Application of Droplet Digital PCR for SARS-CoV-2: Extraction-Free Process for Environmental Swabs

Raymond Abayan
Bio-Rad Laboratories, Inc., Digital Biology Center, 5731 W Las Positas Blvd, Pleasanton, CA 94588

Droplet Digital PCR

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Abstract

This application note will discuss combining the Bio-Rad SARS-CoV-2 Droplet Digital PCR (ddPCR) Kit with an extraction-free process for the testing of surface swab samples. Previously, the SARS-CoV-2 ddPCR Kit has been validated for use with the QIAamp Viral RNA Mini Kit (QIAGEN, catalog #52906) and the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific Inc., catalog #A42352). The QIAGEN extraction method is a silica column and vacuum-based method, allowing for 24 samples to be extracted at a time. The Thermo Fisher Scientific extraction method is based on paramagnetic beads and up to 96 samples can be processed at a time.

In situations where there are large numbers of samples or when there is a shortage of these extraction kits, requiring RNA extraction may slow turnaround times from sample preparation to results. Here we show a method and the resulting data of RNA sample preparation that skips extraction (extraction-free) to help improve turnaround times for processing large numbers of samples and avoid any delay due to a shortage of commercially available kits.

Initially, we determined the most effective protocol for extraction-free sample processing by comparing three methods. Subsequently, we improved the most effective process to work with our ddPCR chemistry and determined the lowest concentration that the SARS-CoV-2 ddPCR Kit could achieve with this method. We also compared the optimized method with a QIAGEN extraction run in parallel to illustrate the efficiency of the extraction-free method. Lastly, we calculated results for various input sample volumes in ddPCR reactions. Ultimately, differences in operators and input samples may lead to different results, but the goal of this study was to determine the effectiveness of applying this extraction-free method to environmental swab samples where high sample loads are common and necessary.

Introduction

Due to the instability and varying concentrations of viral RNA in environmental samples, most extraction-free methods in the past have been unsuccessful in isolating and maintaining RNA for downstream analysis with PCR. However, while extraction-free methods have improved recently, they rely on lysing the viral capsid with Proteinase K and cleaning up the sample in one or two steps, which still requires multiple pipetting steps. The method described here is based on Proteinase K lysis but requires no cleanup step and is optimized for use with Droplet Digital PCR for the detection of SARS-CoV-2.

BIO-RAD

The Bio-Rad SARS-CoV-2 ddPCR Kit is a reverse transcription (RT) ddPCR test designed to detect RNA from SARS-CoV-2. The oligonucleotide primer and probe sequences for detection of SARS-CoV-2 are the same as those reported by the Centers for Disease Control and Prevention (CDC) and were selected from regions of the viral nucleocapsid (*N*) gene. The panel includes two primer/probe sets designed for specific detection of the 2019-nCoV and an internal control primer/probe set to detect the human RNase P gene (*RP*) in control samples and clinical specimens. The Bio-Rad SARS-CoV-2 ddPCR Kit includes these three sets of primers/probes as a single multiplex assay to enable a one-well reaction.

Materials and Methods

Extraction-Free Protocol

A heat-inactivated 2019 novel coronavirus stock sample (American Type Culture Collection [ATCC], catalog #VR-1986HK) was used and was estimated to have a concentration of ~390,000 copies/µl postextraction with the QIAGEN QIAamp Kit and quantification by Droplet Digital PCR.

The extraction-free method described by Marzinotto et al. (2020; only preprint available, not peer reviewed) was tested. Here we followed the guidance of the protocol in the paper using Proteinase K from *Tritirachium album* (Sigma-Aldrich, Inc., P2308-10MG) resuspended in Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium and without phenol red (Sigma-Aldrich, 55037C) for a final solution concentration of 30 mg/ml. Of this 30 mg/ml Proteinase K in HBSS, 10 µl was added to 100 µl of broth sample (mixed by pipet) in a ddPCR 96-Well Plate (Bio-Rad Laboratories, Inc., catalog #12001925) and sealed with a removable foil seal (Bio-Rad, #MSF1001).

The plate was kept on ice or on a 4ºC plate holder while working with the Proteinase K solution to protect the RNA from degradation at room temperature. The plate was then heated in a C1000 Touch Thermal Cycler (Bio-Rad, #1851197) with the protocol described in Table 1.

Table 1. Extraction-free thermal cycling protocol.

Cycling Step	Temperature, °C	Time, min	Number of Cycles
1	55	15	1
2	98	5	1
3	4	2	1
4	4	Infinite	Optional

The plate should be held at 4ºC for at least 2 minutes, but can be held until the ddPCR Plate is ready. Briefly centrifuge the plate to bring any solution off the sides of the well or the foil seal before moving to the SARS-CoV-2 ddPCR reaction setup.

The sampling medium tested was the PUR-Blue Swab Sampler in High Capacity (HiCap) Neutralizing Broth (World Bioproducts LLC, BLU-1HC-S). Broth from the swab collection tubes was spiked with a 1:1,000 dilution of the SARS-CoV-2 viral stock for an estimated neat concentration of 390 copies/µl. This was then serially diluted 1:10, 1:20, 1:40, and 1:100 in DNA LoBind 1.5 ml Tubes (Eppendorf AG, catalog #022431021) with HiCap Broth to achieve a final concentration of 39, 19.5, 9.75, and 3.9 copies/µl, respectively.

The same viral concentrations were tested with nuclease-free water (NFW) in parallel as an extraction-free process control. The Exact Diagnostics SARS-CoV-2 Positive Standard diluted 1:1 in HiCap Broth was also included as an additional positive process control. To maintain droplet stability with concentrated broth samples, preliminary studies found that a broth dilution of 1:20 in NFW was optimal before the addition of Proteinase K (Figure 1).

SARS-CoV-2 ddPCR Protocol

A positive ddPCR control was prepared by diluting the Exact Diagnostics SARS-CoV-2 Positive Standard 1:10 in 100 µl of NFW then combining with master mix in a separate well. QIAGEN extracted viral RNA and extraction-free viral samples were run in the sample plate in parallel with the Exact Diagnostics SARS-CoV-2 Positive Standard 1:10 dilution as a positive control. NFW was used as the no template control (NTC) in each ddPCR Plate.

During the first optimization runs, a volume of 5.5 µl of each sample was added to the master mix composed of 1.1 µl 2019-nCoV CDC ddPCR Triplex Assay, 2.2 µl reverse transcriptase, 5.5 µl supermix, 1.1 µl dithiothreitol, and 6.6 µl NFW. Once the extraction-free process was defined, different input samples of 5.5, 7.7, 9.9, and 12.1 µl were added to ddPCR reactions. For these inputs, the NFW volume must be adjusted to maintain a total reaction volume of 22 µl. It is recommended that while preparing the master mix, all reagents should be kept on ice or a 4ºC cold block before adding to the plate.

Twenty-two microliters (22 µl) from the sample and master mix RT-ddPCR mixtures were loaded into the wells of a ddPCR 96-Well Plate. The mixtures were then fractionated into up to 20,000 nanoliter-sized droplets in the form of a water-in-oil emulsion in the Automated Droplet Generator (Bio-Rad, #1864101) with Automated Droplet Generation Oil for Probes (Bio-Rad, #1864110). The ddPCR 96-Well Plate containing droplets was sealed with foil using a plate sealer and thermal cycled in a C1000 Touch Thermal Cycler (Bio-Rad, #1851197) to achieve reverse transcription of RNA followed by PCR amplification of cDNA, as described in Table 2.

Table 2. SARS-CoV-2 ddPCR thermal cycling protocol.

Cycling Step	Temperature, °C	Time	Number of Cycles
Reverse transcription	50	60 min	1
PCR enzyme activation	95	10 min	1
Template denaturation	94	30 sec	40
Annealing/extension	55	60 sec	
Droplet stabilization	4	30 min	1
Hold (optional)	4	Overnight	1

Following PCR, the plate was loaded into the QX200 Droplet Reader (Bio-Rad, #1864003), and the droplets in each well were singulated and flowed past a two-color fluorescence detector. The fluorescence intensity of each droplet was measured in FAM and HEX channels, and droplets were determined to be positive or negative for each target in the Bio-Rad SARS-CoV-2 ddPCR Kit: *N1*, *N2*, and *RP*. The fluorescence data were then analyzed by QuantaSoft 1.7 and QuantaSoft Analysis Pro 1.0 Software to determine the presence of SARS-CoV-2 *N1* and *N2* in the specimen.

Results

Droplet Count Optimization

In the samples that were extracted with QIAGEN the HiCap Broth did not have any effect on the ddPCR reaction (Figure 1) since the extraction results in purified RNA eluted in water. However, following the Marzinotto method with concentrated broth, we encountered lower droplet counts going into droplet generation and this ultimately impacted the ddPCR reaction. Although the composition of the broth is proprietary, it is likely to include components that are known to inhibit droplet formation, such as ethanol, chlorine compounds, and/or peptones. Additionally, we observed that the broth had an effect in the FAM and HEX signal amplitudes, creating an undesired shift in the clusters.

In order to determine the effect of the broth mixture on the droplet chemistry and assay performance, we compared different levels of broth dilutions in NFW in a total volume of 100 µl of input sample. Both spiked broth and spiked NFW samples were processed in duplicate with the Marzinotto method, and ran in triplicate ddPCR wells. Figure 2 shows ddPCR data on the effects of the HiCap Broth on droplet counts compared to the control NFW with the dilutions mentioned previously, confirming that diluting the broth improves droplet formation and returns the assay to the correct channel amplitudes for each target. Figure 2 shows the 2-D amplitude plot of the 1:20 broth dilution compared to the virus spiked in NFW control, in which the clusters for both wells overlap as expected.

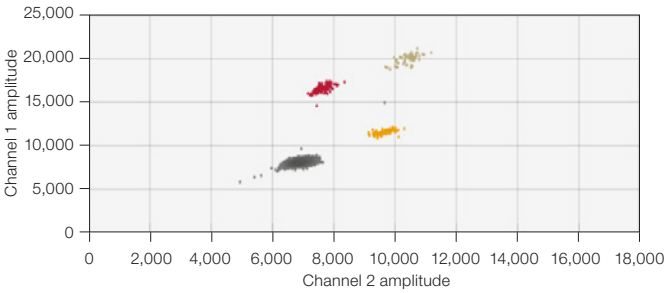

Fig. 1. 2-D amplitude plot showing two ddPCR wells, one with the virus spiked in diluted broth (1:20 broth/NFW) compared to one with virus spiked only in NFW. The clusters of both conditions overlap and show the amplitude for each target in FAM and HEX as expected.

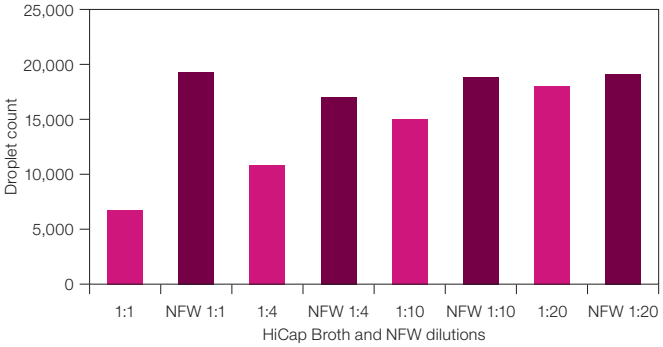

Fig. 2. Effect of HiCap Broth on number of accepted changes. Accepted event averages of triplicate ddPCR reactions at each dilution were compared to a process replicate diluted only in NFW. The 1:20 dilution of the broth in NFW shows equivalent droplet counts compared to the NFW controls. (■), dilution in Hi-Cap Broth; (■), dilution in NFW.

Method Performance

As described earlier, the contrived samples were tested by spiking inactivated RNA virus to create the following dilutions: 39, 19.5, 9.75, and 3.9 copies/µl of the starting sample to determine the lowest concentration that the extraction-free method could determine reliably.

The virus-spiked dilutions were processed using the extraction-free method (at a 1:20 dilution of broth to NFW). They were run side by side on the same ddPCR Plate with QIAGEN extracted samples serving as a control.

Additional process controls included duplicate virus spiked in NFW and QIAGEN extracted 1:10 Exact Diagnostics SARS-CoV-2 Positive Standard for the extraction-free and QIAGEN extraction methods, respectively. The SARS-CoV-2 ddPCR reaction was performed as described previously.

The 2-D amplitude plots and concentrations of the extraction-free and QIAGEN extracted samples were compared, as shown in Figure 3 and Table 3.

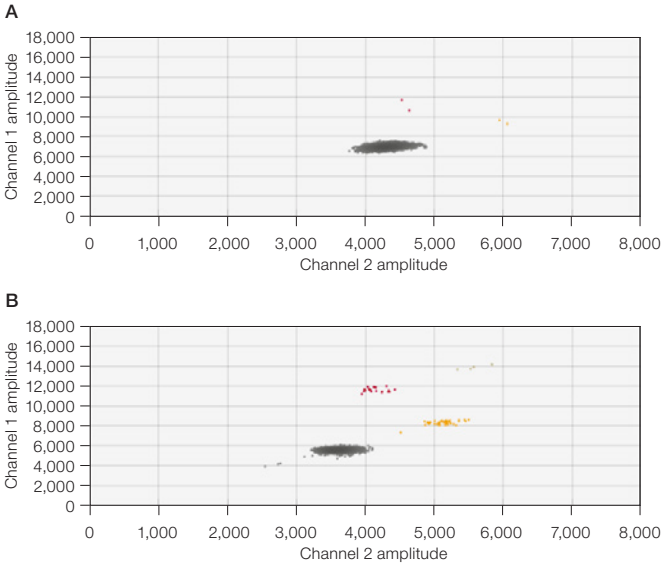

Fig. 3. Extraction-free and QIAGEN 2-D plots of sample dilutions containing 3.9 copies/µl. A, the lowest viral concentration detected with the extraction-free method was 3.9 copies/µl; **B**, QIAGEN extracted sample at the same concentration. The human *RP* target was not present since the inactivated virus does not contain a human background.

Table 3. Comparison of ddPCR concentrations for the extraction-free vs. QIAGEN extracted samples (n = 6 process replicates per concentration for each method).

Sample Concentration, copies/µl	Method	*N1*,		*N2*,	
		copies/µl	copies/ 20 µl reaction	copies/µl	copies/ 20 µl reaction
39	Extraction-free	0.57	11.30	0.72	14.34
	QIAGEN extracted	25.81	516.28	28.27	565.47
19.5	Extraction-free	0.30	5.95	0.22	4.35
	QIAGEN extracted	12.98	259.58	14.77	295.31
9.75	Extraction-free	0.11	2.26	0.11	2.29
	QIAGEN extracted	6.17	123.37	6.89	137.77
3.9	Extraction-free	0.07	1.39	0.07	1.40
	QIAGEN extracted	2.60	52.02	3.20	63.96

The validation data for the SARS-CoV-2 ddPCR Kit show that the limit of detection using the QIAGEN QIAamp Kit is 0.33 copies/µl for the *N1* target and 0.35 copies/µl for the *N2* target when used with nasopharyngeal swabs. Previous experiments with environmental samples in HiCap Broth demonstrate similar concentration levels (data not shown). So the lowest concentration tested in this study, 3.9 copies/µl, is well above the limit of detection for the test when the RNA is extracted from the sample.

For the extraction-free method, the lowest concentration at which both viral targets in this sample set, *N1* and *N2*, could be detected is 3.9 copies/µl. This ATCC sample has no human background, therefore the negative concentrations of *RP* achieved are as expected.

Recommended Sample Volume for the ddPCR Reaction

As shown previously, the assay has detected virus material at the 3.9 copies/µl level when using this extraction-free method. To confirm the reproducibility of the detection, a broth sample spiked with 3.9 copies/µl of ATCC material was processed with the Marzinotto method in 18 replicates followed by single ddPCR reactions. These 18 replicates were processed in Droplet Digital PCR with varying input sample volumes (5, 7, 9, and 11 µl) spiked into the ddPCR reaction. Exact Diagnostics SARS-CoV-2 Positive Standard diluted 1:1 in HiCap Broth was processed in parallel and used as a positive control for clustering these replicates. Samples of broth that were not spiked were also processed as a negative control for the process. The Exact Diagnostics SARS-CoV-2 Positive Standard diluted 1:10, as previously mentioned, was used as a ddPCR positive control, and NFW was used as a ddPCR negative control.

The 2-D amplitude plots and concentrations of the different input sample volumes in ddPCR reactions were compared as shown in Figure 4, Figures 5A–D, and Table 4.

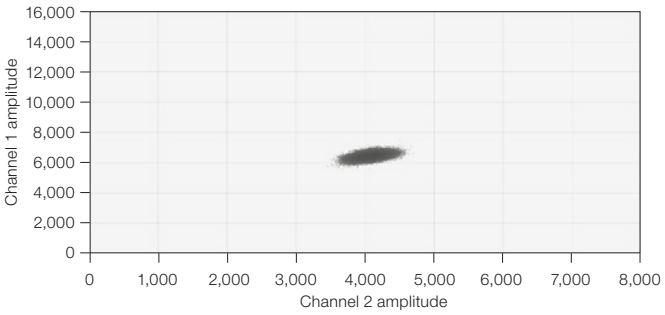

Fig.4. Negative process control (unspiked HiCap Broth, n = 8 process replicates). As expected, no positive droplets for any of the targets (*N1*, *N2*, and *RP*) were detected in the clean broth sample after processing with the Marzinotto method and Droplet Digital PCR.

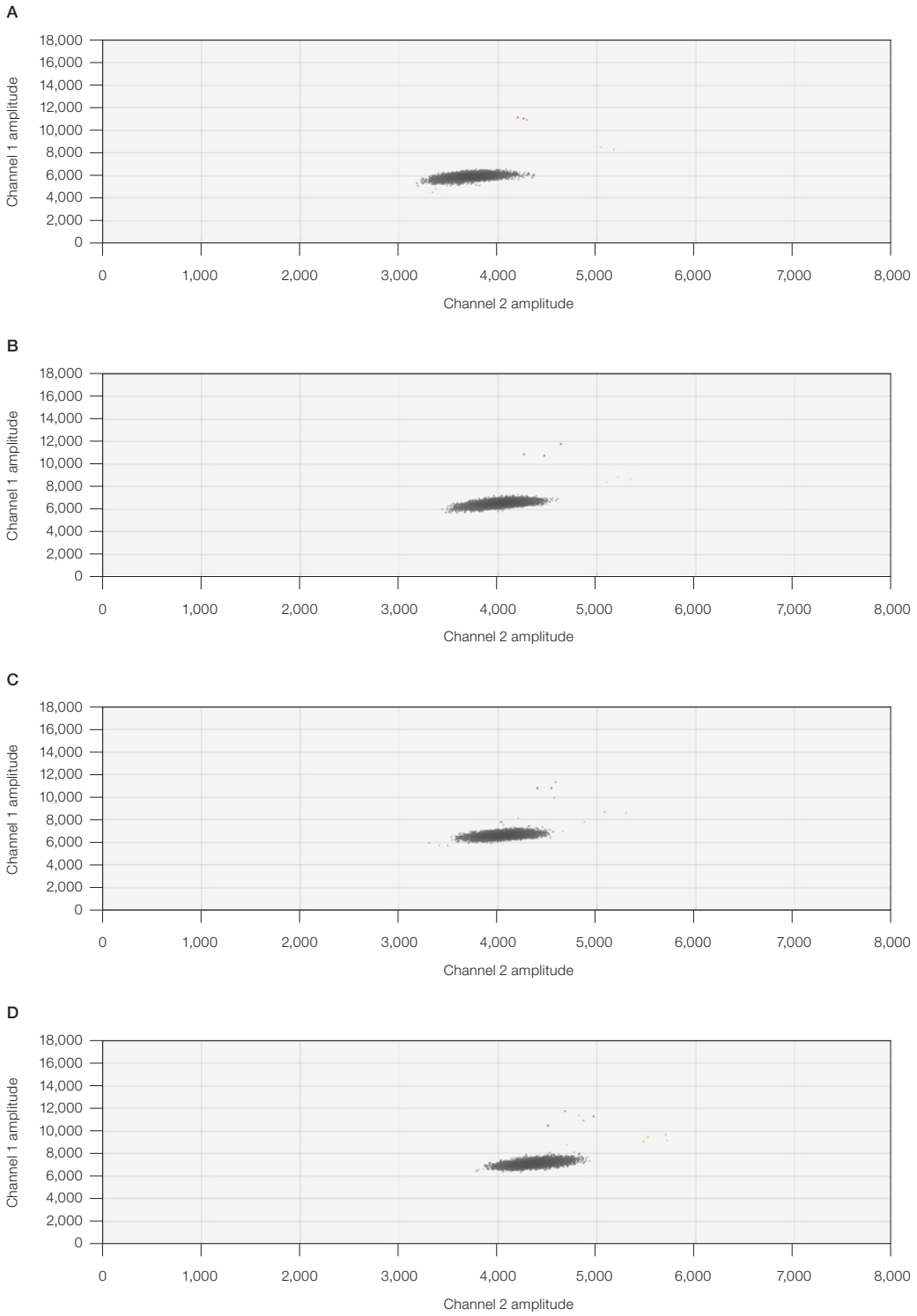

Fig. 5. 2-D plots of input sample variation in ddPCR reactions. Representative 2-D plots (2-well overlay) at the 3.9 copies/µl sample dilution level, which is the lowest concentration detected for the extraction-free method with **A**, 5; **B**, 7; **C**, 9; and **D**, 11 µl input sample volumes into Droplet Digital PCR.

Table 4. Average ddPCR concentrations at different input volumes (n = 18 sample replicates per input volume).

Input Sample, µl	*N1*, copies/20 µl reaction	*N2*, copies/20 µl reaction
5	0.8	1.2
7	1.0	1.6
9	1.8	2.0
11	2.6	2.6

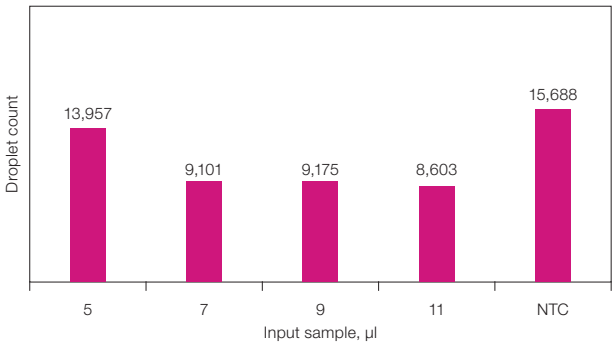

Fig. 6. Effect of input sample volume on droplet counts in Droplet Digital PCR. The NTC (n = 8 sample replicates) was used as reference for this ddPCR reaction (n = 20 sample replicates for all other input samples). (■), average of accepted droplets.

Adding more sample into the ddPCR reaction impacts droplet counts, as expected, due to more broth in the ddPCR reaction. This is shown in Figure 6. Despite lower accepted events, cluster separation for all but the 11 µl concentration was not significantly affected and, therefore, did not impede the test's ability to amplify the targets clearly.

From this, we recommend to use 9 µl input sample in ddPCR reactions (9.9 µl when including recommended overage) and 3.3 µl of NFW when preparing the master mix. All other components should be added as described previously.

Discussion

The primary focus of this study was to explore the feasibility of applying an extraction-free sample preparation method to the established SARS-CoV-2 ddPCR Kit to detect the virus on surfaces. The data shown were obtained using spiked environmental swabs as contrived samples. This serves as a guide for applying the method to a ddPCR workflow.

Since it is important to have extraction controls during RNA extraction, we recommend to use a control similar to the one used here by having a positive and a negative standard to spike clean broths with, and to run them along with the other samples. This method would allow an operator to process up to 92 samples with two process controls (positive and negative) and two ddPCR controls (positive and NTC) in one run.

As expected, when using an extraction-free method, some loss of sensitivity is a trade-off for the ease of the procedure. A 96-well plate can be processed in less than 30 minutes at minimal cost. For environmental samples, this trade-off may be acceptable given that they are not clinical specimens and it is not clear yet what viral concentration is pathogenic when found on surfaces.

If applying the methods and results discussed in this note to another sample collection method or broth type, it is important to first determine how much the broth needs to be cleaned or diluted before focusing on detection. It is also important to carry out an experiment for input volumes in ddPCR reactions similar to the one described here in order to determine the effective input for other sample mediums. The chemical composition of broths often do not interact positively with the chemicals used during droplet generation, which could lead to poor amplification and low droplet/event counts.

Conclusions

With RNA extraction sample preparation, it is evident that manual extractions are time-consuming and fatiguing to operators. Typical magnetic bead, spin-column, and vacuum purification methods are slow and require various different consumables, which could lead to more variability throughout the process. Variability can be overcome by a faster extraction-free method that produces similar results and requires fewer upstream consumables. There is also less variability because there are fewer repeated actions and less time spent processing samples. Here we present results for a previously reported extraction-free method, optimized specifically for environmental samples collected in broth media with the SARS-CoV-2 ddPCR Kit.

Reference

Marzinotto S et al. (2020). A streamlined approach to rapidly detect SARS-CoV-2 infection, avoiding RNA purification. medRxiv 2020.04.06.20054114 [not peer-reviewed].

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