Wastewater surveillance

Analyzing wastewater samples for SARS-CoV-2 targets using the QuantStudio Absolute Q Digital PCR System

Background

Wastewater-based epidemiology (WBE) enables tracking of biomarkers for specific pathogens to monitor for disease outbreak and spread. WBE's utility in disease surveillance has proven to be effective in monitoring for rare cases of disease [1]. It has also proven useful for understanding community-level prevalence of pathogens, including SARS-CoV-2, and predicting future caseloads, serving as a type of biomarker test for a whole community [2]. The major challenge of WBE is that its base material, nucleic acid biomarkers in wastewater, is extremely rare compared to the availability of biomarkers in blood, buccal swabs, and other biological materials. Thus, the tools, processes, and reagents used in WBE need to be much more sensitive and much more suitable to generate usable results from very low initial copy numbers. To effectively monitor the quantity of SARS-CoV-2 in wastewater samples, it is critical to maximize the amount of information per reaction, minimize reagent waste, and control for external factors such as population size and sample processing efficiency.

Measuring SARS-CoV-2 targets alongside robust controls can provide tangible metrics for normalization of results and help limit the impact of sample preparation variability. Using the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR (dPCR) System, up to four optical channels can be leveraged to provide simultaneous multiplex detection of wastewater-relevant targets.

In this study, we demonstrate the detection and quantification of the N1 and N2 SARS-CoV-2 targets alongside the human fecal normalization control, pepper mild mottle virus (PMMoV), from four wastewater samples collected by the University of Arizona's WEST Center during their WBE testing efforts.

Workflow features

- Quantification of three wastewater-specific genomic targets in a single dPCR reaction
- Single-instrument qPCR-like workflow with <5 minutes of hands-on preparation
- Digital PCR results generated in 90 minutes

Methods

The Combinati SARS-CoV-2 Wastewater Surveillance assay (Cat. No. A52689) was used in this study. This assay detects two genetic targets, N1 (Applied Biosystems™ FAM™ dye–labeled probe) and N2 (Applied Biosystems™ HEX™ dye–labeled probe) in the SARS-CoV-2 N gene and a human fecal normalization control target in PMMoV (Cy®5 dye–labeled probe). N1 and N2 have been reported to be sensitive and specific for quantifying SARS-CoV-2 RNA in wastewater [2]. The plant pathogen PMMoV, an indicator of human fecal pollution, is widespread and abundant in wastewater from the United States, making PMMoV an effective control.

Sample selection and testing

Table 1. Reaction setup for dPCR.

Reagent	Final concentration	Reaction volume
Combinati 1-Step RT Master Mix (4X)	1X	2.25 µL
SARS-CoV-2 Wastewater Surveillance Assay (20X)	1X	0.45 µL
Extracted RNA	Variable	2.0 µL
Water	–	4.3 µL

Reagent table denotes volumes used to prepare dPCR reactions for wastewater sample testing experiments.

Four wastewater samples with previous dPCR tests for the N1 and N2 SARS-CoV-2 targets were selected for this study. Two microliters of each extracted wastewater RNA sample were tested using the Combinati SARS-CoV-2 Wastewater Surveillance kit. After preparing the dPCR mix (Table 1), 9 µL of the reaction mixture was loaded into the Applied Biosystems™ QuantStudio™ MAP16 Digital PCR Plate followed by an overlay of 15 µL of isolation buffer. The prepared plate was then loaded on the QuantStudio Absolute Q dPCR System and run using the dPCR thermal protocol in Table 2.

Table 2. Run parameters for dPCR reaction

Step	Cycles	Temperature	Duration (MM:SS)
Reverse transcription	1	50°C	10:00
Hot start	1	95°C	05:00
Denaturation	45	95°C	00:05
Annealing/extension		55°C	00:30

The targets of the 3-plex assay are the N1 (FAM dye) and N2 (HEX dye) SARS-CoV-2 targets and human fecal control from PMMoV (Cy5 dye).

Results

Detection of SARS-CoV-2 and wastewater-related targets in a single reaction

This dPCR assay leverages three of the available optical channels for target multiplexing, using a separate color channel to detect each of the assay targets. The number of FAM dye–positive and the number of HEX dye–positive microchambers are used to calculate the absolute quantity of the N1 and N2 targets, respectively (Figure 1A). Subsequently, the number of Cy5 dye–positive microchambers are used to calculate the quantity of PMMoV targets (Figure 1B). The concentrations reported by the Applied Biosystems™ QuantStudio™ Absolute Q™ Analysis Software for each sample are listed in Table 3.

Of the four wastewater RNA samples tested using the SARS-CoV-2 Wastewater Surveillance 3-plex assay, the presence or absence of the N1 and N2 targets corroborated previous dPCR testing. The concentration for the PMMoV target varied for each of the samples (Figure 1).

PMMoV human fecal control enables cross-sample normalization

To normalize the dPCR data, we first calculated the concentration of each RNA sample using the following formula:

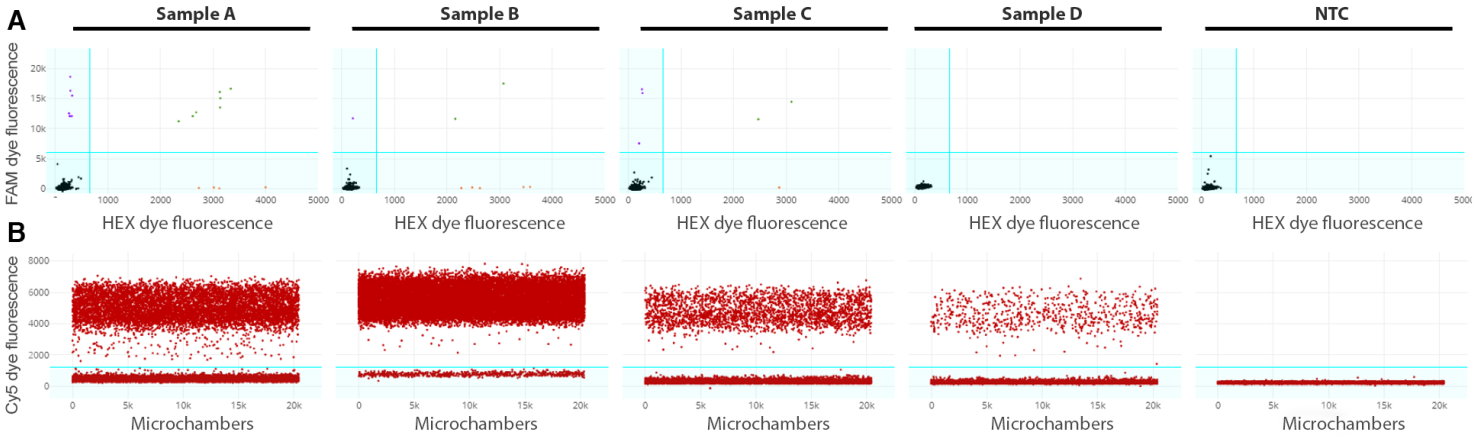

Figure 1. Digital PCR plots for the SARS-CoV-2 Wastewater Surveillance 3-plex assay. (A) Two-dimensional microchamber fluorescence plot in the FAM dye and HEX dye channels for the detection of SARS-CoV-2 N1 and N2 targets, respectively. Single-positive FAM dye and HEX dye microchambers are denoted in purple (upper left quadrant) and orange (lower right quadrant), respectively, and double-positive microchambers are denoted in green. **(B)** Microchamber fluorescence in the Cy5 dye channel denoting microchambers positive for the PMMoV target.

Table 3. Concentration of targets reported by the QuantStudio Absolute Q Analysis Software.

Sample	Total count	N1 (cp/µL)	95% CI	FAM dye–positive	N2 (cp/µL)	95% CI	HEX dye–positive	PMMoV (cp/µL)	95% CI	Cy5 dye–positive
Sample A	20,426	1.29	0.58	11	1.29	0.58	11	1,195.75	26.15	8,020
Sample B	20,209	0.83	0.43	7	0.83	0.43	7	8,102.26	174.09	19,520
Sample C	20,478	0.35	0.24	3	0.35	0.24	3	321.32	12.2	2,568
Sample D	20,464	0	0	0	0	0	0	84.07	5.98	705
NTC	20,480	0	0	0	0	0	0	0	0	0

$$\text{Sample concentration} = \frac{\text{dPCR concentration } \frac{\text{cp}}{\mu\text{L}} \times 9\ \mu\text{L PCR volume}}{2\ \mu\text{L wastewater RNA input per reaction}}$$

We then calculated the ratio of SARS-CoV-2 target concentration to the PMMoV target concentration. Subsequently, we normalized the dataset to the median concentration of PMMoV across the 4 samples tested (3,413.2 cp/µL). Finally, using the equation below, we calculated the normalized concentration of each SARS-CoV-2 target in copies per microliter (cp/µL).

$$\text{Normalized concentration} = \frac{\text{SARS-CoV-2 target (cp/µL)}}{\text{PMMoV target (cp/µL)}} \times \text{median PMMoV target (cp/µL)}$$

Figure 2 compares the pre- and post-normalization results of the N1 and N2 SARS-CoV-2 targets. Notably, the PMMoV target normalization reveals that sample C had the highest normalized quantity of SARS-CoV-2 targets compared to the PMMoV target.

Summary

WBE offers a flexible solution for routine monitoring of communities for the presence of disease-related biomarkers. To effectively compare datasets over time or even between communities, it is essential that the selected measurement techniques provide consistent and accurate quantification while introducing as few variables as possible. dPCR, which provides absolute quantification of targets without standard curves, enables the quantification of all targets (including controls) to produce more accurate and more broadly comparable wastewater datasets, even when upstream preparation methods vary.

This study showcases the use of the QuantStudio Absolute Q Digital PCR System to detect two SARS-CoV-2 targets alongside a human fecal normalization target in wastewater samples collected on a university campus. The resulting data may be used to help make more consistent data comparisons. The QuantStudio Absolute Q Digital PCR System offers a simplified dPCR workflow with less than 5 minutes of hands-on time, which is comparable to a qPCR workflow. With an industry-leading >95% sample utilization per dPCR reaction, the QuantStudio Absolute Q dPCR System provides accurate, consistent, and reproducible absolute quantification of wastewater-relevant targets.

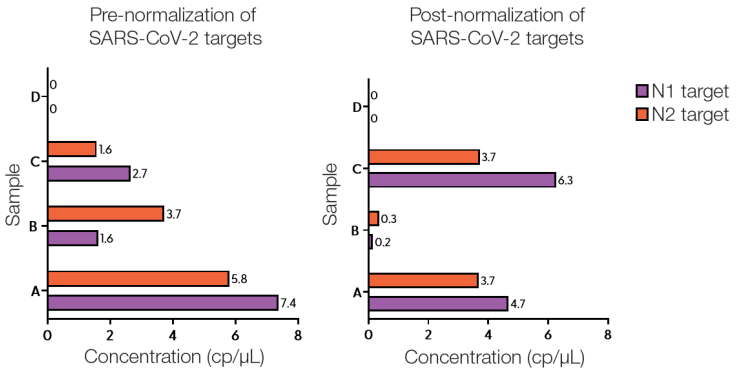

Figure 2. Concentration of SARS-CoV-2 N1 and N2 targets before and after normalization to the median concentration of PMMoV.

Acknowledgements

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References

1. Asghar H, OM Diop, G Weldegebriel, et al. (2014) Environmental surveillance for polioviruses in the Global Polio Eradication Initiative. *J Infect Dis* 210 Suppl 1:S294–303.
2. Larsen DA, KR Wigginton (2020) Tracking COVID-19 with wastewater. *Nat Biotechnol* 38(10):1151–1153.