessitates the development of reliable tests for sensitive and accurate detection of SARS-CoV-2. Particularly, early diagnosis is vitally important for disease containment and minimizing transmission through prompt isolation of patients and supporting them with timely treatment. The SARS-CoV-2 genome consists of a single linear positive-strand RNA segment of approximately 30,000 bases comprising ORF1a, ORF1b, S, E, M, RNA-dependent RNA Polymerase (RdRP), N1, and N2 sequences [14,15]. Presently, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the gold standard method for the diagnosis of COVID-19 worldwide [16]. Various RT-qPCR assays have been developed to detect SARS-CoV-2 RNA through amplification of 2 or 3 distinct segments [17]. While qPCR is robust in many settings, reports of false negative cases have had adverse implications for the prompt isolation of positive cases and management of the disease in general. False negative results can be attributed to different factors including low viral load, premature testing in the course of infection and low analytical sensitivity [5,18,19]. In light of these challenges, other methodologies including dPCR have been explored for SARS-CoV-2 detection to complement the currently available tests [20]. In this study, a TaqMan-based dPCR assay optimized on the Clarity Plus™ system was evaluated for SARS-CoV-2 detection and the results demonstrate that this technology can potentially be employed in clinical settings for detection and absolute quantification of low copy viral load.

Table 1a
Linearity analysis of the SARS-CoV-2 RT-dPCR assay (N1) using Clarity Plus™. Analyses of the serial dilutions ranging from 0.02 to 30,000 copies/µl were performed in duplicates across three independent experiments.

Expected N1 Concentration (Copies/µl)	Measured Concentration Run 1	Measured Concentration Run 2	Measured Concentration Run 3	Mean of measured concentration (Copies/µl)	Relative Uncertainty[a] (%)
30,000	22272.25	21418.38	20045.35	21245.33	5.3
20,000	18924.60	18887.03	18498.95	18770.19	1.3
10,000	10502.90	10048.33	9935.72	10162.32	3.0
2000	1774.03	1827.02	1888.77	1829.94	3.0
200	205.88	198.63	204.38	202.96	1.8
20	22.39	21.55	22.16	22.03	2.0
2	1.79	2.48	1.75	2.01	2.0
1	0.97	1.32	1.06	1.12	16.2
0.2	0.31	0.11	0.21	0.21	47.6
0.02	0.17	0.15	0.10	0.14	25.7
No Template Control	0.00	0.00	0.00	0.00	–

The results represent the mean measured concentration of two replicates within each experimental run. Mean of measured concentration represents the mean concentration of the three runs.
[a] Relative Uncertainty (%) = (Standard Deviation of measured concentration/Mean of measured concentration) × 100

Table 1b
Linearity analysis of the SARS-CoV-2 RT-dPCR assay (ORF1ab) using Clarity Plus™. Analyses of the serial dilutions ranging from 0.02 to 30,000 copies/µl were performed in duplicates across three independent experiments.

Expected ORF1ab Concentration (Copies/µl)	Measured Concentration Run 1	Measured Concentration Run 2	Measured Concentration Run 3	Mean of measured concentration (Copies/µl)	Relative Uncertainty[a] (%)
30,000	20274.11	22866.26	21324.75	21488.37	6.07
20,000	17067.08	17527.18	16326.37	16973.54	3.57
10,000	9020.12	8711.51	8579.86	8770.50	2.58
2000	1662.72	1696.34	1684.34	1681.13	1.01
200	175.54	172.23	158.37	168.71	5.40
20	20.30	17.66	17.09	18.35	9.33
2	1.43	2.05	1.29	1.59	25.44
1	0.97	0.81	1.08	0.95	14.24
0.2	0.28	0.18	0.58	0.35	60.05
0.02	0.18	0.19	0.21	0.19	7.90
No Template Control	0.00	0.00	0.00	0.00	–

The results represent the mean measured concentration of two replicates within independent experimental runs. Mean of measured concentration represents the mean of three runs.
[a] Relative Uncertainty (%) = (Standard Deviation of measured concentration / Mean of measured concentration) × 100

Table 2a
Linearity analysis of the SARS-CoV-2 RT-dPCR assay (N1) using Clarity Plus™. Analyses of the serial dilutions ranging from 0.02 to 30,000 copies/µl were performed in duplicates across three independent experiments.

Expected Concentration (Copies/µl)	Measured Concentration Run 1		Relative Uncertainty[a] (%)	Measured Concentration Run 2		Relative Uncertainty[a] (%)	Measured Concentration Run 3		Relative Uncertainty[a] (%)
30,000	23590.46	20954.04	8.37	22618.00	20218.76	7.92	19069.93	21020.77	6.88
20,000	19149.16	18700.05	1.68	18074.79	19699.26	6.08	18079.42	18918.48	3.21
10,000	10409.95	10595.85	1.25	10081.11	10015.54	0.46	10295.95	9575.48	5.13
2000	1792.33	1755.73	1.46	1809.06	1844.98	1.39	1817.38	1960.16	5.35
200	207.07	204.70	0.81	198.54	198.72	0.06	212.81	195.96	5.83
20	22.56	22.23	1.04	20.80	22.30	4.92	21.17	22.90	5.55
2	1.91	1.68	9.06	2.82	2.15	19.06	1.42	2.08	26.67
1	0.97	0.97	0	1.51	1.13	20.36	1.06	1.06	0
0.2	0.41	0.21	45.62	0.15	0.07	51.43	0.20	0.21	3.45
0.02	0.14	0.21	28.28	0.07	0.23	75.42	0.13	0.07	42.43
No Template Control	0.00	0.00	–	0.00	0.00	–	0.00	0.00	–

The results represent the individual measured concentration of two replicates within each independent run.
[a] Relative Uncertainty (%) = (Standard Deviation of measured concentration / Mean of measured concentration) × 100

2. Materials & methods

2.1. Reagent preparation and dPCR analysis

Each 15 µl Clarity Plus™ COVID-19 reverse transcription (RT)-dPCR reaction mix consisted of 7.5 µl Clarity Plus™ COVID-19 Probe RT-dPCR Mastermix (2x), 0.75 µl Clarity Plus™ COVID-19 RT Mix (20x), 0.75 µl Clarity Plus™ JN solution (20x), 1 µl Clarity Plus™ COVID-19 Primer & Probe Mix (N1-FAM, ORF1ab-Quasar670 and human RNaseP-HEX) and 5 µl RNA Sample/Positive control/NTC following manufacturer's instructions (JN Medsys; Catalogue number: 10028). Clarity Plus™ JN solution is a proprietary formula optimized for robust dPCR performance on the Clarity Plus™ high-density chips. The N1 and ORF1ab probes and primers used were custom-designed by JN Medsys. The prepared mix was placed in the thermocycler for reverse transcription at 55 °C for 15mins and a continuous 4 °C step till the sample is ready to be partitioned. Using the Clarity™ auto loader, the mix was delivered onto the chip where it was sub-divided into 40,000 partitions. Each chip was

Table 2b
Linearity analysis of the SARS-CoV-2 RT-dPCR assay (ORF1ab) using Clarity Plus™. Analyses of the serial dilutions ranging from 0.02 to 30,000 copies/µl were performed in duplicates across three independent experiments.

Expected Concentration (Copies/µl)	Measured Concentration Run 1		Relative Uncertainty[a] (%)	Measured Concentration Run 2		Relative Uncertainty[a] (%)	Measured Concentration Run 3		Relative Uncertainty[a] (%)
30,000	23295.77	17252.46	21.08	23599.31	22133.22	4.53	21493.69	21155.82	1.12
20,000	16655.78	17478.38	3.41	17613.36	17441.00	0.70	16061.96	16590.78	2.29
10,000	9005.66	9034.58	0.23	8690.26	8732.76	0.34	8452.98	8706.74	2.09
2000	1644.24	1681.20	1.57	1780.67	1612.01	7.03	1684.34	1684.33	0.00
200	176.66	174.42	0.90	176.43	168.02	3.45	153.89	162.85	4.00
20	19.67	20.93	4.39	18.70	16.63	8.29	18.12	16.06	8.52
2	1.30	1.55	12.41	2.08	2.02	2.07	1.11	1.48	20.20
1	1.19	0.76	31.19	0.42	1.20	68.09	0.98	1.19	13.69
0.2	0.21	0.34	33.43	0.07	0.30	87.91	0.14	1.02	107.29
0.02	0.07	0.28	84.85	0.16	0.22	22.33	0.42	0.00	141.42
No Template Control	0.00	0.00	–	0.00	0.00	–	0.00	0.00	–

The results represent the individual measured concentration of two replicates within each independent run.
[a] Relative Uncertainty (%) = (Standard Deviation of measured concentration / Mean of measured concentration) × 100

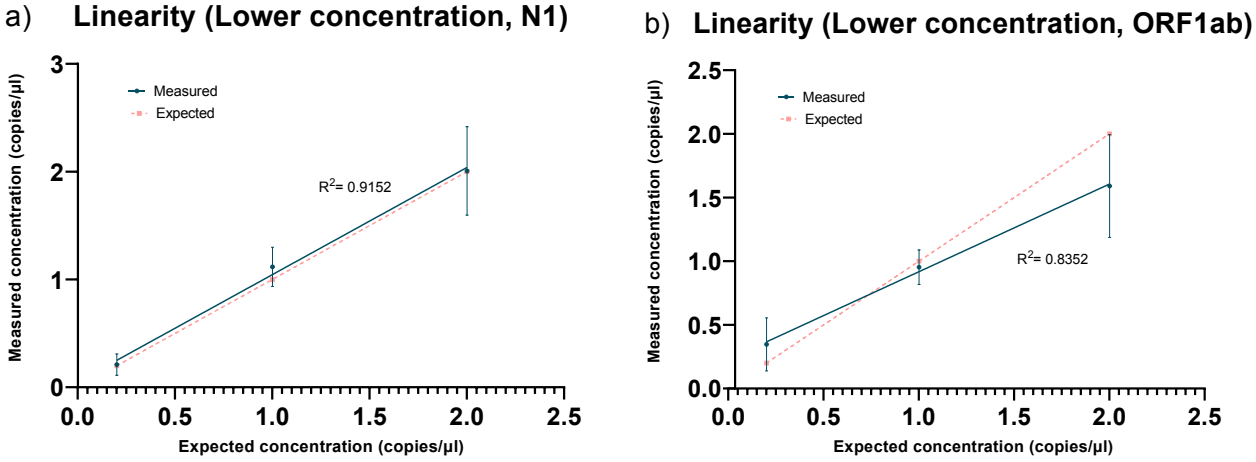

Fig. 3. Linearity of the SARS-CoV-2 RT-dPCR assay analyzed with serially diluted N1 (a) and ORF1ab (b) synthetic RNA at 0.2, 1, and 2 copies/µl.

Table 3a
Limit of Quantification (LoQ) of N1 SARS-CoV-2 RT-dPCR assay using Clarity Plus™. Three independent experimental runs were performed with a series of N1 concentrations ranging from 0.125 to 1 copies/µl.

Expected Concentration (Copies/µl)	Measured Concentration Run 1	Measured Concentration Run 2	Measured Concentration Run 3	Mean of measured concentration (Copies/µl)	Relative Uncertainty[a] (%)
1	0.97	1.02	0.96	0.98	3.2
0.5	0.49	0.51	0.48	0.49	3.1
0.25	0.26	0.11	0.22	0.20	39.4
0.125	0.23	0.19	0.18	0.20	13.2
No Template Control	0.00	0.00	0.00	0.00	–

The results represent the mean measured concentration of two replicates within each independent run. Mean of measured concentration represents the mean of three runs.
[a] Relative Uncertainty (%) = (Standard Deviation of measured concentration / Mean of measured concentration) × 100

Table 3b
Limit of Quantification (LoQ) of ORF1ab SARS-CoV-2 RT-dPCR assay using Clarity Plus™. Three independent experimental runs were performed with a series of ORF1ab concentrations ranging from 0.125 to 1 copies/µl.

Expected Concentration (Copies/µl)	Measured Concentration Run 1	Measured Concentration Run 2	Measured Concentration Run 3	Mean of measured concentration (Copies/µl)	Relative Uncertainty[a] (%)
1	0.94	0.91	0.81	0.89	7.68
0.5	0.45	0.63	0.29	0.46	37.25
0.25	0.44	0.15	0.26	0.28	51.67
0.125	0.26	0.23	0.40	0.30	30.59
No Template Control	0.00	0.00	0.00	0.00	–

The results represent the mean measured concentration of two replicates within each independent run. Mean of measured concentration represents the mean of three runs.
[a] Relative Uncertainty (%) = (Standard Deviation of measured concentration / Mean of measured concentration) × 100

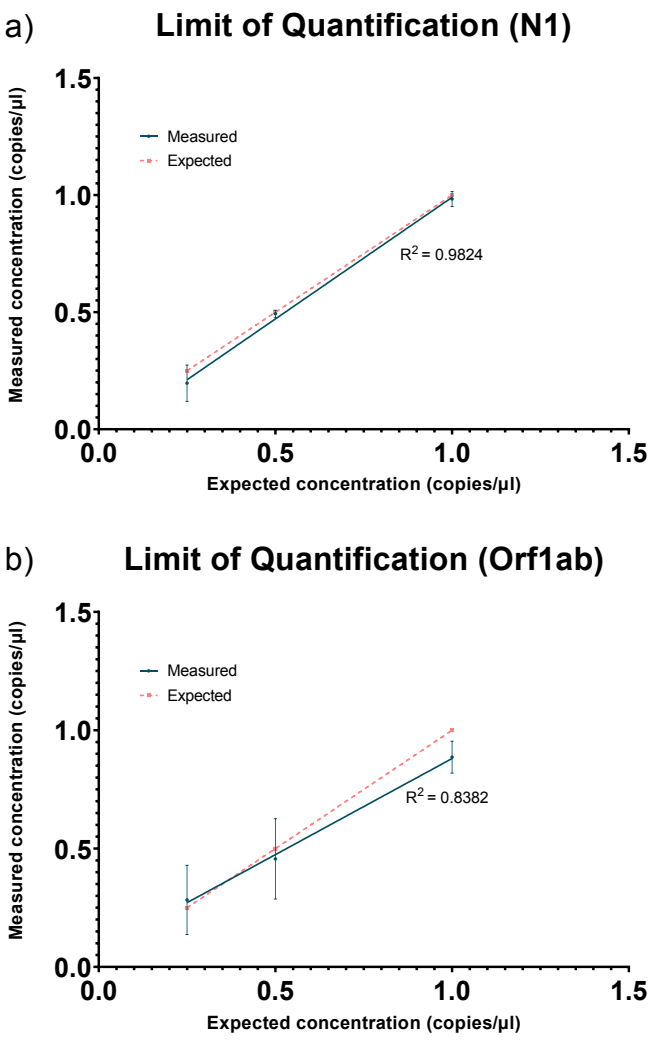

Fig. 4. LoQ analyses of the SARS-CoV-2 RT-dPCR assay with serially diluted N1 (a) and ORF1ab (b) synthetic RNA.

Table 4a
Preliminary LoD determination of the N1 SARS-CoV-2 RT-dPCR assay using RNA extracted from AccuPlex™ SARS-CoV-2 reference material.

	Dilution /Expected Concentration (Copies/µl)					
No template control	**0x**	**2x**	**4x**	**8x**	**16x**	**32x**
	1.8	0.9	0.45	0.225	0.1125	0.05625
	Measured Concentration (Copies/µl) in triplicates					
0.00	1.74	1.38	0.53	0.14	0.07	0.26
0.00	2.17	1.57	0.78	0.43	0.21	0.29
0.00	1.29	1.54	0.89	0.41	0.07	**0.00**
	Mean Concentration (Copies/µl)					
0.00	1.73	1.50	0.73	0.33	0.09	0.18

The results represent the measured concentrations of three replicates for each dilution. Mean concentration represents the mean concentration of each triplicate.

then sealed with the Clarity™ Sealing Enhancer and 245 µl Clarity™ Sealing fluid (Fig. 1). All sealed chips are subjected to thermal cycling using the following parameters: Initial 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 50 s and 55 °C for 90 s, and final hold at 70 °C for 5 min (ramp rate = 2.5 °C/s). After PCR amplification, the tube strips were transferred to the Clarity Plus™ Reader, which was set to detect N1 gene on the FAM channel and ORF1ab gene on the Cy5 channel. The data were analyzed with the Clarity™ software (version 4.1), and a

Table 4b
Preliminary LoD determination of the ORF1ab SARS-CoV-2 RT-dPCR assay using RNA extracted from AccuPlex™ SARS-CoV-2 reference material.

	Dilution /Expected Concentration (Copies/µl)					
No template control	**0x**	**2x**	**4x**	**8x**	**16x**	**32x**
	1.8	0.9	0.45	0.225	0.1125	0.05625
	Measured Concentration (Copies/µl) in triplicates					
0.00	1.60	0.72	0.53	0.34	0.00	0.13
0.00	1.66	1.09	0.39	0.28	0.21	0.00
0.00	1.78	1.00	0.41	0.14	0.22	0.00
	Mean Concentration (Copies/µl)					
0.00	1.68	0.97	0.44	0.25	0.14	0.04

The results represent the measured concentrations of three replicates for each dilution. Mean concentration represents the mean concentration of each triplicate.

Table 5a
Comparison of the N1 SARS-CoV-2 assay on both dPCR (Clarity Plus™) and qPCR (QuantStudio® 3) platforms. Analyses were performed on 0.225 copies/µl and 0.1125 copies/µl dilutions in 20 replicates.

	Clarity Plus™ (N1)		QuantStudio® 3 (N1)	
Expected Concentration (Copies/µl)	0.225	0.1125	0.225	0.1125
Replicate	Measured Concentration (Copies/µl)		Measured Concentration (Cq)	
1	0.13	0.07	33.05	35.66
2	0.34	0.07	33.40	**Undetermined**
3	0.19	0.13	33.79	35.38
4	0.68	0.21	35.00	33.52
5	0.35	0.07	31.80	35.85
6	0.36	0.14	32.53	34.06
7	0.41	0.20	32.63	**Undetermined**
8	0.28	0.42	32.63	32.55
9	0.22	**0.00**	32.64	**Undetermined**
10	0.35	0.14	32.20	32.64
11	0.34	0.32	32.79	33.59
12	0.36	**0.00**	32.63	**Undetermined**
13	0.27	0.07	32.02	**Undetermined**
14	0.08	0.13	32.57	34.17
15	0.34	**0.00**	32.30	35.62
16	0.51	0.19	32.89	32.59
17	0.34	0.27	32.31	35.92
18	0.33	0.07	32.89	31.44
19	0.20	0.13	33.44	34.61
20	0.28	0.28	32.54	35.91
NTC	0.00	0.00	–	–
Detection Rate	100%	85%	100%	75%

proprietary algorithm was used for setting individual thresholds based on fluorescent intensities to determine the proportion of positive partitions. Using this information, the software determines the DNA copies per microliter of dPCR mix using Poisson statistics. The mean partition volume of 0.31 nl was used for copy number calculation.

2.2. SARS-CoV-2 RT-dPCR assay linearity and limit of quantification (LoQ) analyses

The linearity and LoQ of the SARS-CoV-2 RT-dPCR assay were evaluated by quantifying serial dilutions of the N1 and ORF1ab synthetic RNAs (GenScript). Prior to dilution, copy number concentration of the stock RNA was determined using the Clarity™ dPCR system. Linearity analyses were performed across the expected concentration range from 0.02 to 30,000 copies/µl reaction. The LoQ was determined by quantifying RNA dilutions ranging from 0.125 to 1 copies/µl reaction. Three independent linearity and LoQ analyses were conducted in duplicate reactions.

Table 5b
LoD confirmation of the ORF1ab SARS-CoV-2 assay on Clarity Plus™. Analyses were performed on 0.225 copies/µl and 0.1125 copies/µl dilutions in 20 replicates.

	Clarity Plus™ (ORF1ab)	
Expected Concentration (Copies/µl)	0.225	0.1125
	Measured Concentration (Copies/µl)	
1	0.07	**0.00**
2	0.21	**0.00**
3	0.32	0.13
4	0.45	0.21
5	0.14	**0.00**
6	0.07	0.07
7	0.20	**0.00**
8	0.14	**0.00**
9	0.15	0.14
10	0.35	0.21
11	0.27	0.06
12	**0.00**	**0.00**
13	0.40	**0.00**
14	0.31	0.13
15	0.14	0.07
16	0.08	**0.00**
17	0.14	**0.00**
18	0.33	**0.00**
19	0.26	0.07
20	0.14	0.21
NTC	0.00	0.00
Detection Rate	95%	50%

2.3. Limit of detection (LoD) of the SARS-CoV-2 RT-dPCR assay

The AccuPlex™ SARS-CoV-2 positive reference material (LGC SeraCare; Catalogue Number: 0505–0126) was first extracted using the EX3600 Automated Nucleic Acid Extraction System (Liferiver™) according to manufacturer's instructions. The extracted RNA stock was subsequently quantified with Clarity Plus™. To determine the preliminary LoD, a two-fold serial dilution of the stock RNA was performed and quantified in triplicates using Clarity Plus™. The LoD of the assay was then verified on both the Clarity Plus™ dPCR and QuantStudio® 3 qPCR (Thermo Fisher Scientific) platforms using 20 replicates of the selected RNA dilutions. Digital PCR data is presented for both the N1 and ORF1ab targets, while qPCR data is presented only for the N1 gene since QuantStudio® 3 qPCR system is not equipped to detect Quasar670 fluorescence. The dPCR assay conditions followed that described in Section 2.1. For the qPCR assay, each reaction mix consisted of 10 µl Clarity Plus™ COVID-19 Probe RT-dPCR Mastermix (2x), 1 µl Clarity Plus™ COVID-19 RT Mix (20x), 1.33 µl Clarity Plus™ COVID-19 Primer & Probe Mix, 5 µl RNA Sample/Positive control/ NTC and nuclease free water to a final volume of 20 µl. The samples were loaded onto a 96-well plate prior to amplification on QuantStudio® 3 using the following parameters: Initial cycle of 55 °C for 15 min, 95 °C for 2 min, 40 cycles of 95 °C for 3 s and 55 °C for 30 s (ramp rate 1.6 °C/s). After each run, the results were analyzed with the Design and Analysis software (version 2.4.3; Thermo Fisher Scientific).

2.4. Detection and quantification of contrived nasopharyngeal samples using SARS-CoV-2 RT-dPCR assay

The clinical performance of the SARS-CoV-2 RT-dPCR assay was assessed using contrived human nasopharyngeal samples (10 positive and 2 negative samples). Nasopharyngeal swab from healthy volunteer was first collected into the viral transport medium [VTM, (ABclonal Biotechnology, Catalog Number: 43903)]. The VTM is then spiked with equal volume of the AccuPlex™ SARS-CoV-2 positive or negative reference material (LGC Seracare; Catalogue Number: 0505–0119 & 0505–0123). Viral RNA from the contrived samples were subsequently extracted using the EX3600 Automated Nucleic Acid Extraction System (Liferiver™) according to manufacturer's instructions. The extracted viral RNA samples were then quantified using Clarity Plus™.

2.5. Statistical analysis

All linear regression analysis on the data obtained from Clarity Plus™ was performed on GraphPad Prism® (version 8.0.1).

3. Results and discussion

3.1. Performance of the SARS-CoV-2 RT-dPCR assay using Clarity Plus™

Linearity of the SARS-CoV-2 RT-dPCR assay was first examined using N1 and ORF1ab synthetic RNA samples. A dilution series ranging from 0.02 to 30,000 copies/µl was obtained from a $3x10^{12}$ copies/µl stock. Three independent experiments were performed to quantify these RNA dilutions using the Clarity Plus™ system. The mean measured concentrations of the N1 and ORF1ab RNA dilutions from three independent runs are shown in Tables 1a and b. The measured concentrations of N1 and ORF1ab were found to be closely associated with their expected concentrations except for two dilutions at 0.02 and 30,000 copies/µl, respectively. When the measured concentrations (0.2 to 20,000 copies/µl) were plotted against their expected values, linear regression analysis revealed an R^2 value of 0.998 for both N1 (Fig. 2a) and ORF1ab (Fig. 2b) across a dynamic range of over six orders of magnitude. These results indicate excellent linearity across a dynamic range of 0.2 to 20,000 copies/µl.

Inter- and intra-assay precision of the SARS-CoV-2 RT-dPCR assay on Clarity Plus™ were also evaluated based on relative uncertainty (RU) between data values. As summarized in Tables 1a and b, the mean measured N1 and ORF1ab concentrations from three independent experiments are well correlated with a RU of <10%, except for the lowest four concentrations (0.02, 0.2, 1, and 2 copies/µl). Within the respective assays (Tables 2a and 2b), the data of N1 and ORF1ab similarly showed good intra-assay precision between duplicates (RU < 10%), except those from the lower concentrations (0.02, 0.2, 1, 2 copies/µl). Higher RU values observed for these concentrations do not accurately portray the relationship between data points since a minor variation of 0.1 and 0.2 copies/µl will result in a 50% variation in RU. Furthermore, linear regression analysis on the lower concentrations of 0.2, 1 and 2 copies/µl showed an R^2 value of 0.915 for N1 and 0.835 for ORF1ab which indicates good linearity despite exhibiting high RU values (Fig. 3).

3.2. Limit of quantification (LoQ) of the SARS-CoV-2 RT-dPCR assay

LoQ of the SARS-CoV-2 RT-dPCR assay was evaluated with four serial dilutions of the N1 and ORF1ab synthetic RNA ranging from 0.125 to 1 copies/µl. Three independent analyses were performed, and the results are summarized in Tables 3a and b. The mean measured concentrations of the first three dilutions were found to be closely associated with their expected concentrations of 1, 0.5, and 0.25 copies/µl [$R^2 = 0.982$ (N1) and $R^2 = 0.838$ (ORF1ab), Fig. 4]. For the lowest dilution sample (expected concentration of 0.125 copies/µl), its mean measured concentration did not exhibit a 2-fold relationship with the previous dilution, and hence, the LoQ of the assay was established to be 0.25 copies/µl.

3.3. Limit of detection (LoD) of the SARS-CoV-2 RT-dPCR assay

The preliminary LoD of the SARS-CoV-2 RT-dPCR assay was determined by quantifying two-fold serial dilutions of the RNA extracted from the AccuPlex™ SARS-CoV-2 positive reference material in triplicates. The stock concentration of the extracted RNA was determined to be 1.8 copies/µl using the Clarity Plus™ system. From the results shown in Table 4a, the N1 assay successfully detected three out of three replicates (100% detection) for all concentrations down to 0.1125 copies/µl. For the ORF1ab assay (Table 4b), a 100% detection rate was observed for

Table 6
SARS-CoV-2 RT-dPCR analyses on contrived human nasopharyngeal samples.

Samples	Number of Positive Partition	Total Partition Number	Ratio[a]	Measured N1 Concentration[b] (Copies/µl)	Number of Positive Partition	Total Partition Number	Ratio[a]	Measured RNase P Concentration[b] (Copies/µl)
Contrived Positive	27	42,570	0.00063	2.05	1380	42,570	0.03242	106.30
Contrived Positive	24	38,252	0.00063	2.02	1133	38,140	0.02971	97.28
Contrived Positive	21	40,808	0.00051	1.66	1050	40,808	0.02573	84.09
Contrived Positive	21	43,274	0.00049	1.57	1232	43,274	0.02847	93.17
Contrived Positive	16	39,614	0.00040	1.30	1121	39,614	0.02830	92.60
Contrived Positive	11	42,973	0.00026	0.83	1131	42,973	0.02632	86.04
Contrived Positive	9	40,736	0.00022	0.71	1051	40,736	0.02580	84.32
Contrived Positive	31	42,610	0.00073	2.35	1375	42,610	0.03227	105.81
Contrived Positive	15	39,425	0.00038	1.23	1058	39,425	0.02684	87.75
Contrived Positive	17	41,768	0.00041	1.47	1145	41,754	0.02742	89.70
Contrived Negative	0	43,580	0.00000	0.00	1072	43,580	0.02460	80.34
Contrived Negative	0	43,543	0.00000	0.00	1056	43,543	0.02425	79.20
No template control	0	39,353	0.00000	0.00	0	39,353	0.00000	0.00
No template control	0	43,160	0.00000	0.00	0	43,160	0.00000	0.00

Samples	Number of Positive Partition	Total Partition Number	Ratio[a]	Measured ORF1ab Concentration[b] (Copies/µl)
Contrived Positive	15	42,570	0.00035	1.14
Contrived Positive	15	38,140	0.00039	1.27
Contrived Positive	13	40,808	0.00032	1.03
Contrived Positive	18	43,274	0.00042	1.34
Contrived Positive	14	39,614	0.00035	1.14
Contrived Positive	10	42,973	0.00023	0.75
Contrived Positive	17	40,736	0.00042	1.35
Contrived Positive	15	42,610	0.00035	1.14
Contrived Positive	17	39,425	0.00043	1.39
Contrived Positive	19	41,754	0.00046	1.47
Contrived Negative	0	43,580	0.00000	0.00
Contrived Negative	0	43,543	0.00000	0.00
No template control	0	39,353	0.00000	0.00
No template control	0	43,160	0.00000	0.00

[a] Ratio = Number of Positive Partition / Total Partition Number
[b] Measured Concentration (Copies/µl) = −ln (1 − Ratio) / Partition Volume × 1000. Partition volume used is 0.31 nL.

concentrations down to 0.225copies/µl. Further analysis of the N1 results revealed that while three out of three replicates were detected at 0.1125 copies/µl, two replicates detected only one positive partition which corresponds to a measured concentration of 0.07 copies/µl. At such a low concentration, it is anticipated that < 95% detection rate will be obtained for LoD confirmation using 20 replicates. Hence, the preliminary LoD was set at 0.225copies/µl for both assays consequently.

Following which, LoD confirmation of the N1 SARS-CoV-2 assay was performed using both the Clarity Plus™ dPCR and QuantStudio® 3 qPCR platforms. A series of 20 replicates for 0.225 copies/µl and 0.1125 copies/µl were tested. Results summarized in Table 5a indicate that 20 out of 20 replicates (100% detection rate) were detected at the concentration of 0.225 copies/µl on both platforms. At the lower concentration of 0.1125 copies/µl, Clarity Plus™ successfully detected 17 out of 20 replicates while the qPCR platform detected 15 replicates. The results imply that if the concentration of viral RNA extracted from clinical samples is indeed as low as 0.1125 copies/µl, the SARS-CoV-2 assay will successfully detect 85% of the samples using Clarity Plus™, as compared to 75% using the qPCR platform. While ORF1ab detection was not compared across both platforms, the results obtained with Clarity Plus™ further confirmed that the assay has a LoD of 0.225 copies/µl (Table 5b).

Based on these results, the SARS-CoV-2 assay demonstrated a slightly higher sensitivity when performed on the Clarity Plus™ dPCR system as compared to the qPCR platform. Interestingly, this finding differs from some reports where dPCR using droplet-based systems demonstrated higher sensitivities than the comparator COVID-19 RT-qPCR assays [21–23]. These conclusions might need to be further assessed as the differences observed could plausibly be attributed to the variations in sample volume input and varying assays used for the comparison studies. Nonetheless, the SARS-CoV-2 RT-dPCR assay when coupled with Clarity Plus™ has an additional advantage of providing direct absolute quantification of the viral RNA which could be useful for monitoring disease progression and therapeutic effects.

3.4. Detection and quantification of SARS-CoV-2 in contrived nasopharyngeal samples

The clinical performance of the SARS-CoV-2 RT-dPCR assay was subsequently assessed with 10 contrived positive and 2 contrived negative human nasopharyngeal samples. The contrived samples were prepared by spiking equal ratio of VTM containing nasopharyngeal swab

sample with the AccuPlex™ SARS-CoV-2 positive or negative reference materials. RNA from these samples were subsequently extracted using the EX3600 automated nucleic acid extractor and quantified using Clarity Plus™. A positive sample is indicated by the detection of N1 and/or ORF1ab (SARS-CoV-2) and RNase P (human nasopharyngeal sample) targets. On the other hand, samples with only RNase P detected are deemed negative for SARS-CoV-2. As shown in Table 6, the SARS-CoV-2 RT-dPCR assay was able to successfully detect and quantify N1, ORF1ab and RNase P for all the contrived positive samples and only RNase P in the contrived negative samples. These results demonstrate that the SARS-CoV-2 RT-dPCR assay is applicable for use in clinical settings, where PCR inhibitors could copurify with the viral RNA extracted from patient specimens.

4. Conclusion

This study illustrates the performance of a RT-dPCR assay for SARS-CoV-2 detection and quantification. Coupled with the Clarity Plus™ dPCR system, this assay demonstrated excellent linearity across 6 orders of magnitude along its dynamic range for both N1 and ORF1ab targets, and successfully detected low-copy viral RNA samples with high inter- and intra-assay precision. In light of these advantages, RT-dPCR using Clarity Plus™ can potentially be employed in clinical settings as a molecular diagnostic tool for effective detection of SARS-CoV-2 in patients with low viral load and/or monitoring effectiveness of treatment through absolute quantitation of viral load. In addition, this technology can also be valuable for environmental surveillance of COVID-19 where reliable viral load quantification tools are required. Detection of SARS-CoV-2 in environmental samples, such as wastewater, is critical since it can provide early warning signs for disease transmission and outbreaks in the community.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2021.07.005.

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