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Journal of Clinical Virology 153 (2022) 105216

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

A reverse-transcription droplet digital PCR assay to detect and quantify SARS-CoV-2 RNA in upper respiratory tract specimens

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ARTICLE INFO

Keywords:
SARS-CoV-2
Droplet digital PCR
RNA quantification

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense, single-stranded RNA virus that causes coronavirus disease 2019 (COVID-19). Symptoms are variable and range from asymptomatic or mild to severe (i.e., pneumonia) in both healthy and immunocompromised patients. We developed a reverse-transcription droplet digital PCR (RT-ddPCR) assay for quantification of SARS-CoV-2 RNA in clinical nasopharyngeal and oropharyngeal swab specimens and evaluated the assay, including reproducibility, agreement of results, analytical measurement range, linearity, analytical sensitivity, and analytical specificity. This quantitative assay had a LoD of 218 copies/mL of viral transport media, with a linear quantification range from 500 to 5,000,000 copies/mL (R^2 of 0.9817 and 0.9853 for *N1* and *N2* targets, respectively). Qualitative agreement of categorical results was 90.5% (57/63) between the reference and RT-ddPCR assays. Quantitative agreement between the two assays showed correlation, with R^2 of 0.9726 and 0.9713 for *N1* and *N2* targets, respectively. No cross-reactivity with common coronavirus strains was detected. This SARS-CoV-2 quantitative RT-ddPCR assay may be a useful tool for a variety of applications including identification of patients with low viral load and serial monitoring of viral load in respiratory tracts specimens of patients for evaluation of the efficacy of therapy for COVID-19.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, is typically present in highest amount in the nasopharynx during the first 3 to 5 days of symptomatic illness[1-3]. The impact of viral load on SARS-CoV-2 severity and progression is not currently well-established; however, recent studies have suggested that higher viral load at diagnosis may be associated with higher mortality, but also an earlier antibody response[4].' [5] Additionally, viral load at onset may influence transmission[6]. Clinical assays that quantify the viral load will help to better understand the underlying biology of SARS-CoV-2 infections, such as viral load required for illness and infectiousness, as well as to monitor therapeutic response in both clinical and investigative settings. Since the clinical relevance of low viral titers in patients remains unclear, more accurate detection of the virus may help facilitate understanding of transmission dynamics.

The gold standard for diagnosis of COVID-19 is the qualitative real-time PCR (rtPCR) of SARS-CoV-2, as recommended by US Center for Disease Control and Prevention (CDC)[7]. Although in general the crossing threshold (C_T) values of these assays may provide an estimate of viral load, the C_T values vary among methods[8].' [9] Furthermore, typical rtPCR assays may be prone to effect of inhibitors, which could further impact C_T. Droplet digital PCR (ddPCR) has been demonstrated to have higher sensitivity and specificity than traditional real-time PCR for the detection of SARS-CoV-2[10]. We describe the development and performance characteristics of a quantitative test for SARS-CoV-2, modified from the Bio-Rad SARS-CoV-2 ddPCR Kit (Bio-Rad Laboratories, Inc., Pleasanton, CA) that received emergency use authorization (EUA) from the U.S. Food and Drug Administration for qualitative detection of SARS-CoV-2 in upper respiratory tract specimens during the COVID-19 pandemic.

Abbreviations: ddPCR, Droplet digital PCR; rtPCR, Real-time PCR; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; C_T, Crossing threshold; RT-ddPCR, Reverse-transcription droplet digital PCR; PBS, Phosphate-buffered saline; NP, Nasopharyngeal; OP, Oropharyngeal; LoD, Limit of detection.

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https://doi.org/10.1016/j.jcv.2022.105216
Received 19 June 2021; Received in revised form 29 May 2022;
Available online 8 June 2022

2. Materials and methods

2.1. Clinical specimens

A total of 93 residual nasopharyngeal (NP) and oropharyngeal (OP) swab specimens collected in MicroTest™ M4RT® Multi-Microbe Media (Remel, Inc., Lenexa, KS) or phosphate-buffered saline (PBS) for routine clinical testing were used for accuracy, precision, stability, and linearity studies.

2.2. Reference material and assay controls

Heat-inactivated 2019-nCoV/USA-WA1/2020 virions (ATCC® VR-1986HK™; American type culture collection, Manassas, VA) were used to prepare various concentrations (as genomic copies/mL) of SARS-CoV-2 RNA in PBS. EDX SARS-CoV-2 negative and positive (200,000 and 2000 copies/mL) reference standards (Bio-Rad Laboratories, Inc., Hercules, CA) were used as controls in all assay runs.

2.3. Nucleic acid extraction and purification

For extraction and purification of total nucleic acid (TNA), 200 μL of the transport media was added directly to 2 mL lysis buffer in each easyMAG cartridge in the biosafety level 2 cabinet, where the cartridges are left standing at ambient temperature for 10 min, before loading onto the easyMAG instrument system (bioMérieux, Inc., Durham, NC). System protocol Workflow 4 (B41-t) was used with an elution volume of 50 μL. Processing and extracting 24 samples (including assay controls) took 1.5 hr, with 37.4 min of total hand-on time

2.4. SARS-CoV-2 RT-ddPCR assay

Simultaneous quantification of 2 SARS-CoV-2 nucleocapsid (*N*) target sequences (*N1* and *N2*) and human RNase P-encoding (*RPP30*) sequence was performed using the Bio-Rad SARS-CoV-2 ddPCR Kit with the QX200 AutoDG Droplet Digital PCR System (QX200 AutoDG System; Bio-Rad Laboratories, Hercules, CA). Reaction mixtures of 22 μL each were prepared as follows: 5.5 μL of 4x One Step-RT-ddPCR Supermix, 2.2 μL of reverse transcriptase, 1.1 μL of dithiothreitol, 1.1 μL of the 20 × 2019-nCoV CDC ddPCR triplex probe TaqMan assay, and 12 uL of TNA. Of this 22-uL reacture mixture, 20 uL were loaded onto the instrument for testing. Final concentrations of primers and probes were 900 and 250 nmol/L, respectively. The PCR plates were placed on the QX200 AutoDG, followed by RT-ddPCR amplification as follows: 25 °C for 3 min and 50 °C for 60 min, followed by the PCR steps: 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 55 °C for 1 min, and a final enzyme deactivation at 98 °C for 10 min. Targets in the droplets were counted by the QX200 Droplet Reader, and signal data were analyzed using QuantaSoft Analysis Pro-software version 1.0.596 (Bio-Rad Laboratories, Inc., Hercules, CA). Preparation of amplification plate, amplification, detection, and data analysis required 4.5 hr and 7.5 hr for 24- and 96-sample assay runs, respectively.

2.5. Assay performance characteristics

2.5.1. Specimen stability

Three residual clinical SARS-CoV-2 RNA-positive specimens in PBS transport media containing high, mid-range, and low levels of SARS-CoV-2 RNA were tested in duplicate on days 0, 1, and 3 of being held at ambient temperature (25 °C), on day 10 at 4 °C, and on day 30 of storage at −70 °C.

2.5.2. Reproducibility

Intra- and inter-assay precision was evaluated using 3 pooled swab samples containing high, medium, or low levels of SARS-CoV-2 RNA in PBS transport media. Each pooled sample was tested in 6 replicates within a run for intra-assay precision. For inter-assay precision, each pool was tested in duplicate per assay run for 3 runs (total of 6 replicate results).

2.5.3. Agreement of results

Forty-two unique, known-positive and 21 known-negative residual clinical swab specimens in transport media previously tested with the FDA EUA Abbott RealTi*m*e SARS-CoV-2 assay (Abbott rtPCR; Abbott Molecular Inc, Des Plaines, IL) were used for comparative testing. These previously tested positive specimens have Abbott rtPCR target C_T values in the following ranges: <15, 15 to 20, >20 to 25, >25 to 30, >30 to 35. A 7-member panel of SARS-CoV-2 RNA was prepared from duplicates of 10-fold serial dilutions of ATCC® VR-1986HK™ from 10^7 to 10^1 copies/mL and tested with Abbott rtPCR to generate a standard curve correlating target C_T values to known concentrations of SARS-CoV-2 RNA. This standard curve was used to quantify SARS-CoV-2 RNA in all 63 residual clinical specimens.

2.5.4. Analytical measurement range and linearity

The quantification range and linearity were determined by testing duplicate panels of the ATCC® VR-1986HK™, diluted 10-fold serially from 10^7 to 10^1 copies/mL in PBS.

2.5.5. Analytical sensitivity

Ten panels with SARS-CoV-2 RNA levels at 2000, 1000, 500, 250, 125 and 0 copies/mL were prepared with ATCC® VR-1986HK™ diluted in PBS to determine the limit of detection (LoD).

2.5.6. Analytical specificity

Cross-reactivity of common respiratory tract pathogens (bacteria, fungi, and viruses) was evaluated extensively with *in silico* analyses by the Bio-Rad kit manufacturer. The analytical specificity of the quantitative RT-ddPCR assay was verified by testing 4 inactivated non-SARS-related human coronaviruses (HCoV 229, HCoV OC43, HCoV HKU1, and HCoV NL63; ZeptoMetrix, Inc., Buffalo, NY).

2.5.7. Carryover

Sample-to-sample carryover in the QX200 AutoDG System was evaluated by testing a series of “no template control” samples and purified eluate from the EDX SARS-CoV-2 reference standard of 200,000 copies/mL, placed in alternating wells of the PCR reaction plate.

2.6. Data analysis

The QX200 AutoDG system counts acceptable droplets and measures the fluorescence emissions from each droplet using 2 channels (FAM and HEX). Droplets of different color and intensity are displayed on 2-dimensional plots, allowing counting of negative droplets as well as those positive for *N1, N2, RPP30*, or a combination of targets (see Supplementary Fig. S1). Concentrations of each target in the sample were determined with Poisson statistics. The following formula was used to convert target copies per 20-μL well into copies/mL of transport media of the original sample.

$$\text{Copies per mL media} = \frac{Copies\,per\,20-\mu L\,well}{10.9\,\mu L\,TNA\,input} \times \frac{50\,\mu L\,TNA\,elution\,volume}{0.2\,mL\,media\,volume}$$

For precision and stability experiments, average standard deviation (SD) among the replicates from each 3 pooled specimens tested was calculated for both *N1* and *N2* targets.

Deming regression and Bland-Altman plots were utilized to compare quantitative results for the 42 known SARS-CoV-2 RNA-positive clinical NP and OP swab specimens as measured by the quantitative RT-ddPCR with those generated with the calibrated Abbott rtPCR. Ordinary linear regression analysis was used to correlate the mean observed and

expected viral loads in the linearity study. LoD at 95% detection rate with 95% confidence interval was determined by probit logistic regression analysis (MedCalc®; MedCalc Software Ltd., Ostend, Belgium).

3. Results

3.1. Specimen stability

SARS-CoV-2 viral load remained stable in PBS transport media containing NP or OP swabs up to 3 days at 25 °C, 10 days at 4 °C, and 30 days at −70 °C, with maximum change of up to 0.13 $\log_{10}$ copies/mL at all testing time points.

3.2. Reproducibility

Replicates from the high-positive pooled specimen generated results beyond the upper limit of quantification (ULoQ) and they were excluded from further analysis. Mean SD among the 6 replicates from the remaining 2 pooled specimens tested for intra-assay precision were 1.1 (or 0.03 $\log_{10}$) copies/mL for both *N1* and *N2* targets, while the mean SD for inter-assay runs ranged from 1.1 (or 0.3 $\log_{10}$) to 1.2 (or 0.08 $\log_{10}$) copies/mL.

3.3. Agreement of results

Total agreement of qualitative results (detected vs. not detected) was observed for 42 known-positive and 21 known-negative clinical swab specimens previously tested with Abbott rtPCR (Table 1). Overall agreement of categorical results, based on range of viral load, was 90.5% (57/63) between the reference Abbott rtPCR and RT-ddPCR assays (Table 1). Among the 42 clinical NP and OP swab specimens, 20 yielded quantifiable results within the linear range of both assays. Deming regression analysis and Bland-Altman plots of the comparison are shown in Fig. 1, with coefficients of determination, R^2, being 0.9726 and 0.9713 for RT-ddPCR results of the *N1* and *N2* targets, respectively. For the *N1* target, the Abbott rtPCR yielded an overall mean difference of 0.72 $\log_{10}$ copies/mL as compared to RT-ddPCR assay, with 95% (±1.96 SD) of the result differences falling in the range from 0.15 $\log_{10}$ to 1.30 $\log_{10}$ copies/mL. Similarly, for the *N2* target, Abbott rtPCR yielded an overall mean difference of 0.69 $\log_{10}$ copies/mL as compared to RT-ddPCR assay, with 95% (±1.96 SD) of the result differences within the range of 0.11 $\log_{10}$ to 1.27 $\log_{10}$ copies/mL.

3.4. Analytical measurement range and linearity

Linear regression analysis of the mean observed versus expected SARS-CoV-2 RNA results across the quantification range of the RT-ddPCR assay is shown in Supplemental Fig. S2, with coefficients of determination, R^2, of 0.9817 and 0.9853 for the for the *N1* and *N2* targets, respectively. Serial dilution of ATCC® VR-1986HK™ showed linear quantification from 500 to 5000,000 copies/mL. The lower limit of quantification (LLoQ) was established at 500 copies/mL based on a 100% detection rate and and SD of < 0.25 $\log_{10}$ copies/mL from expected results.

Table 1
Comparison of results between the RT-ddPCR and Abbott rtPCR assays for quantification of SARS-CoV-2 RNA.

RT-ddPCR (LoD = 191 cp/mL)	Abbott RealTime SARS-CoV-2 (LoD = 100 cp/mL)		
	TND	500 – 10^7 cp/mL	>10^7 cp/mL
TND	21	0	0
500 – 10^7 cp/mL	0	20	6 [a]
>10^7 cp/mL	0	0	16

cp/mL, copies/mL; LoD, limit of detection; TND, target not detected.
[a] The Abbott rtPCR assay results ranged from 7.02 log to 7.30 log cp/mL, while the RT-ddPCR results ranged from 6.77 log to 6.96 log cp/mL for *N1* and *N2* targets. All differences were < 0.50 log cp/mL between the 2 assays.

3.5. Analytical sensitivity

The LoD was established at 218 copies/mL (95% CI, 109 to 328 copies/mL) of the transport media based on 95% detection rate (Table 2, Supplemental Fig. S3).

3.6. Analytical specificity

No amplification of either nucleocapsid targets was present (i.e., 0 copies/mL) for any of the other coronavirus strains tested, indicating no cross-reactivity.

3.7. Carryover

All no-template control samples showed "target not detected" results (i.e. 0 copies/mL).

4. Discussion

This study describes the development and performance characteristic of an RT-ddPCR assay designed for quantification of SARS-CoV-2. The findings show that it is a reliable and accurate assay with reproducible results. Qualitative analysis demonstrated 100% concordance with the Abbott rtPCR used clinically in our laboratory. Currently there is no gold standard quantitative test for SARS-CoV-2 viral load. Results of this assay compared well to those of the Abbott rtPCR calibrated with a 7-level panel of a viral reference standard. Quantitative values were slightly higher with the Abbott rtPCR, possibly due to its use of a single fluorophore on the 2 target probes. Serial dilution studies using the panel of reference viral standard showed the RT-ddPCR assay to be linear from 500 to 5000,000 copies/mL. Similar to other assays that have recently been evaluated[11], this assay is highly specific.

The amount of SARS-CoV-2 present in a given clinical specimen (ie, viral load) depends on many factors, such as timing of obtaining clinical specimen in relation to onset of symptoms, specimen type (eg, sputum, NP, OP, anterior nares, saliva), adequacy of sampling, transport media, duration of transport and storage before testing, number of freeze-thaw cycle before testing, assay design (primer and probe sequences, amplification and detection conditions). Studies have indicated that viral load may be highest in sputum, followed by OP and NP swabs.' [10] Therefore, if this assay were used for monitoring of patients, it will be important to ensure collection of the same specimen type for serial measurements.

This assay has multiple advantages. First, this assay is quantitative, allowing monitoring viral burden at a specific anatomic site during the course of infection with or without treatment and monitoring responses in clinical trial studies of new therapeutic agents. Secondly, since this assay uses an existing commercial kit and associated software, clinical laboratories can perform both qualitative and quantitative assays using the same equipment and reagents. Thirdly, this assay demonstrated similar analytical sensitivity as other assays, with an LoD established at 218 copies/mL for 95% detection rate. Such sensitivity was achieved in part because the assay amplifies two viral targets, which may also be beneficial if the virus acquires a mutation that would cause one target to drop out. The TNA extraction platform is independent of the RT-ddPCR assay, allowing for flexibility in selection of an extraction platform and setting extraction parameters to optimize sensitivity. Finally, the RT-ddPCR assay allows users to visualize individual positive droplets plotted on a chart and to determine whether a given droplet is positive for one or multiple targets. In contrast, a typical RT-PCR assay generates only an amplification curve and target C_T value.

This test has several limitations. First, although the limit of detection

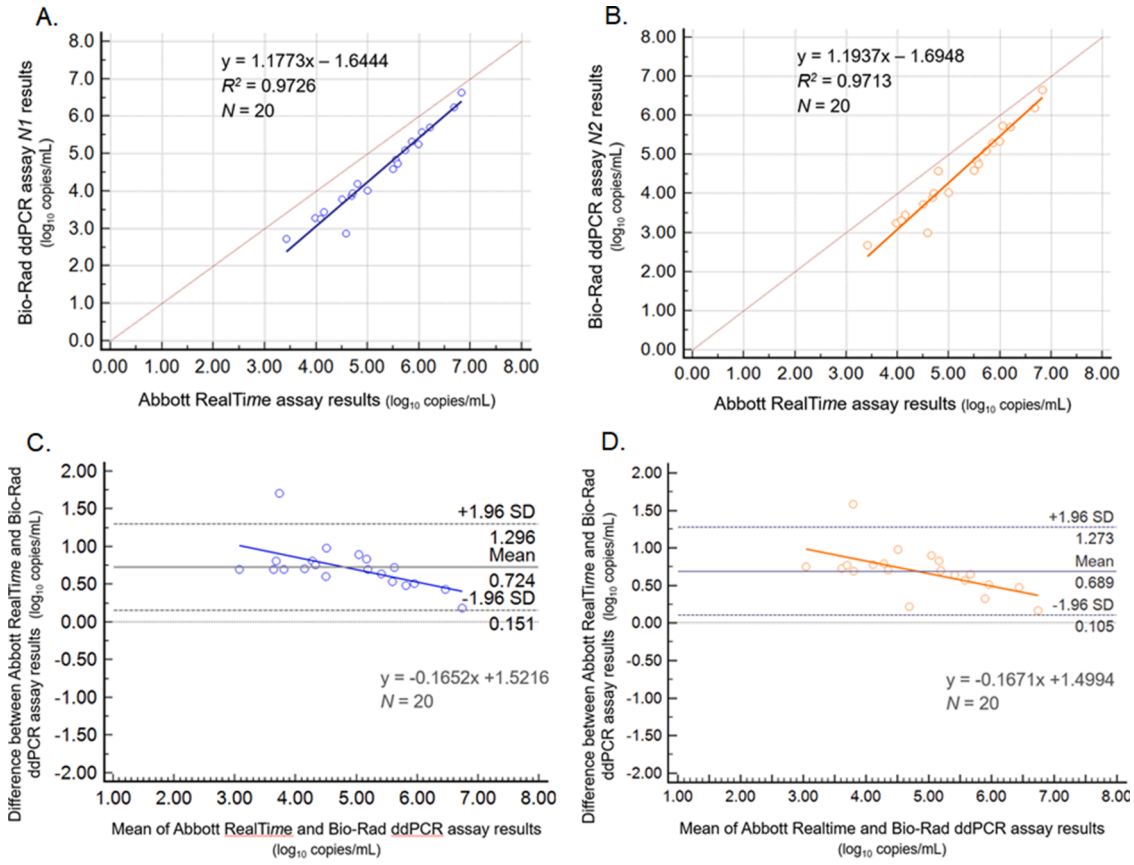

Fig. 1. Correlation between results obtained from the RT-ddPCR and Abbott rtPCR assay. Results in $\log_{10}$ copies/mL for Abbott rtPCR assay (x-axis) and the RT-ddPCR assay (y-axis) were correlated by Deming regression analysis for the SARS-CoV-2 *N1* (A) and *N2* (B) targets of the RT-ddPCR assay. The overall mean ± 1.96 SD of the differences in the corresponding results from both assays were determined by Bland-Altman plots for the *N1* (C) and *N2* (D) targets.

Table 2
Detection rate at varying concentrations of ATCC® VR-1986HK™ tested with RT-ddPCR.[a].

SARS-CoV-2 RNA level (copies/mL)	No. replicates tested	No. replicates detected	% Detected
2000	10	10	100%
1000	10	10	100%
500	10	10	100%
250	10	9	90%
125	10	9	90%
0	10	0	0%

[a] Limit of detection at 95% detection rate was determined to be 218 copies/mL (95% CI, 109 to 328 copies/mL).

was established at 218 copies/mL of transport media, which was lower than the LoD of 11 assays compared in a recent study (range, 240 to 31,151 genome copies/mL)[11]. However, the lower LoQ was established to be 500 copies/mL, which potentially could be modulated by further altering the amount of TNA eluate added to the RT-ddPCR reaction. In addition, as TNA are extracted using a platform separate from RT-ddPCR, extraction parameters can be modified to decrease the elution volume, allowing for increased concentration of eluate for RT-ddPCR. Another limitation is that only NP and OP specimens were validated in this study. However, this assay could be modified in the future to quantify SARS-CoV-2 in other specimen types, including saliva, stool, tissue, or municipal wastewater that may be of clinical and epidemiological interest. Of note, the clinical significance of the viral load results generated remains to be validated. Availability of such a quantitative assay will allow for future clinical studies to better understand the clinical implications of SARS-CoV-2 viral load and whether such measurement is a useful marker for evaluation of therapeutic response.

Due to the widespread nature of the COVID-19 pandemic, individuals with training and responsibilities only for human molecular genetics tests but may not routinely participate in molecular microbiology laboratory oversight have become involved in molecular testing for SARS-CoV-2. There are a few important considerations for this assay, as well as other molecular assays for SARS-CoV-2, that may not apply to hereditary or somatic molecular diagnostics. First, this test requires biosafety level 2 cabinets to process clinical specimens due to their infectious nature. Second, it is important to remember that specimens with a very high viral load may result in the *N1* and/or *N2* targets outcompeting the human internal control target, *RPP30*, in the RT-ddPCR assay. Therefore, a test result may be positive – particularly in the qualitative setting – even when the internal control target fails to amplify. Finally, most assays for SARS-CoV-2 fall under microbiology checklists of clinical laboratory accrediting agencies, rather than the molecular genetics checklist. Therefore, clinical laboratories should be aware that different requirements exist for assay performance verification and release of test results for patient care purposes.

In summary, we have modified a qualitative, commercially available assay to perform quantitative measurement of SARS-Co-V-2 viral load. This assay is reliable, accurate and reproducible, and it can be used to further our understanding of the disease course of SARS-CoV-2 as well as in the development of novel therapeutic modalities.

Funding statement

No funding was solicited for this study.

Supplementary data

Supplementary material related to this article can be found in the online version of this publication.

Declaration of Competing Interests

All investigators of this work presented in this manuscript have no conflict of interest to declare.

Acknowledgements

We kindly thank the staff of Division of Laboratory Genetics and Genomics and the Clinical Virology Laboratory for their assistance in this work.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105216.

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