

Digital Droplet PCR for SARS-CoV-2 Resolves Borderline Cases

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ABSTRACT

Objectives: *The Bio-Rad SARS-CoV-2 ddPCR Kit (Bio-Rad Laboratories) was the first droplet digital polymerase chain reaction (ddPCR) assay to receive Food and Drug Administration (FDA) Emergency Use Authorization approval, but it has not been evaluated clinically. We describe the performance of ddPCR—in particular, its ability to confirm weak-positive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) results.*

Methods: *We clinically validated the Bio-Rad Triplex Probe ddPCR Assay. The limit of detection was determined by using serial dilutions of SARS-CoV-2 RNA in an artificial viral envelope. The ddPCR assay was performed according to the manufacturer's specifications on specimens confirmed to be positive (n = 48) or negative (n = 30) by an FDA-validated reverse transcription–polymerase chain reaction assay on the m2000 RealTime system (Abbott). Ten borderline positive cases were also evaluated.*

Results: *The limit of detection was 50 copies/mL (19 of 20 positive). Forty-seven specimens spanning a range of quantification cycles (2.9–25.9 cycle numbers) were positive by this assay (47 of 48; 97.9% positive percent agreement), and 30 negative samples were confirmed as negative (30 of 30; 100% negative percent agreement). Nine of 10 borderline cases were positive when tested in triplicate.*

Conclusions: *The ddPCR of SARS-CoV-2 is an accurate method, with superior sensitivity for viral RNA detection. It could provide definitive evaluation of borderline positive cases or suspected false-negative cases.*

Key Points

- This study addresses how well a novel platform such as ddPCR works for detecting SARS-CoV-2.
- The ddPCR is one of the most sensitive assays available and can resolve borderline positive cases.
- Interestingly, high-viral-load samples create a smear artifact not typically observed in ddPCR.

The global coronavirus disease 2019 (COVID-19)¹ pandemic continues to pose a serious global public health threat. The COVID-19 pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),² is a single-stranded RNA *Betacoronavirus* with a 26-kilobase genome. Molecular detection of SARS-CoV-2 targeting the viral genes (eg, *Orf1a/b*, *E*, *S*, *N* genes) is currently the gold standard for assessing acute infection.^{3–7}

In the United States, the first clinical assay for SARS-CoV-2 was developed by the Centers for Disease Control and Prevention (CDC)³ and approved under a Food and Drug Administration (FDA) Emergency Use Authorization (EUA). As of the first quarter of 2021, multiple testing platforms have obtained EUA and been clinically implemented to diagnose SARS-CoV-2 infection. Although these point-of-care tests are rapid, many are limited by moderate sensitivity and high false-negative rates.⁸ Real-time polymerase chain reaction (RT-PCR) platforms can detect low levels of virus with high throughput, but weak positives (cycle number [CN] > 35) can be difficult to distinguish from technical artifacts after many PCR cycles. Droplet digital PCR (ddPCR) is an orthogonal method designed to detect and measure precise copy numbers of nucleic acid, but it can

be applied to detect extremely low levels of nucleic acid, as well. The Bio-Rad SARS-CoV-2 ddPCR Kit (Bio-Rad Laboratories) was the first assay in this class to receive EUA (Precigenome is the only other company with a ddPCR EUA-approved assay), but data characterizing its clinical performance are limited. One study observed that ddPCR has a sensitivity of 93.33% and a specificity of 100% for both the N1 and N2 gene regions of SARS-CoV-2.⁹ In this study, we report the limit of detection, artifactual findings, and correlation with other clinically validated methods. Overall, ddPCR is a highly sensitive approach capable of resolving borderline results with reasonable throughput and cost. With its sensitivity, it is well suited to pooled testing as well.

Materials and Methods

RNA Extraction

In Biosafety Level 2 containment, 400 μ L of residual nasopharyngeal (NP) swab samples in universal viral transport medium (VTM) (3 mL initial collection, VTM; BD Catalog No 220529 [Becton Dickinson]) or spiked control material in VTM were transferred to conical tubes containing 400 μ L lysis buffer, 40 μ L proteinase K, and 7.5 μ L carrier RNA for lysis/virus inactivation (20 minutes at 56°C) before extraction. Total nucleic acids from pooled or individual residual NP collections and controls were obtained using a Maxwell RSC instrument (Promega), with an offboard lysis protocol and the following volume parameters: 400 μ L input, 500 μ L magnetic silica, and 35 μ L output/elution.

Control Material and Patient Specimens

Control material for endemic SARS-CoV-2 coronavirus strains was obtained from AccuPlex SARS-CoV-2 Reference Material Kit (SeraCare).

Residual deidentified patient samples from a clinical laboratory performing testing with the m2000 RealTime SARS-CoV-2 EUA test (Abbott) included both positive ($n = 30$) and negative ($n = 30$) samples. All CN values come from the m2000 system unless specified as Alinity CN, which designates a value coming from the FDA EUA Alinity m SARS-CoV-2 assay (Abbott). The transport medium of these samples was a formulation of VTM. Each patient sample underwent freeze-thaw cycles at least twice before our extraction and PCR. Positive patient specimens, when indicated, were diluted with nuclease-free water after extraction. Borderline positive cases were defined as “Alinity CN > 38” that repeated weak positive by

the Alinity SARS-CoV-2 assay ($n = 10$). Only borderline cases were run in triplicate.

Droplet Digital PCR

All procedures followed the manufacturer’s instructions for the SARS-CoV-2 ddPCR Kit unless otherwise specified.¹⁰ A mastermix was prepared from the Bio-Rad 1-Step RT-ddPCR Advanced Kit for Probes and ddPCR Expert Design Assay 2019-nCoV CDC probe. In a 96-well PCR plate, 13 μ L of extracted patient sample or control (representing 149 μ L of original) were added to 9 μ L of mastermix for each reaction. At least 6,000 and 10,000 total droplets were required for positive and negative samples, respectively. After droplet generation, the samples were placed on a Bio-Rad C1000 Touch Thermal Cycler and underwent reverse transcription and PCR amplification. When droplet stabilization had occurred, the plate was immediately read on a Bio-Rad QX200 Droplet Reader and evaluated using the QuantaSoft Analysis Pro 1.0 software.

Primers and probes for the CDC N1 and N2 gene targets were present, along with ribonuclease P protein subunit p30 (RPP30) as an internal control for endogenous human RNA. The N1 probe was labeled with HEX, the N2 probe was labeled with HEX and carboxyfluorescein (FAM), and the RPP30 probes were labeled with higher levels of the FAM fluorophore. Therefore, positive results led to unique clusters of populations. Sometimes, droplets were positive for multiple targets; in this case, the software could anticipate the location and apply gating. Each case was reviewed for automated droplet count and then reviewed manually to determine whether positive droplets fell outside gating parameters. At least 1 of the N1 and N2 droplets (or ≥ 3 N1 or N2 droplets) was required for a positive result, and at least 10 RPP30 droplets were required for a valid result. A result was considered invalid if (1) fewer than 10 RPP30 droplets were detected; (2) the sample was negative, with fewer than 10,000 negative droplets; or (3) the sample was positive, with fewer than 6,000 droplets.

Results

Limit of Detection

To determine the limit of detection of the assay, triplicate serial dilutions of synthetic SARS-CoV-2 viral particles were prepared (AccuPlex SARS-CoV-2 Reference Material Kit positive reference) ranging from 500 copies/mL to 1 copy/mL (Table 1). When all triplicate samples were detected, an extended replicate series of 20 samples was performed (Figure 1A). We found a 95% positive rate

(19 of 20) at 50 copies/mL, which was 20-fold lower than the kit manufacturer reported. The 1 sample that failed had 1 droplet positive from N1, which was below our cutoff but suggested that even better sensitivity may be possible.

Accuracy

Residual SARS-CoV-2-positive patient specimens were tested undiluted or diluted in VTM. Thirty positive and 30 negative clinical samples confirmed by an FDA-approved and clinically validated RT-PCR assay (m2000 RealTime System) were used along with 20 diluted positive specimens. Two positive specimens failed quality metrics (droplet count criteria) and were excluded. All specimens were tested in a blinded manner and showed sufficient expression of the internal control (*RPP30*). All 30 negative and 47 of 48 positive specimens were concordant with the reference quantitative PCR (qPCR)

Table 1
Summary of Limit-of-Detection Analysis Results

Concentrations, copies/mL ^a	No. of Copies per Reaction ^b	N1 ^c	N2 ^c	Total ^c
500	74.3	3/3	3/3	3/3
100	14.8	3/3	3/3	3/3
50	7.4	3/3	3/3	3/3
25	3.7	1/3	1/3	1/3
10	1.5	1/3	2/3	1/3
1	0.1	0/3	0/3	0/3
VTM	0	0/3	0/3	0/3

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VTM, viral transport medium.

^aStarting copy numbers of SARS-CoV-2.

^bTheoretical calculated viral copy number in each individual reaction.

^cN1, N2, and total positivity rates (positive if at least 3 droplets are detected). The ability to detect SARS-CoV-2 falls at viral loads less than 50 copies/mL.

method (97.9% positive percent agreement [PPA] and 100% negative percent agreement [NPA]). The 1 positive specimen that failed had 2 positive N2 droplets and corresponded to a high CN value (25.23), which was above the reference assay's cutoff.

High-Viral-Load Artifacts

The positive samples for the accuracy study spanned a range of CN values, from 3 to more than 25. Some of the samples exhibited a smear of droplet positions that did not fall into any of the predicted categories for the N1, N2, or *RPP30* primers (Figure 2B) nor into any gates where a combination of primers would be expected (Figure 2A). This smear was highly positive for both FAM and HEX and was observed in samples with low CN values (<10), consistent with high viral loads (Figure 2C). This phenomenon likely arises from droplets having transcripts labeled with multiple probes for N1 and N2. This unusual finding of a smear population is not commonly reported from ddPCR assays and likely reflects the technology's deployment for the detection of low-prevalence, inherited genetic variants.

Lastly, inhibitors may be present and will present as a smear of droplets from the expected gate position downwards (Figure 2D).

Correlation of ddPCR Results to CN Values

A comparison of ddPCR for the detection of SARS-CoV-2 to approved RT-PCR methods has not been described in a clinical laboratory setting. Our current RT-PCR assay has a limit of detection of 100 copies/mL, with a CN value (CN is equivalent to cycle threshold) of 25. Differences in N1 and N2 primer sensitivity have been reported for the RT-PCR method, so we evaluated

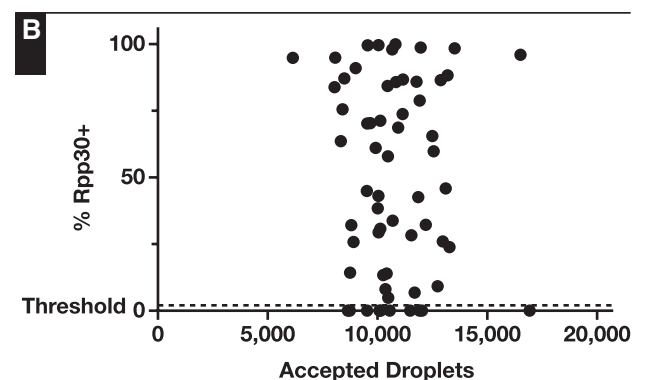
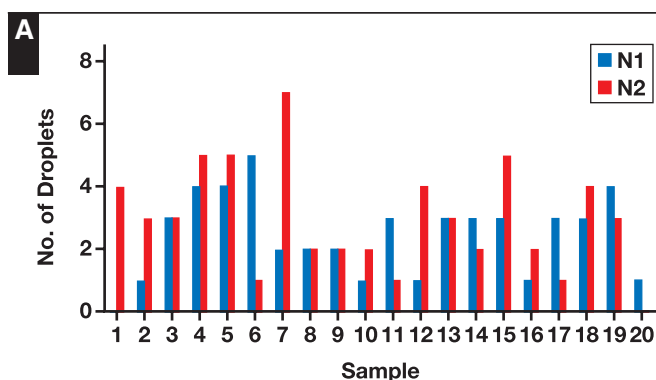


Figure 1 **A**, Limit-of-detection study with a 95% (19 of 20) positivity rate at a concentration of 50 copies/mL. Sample 20 failed, with only 1 positive droplet below our cutoff. **B**, Rate of positivity of internal control (ribonuclease P protein subunit p30 [*RPP30*]) compared with the number of accepted droplets. The total accepted droplet count is not related to percent positive internal control. Samples with *RPP30* < 5% had high-viral-load artifact present.

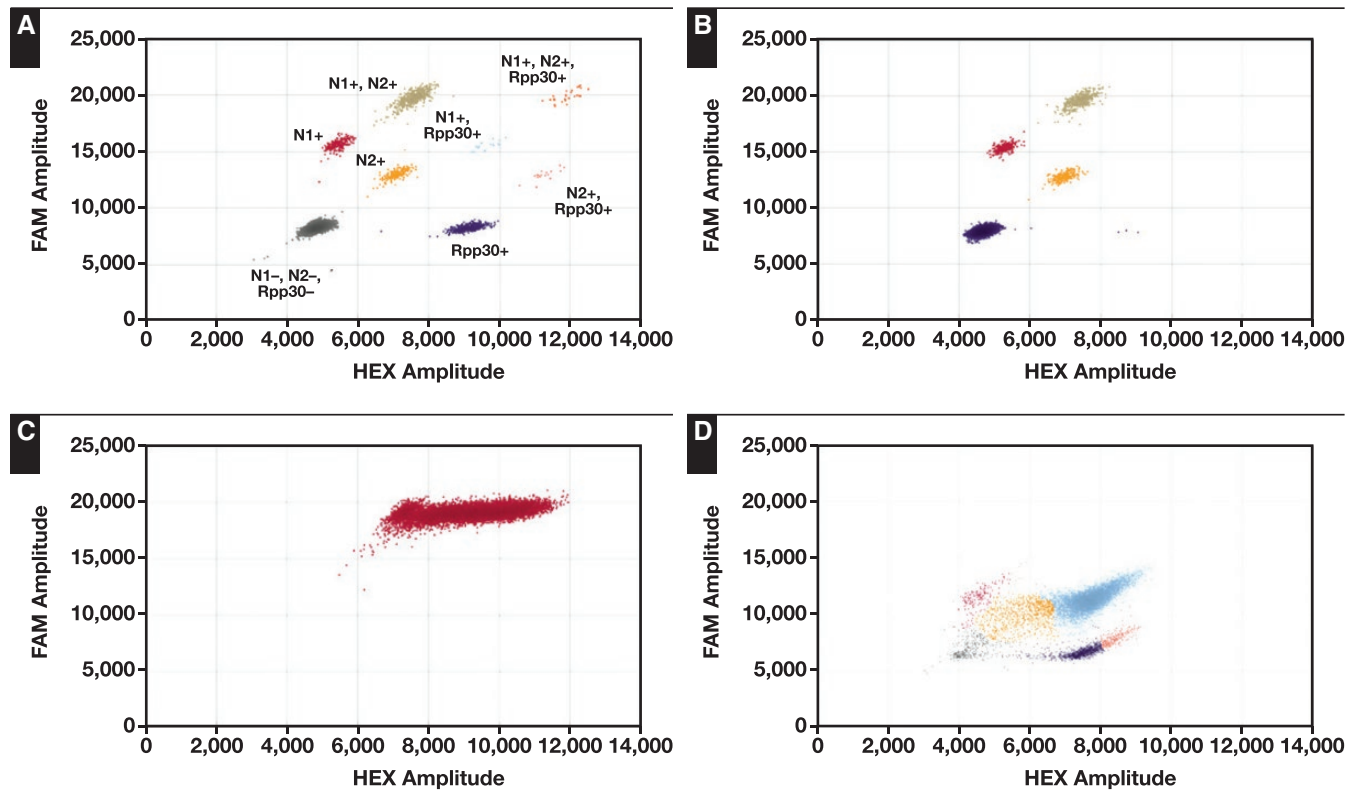


Figure 2 Examples of severe acute respiratory syndrome coronavirus 2–positive samples. **A**, All possible clusters displayed and labeled. Each is discrete and easily distinguishable. **B**, A typical plot of a positive and **C**, smear artifact observed in cases with high viral load. **D**, Artifact caused by inhibitors in a positive sample.

whether differences existed with the ddPCR testing modality as well.

When low CN values were present, the N1 target still worked well, with a high number of positive droplets, but the N2-positive droplets would often be absent **Figure 3A** and **Figure 3B**. When the number of N1 and N2 droplets in the same specimen were compared, a bias for high N1 and low N2 was again observed **Figure 3C**. To determine whether the observed bias for N1-positive droplets is a function of primer or probe compatibility or artifactual, we manually examined the 2-dimensional plots of droplets. For each case of N1 or N2 bias, the result stemmed from smear artifact, which fell into the N1 gate. Thus, there is no apparent bias of N1 to N2 targets.

If these samples were excluded, the linear regression of N1-positive to N2-positive droplets (ddPCR) formed a line, with a slope of 0.94 ± 0.01 ($R^2 = 0.99$), indicating strong correlation between probes (**Figure 3C**). Similarly, when samples with low CN values (<10) were excluded, N1 and N2 had good correlation to CN value (**Figure 3D**; $R^2 = 0.815$ and 0.806 , respectively).

Quality Metrics

Several important quality metrics for ddPCR exist, and they must be considered for a run to be called valid. First, the total number of counted droplets must be over a certain threshold to reach reported sensitivity levels. Internal control RPP30-positive droplets must also be present except when artifact was observed **Figure 1B**. These cases were considered valid because they were clearly positive for SARS-CoV-2, but they were unquantifiable for RPP30 droplets, which typically require 10 droplets counted.

Variability between runs could emanate from emulsion preparation, which would affect the whole plate. Therefore, we measured variability in droplet formation among each run. There was minimal variability in the number of accepted drops ($11,022 \pm 1,404$) within a run (coefficient of variation [CV], 13%) and between runs (CV, 9%).

Precision Studies

For interrun tests, the mean (SD) SARS-CoV-2 droplet number for the plasmid control (N1 and N2; 4,760,000 copies/mL) and negative control (RPP30) was

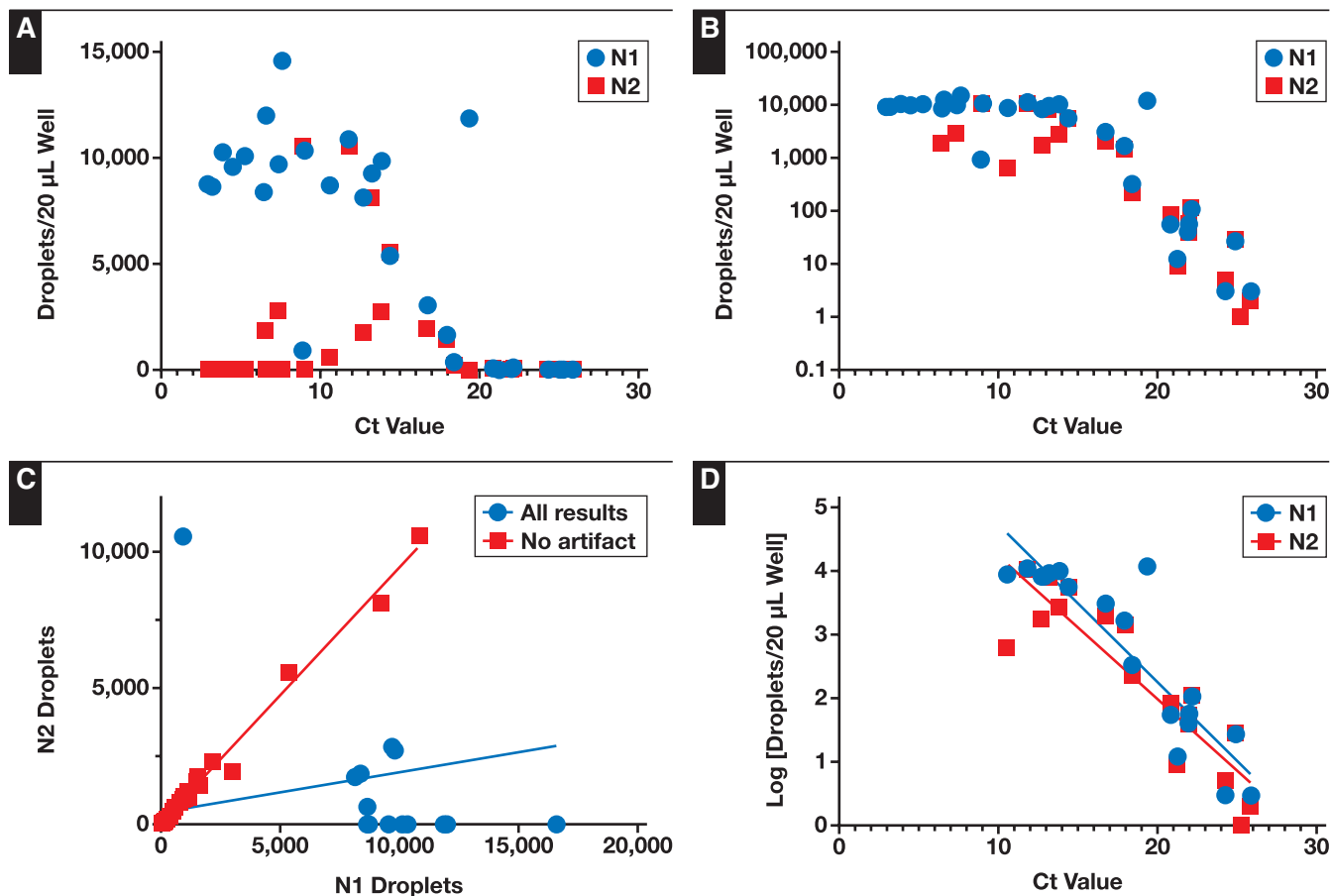


Figure 3 **A**, **B**, and **D** compare the concentration of positive droplets vs the cycle threshold (Ct) values for corresponding polymerase chain reaction results. **A**, Bias toward N1 droplets called positive and N2 called negative at lower Ct values. **B**, Saturation effect observed at Ct values < 10. **C**, Comparison of N1-positive droplets with N2-positive droplets in every sample (blue circles) (slope = 0.15; $R^2 = 0.088$). Bias toward N1 or N2 is eliminated when smear artifact data are excluded (red squares) (slope = 0.94; $R^2 = 0.992$). **D**, Linear relationship for N1 (slope = -0.2475 ; $R^2 = 0.81$) and N2 (slope = -0.2244 ; $R^2 = 0.81$) compared with Ct values > 10.

1,237 (190) for N1, 1,095 (171) for N2, and 1,096 (120) for RPP30. Mean CV was 15%, 16%, and 11% for N1, N2, and RPP30, respectively. No carryover was observed between positive and negative controls nor between high-viral-load samples and adjacent negative samples.

Resolving Borderline Positive Cases

Specimens with amplification at 38 to 41 CN on the Alinity assay often have poor amplification characteristics and must be repeated to determine whether the sample was a true positive or a false positive. Many times, however, the repeat is positive but still weak, with a high CN value and poor amplification. To resolve this situation, an orthogonal, sensitive assay is required because the implications of a positive result can include quarantine, loss of ability to work, or delay in a medical procedure. Ten specimens with CN greater than 38 repeating weakly positive (CN > 38) were selected and performed on the

ddPCR assay. When run as a single specimen, only 50% (5 of 10) of the samples were positive, but repeating the specimens 2 additional times for a total of 3 runs led to detection of positivity in 90% of samples (9 of 10). The “3 positive droplet” criterion was applied additively across all 3 runs. Although the numbers of droplets were at low levels, this finding is consistent with low concentrations of SARS-CoV-2 RNA. Running samples in triplicate improved the sensitivity from 50% to 90%, which resolved our concern regarding repeat weak-positive Alinity samples. Now, we can observe these cases with low viral loads with little concern that a false positive will be present.

Discussion

This clinical validation of ddPCR for COVID-19 molecular testing demonstrates interesting potential artifacts, potentially high throughput, and increased sensitivity (50

copies/mL) compared with an approved and validated qPCR method (100 copies/mL). The sensitivity (97.9% PPA), specificity (100% NPA), precision (9%-13% CV), same-day turnaround time (8 hours for a 96-well plate), and relatively low cost (\$29.49 per sample for a full plate; reagent cost and tech time) make the platform appealing for use in pooled sample testing, where high throughput without sacrificing sensitivity is a priority. Overall, ddPCR results for N1 and N2 correlated well with each other and with the qPCR method when viral load was greater than 10 Ct cycles, but cases in which only N1 or N2 have high positive droplet counts warrant manual inspection for potential artifact.

Many of the advantages of ddPCR are derived from its use of end point quantification rather than quantification by real-time amplification used by qPCR.¹¹ This qualitative measurement for each droplet confers increased resistance to inhibitors,¹² reduced susceptibility to poor amplification efficiency,¹³ and compartmentalization of the individual droplets for a higher confidence of low positive results¹⁴ because of lower competition for reaction resources with higher-frequency targets.¹⁵

To ensure optimal detection, a minimum number of accepted droplets is required for analysis. Our assay requires that a sample have at least 6,000 accepted droplets to be interpreted as SARS-CoV-2 positive and at least 10,000 to be interpreted as SARS-CoV-2 negative. Samples that do not meet the criteria for SARS-CoV-2 detected with fewer than 10,000 droplets were called indeterminate and repeated. Most cases in routine clinical use require a single run in a single well. Detecting human RPP30 served as an internal control for intact RNA collection from a patient. A negative RPP30 can suggest several errors with the sample collection, reagents, or instrument. We noticed that RPP30 could be falsely negative when high-viral-load artifact was present. Positive N1 and/or N2 signals with negative RPP30 suggests low human cell number but still could be resulted as SARS-CoV-2 detected.

Droplet digital PCR technology has been applied widely to circulating tumor DNA detection, but a role in infectious disease and specifically viruses has not been as extensively explored. Examples of viruses that have been detected using ddPCR include hepatitis B virus (HBV), human immunodeficiency virus (HIV), influenza virus, human papillomavirus (HPV), and cytomegalovirus.^{11,16-21} This technique has been applied to measuring HBV DNA copy number,¹⁶ HIV RNA for viral load,¹⁹ high-sensitivity detection of influenza A virus, and HPV DNA in cytology and formalin-fixed samples.²² These studies demonstrated increased sensitivity and specificity vs other analytical methods, such as qPCR and serologic testing.¹⁶

Multiple studies have shown that ddPCR testing has improved sensitivity and specificity compared with RT-qPCR for the diagnosis of SARS-CoV-2 infection, especially in specimens with low viral load.²³⁻²⁷ In particular, some of the negative RT-PCR samples that were identified as positive using ddPCR assay were confirmed by RT-PCR in follow-up tests.²⁶ It has been reported that ddPCR can also be used to quantitatively monitor a patient's viral load, evaluate disease progression,²⁷ and directly detect SARS-CoV-2 in crude lysate.^{9,28} The limits of detection of ddPCR SARS-CoV-2 assays were reported to be between 1.8 and 2.9 copies per reaction,^{26,29,30} slightly lower than the data reported here.

As ddPCR can detect low levels of SARS-CoV-2 with good sensitivity and specificity, it could potentially be used as a reflex test for equivocally positive RT-PCR results. The clinical significance of low levels of SARS-CoV-2 is still controversial, however. Persistent, asymptomatic SARS-CoV-2 nucleic acid is present in a significant percentage of patients with COVID-19,³¹ which can delay discharge from the hospital without strong evidence that they are still infectious. Wölfel et al reported that live virus could not be isolated from samples collected after day 8 of symptom onset in patients with COVID-19 despite ongoing high viral RNA load.³² Another study by Bullard et al reported that infectious virus was detected only on respiratory specimens with a high concentration of viral RNA (RT-PCR positive at CT < 24).³³ Therefore, it is important to incorporate the ddPCR test results into the clinical contexts of these patients.

Thus, the usefulness of this assay may be best suited to pooled sample testing, where high sensitivity allows for significant pooling. Twenty samples of pooled RNA in this platform would have a theoretical lower limit of detection of ~1,000 copies/mL, sufficient to reliably detect a single positive patient. In SARS-CoV-2 assays, 500 to 1,000 copies/mL is a common benchmark for sensitivity.^{10,34} False negatives are a concern for pooled specimen testing, but studies^{35,36} have found that most borderline positive results occur in convalescent patients after 10 to 14 days of symptoms, when infectiousness is thought to be low, because viable virus could not be cultured.^{37,38} This finding is important for testing large numbers of asymptomatic patients, where a low positivity rate would be expected. Positivity rates would need to be adjusted with pooling numbers to balance the number of retests required.^{39,40}

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Disclosure: J.A.S. owns stock in Myriad Genetics.

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