Detection of SARS-CoV-2 in fecal samples and wastewater

Introduction

A novel coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in Wuhan, China, in December 2019 and has rapidly spread, creating a worldwide crisis.

Studies have shown that SARS-CoV-2 can be spread through large respiratory droplets (Kampf et al., 2020) and contact with infected individuals (Chan et al., 2020). Although the virus primarily resides in the upper respiratory tract, it could also be detected in stool.

Wastewater (or sewage) surveillance is a useful tool for monitoring the regional spread of viral and bacterial pathogens (Sims and Kasprzyk-Hordern, 2020). Early work has shown that wastewater surveillance at different levels of operations (e.g., dorms or accomodations at the university or the entire city) could reliably detect SARS-CoV-2 before it became widespread in a population (Lodder and de Roda Husman, 2020).

The initial step in processing wastewater or sewage samples is removal of large debris and particles that can negatively impact the downstream analysis steps. Larger particles can be depleted from the samples by pelleting using centrifugation at 4,650–4,750 x *g* for 30 min (Medema et al., 2020 and Ahmed et al., 2020) or by sequential filtration through 20 µm and 5 µm filters (Nemudryi et al., 2020).

It is important to harvest optimal volumes of wastewater samples for the detection of SARS-CoV-2 and other viruses. Based on reports, 50–200 mL of untreated wastewater sample is typical for detection of viruses (Medema et al., 2020 and Ahmed et al., 2020). The wastewater samples can be concentrated using one of the following approaches: ultracentrifugation at 200,000 x *g* for 1 hour (Wurtzer et al., 2020); precipitation with polyethylene glycol (PEG) 8000 (Wu et al., 2020); membrane filtration with a 0.45 µm pore size filter (Ahmed et al., 2020 and Nemudryi et al., 2020); and concentration on a 10–100 kDa molecular weight cut-off (MWCO) membrane (Medema et al.,2020, Ahmed et al., 2020, and Nemudryi et al., 2020). Some studies have used primary sewage sludge without concentration (Peccia et al., 2020).

Once the samples are concentrated, viral RNA or DNA is purified using either a spin column–based kit (for low throughput) or magnetic bead–based kits (for high throughput). Next, viral nucleic acid is detected by downstream qPCR analysis.

In the following study, robust workflows for SARS-CoV-2 detection in fecal samples, rectal swabs, and wastewater samples were developed using the Applied Biosystems™ MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Cat. No. A42357), automated on the Thermo Scientific™ KingFisher™ Flex Purification System.

Methods

- Inactivated SARS-CoV-2 was obtained from BEI resources (Cat. No. N-52287).
- Human fecal (stool) and rectal swab samples were obtained from Discovery Bio Sciences, and total nucleic acid was purified in duplicate using the MagMAX Microbiome Ultra kit. Input amounts of 100 mg of fecal samples and rectal swabs, stored in 200 µL preservative, were used. The KingFisher Flex Purification System with 96 Deep-Well Head (Cat. No. 5400630) was utilized for isolation of total nucleic acid from human fecal (stool) and rectal swab samples. Viral and microbial nucleic acid were eluted in 200 µL volume for fecal samples and 50 µL volume for rectal swabs.
- Two methods were used for detection of SARS-CoV-2 levels in wastewater samples (Figure 1):
  - **Direct method for smaller volume:** First, heat-inactivated wastewater samples were subjected to bead beating. Depending on the wastewater volume input, the samples can be processed in several plate wells and then pooled (Figure 1A). Following this step, viral RNA was purified from the supernatants with the MagMAX Microbiome Ultra kit on the KingFisher Flex Purification System using 24 deep-well plates (Cat. No. 95040470) with 2 mL supernatant per well.
  - **Filtration/concentration method for larger volume:** First, heat-inactivated wastewater samples were aliquoted into 50 mL conical tubes and centrifuged at 4,560 x *g* for 30 min to eliminate large debris that can clog the filter. Following centrifugation, the supernatant was filtered through a 0.45 µm filter unit to capture the viral particles. Next, the filter was cut into pieces with a razor blade and subjected to bead beating. Lastly, viral RNA was purified from the supernatants with the MagMax Microbiome Ultra kit on the KingFisher Flex Purification System using a 96 deep-well plate (Figure 1B).
- Purified viral RNA was analyzed for the presence of SARS-CoV-2 using the Applied Biosystems™ TaqMan® 2019-nCoV Assay Kit v1 (Cat. No. A47532), TaqMan® Fast Virus 1-Step Master Mix (Cat. No. 4444432), and QuantStudio™ 12K Flex Real-Time PCR System (4471134).

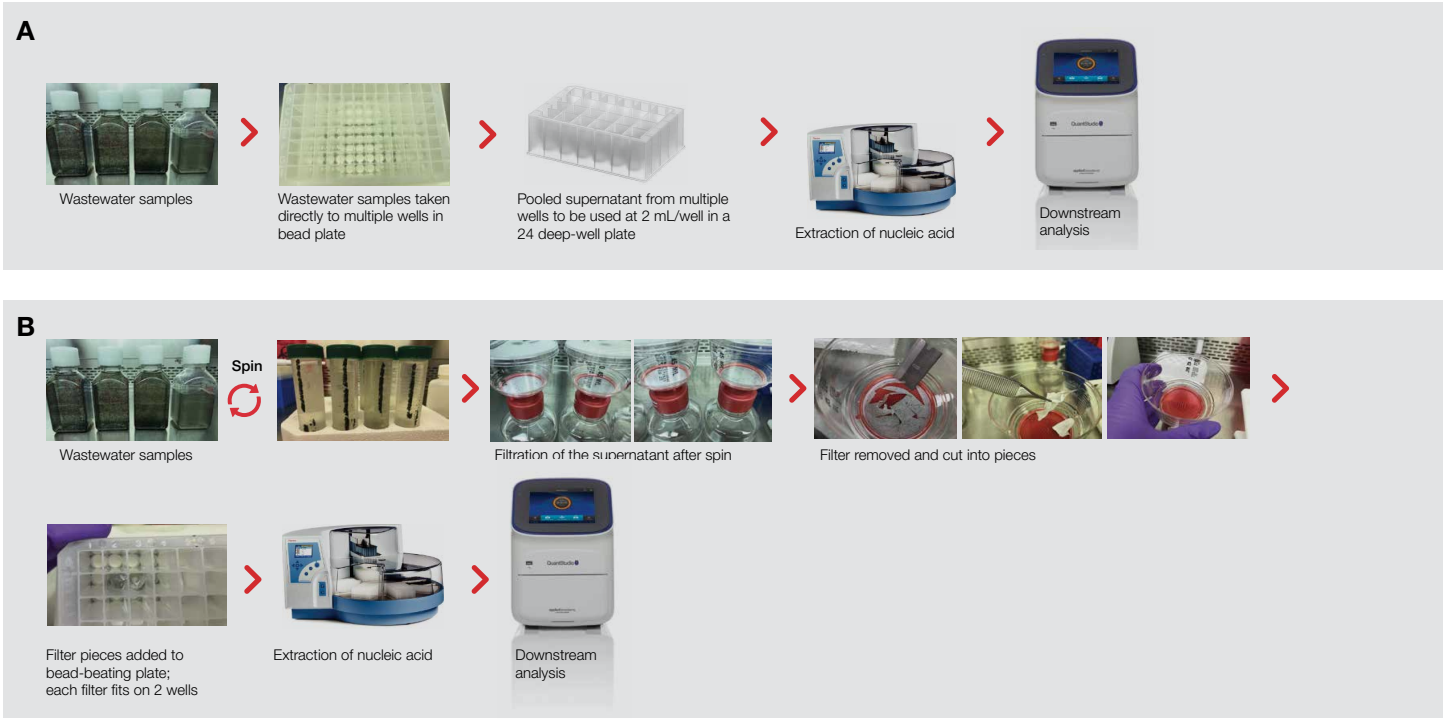

Figure 1. Workflows for analysis of SARS-CoV-2 levels in wastewater samples. (A) "Direct" method for small-volume samples. **(B)** "Filtration/concentration" method for large-volume samples.

Results

Testing for SARS-CoV-2 in fecal samples and rectal swabs

First, fecal samples from 10 donors and rectal swabs (preserved in storage media) from 12 donors were spiked with inactivated SARS-CoV-2 virus. Next, viral RNA was extracted using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit, and qPCR analysis was performed. TaqMan Assays for the following viral genes were utilized: N gene, *orf1ab*, and S gene. To assess carryover of PCR inhibitors in the samples, Applied Biosystems™ Xeno™ Internal Positive Control (IPC) RNA was spiked into the fecal and rectal swab samples following bead beating. The data were compared to the controls (Figures 2–5). SARS-CoV-2 was efficiently detected in fecal and rectal swab samples derived from all 10 and 12 donors, respectively, as indicated by the change in C_t values following four decreasing spike-in amounts. None of the samples showed inhibitor carryover according to the Xeno assay results, indicating that the MagMax Microbiome Ultra kit enables efficient purification of RNA/DNA from the challenging sample types.

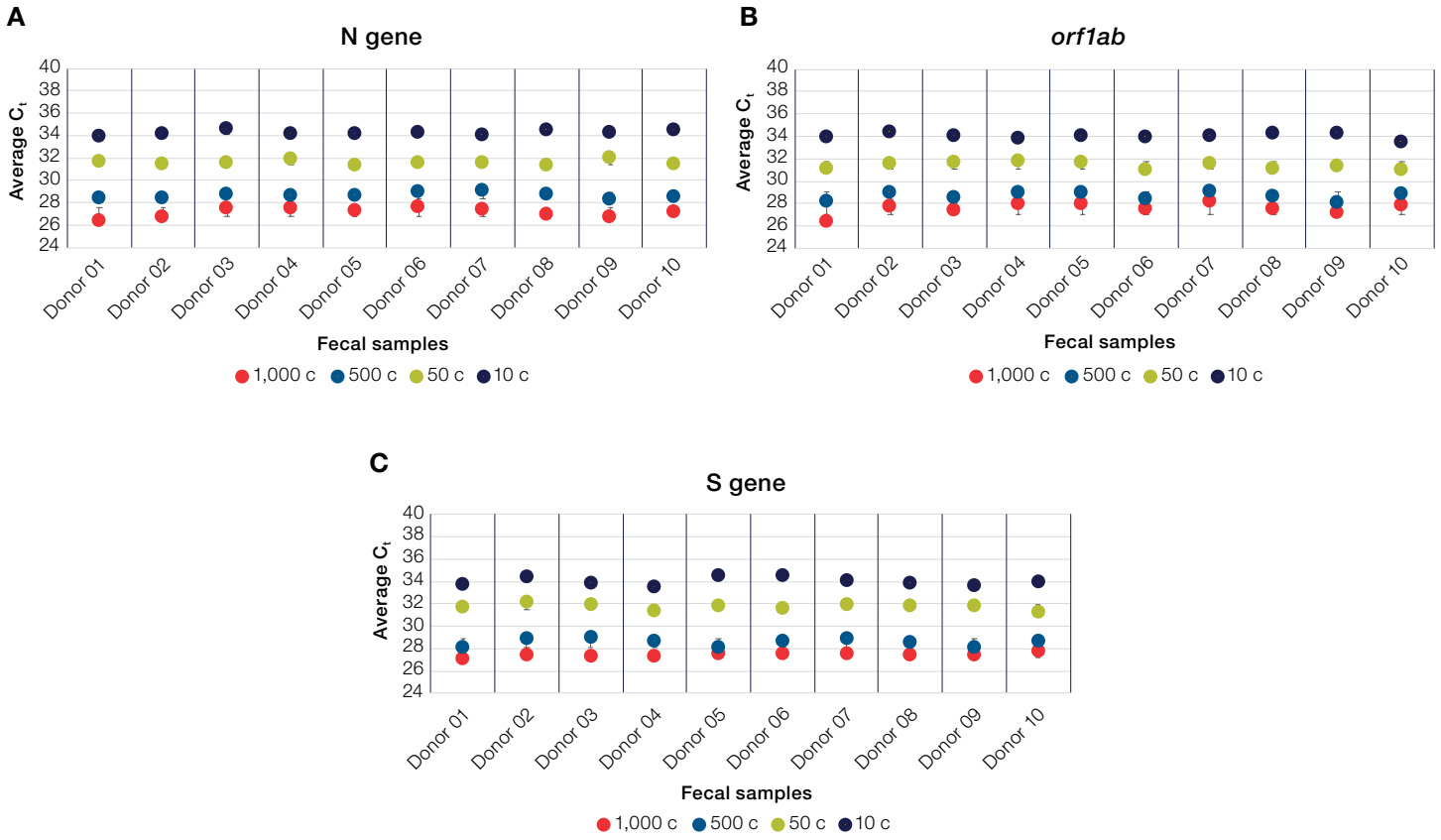

Figure 2. Detection of SARS-CoV-2 in fecal samples from 10 donors. Fecal samples (100 mg) were spiked with inactivated SARS-CoV-2 virus (1,000, 500, 50, and 10 copies) and nucleic acid was extracted in duplicate using the MagMAX Microbiome Ultra kit with a bead-beating plate. 25 µL of the 50 µL eluate were tested by RT-qPCR using the TaqMan 2019-nCoV Assay Kit v1. The following genes were analyzed: **(A)** N gene, **(B)** *orf1ab*, and **(C)** S gene. All negative samples had undetermined C_t values (>40; data not shown).

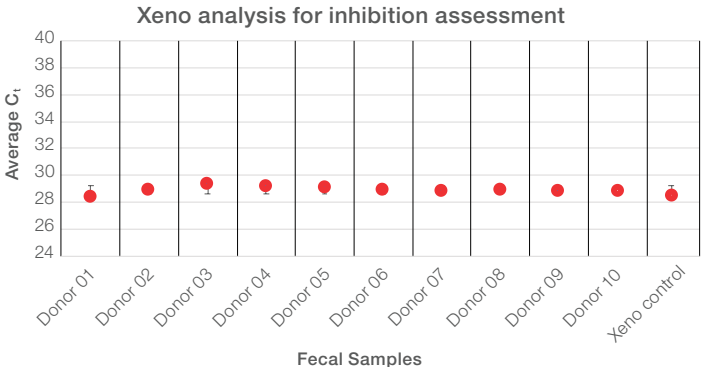

Figure 3. Evaluation of PCR inhibition in fecal-derived nucleic acid samples. 100,000 copies of Xeno IPC RNA were spiked into fecal-derived samples after bead beating, then nucleic acid extraction was carried out using the MagMAX Microbiome Ultra kit. Total nucleic acid was eluted in 200 µL volume and 40% of the input volume was used for the TaqMan Assay with Xeno control. None of the samples had PCR inhibitor carryover as indicated by C_t values that were similar to the Xeno control.

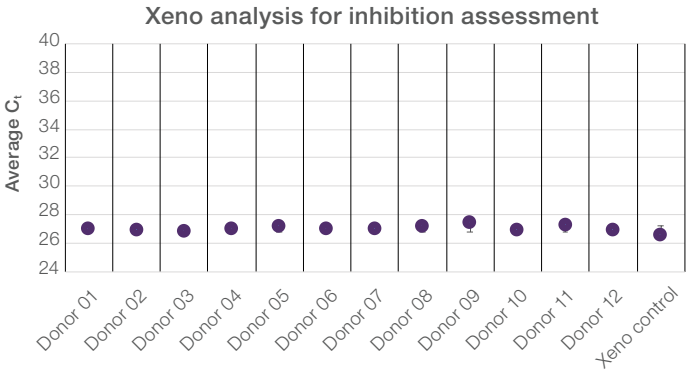

Figure 5. Evaluation of PCR inhibition in rectal swab–derived nucleic acid samples. 100,000 copies of Xeno IPC RNA were spiked into rectal swab samples after bead beating, then extraction was carried out using the MagMAX Microbiome Ultra kit. Total nucleic acid was eluted in 50 µL volume and 40% of the input volume was used for a TaqMan Assay. None of the samples had PCR inhibitor carryover as indicated by C_t values that were similar to the Xeno control.

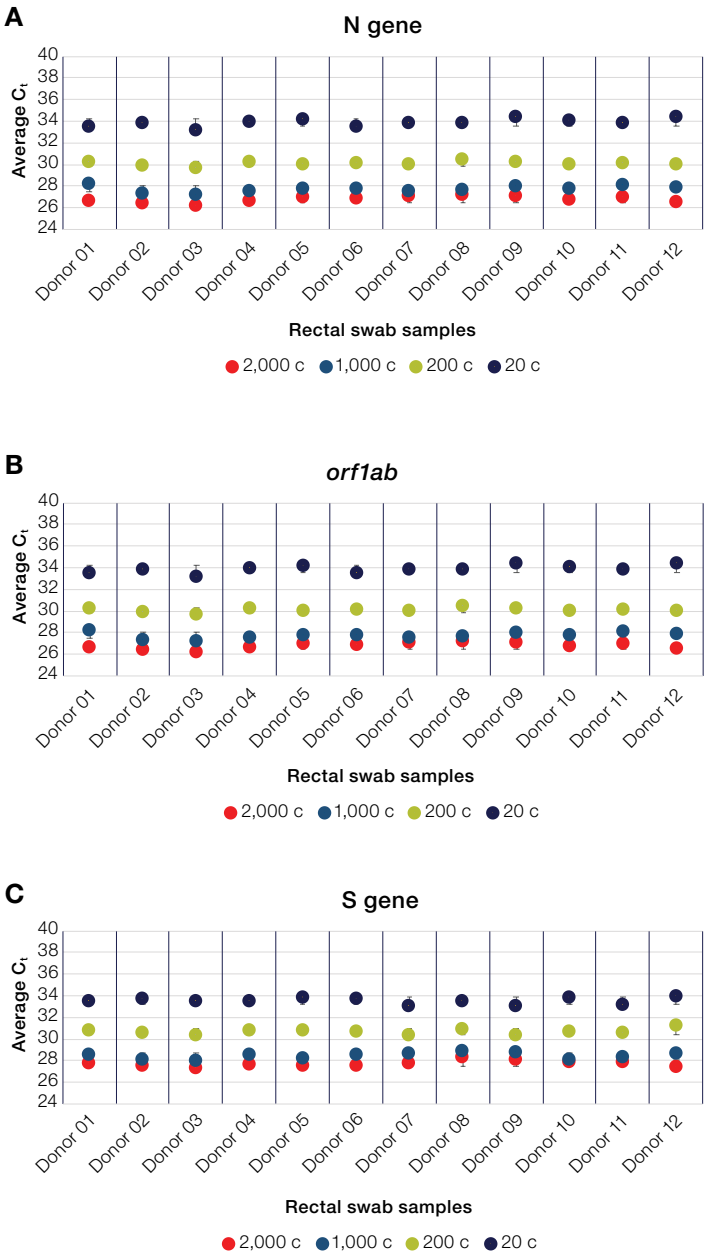

Figure 4. Detection of SARS-CoV-2 in rectal swab samples from 12 donors. Rectal swab samples (in 200 µL preservative) were spiked with inactivated SARS-CoV-2 virus (2,000, 1,000, 200, and 20 copies) and nucleic acid was extracted in duplicate using the MagMAX Microbiome Ultra kit with bead-beating homogenizer. After elution of nucleic acid in 50 µL volume, 25 µL of it were tested by RT-qPCR using the TaqMan 2019-nCoV Assay Kit v1. The following genes were analyzed: **(A)** N gene, **(B)** *orf1ab*, and **(C)** S gene. All negative samples had undetermined C_t values (>40; data not shown).

Detection of SARS-CoV-2 in wastewater samples

Raw untreated wastewater influent samples—i.e., before primary, secondary, and tertiary treatments—were collected at two different wastewater reclamation facilities in north Georgia, USA. Sample bottles were rinsed with deionized water, three times, prior to collection. Two 250 mL high-density polyethylene (HDPE) containers were used to collect samples at each site. Personal protective equipment (PPE) associated with collection included N95 face masks, nitrile gloves, and safety glasses. Confined space entry was not required for sample collection. Grab samples were obtained via automatic sampler at permitted influent sites at each facility using an autosampler with a pump head, a peristaltic pump connected to sample tubing, and a tubing strainer lowered into the wastewater stream or flow. The wastewater sample line was cleared prior to collection by flushing the automatic sampler line. The harvested samples were characterized based on the parameters shown in Table 1.

The harvested wastewater samples were heat-treated at 65˚C for 1 hr to inactivate any pathogens potentially present. The two different methods ("Direct" and "Filtration/concentration" protocols), described above in the "Methods" section, were used for isolation of viral and bacterial nucleic acid from small (0.5–10 mL) and large (up to 1 L) sample volumes, respectively. For either method, RT-qPCR tests for SARS-CoV-2 were performed using TaqMan Assays for the following genes: N gene, *orf1ab*, and S gene.

Initial tests showed that all four wastewater samples contained low levels of SARS-CoV-2 (Figure 6). Phosphate-buffered saline (PBS) was used as a negative control, which resulted in expected undetermined C_t values (>40) for all targets. Both protocols performed well, yielding efficient detection of the virus.

Next, known amounts of inactivated SARS-CoV-2 (4,000 and 8,000 copies) were spiked into wastewater samples (5 mL), and samples were processed following the "Direct" protocol. Results in Figure 7 show that SARS-CoV-2 was efficiently recovered and detected for both spike-in amounts. The Xeno assay displayed C_t values similar to control, indicating that no PCR inhibitors were carried over to the eluate during the nucleic acid purification.

Finally, known amounts of inactivated SARS-CoV-2 (2,500 and 5,000 copies) were spiked into wastewater samples (50 mL), and were processed following the "Filtration/concentration" protocol. Results in Figure 8 show that SARS-CoV-2 was efficiently recovered and detected for both spike-in amounts. The Xeno assay displayed C_t values similar to control, indicating that no PCR inhibitors were carried over to the eluate during the nucleic acid purification.

Following filtration, the flow-through sample was also tested for SARS-CoV-2 levels and all three targets were "undetected" (C_t values >40)—indicating that all viral particles were successfully captured on the filter.

Table 1. Analytical characterization of wastewater samples using the Thermo Scientific™ Orion™ Versa Star Pro™ pH/ISE/Conductivity/Dissolved Oxygen Multiparameter Benchtop Meter.

Samples	pH	Conductivity (µs/cm)	Oxidation-reduction potential (ORP) (mV)	Ion selective electrodes (ISE) (PPM)
Wastewater sample 1	6.99	1,255	341	34
Wastewater sample 2	6.97	1,104	337	38
Wastewater sample 3	6.78	812	191	18
Wastewater sample 4	7.02	885	211	18
Nuclease-free water	6.99	2	289	1.5

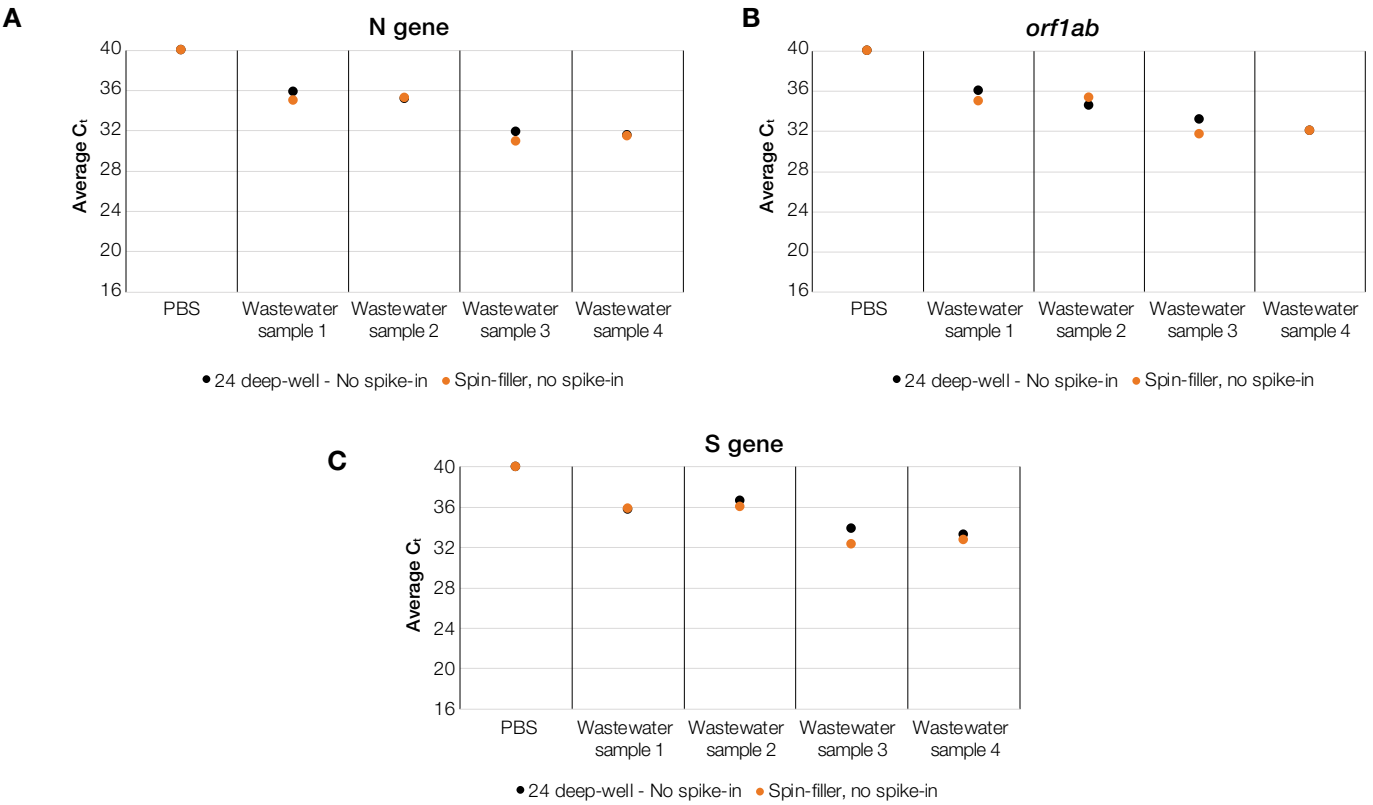

Figure 6. Testing for the presence of SARS-CoV-2 in wastewater samples. Viral/microbial nucleic acid was purified from the heat-inactivated wastewater samples following two protocols—directly with the MagMax Microbiome Ultra kit on the KingFisher Flex Purification System (black dots), or with initial sample concentration on a filter (green dots) followed by purification using the MagMAX Microbiome Ultra kit on the KingFisher Flex Purification System. Next, RT-qPCR was performed with TaqMan Assays for the following genes: **(A)** N gene, **(B)** *orf1ab*, and **(C)** S gene.

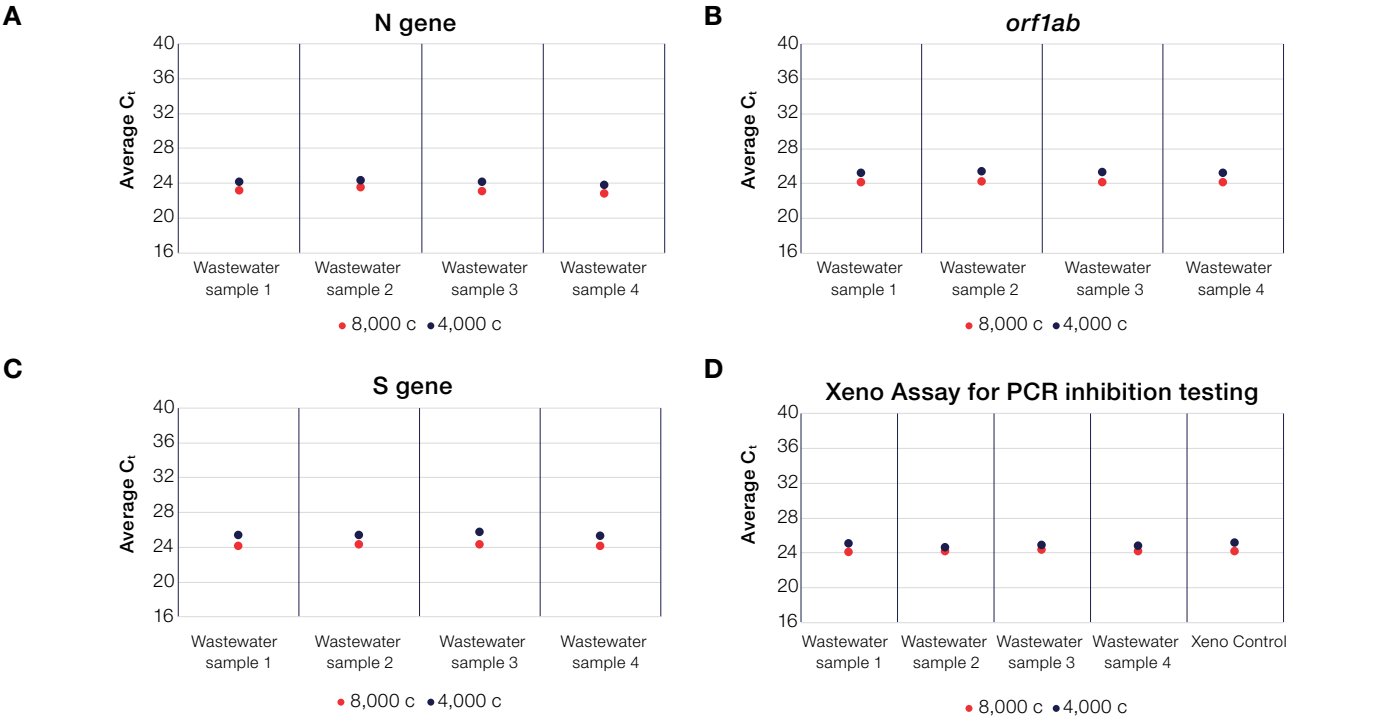

Figure 7. Detection of spiked-in inactivated SARS-CoV-2 in wastewater samples (4,000 and 8,000 copies): "Direct" protocol. Viral/microbial nucleic acid was purified from the wastewater samples with the MagMAX Microbiome Ultra kit on the KingFisher Flex Purification System following the "Direct" protocol. RT-qPCR was performed with TaqMan Assays for the following genes: **(A)** N gene, **(B)** *orf1ab,* and **(C)** S gene. **(D)** A TaqMan Assay with Xeno control was used for evaluation of PCR inhibition in wastewater-derived nucleic acid samples.

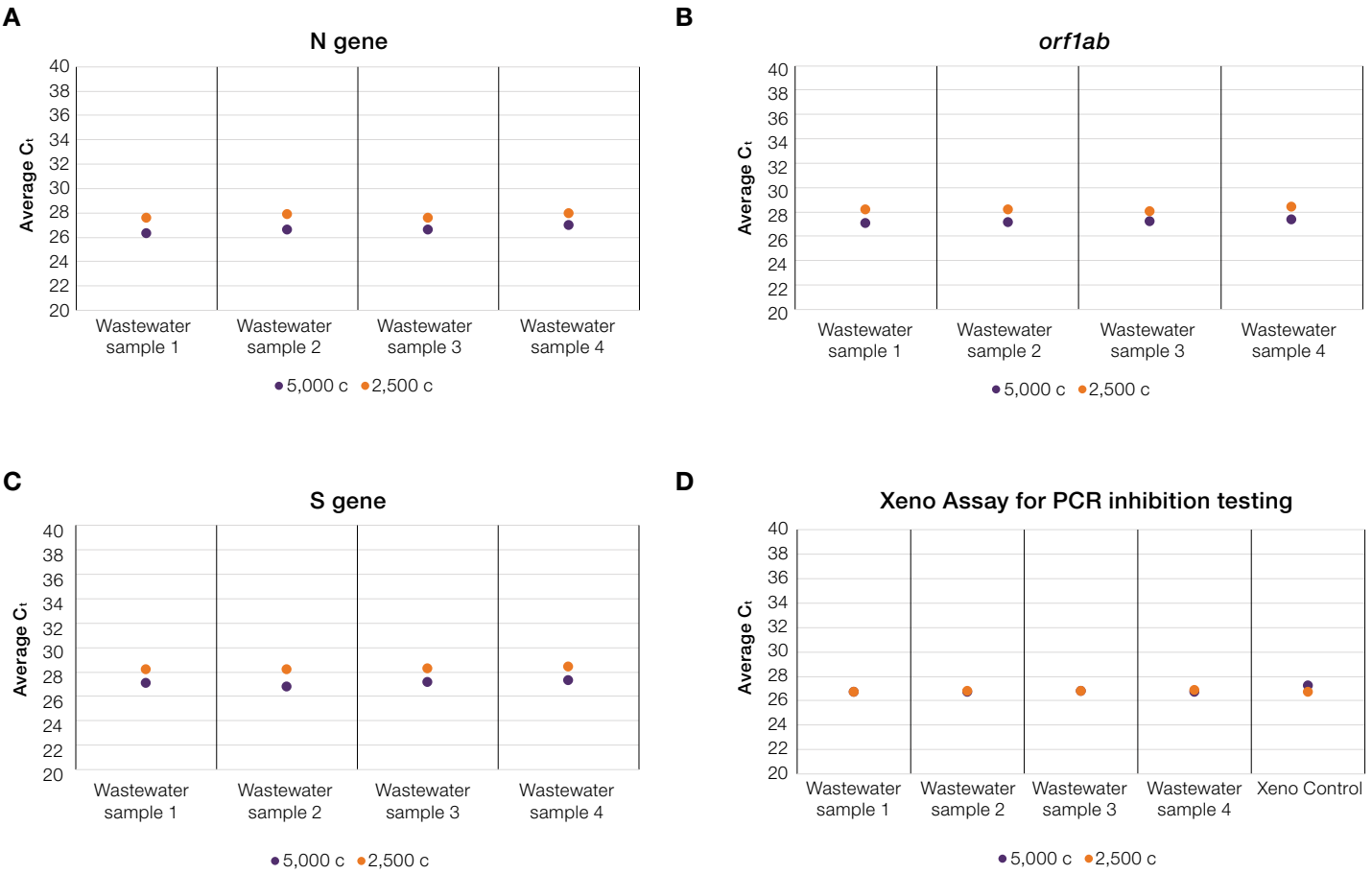

Figure 8. Detection of spiked-in inactivated SARS-CoV-2 in wastewater samples (2,500 and 5,000 copies): "Filtration/concentration" protocol. Viral/microbial nucleic acid was concentrated on the filter and purified with the MagMax Microbiome Ultra kit on the KingFisher Flex Purification System. RT-qPCR was performed with TaqMan Assays for the following genes: **(A)** N gene, **(B)** *orf1ab,* and **(C)** S gene. **(D)** A TaqMan Assay with Xeno control was used for evaluation of PCR inhibition in wastewater-derived nucleic acid samples.

Conclusion

The MagMAX Microbiome Ultra Nucleic Acid Isolation kit allows efficient processing of fecal samples, rectal swabs, and wastewater samples for SARS-CoV-2 testing. The RNA/DNA purification process can be automated on the KingFisher Flex Purification System. The workflows for testing SARS-CoV-2 described in this study can potentially be utilized to study other viruses and microbial pathogens.

Note: Custom script available for 24-deep well plate on the KingFisher Flex Purification System. Submit a request to the technical support team by visiting **thermofisher.com/contactus**

References

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