APPLICATION NOTE

COMBINATI

Multiplexed microfluidic array partitioning (MAP) based digital PCR testing of qPCR positive, negative and inconclusive COVID-19 samples using the Combinati Absolute Q™

Background

In response to the global outbreak of coronavirus disease 2019 (COVID-19) there is a high demand for sensitive, accurate and consistent tests. Although RT-qPCR has served as the standard of care diagnostic test for the detection of SARS-CoV-2 infection, RT-dPCR (reverse transcription digital PCR) has recently been shown to outperform the traditional method in terms of sensitivity and accuracy.[1,2]

False negative and questionable negative rates using the current screening methodologies (RT-qPCR) have varied over the course of the pandemic and have been reported to be as high as 20%.[3,4] Because of this, asymptomatic patients are at an elevated risk of unknowingly spreading the disease. In addition to the need for more sensitive screening methods, a technology enabling higher accuracy will be critical for screening in determining more accurate rates of re-infection.

A highly sensitive, orthogonal test method to help resolve questionable negatives will increase overall testing accuracy and may also help reduce community transmission. The Combinati Absolute Q with its industry leading accuracy is ideally suited for the disambiguation of questionable negative test outcomes.

The goal of this study was to compare the results obtained using the CDC RT-qPCR assay with a dPCR test on a series of clinical samples. In collaboration with USC Clinical Laboratories, Molecular Pathology at University of Southern California, nucleic acids extracted from 19 clinical specimens from individuals who tested negative or were diagnosed with COVID-19 were tested on the Combinati Absolute Q dPCR Platform using the |Q|™ SARS-CoV-2 Triplex Assay.

Workflow Features:

- Single instrument and single consumable for 90 minute detection of SARS-CoV-2 Viral RNA targets
- |Q| SARS-CoV-2 Triplex assay combines N1, N2 and human RnaseP targets into a single reaction
- Microfluidic array partitioning technology enables 5% dead volume
- Limit of detection (LoD) of 200 copies per mL

The Absolute Q Digital PCR platform is a partition array-based digital PCR platform that leverages a single instrument and single consumable to perform all steps necessary for digital PCR (dPCR). The Combinati platform offers advantages over existing digital PCR platforms in terms of partitioning consistency, low sample dead volume, ease of workflow, and minimum hands-on steps. The MAP consumable relies on a fixed, injection-molded microchamber array that defines both the precise volume and total number of the picoliter-scale partitions. The instrument supports absolute quantification in four different optical channels, three of which are utilized for the |Q| SARS-CoV-2 Triplex Assay.

The Combinati |Q| SARS-CoV-2 Triplex Assay is a single tube solution for SARS-CoV-2 identification with a limit of detection of 200cp/mL in pre-extraction samples. Pairing the assay with the true 1-step RT-dPCR workflow of the Absolute Q dPCR platform enables integration of sample digitization, reverse transcription, PCR and data collection into a single instrument and can be completed in approximately 80 minutes.

Materials and Methods:

Sample Preparation

Nasopharyngeal samples from 19 patients were collected and nucleic acid was extracted using the QiaCube on the same day. All samples were tested using the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. Out of the 19 samples, 7 were RT-qPCR positive, 7 were RT-qPCR negative and 5 were RT-qPCR inconclusive. RT-qPCR inconclusive is defined by CDC guidelines as a sample which generates a signal for either the N1 or N2 targets with a Ct value under 40, but not both.

Digital PCR Reagent Preparation

Reagents for the |Q| SARS-CoV-2 Triplex Assay were prepared according to Table 1. For each sample, the maximum volume of extracted nucleic acid was added to each reaction. After reagent preparation, 9µL of the prepared PCR mix was loaded into a well of the MAP16 dPCR plate followed by an overlay of 12µL of isolation buffer. Each sample was run in duplicate. For each MAP16 plate run, a positive and a negative control were run alongside the RNA samples.

Reagent	Volume per reaction
Combinati 4X 1-step MM	2.25 µL
|Q| SARS-CoV-2 Triplex Assay (10X)	0.9 µL
Patient RNA Sample	5.85 µL

Table 1. PCR mix preparation table for |Q| SARS-CoV-2 Triplex Assay on the Absolute Q. A final volume of 9µL of prepared PCR mix is loaded into a single well of the MAP16 consumable.

Using the Combinati Absolute Q Control Software, the data was analyzed and concentrations of each target were reported in copies per reaction. All digital PCR plots were generated using the Absolute Q Control Software.

Results Interpretation

A positive control (Exact Diagnostics SARS-CoV-2 Standard) and no template controls were run with each MAP16 plate run. The NTC reactions for the probe mix must not exhibit positive signals in any channel (FAM, HEX or Cy5) for any of the three targets tested, N1, N2 or RP. The positive control must exhibit positive signals in all channels (FAM, HEX or Cy5) for the three targets tested. After the positive and negative controls were examined, they were determined to be valid and acceptable. Given that all controls perform as expected, each target was assessed for the presence of the intended target. If 2 or more positive partitions were detected, that target was considered positive. In alignment with the CDC EUA recommendations, COVID-19 positive samples must have positive signals in both the N1 and N2 targets. If 2 or more partitions are detected in either N1 or N2, but not both, the sample is considered inconclusive.

Results:

Absolute Q dPCR enables absolute quantification without the need of a standard curve.

Fourteen of the samples included in this study were called definitively positive or negative for SARS-CoV-2 targets by RT-qPCR. Using the Absolute Q dPCR platform and kit, 13 out of 14 were in agreement between the two platforms (Table 2).

	qPCR positive	qPCR negative
dPCR positive	7	1
dPCR negative	0	6

Table 2. Results of positive and negative call associations between original CDC EUA qPCR and |Q| SARS-CoV-2 Triplex Assay dPCR.

COMBINATI
info@combinati.com | www.combinati.com

Figure 1 shows examples of dPCR results obtained from a sample which tested positive by dPCR (left) and a sample which tested negative (below).

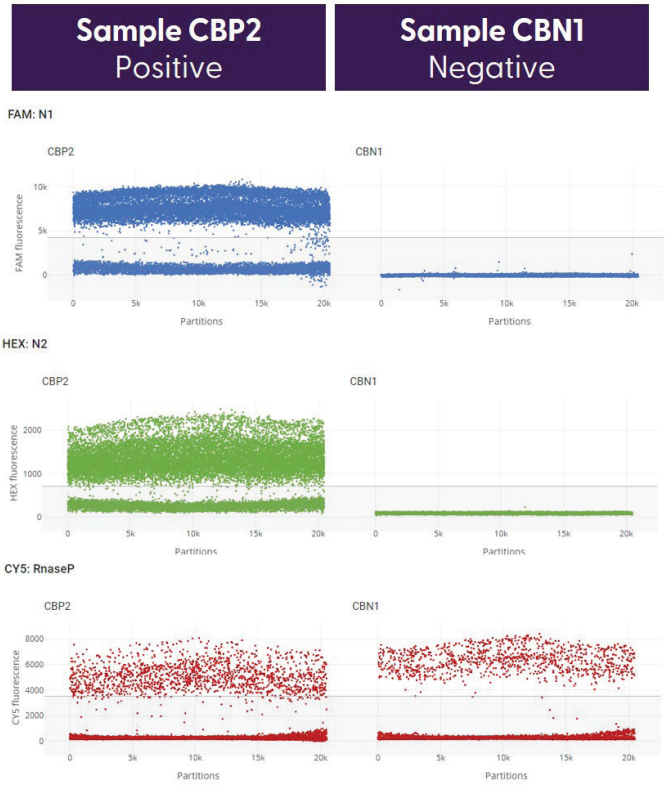

Figure 1: Digital PCR plots from a positive (left) and negative (right) SARS-CoV-2 clinical sample for the triplex assay targets N1 (blue), N2 (green) and Human RnaseP (red).

For all positive samples, the reported Ct values were inversely correlated with the absolute quantification (R2 N1: 0.63 p=0.03 and, R2 N2: 0.79 p = 0.01) of the SARS-CoV-2 targets identified through dPCR (Figure 2).

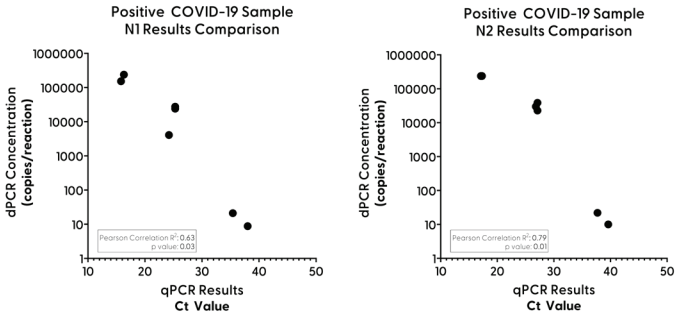

Figure 2. Digital PCR enables absolute quantification of target molecules without a required standard curve. The correlation is shown between the concentration of each target A) N1 and B) N2 using the Absolute Q dPCR platform and the Ct values identified using the CDC EUA N1 and N2 qPCR simplex assays.

Identification of qPCR false negative

Sample CBN3 was collected from a symptomatic patient and tested negative by RT-qPCR with CT values for N1 and N2 of undetected and 42.5 respectively. However, the same sample tested positive by dPCR with 2.3 and 2.9 copies per reaction for N1 and N2 respectively (Figure 3).

The human Rnase P gene was detected across both platforms (qPCR: Ct 30.5 and Absolute Q dPCR: 850 cp/reaction).

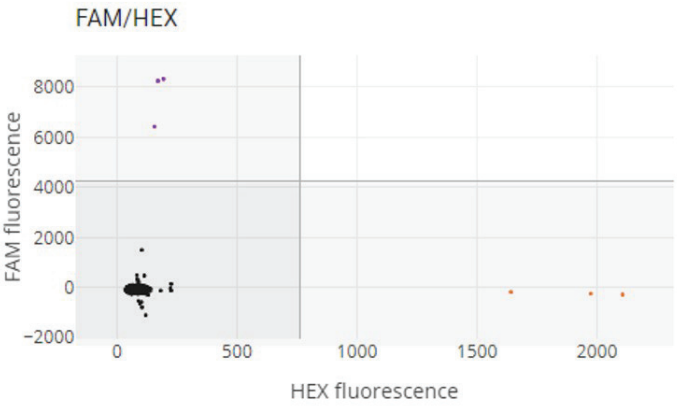

Figure 3. Digital PCR results showing positive partitions which contain the N1 (FAM) and N2 (HEX) SARS-CoV-2 targets for sample CBN3 which was originally identified as SARS-CoV-2 negative by qPCR.

info@combinati.com | www.combinati.com

Resolution of Inconclusive Samples

Five of the clinical RNA samples which were collected from symptomatic individuals generated inconclusive SARS-CoV-2 results using the CDC EUA qPCR test. For all 5 samples, N1 was the only target detected via qPCR with Ct values ranging from 36.8 to 40. Only sample CB15-p generated detectable signal from N2, but the Ct of 41 is past the positivity criteria of Ct 40. When tested on the Absolute Q, 4 out of the 5 inconclusive cases were resolved. Samples CBI1-n and CBI2-n were deemed negative by the Absolute Q, showing no positive partitions for either N1 or N2 across both replicates. Samples CBI3-n and CBI5-p were positive for SARS-CoV-2 targets on the Absolute Q for both N1 and N2 targets (Table 4). Digital PCR results from CBI4-p were consistent with the qPCR results with a concentration of 3.5 copies per reaction of the N1 target and no positive partitions from the N2 target. RnaseP was detected in all five samples across both testing platforms.

	Absolute Q		qPCR	
Sample	**N1**	**N2**	**N1**	**N2**
CBI1-n	ND	ND	38.34	ND
CBI2-n	ND	ND	38.7	ND
CBI3-n	30.5 (±0.5)	27.5 (±0.4)	40	ND
CBI4-n	3.5 (±0.2)	ND	36.8	ND
CBI5-n	14.1 (±0)	10.5 (±0)	37.7	41

Table 4. Comparison of Absolute Q dPCR and qPCR testing results from 5 inconclusive SARS-CoV-2 samples. Absolute Q quantitative results are reported in average copies per dPCR reaction (±STDEV) across 2 technical replicates. The qPCR Ct values are those reported from the CDC qPCR test. ND indicates the signal was not detected from that target.

Summary:

In response to the global outbreak of COVID-19, digital PCR (dPCR) has been recognized as an exceptional tool for detection of SARS-CoV-2 viral targets. By enabling absolute quantification of the total number of target molecules in a sample, detection of extremely rare viral RNA molecules which may otherwise be missed in a bulk qPCR reaction is possible.

In this study, we found complete agreement between all qPCR positive samples, and 6 out of 7 qPCR negative samples. The single discordant sample which was identified as positive by dPCR produced a Ct above the threshold allowed on qPCR and was subsequently deemed negative. Additionally, statistically significant correlation between RT-qPCR Ct values and RT-dPCR absolute quantification values were shown. The |Q| SARS-CoV-2 Triplex assay, detection and quantification of both viral targets were obtained using a single dPCR reaction without the need for a standard curve.

In addition, the Absolute Q was used to resolve 4 out of 5 inconclusive samples. Two of these samples were called positive by dPCR and two were called negative. The single remaining inconclusive sample demonstrated consistent signal of N1 and absence of N2 across both testing platforms.

The Absolute Q dPCR platform has unparalleled consistency in partition generation and the lowest sample dead volume of all dPCR platforms. These features enable more sample volume to be analyzed with each reaction to achieve higher sensitivity and better accuracy for rare target detection. Combined with minimal hands-on time and 4-color multiplexity, the Absolute Q allows digital PCR to be smoothly integrated into any lab workflow.

References

Suo T, Liu X, Guo M, et al. ddPCR: a more sensitive and accurate tool for SARS-CoV-2 detection in low viral load specimens. medRxiv. March 2020:2020.02.29.20029439. doi:10.1101/2020.02.29.20029439

Dong L, Zhou J, Niu C, et al. Highly accurate and sensitive diagnostic detection of SARS-CoV-2 by digital PCR. medRxiv. March 2020:2020.03.14.20036129. doi:10.1101/2020.03.14.20036129

Woloshin, Steven, et al. "False Negative Tests for SARS-CoV-2 Infection - Challenges and Implications: NEJM." New England Journal of Medicine, 22 May 2020, www.nejm.org/doi/full/10.1056/NEJMp2015897.

Yu F, et al., "Quantitative Detection and Viral Load Analysis of SARS-CoV-2in Infected Patients", Clin Infect Dis, 2020

COMBINATI
info@combinati.com | www.combinati.com