Wastewater surveillance

Accurate quantification of SARS-CoV-2 in wastewater samples with the QuantStudio Absolute Q Digital PCR System

Keywords
SARS-CoV-2, sewage, wastewater surveillance, wastewater-based epidemiology, digital PCR

Summary

- Fast time to results—one easy-to-use instrument, 5 minutes of hands-on time, and delivery of results in less than 2 hours
- More flexibility—microfluidic array plate (MAP) technology enables effortless and consistent sample digitization with the flexibility to run as few as 4 samples at a time
- Multiplexing capability—detect up to 4 targets in a single reaction

Introduction

Wastewater-based epidemiology (WBE) is the systematic sampling and analysis of wastewater influent to detect specific pathogens [1]. The importance of WBE surveillance gained significant attention with the emergence of SARS-CoV-2. WBE surveillance for SARS-CoV-2 can allow community-level trends to be evaluated so that public health measures can be efficiently implemented to limit transmission [2].

Quantitative real-time PCR (qPCR) has been widely used for the detection and relative quantification of SARS-CoV-2. However, sensitive and accurate quantification of the virus is necessary in wastewater surveillance efforts to detect changes in viral quantities at extremely low concentrations. Optimized digital PCR (dPCR) is required to precisely quantify SARS-CoV-2. dPCR complements existing qPCR methods for SARS-CoV-2 detection, particularly with samples that contain extremely low concentrations of the virus. dPCR technology also has significant advantages over common PCR platforms, including unparalleled sensitivity, high precision, reliable performance, and the possibility of absolute quantification without a standard curve [3].

Individuals infected with SARS-CoV-2 shed the virus in their feces, and the virus has been detected in wastewater [4]. Since the virus is very dilute in large volumes, the effectiveness of the extraction method used to isolate SARS-CoV-2 RNA is pivotal for wastewater surveillance. The Applied Biosystems™ MagMAX™ Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment can be used to isolate SARS-CoV-2 RNA from dilute samples. This kit includes Applied Biosystems™ Dynabeads™ Wastewater Virus Enrichment beads, which are optimized for enriching intact SARS-CoV-2 from very low concentrations.

The high sensitivity and robust performance of the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System enables accurate quantification of SARS-CoV-2 in wastewater without requiring standard curves. In this application note, we present a simple and complete workflow for wastewater surveillance from sample collection to SARS-CoV-2 quantification. Viral nucleic acid was extracted from real wastewater influents using a protocol that enables fast, easy, automated, and reliable sample processing without any inconvenient filtration, precipitation, or bead-beating steps. Absolute quantification of SARS-CoV-2 was achieved using the QuantStudio Absolute Q Digital PCR System and the Combinati™ SARS-CoV-2 Wastewater Surveillance Kit to target the SARS-CoV-2 N1 and N2 genes. A pepper mild mottle virus (PMMoV) control was used for fecal normalization.

Materials and methods

Wastewater sample collection

Raw wastewater influent was collected at wastewater facilities in northern Georgia (USA) using four 1 L high-density polyethylene containers. Four grab samples were obtained at permitted influent sites and shipped to Austin, Texas. Upon arrival, the wastewater samples were stored at 4°C until they were heat-inactivated at 56°C for 3 hours before processing.

Sample processing

To prepare 10 mL samples for processing, 15 mL wastewater replicates from each of the four collection sites were centrifuged for 10 minutes at 10,000 x *g*. The clarified supernatants were transferred to new tubes in 10 mL aliquots and spiked with 200, 2,000, or 20,000 copies of inactivated SARS-CoV-2. The extraction controls consisted of 10 mL samples from each collection site that were not spiked. The samples were transferred in 5 mL aliquots to two 24 deep-well plates, one of which contained 100 µL of Dynabeads Wastewater Virus Enrichment beads per well for sample concentration. The samples were then processed on the Thermo Scientific™ KingFisher™ Flex Purification System (Figure 1). The concentrated viral particles were eluted into a single plate that contained 500 µL per well of lysis buffer from the MagMAX Wastewater Ultra Nucleic Acid Isolation Kit. Proteinase K, binding buffer, and magnetic beads were added to the concentrated samples, and the extraction script was initiated. At the end of the run, all of the viral nucleic acid from each sample was eluted in 100 µL of elution buffer.

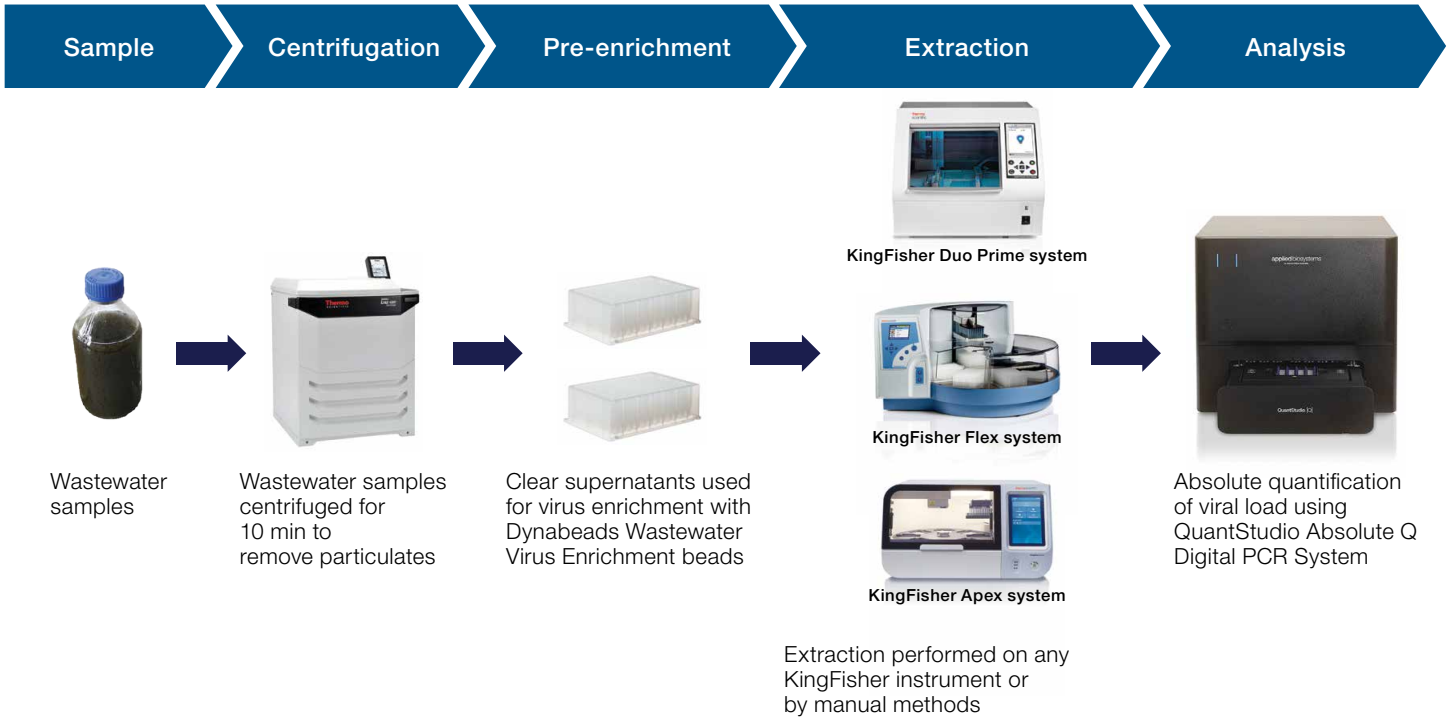

Figure 1. Workflow for processing 10 mL wastewater samples. The workflow includes SARS-CoV-2 enrichment with Dynabeads Wastewater Virus Enrichment beads.

Absolute quantification of SARS-CoV-2 in processed wastewater samples

The absolute viral load in each sample was quantified using the Combinati SARS-CoV-2 Wastewater Surveillance Kit and the QuantStudio Absolute Q Digital PCR System (Figure 2). The proprietary technology of the Applied Biosystems™ QuantStudio™ Absolute Q™ MAP16 Plate enables consistent digitization of 20,000 precisely defined microchambers. The architecture of the MAP16 plate also provides the flexibility to run 4, 8, 12, or 16 samples at once (Figure 3). Following nucleic acid isolation, 2 µL of each sample was added to a reaction mixture containing 2.25 µL Combinati™ 1-Step RT Master Mix (4X), 0.45 µL SARS-CoV-2 wastewater surveillance assay mix (20X), and 4.3 µL nuclease-free water. The 9 µL reaction mixtures were loaded into individual wells in the MAP16 dPCR plate with 20,000 precisely defined microchambers. After adding 15 µL of isolation buffer to each well, the wells were covered with gaskets. The prepared MAP16 plate was then loaded onto the QuantStudio Absolute Q Digital PCR system. The dPCR cycling conditions are shown in Table 1. The triplex assay employed three optical channels of the QuantStudio Absolute Q system to detect the SARS-CoV-2 N1 gene (FAM label), SARS-CoV-2 N2 gene (HEX label), and the PMMoV normalization control for human fecal samples (Cy5 label). The QuantStudio Absolute Q digital PCR system enabled all necessary steps to be performed on one instrument, and results were generated in less than 2 hours.

Table 1. Cycling conditions for dPCR on the QuantStudio Absolute Q Digital PCR System.

Number of cycles	Conditions
1	10 min at 50°C
1	5 min at 96°C
40	5 sec at 95°C, 30 sec at 55°C

Figure 2. Simplified workflow for the QuantStudio Absolute Q Digital PCR System. The reaction mixtures are loaded onto the MAP16 dPCR plate and run on one instrument that integrates all PCR steps, including digitization, thermal cycling, and data acquisition.

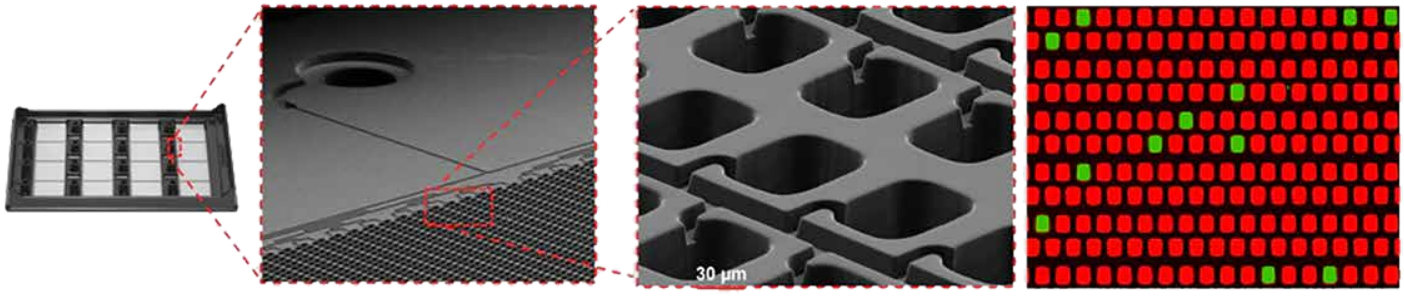

Figure 3. QuantStudio Absolute Q MAP16 Plate.

Results

The concentrations of the SARS-CoV-2 N1 and N2 genes and PMMoV in the extraction controls and samples spiked with 20, 200, or 20,000 copies of inactivated SARS-CoV-2 are shown in Table 2. The extraction controls generated microchamber fluorescence in the FAM and HEX dye channels, which suggested that low numbers of SARS-CoV-2 were present in all four samples. Representative two-dimensional (2D) fluorescence plots showing the numbers of microchambers positive for the FAM and HEX labels, used to calculate the absolute quantities of the N1 and N2 genes, are shown in Figure 4A.

Fluorescence in the Cy5 dye channel for the PMMoV fecal indicator is shown in Figure 4B. The reported PMMoV concentrations varied in all samples tested (Table 2). The median PMMoV concentration was used for internal normalization of the dPCR data to ensure accurate quantification of viral concentration. Briefly, the concentrations of the SARS-CoV-2 N1 and N2 targets and PMMoV were multiplied by the reaction volume (9 µL) to calculate the total copy number per reaction. The total copy number per reaction was then divided by 2 µL, which was the extracted RNA input per reaction. The concentrations of the N1 and N2 targets were then divided by the PMMoV concentration. The normalized target concentrations were calculated by multiplying these ratios by the median PMMoV concentration (7,088 copies/µL) for the dataset.

Table 2. Measured concentrations of the SARS-CoV-2 N1 and N2 genes and PMMoV fecal indicator reported by QuantStudio Absolute Q software.

Name	Total*	Dye	Target	Concentration (copies/µL)	95% CI	Positives	Dye	Target	Concentration (copies/µL)	95% CI	Positives	Dye	Target	Concentration (copies/µL)	95% CI	Positives
S1_0	20,470	FAM	N1	0	0	0	HEX	N2	0.11	0.10	1	Cy5	PMMoV	1,231.42	26.29	8,445
S2_0	20,453	FAM	N1	0.45	0.28	4	HEX	N2	0.34	0.23	3	Cy5	PMMoV	1,217.21	26.10	8,364
S3_0	20,467	FAM	N1	0.57	0.33	5	HEX	N2	0.45	0.28	4	Cy5	PMMoV	1,264.82	26.75	8,616
S4_0	20,452	FAM	N1	0.68	0.37	6	HEX	N2	0.91	0.45	8	Cy5	PMMoV	1,289.84	27.11	8,737
S1_L	20,474	FAM	N1	1.02	0.49	9	HEX	N2	0.68	0.37	6	Cy5	PMMoV	1,397.22	28.57	9,278
S2_L	20,474	FAM	N1	2.28	0.80	20	HEX	N2	1.36	0.59	12	Cy5	PMMoV	1,976.31	36.48	11,756
S3_L	20,466	FAM	N1	1.24	0.56	11	HEX	N2	1.36	0.59	12	Cy5	PMMoV	1,880.46	35.17	11,383
S4_L	20,442	FAM	N1	0.68	0.37	6	HEX	N2	1.13	0.52	10	Cy5	PMMoV	1,187.61	25.70	8,204
S1_M	20,475	FAM	N1	9.06	1.78	80	HEX	N2	10.65	1.95	94	Cy5	PMMoV	2,102.18	38.22	12,218
S2_M	20,478	FAM	N1	8.27	1.69	73	HEX	N2	7.93	1.66	70	Cy5	PMMoV	1,572.85	30.96	10,098
S3_M	20,476	FAM	N1	8.04	1.67	71	HEX	N2	8.61	1.73	76	Cy5	PMMoV	2,477.33	43.50	13,454
S4_M	20,729	FAM	N1	9.20	1.80	81	HEX	N2	10.11	1.90	89	Cy5	PMMoV	2,875.79	49.40	14,531
S1_H	20,467	FAM	N1	91.82	6.16	796	HEX	N2	90.53	6.12	785	Cy5	PMMoV	1,972.6	36.43	11,738
S2_H	20,449	FAM	N1	84.26	5.89	731	HEX	N2	90.02	6.10	780	Cy5	PMMoV	1,577.39	31.05	10,104
S3_H	20,462	FAM	N1	71.22	5.39	620	HEX	N2	71.22	5.39	620	Cy5	PMMoV	1,979.21	36.53	11,760
S4_H	20,452	FAM	N1	32.60	3.57	286	HEX	N2	34.55	3.68	303	Cy5	PMMoV	895.96	21.55	6,564

* The total is the number of microchambers analyzed on the MAP16 plate. S1: sample 1; S2: sample 2; S3: sample 3; S4: sample 4; L: low (20-copy spike); M: medium (200-copy spike); H: high (20,000-copy spike); 0: no spike.

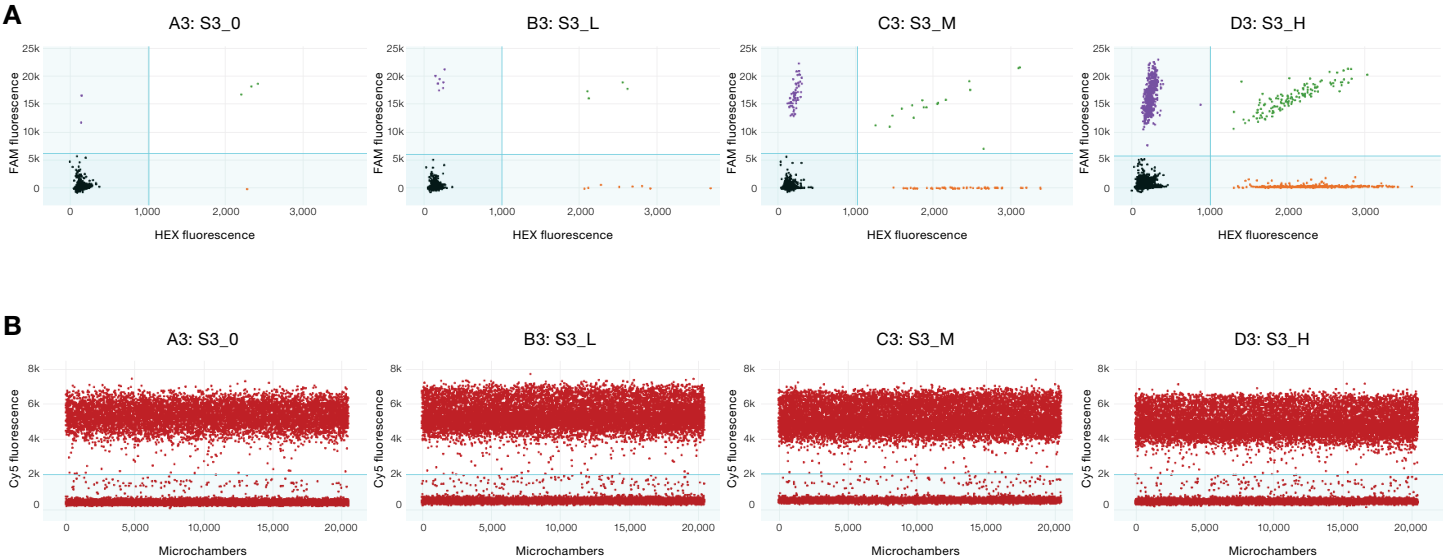

Figure 4. Digital PCR plots from the SARS-CoV-2 wastewater surveillance assay. (A) 2D plots of FAM and HEX fluorescence for the detection of the SARS-CoV-2 N1 and N2 genes, respectively. Data from the FAM-positive microchambers are in purple in the upper left quadrant of each plot, and data from the HEX-positive microchambers are in orange in the lower right quadrants. Data from microchambers that were positive for both labels are in green. The FAM/HEX channel data are shown for sample 3 only; similar plots were obtained with samples 1, 2, and 4. **(B)** Fluorescence in the Cy5 channel from microchambers that were positive for the PMMoV fecal indicator.

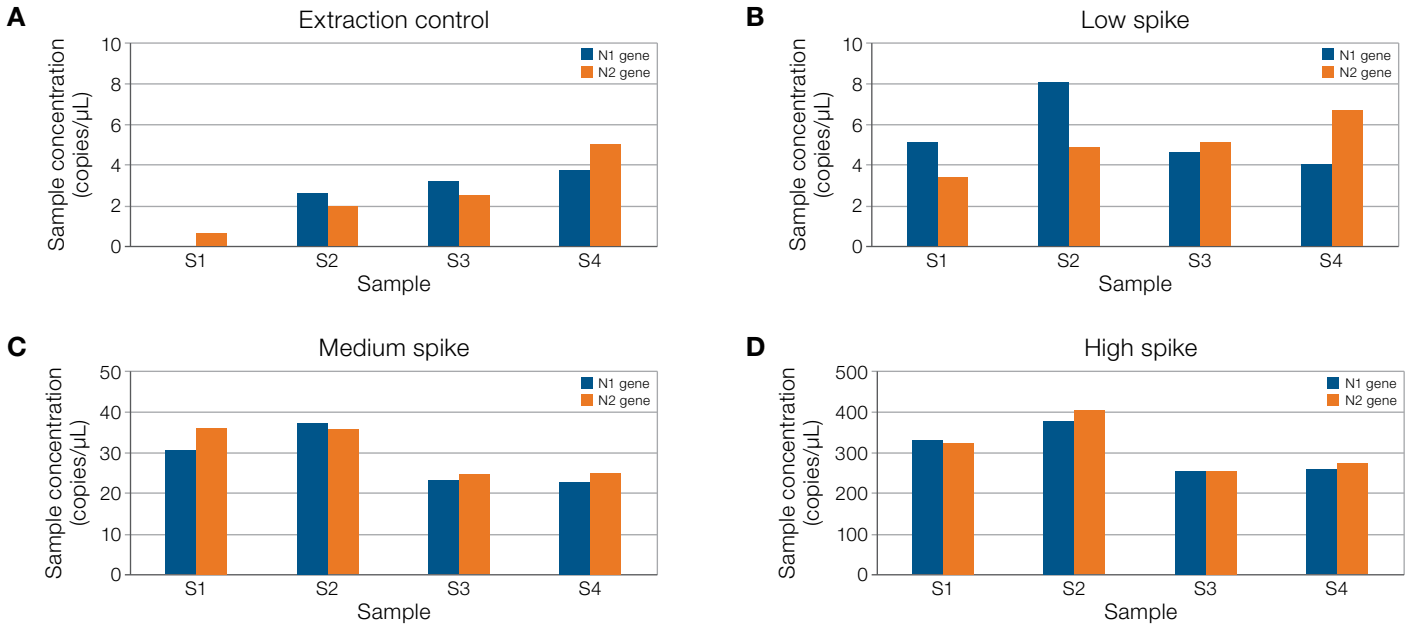

Figure 5. Concentrations of the SARS-CoV-2 N1 and N2 gene targets after normalization to the median concentration of PMMoV.

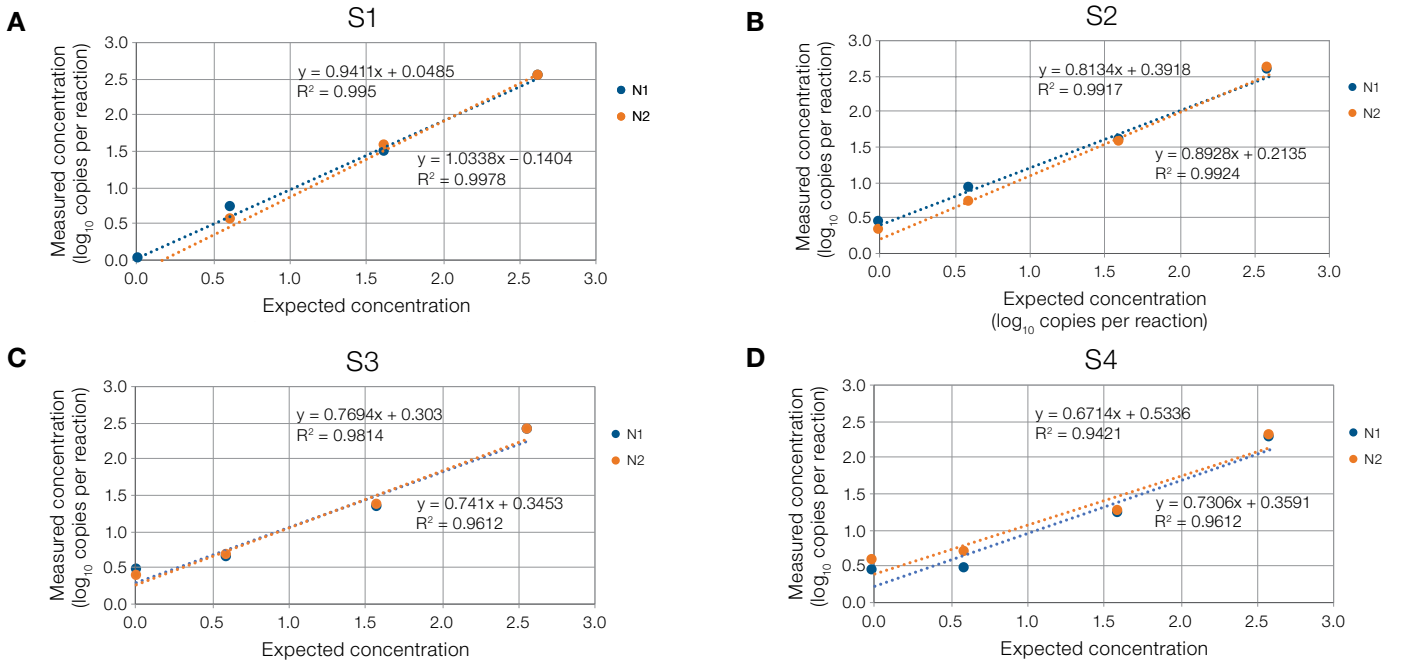

Figure 6. Test of linearity to determine the reportable range for dPCR targeting the SARS-CoV-2 N1 and N2 genes. The expected concentrations were plotted on the x-axes against the dPCR-measured concentrations on the y-axes.

The normalized concentrations of the SARS-CoV-2 N1 and N2 genes in the samples are plotted in Figure 5. As shown in Figure 5, all four extraction controls and samples spiked with 20 SARS-CoV-2 copies had low viral concentrations. This indicated that the Absolute Q dPCR system was sensitive for the detection of SARS-CoV-2 at low concentrations in wastewater. Recovery of SARS-CoV-2 from all spiked samples was linear (Figure 6), which confirmed that the QuantStudio Absolute Q Digital PCR System enables reproducible and accurate quantification of the virus in wastewater samples.

Conclusion

The MagMAX Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment enabled the enrichment of SARS-CoV-2 in wastewater and isolation of SARS-CoV-2 RNA. The high-quality nucleic acid extracted from wastewater can be used for a variety of downstream applications. We have demonstrated successful dPCR with SARS-CoV-2 nucleic acid, with linear recoveries.

References

1. Sims N, Kasprzyk-Hordern B (2020) Future perspectives of wastewater-based epidemiology: monitoring infectious disease spread and resistance to the community level. *Environ Int* 139:105689. doi: 10.1016/j.envint.2020.105689
2. Mousazadeh M, Ashoori R, Paital B et al. (2021) Wastewater based epidemiology perspective as a faster protocol for detecting coronavirus RNA in human populations: a review with specific reference to SARS-CoV-2 virus. *Pathogens* 10(8):1008. doi: 10.3390/pathogens10081008
3. Hall Sedlak R, Jerome KR (2014) The potential advantages of digital PCR for clinical virology diagnostics. *Expert Rev Mol Diagn* 14(4):501-507. doi: 10.1586/14737159.2014.910456
4. Schmitz BW, Innes GK, Prasek SM et al. (2021) Enumerating asymptomatic COVID-19 cases and estimating SARS-CoV-2 fecal shedding rates via wastewater-based epidemiology. *Sci Total Environ* 801:14974. doi: 10.1016/j.scitotenv.2021.149794

Ordering information

Product	Quantity	Cat. No.
Kits, reagents, and dPCR consumables		
MagMAX Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment	100 preps	A52610
Combinati SARS-CoV-2 Wastewater Surveillance Kit	200 reactions	A52689
QuantStudio Absolute Q Isolation Buffer	3 mL	A52730
QuantStudio Absolute Q MAP16 Plate Kit	12 plates	A52865
Instruments		
KingFisher Flex Purification System, KingFisher with 24 Deep-Well Head	1 system	5400640
QuantStudio Absolute Q Digital PCR System, desktop	1 system	A52864

Learn more at **thermofisher.com/magmaxwastewater** and **thermofisher.com/absoluteq**

applied biosystems