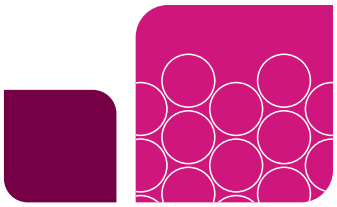
Application of Droplet Digital PCR for SARS-CoV-2: Extraction-Free Process for Nasopharyngeal Swab Specimens

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



Droplet Digital PCR

Bulletin 7390

Abstract

This application note discusses the use of the Bio-Rad SARS-CoV-2 Droplet Digital PCR (ddPCR) Kit with an extraction-free process for the testing of nasopharyngeal swab specimens. 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 one 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, when there is a shortage of these extraction kits, or when laboratory infrastructure is limited, requiring RNA extraction may slow turnaround times from sample preparation to results. Here we demonstrate a method of RNA sample preparation to improve turnaround times when processing large numbers of samples by skipping extraction (extraction-free), avoids any shortages with commercially available kits, and ultimately simplifies workflows for high-volume sample processing.

Initially, we determined the most effective protocol for extraction-free sample processing by comparing two methods. Subsequently, we improved the most effective process to work with our ddPCR chemistry and determined the limit of detection (LOD) that the SARS-CoV-2 ddPCR Kit could achieve with this method. We compared the results and linearity of the optimized method with QIAGEN extractions to illustrate the method's efficiency at two different input sample volumes into Droplet Digital PCR. Lastly, we presented data for different Proteinase K solutions to show flexibility of the method for situations where required supplies are not readily available. Ultimately, the goal of this study was to determine the effectiveness of applying this extraction-free method to nasopharyngeal swab specimens where high sample loads are common, subjects are at high risk, commercially available extraction kits may be in short supply, and fast turnaround times are necessary.

Introduction

Due to the instability of viral RNA, earlier extraction-free methods have been unsuccessful in isolating and maintaining RNA for PCR. In the past, extraction-free methods have focused on lysing the viral capsid with Proteinase K and cleaning up the sample in one or two steps. Any remaining inhibitors from the original sample will have a detrimental effect on downstream PCR applications. However, Droplet Digital PCR relies on endpoint amplification and, as such, it is less negatively impacted by inhibitors remaining in the sample.

BIO-RAD

The Bio-Rad SARS-CoV-2 ddPCR Kit is a reverse transcription (RT) ddPCR test designed to detect RNA in 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 (two primer/probe sets) is designed for specific detection of the 2019-nCoV. An additional primer/probe set to detect the human RNase P gene (*RP*) in control samples and clinical specimens is also included in the panel as an internal control. 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

The sampling medium tested was Universal Transport Medium (UTM) (Copan Diagnostics, Inc., catalog #305C). Positive samples were contrived using heat-inactivated 2019 novel coronavirus stock sample (American Type Culture Collection [ATCC], catalog #VR-1986HK). The stock sample was estimated to have a concentration of ~3.9 x 10^8 copies/ml following extraction with the QIAGEN QIAamp Kit and quantification by Droplet Digital PCR. UTM from the swab collection tubes was spiked with a 1:10^3 dilution of the ATCC SARS-CoV-2 viral stock for a neat concentration of 3.9 x 10^5 copies/ml. This was then serially diluted in DNA LoBind 1.5 ml Tubes (Eppendorf AG, catalog #022431021) with UTM to achieve final concentrations of 4.0, 3.0, and 2.0 x 10^3 copies/ml for an LOD study.

ATCC diluted 1:4 in phosphate buffered saline (PBS) was in parallel as an extraction-free positive process control, and a pool of confirmed negative specimens collected in UTM was used as a negative process control. The Exact Diagnostics SARS-CoV-2 Positive Standard diluted 1:10 in UTM was also included as an additional positive process control.

The extraction-free method by Marzinotto et al. (2020; not peer reviewed) and described previously in Bio-Rad bulletin 7377 was tested, following the protocol using Proteinase K from *Tritirachium album* (Sigma-Aldrich, Inc., P2308-10MG). Proteinase K was 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 media 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).

Keep the plate 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 with 96–Deep Well Reaction Module (Bio-Rad, #1851197) according to the protocol 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 or until the ddPCR reaction plate is ready. The plate was briefly centrifuged to collect any solution off the sides of the well or the foil seal before moving to the SARS-CoV-2 ddPCR reaction setup.

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 nuclease-free water (NFW) then combining with master mix in a separate well. Extraction-free viral samples were run in the sample plate with the Exact Diagnostics SARS-CoV-2 Positive Standard 1:10 dilution as a positive process 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 nuclease-free water. See Table 2.

Table 2. ddPCR master mix reaction components.

Component	Volume, µl	Final Concentration
Supermix	5.5	1x
Reverse transcriptase	2.2	20 U/µl
Dithiothreitol	1.1	15 mM
2019-nCoV CDC ddPCR Triplex Assay	1.1	1x
RNA sample	5.5	100 fg–100 ng per reaction
Nuclease-free water	6.6	–
Total reaction volume	**22**	**–**

Once the extraction-free process was defined, different input samples of 7.7 and 9.9 µl were added to ddPCR reactions. For these inputs, the NFW volume must be adjusted to maintain a total reaction volume of 22 µl. While preparing the master mix, keep all reagents on ice or a 4°C cold block before adding to the plate.

A 22 µl total reaction RT-ddPCR mixture was 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 an 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. The process is described in Table 3.

Table 3. 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 thermal cycling, the plate was loaded into the QX200 Droplet Reader (Bio-Rad, #1864003) and the droplets in each well were run through a microfluidic channel, single file, 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 QX Manager Software Standard Edition, Version 1.1 to determine the presence of SARS-CoV-2 *N1* and *N2* in the specimen.

Results

Method Performance

As described earlier, the contrived samples were created by spiking inactivated virus at the following dilutions: 4.0, 3.0, and 2.0 x 10^3 copies/ml. These dilutions of the starting sample were used to determine the LOD that this method could achieve. Twenty process replicates and ten negative sample replicates were tested with Droplet Digital PCR.

The virus-spiked dilutions were processed using the extraction-free method with an unspiked UTM media sample as a negative control. A positive process control of Exact Diagnostics SARS-CoV-2 Positive Standard spiked in PBS at 2.0 x 10^4 copies/ml was used. The SARS-CoV-2 ddPCR reaction was performed as described previously with input samples of 7.7 and 9.9 µl into Droplet Digital PCR.

The comparison of concentrations (Figure 1) shows the method's performance at 4.0, 3.0, and 2.0 x 10^3 copies/ml of virus and also with negative UTM material. An example 2-D plot showing all possible clusters is included as a reference in Figure 2.

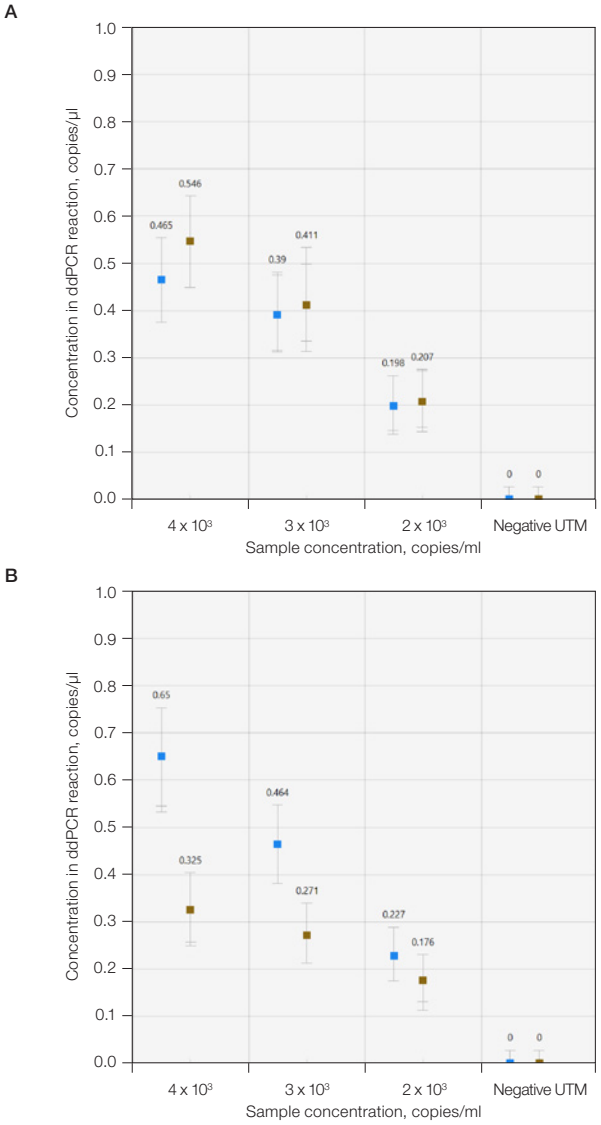


Fig. 1. Comparison of ddPCR concentrations. Input sample volumes into Droplet Digital PCR were **A**, 7.7 µl and **B**, 9.9 µl. The LOD was achieved at a concentration of 2.0 x 10^3 copies/ml. *N1* target (■); *N2* target (■). UTM, Universal Transport Medium.

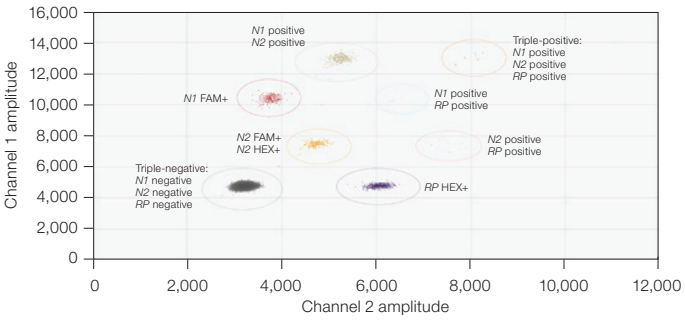


Fig. 2. Reference 2-D plot example describing the cluster locations and designations. There are eight possible cluster combinations when using the SARS-CoV-2 ddPCR Kit. There are three single-positive clusters: *N1* (■); *N2* (■); and *RP* (■). There are three double-positive clusters: *N1* and *N2* (■); *N1* and *RP* (■); *N2* and *RP* (■). Finally, there is one triple-positive cluster, *N1*, *N2*, and *RP* (■), and one triple-negative cluster (■).

The validation data for the SARS-CoV-2 ddPCR Kit show that the LOD using the QIAGEN QIAamp Kit is 200 copies/ml for the *N1* and *N2* targets when used with nasopharyngeal swabs (data not shown). As expected, the lowest concentration detected for this study, 2.0 x 10^3 copies/ml, is well above the LOD for the test when the RNA is extracted from the sample. However, it offers other benefits, such as improved turnaround time, and does not rely on commercial kits that may be in short supply during the COVID-19 pandemic.

For the 7.7 µl input sample into Droplet Digital PCR at the 2.0 x 10^3 copies/ml concentration level, 19 out of 20 process replicates identified more than one droplet of either *N1* or *N2* positive droplets ($\alpha = 0.05$). For the 9.9 µl input RNA sample into Droplet Digital PCR, all 20 identified more than one droplet of either *N1* or *N2* positive droplets ($\alpha = 0.05$) greater than the viral concentration identified in the unspiked UTM sample.

The LOD for the extraction-free method is 2.0 x 10^3 copies/ml for both viral targets *N1* and *N2* in this set with 7.7 and 9.9 µl input samples into Droplet Digital PCR. Lower concentration levels, 400 and 40 copies/ml, were also tested. Less than 50% of positives and zero positives, respectively, were identified.

Comparison of Extraction-Free with Extracted Sample

An initial 1:10^3 dilution of the stock ATCC material in UTM was made for a working concentration of 4 x 10^6 copies/ml. This was then serially diluted at concentrations of 4 x 10^5, 4 x 10^4, 4 x 10^3, 4 x 10^2, and 4 x 10^1 copies/ml. These six levels along with a negative UTM media sample were processed by the extraction-free method in triplicate and tested in Droplet Digital PCR in triplicate (n = 9). These same levels were tested with the QIAamp and MagMAX extraction methods. The linearity of the three methods was compared and validated with the SARS-CoV-2 Kit.

The negative UTM media was used as the negative process control (n = 9) while a 1:10 dilution of the Exact Diagnostics SARS-CoV-2 Standard Positive in UTM media (n = 1) was used as the positive process control. NFW was used as the no template ddPCR control (n = 3) while a 1:10 dilution of the Exact Diagnostics SARS-CoV-2 Standard Positive in NFW (n = 1) was used as the positive ddPCR control.

Input ddPCR samples of 7.7 and 9.9 µl were tested with the extraction-free method and the two extraction methods to show the performance of both input sample volumes. Figure 3 shows the comparison of linearity of all three processes and Table 4 shows concentration data for all three.

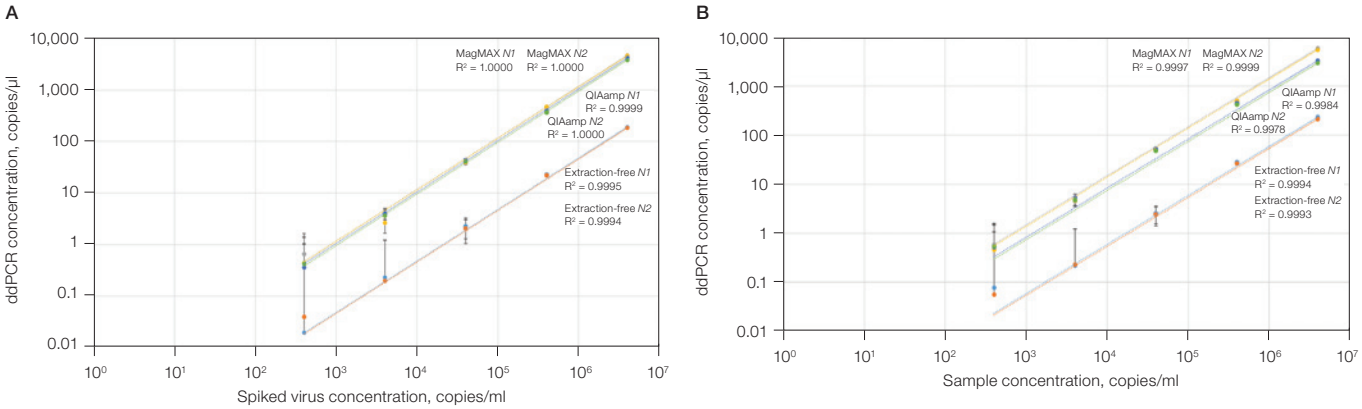


Fig. 3. log-log plots for linearity comparing extraction-free and extraction methods. Five contrived samples were created by serial tenfold dilutions, processed with the extraction-free method, and compared with both validated extraction kits. **A**, linearity results when using 7.7 µl of input sample into Droplet Digital PCR; **B**, linearity results when using 9.9 µl of input sample into Droplet Digital PCR. Both input volumes achieved R^2 >0.999. MagMAX *N1* (●···); MagMAX *N2* (●···); QIAamp *N1* (●···); QIAamp *N2* (●···); extraction-free *N1* (●···); extraction-free *N2* (●···).

Table 4. Summary of linearity data for extraction-free and extraction methods tested using 7.7 and 9.9 µl input samples into Droplet Digital PCR.

ddPCR 7 µl Input Sample	Extraction-Free *N1*	Extraction-Free *N2*	Extraction-Free *RP*	QIAamp *N1*	QIAamp *N2*	QIAamp *RP*	MagMAX *N1*	MagMAX *N2*	MagMAX *RP*
4.0 x 10^6 copies/ml									
Mean concentration, copies/µl	191.97	183.47	11.21	4,285.33	3,920.33	4,835.67	4,786.67	4,676.67	703.67
Standard deviation	15.77	13.65	0.59	498.59	491.44	219.22	484.46	518.34	62.43
4.0 x 10^5 copies/ml									
Mean concentration, copies/µl	23.00	22.23	13.31	393.67	364.67	4,073.33	472.33	477.67	690.33
Standard deviation	1.74	1.81	3.04	49.24	34.53	285.55	82.81	90.16	45.49
4.0 x 10^4 copies/ml									
Mean concentration, copies/µl	2.28	2.04	52.10	44.13	41.00	4,354.00	37.90	39.63	649.67
Standard deviation	0.34	0.45	4.43	17.88	15.03	334.26	2.21	4.31	26.69
4.0 x 10^3 copies/ml									
Mean concentration, copies/µl	0.23	0.20	42.41	4.03	3.60	3,828.67	3.71	2.67	654.67
Standard deviation	0.17	0.08	24.10	0.39	0.14	1,398.10	1.38	0.61	23.71
4.0 x 10^2 copies/ml									
Mean concentration, copies/µl	0.02	0.04	46.27	0.36	0.43	4,584.67	0.66	0.42	681.00
Standard deviation	0.04	0.07	19.52	0.09	0.18	418.12	0.23	0.13	39.34
ddPCR 9 µl Input Sample	**Extraction-Free *N1***	**Extraction-Free *N2***	**Extraction-Free *RP***	**QIAamp *N1***	**QIAamp *N2***	**QIAamp *RP***	**MagMAX *N1***	**MagMAX *N2***	**MagMAX *RP***
4.0 x 10^6 copies/ml									
Mean concentration, copies/µl	235.19	214.39	14.09	3,408.67	2,926.33	3,847.67	6,038.00	5,561.67	937.00
Standard deviation	19.65	23.92	1.61	235.97	173.90	150.64	531.74	153.16	24.06
4.0 x 10^5 copies/ml									
Mean concentration, copies/µl	28.58	26.48	16.14	448.67	416.33	4,786.33	509.00	499.67	915.67
Standard deviation	2.13	1.36	4.10	53.45	45.08	412.33	34.66	33.83	21.73
4.0 x 10^4 copies/ml									
Mean concentration, copies/µl	2.56	2.40	64.73	51.77	48.03	5,312.33	51.57	51.40	855.00
Standard deviation	0.52	0.30	5.43	16.98	17.57	448.13	5.60	2.86	34.07
4.0 x 10^3 copies/ml									
Mean concentration, copies/µl	0.22	0.23	62.42	5.35	4.75	4,403.33	4.46	4.67	863.67
Standard deviation	0.16	0.11	23.66	1.41	0.76	1,053.33	0.25	0.22	98.57
4.0 x 10^2 copies/ml									
Mean concentration, copies/µl	0.08	0.06	66.19	0.51	0.54	4,903.67	0.59	0.45	960.00
Standard deviation	0.07	0.06	11.89	0.13	0.17	152.98	0.53	0.25	162.92

Concentrations reported in Droplet Digital PCR for the extraction-free process are much lower than when the sample is extracted, as expected. However, the linearity of the extraction-free method is comparable to the two extraction methods but at a lower magnitude. While the extraction-free method may be less efficient than the extraction methods, it proves useful for detecting virus material at higher viral concentrations in nasopharyngeal samples, and has a faster turnaround time.

In comparing the ddPCR input sample volumes, 9.9 µl yields higher ddPCR concentrations than 7.7 µl, as expected. For this application, 9.9 µl input sample into Droplet Digital PCR is recommended and would provide consistent results and more sensitive detection.

Using Different Solutions to Resuspend Proteinase K

Finally, the suspension of Proteinase K in both NFW and the recommended HBSS buffer were compared to evaluate the option of a more easily accessible option when HBSS is not available.

All data presented to this point were produced with 30 mg/ml of Proteinase K from *Tritirachium album* in HBSS. However, we were interested to see the results of resuspending the Proteinase K in NFW for situations where the HBSS buffer is not readily available. Here we spiked ATCC virus material into UTM then serially diluted to initial sample concentrations of 2.5 x 10^4, 1.3 x 10^4, 6.3 x 10^3, and 3.1 x 10^3 copies/ml, all of which are above our LOD. Then we tested each concentration with 30 mg/ml Proteinase K in HBSS and 30 mg/ml Proteinase K in NFW with our extraction-free method in triplicate process and duplicate ddPCR reactions (n = 6 sample replicates).

Unspiked UTM was used as a negative process control while ATCC virus diluted 1:4 in PBS was used as a positive process control. A 1:10 dilution of our Exact Diagnostics Positive Standard in NFW was used as a positive ddPCR control and NFW was used as a no template ddPCR control.

Mean droplet counts comparing the two suspension buffers are shown in Figure 4, demonstrating no negative effects on accepted events. Figure 5 shows comparable concentration data for the two buffers.

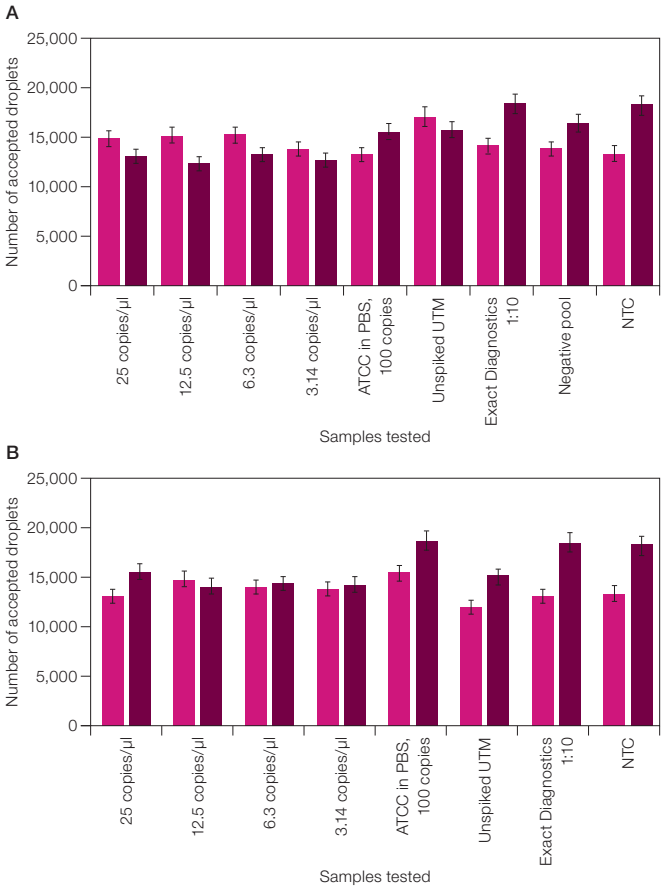


Fig. 4. Mean droplet counts for Proteinase K in HBSS and in NFW. Comparison of suspending Proteinase K in HBSS or NFW and the impact on droplets with two different input samples into Droplet Digital PCR: **A**, 7 µl and **B**, 9 µl. These results represent the mean number of accepted droplets in Droplet Digital PCR (n = 6 per concentration). There are no statistically significant differences between suspensions. HBSS (■); NFW (■). ATCC, American Type Culture Collection; HBSS, Hanks' Balanced Salt Solution; NFW, nuclease-free water; NTC, no template control; PBS, phosphate buffered saline; UTM, Universal Transport Medium.

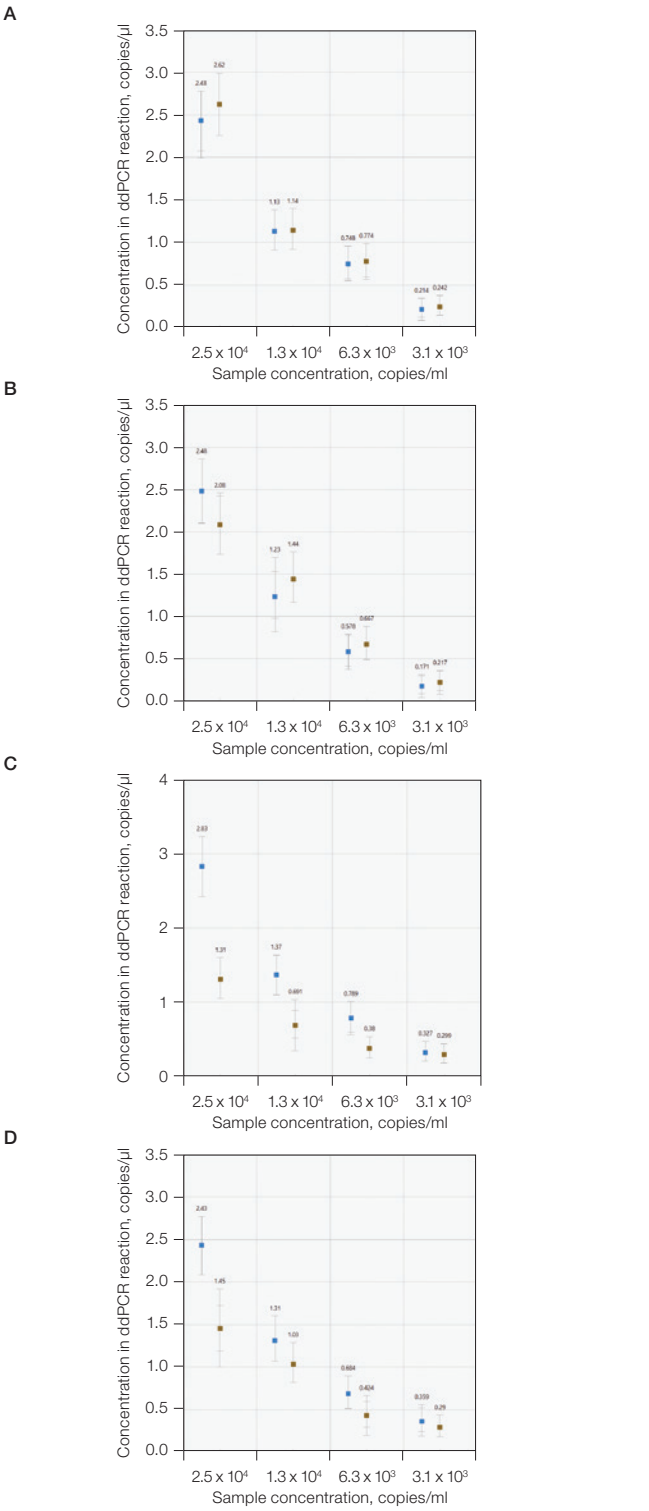


Fig. 5. Eight-well merged concentrations reported in Droplet Digital PCR for dilution levels of 2.5 x 10⁴, 1.3 x 10⁴, 6.3 x 10³, and 3.1 x 10³ copies/ml. Effect on concentration between two different suspension solutions for Proteinase K with two different input samples into Droplet Digital PCR: **A**, 7 µl in HBSS; **B**, 7 µl in NFW; **C**, 9 µl in HBSS; and **D**, 9 µl in NFW. These points represent mean ddPCR concentrations in copies/µl (n = 8 per concentration). Both suspension solutions performed similarly. *N1* target (■); *N2* target (■). HBSS, Hanks' Balanced Salt Solution; NFW, nuclease-free water.

We see that if the Proteinase K is suspended in HBSS or NFW at 30 mg/ml, there is no significant impact on the performance of this method. Run data show that neither the mean number of accepted droplets nor the concentration are significantly different when comparing the two suspension solutions.

Based on these results, if HBSS is not readily available, NFW may be used for resuspension instead and would yield similar results. It is important to note the LOD was identified using HBSS and was not verified with NFW. The lowest detectable concentration with NFW is 3.1 x 10^3 copies/ml, as shown here. Furthermore, there was no testing on Proteinase K in NFW performance over multiple freeze-thaw cycles and this should be tested in the future.

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 in nasopharyngeal sample specimens. The data shown were acquired using contrived samples composed of negative nasopharyngeal swabs in UTM spiked with inactivated virus and should be confirmed with real positive clinical samples in the future. This serves as a guide for applying the method to Droplet Digital PCR, simplifying the workflow, and improving the turnaround time.

Since it is important to have extraction controls during RNA extraction, it is recommended to run a positive and negative standard along with the other samples. This method would allow an operator to process up to 93 samples with three process controls (positive, negative, and NTC) in one run.

As expected, when using an extraction-free method, the ease of the procedure is a trade-off to higher sensitivity. A 96-well plate can be processed in less than 30 minutes at minimal cost, input sample, and reagent usage. For users, this trade-off may serve as a benefit when dealing with samples that may have higher viral loads and/or when more broad screening is optimal.

Different media often have chemical compositions that do not interact positively with the chemicals used during droplet generation, which could lead to poor amplification and low droplet/event counts. Therefore, when applying this application note to different collection media it is important to first determine the effects of the medium on droplet count and cluster phenotypes. If both are negatively impacted, consider diluting the sample before processing to achieve better results.

Conclusions

With RNA extraction sample preparation, it is evident that manual extractions are time-consuming and risk operator fatigue. 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 nasopharyngeal samples collected in UTM media with the SARS-CoV-2 ddPCR Kit.

References

Abayan R (2020). Application of Droplet Digital PCR for SARS-CoV-2: Extraction-free processs for environmental swabs. Bio-Rad Bulletin 7377.

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].

Visit **bio-rad.com/ExtractionFree** for more information.

Bio-Rad
Laboratories, Inc.

Life Science Group

Website *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *00 800 00 24 67 23* ***Belgium*** *00 800 00 24 67 23* ***Brazil*** *4003 0399*
Canada *1 905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *00 800 00 24 67 23* ***Denmark*** *00 800 00 24 67 23* ***Finland*** *00 800 00 24 67 23*
France *00 800 00 24 67 23* ***Germany*** *00 800 00 24 67 23* ***Hong Kong*** *852 2789 3300* ***Hungary*** *00 800 00 24 67 23* ***India*** *91 124 4029300*
Israel *0 3 9636050* ***Italy*** *00 800 00 24 67 23* ***Japan*** *81 3 6361 7000* ***Korea*** *82 2 3473 4460* ***Luxembourg*** *00 800 00 24 67 23*
Mexico *52 555 488 7670* ***The Netherlands*** *00 800 00 24 67 23* ***New Zealand*** *64 9 415 2280* ***Norway*** *00 800 00 24 67 23* ***Poland*** *00 800 00 24 67 23*
Portugal *00 800 00 24 67 23* ***Russian Federation*** *00 800 00 24 67 23* ***Singapore*** *65 6415 3188* ***South Africa*** *00 800 00 24 67 23*
Spain *00 800 00 24 67 23* ***Sweden*** *00 800 00 24 67 23* ***Switzerland*** *00 800 00 24 67 23* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 651 8311*
United Arab Emirates *36 1 459 6150* ***United Kingdom*** *00 800 00 24 67 23*

Bulletin 7390 Ver A US/EG *20-0551 0920 Sig 0220*