Robust detection of SARS-CoV-2 in wastewater samples

Workflows for low, medium, and large sample volumes

Introduction

Wastewater analysis has been widely adopted by scientists as a method to effectively detect and monitor the presence and spread of infectious diseases. With the emergence of SARS-CoV-2 infections, multiple organizations worldwide are working on SARS-CoV-2 surveillance methods using wastewater to monitor regional or city-level spread of the virus [1], analyze individual dorms on campuses to identify asymptomatic carriers, capture emerging mutations, and respond to possible infections. Methods of detection and surveillance for SARS-CoV-2 are outlined in this paper and can potentially be adapted for all pathogens, including viruses, bacteria, and fungi.

Traces of SARS-CoV-2 have been detected in sludge derived from wastewater treatment plants [2], municipal sewage [3], wastewater [4,5], medical wastewater [6], wastewater from commercial cruise ships and commercial passenger aircraft [7], nonpotable water [8], and river water [4,9,10].

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 via centrifugation at 4,650–4,750 x *g* for 30 min [3,7] or by sequential filtration through 20 µm and 5 µm filters [11].

It is important to harvest optimal volumes of wastewater for the detection of pathogens, taking into account the low abundance of viral particles and nucleic acids. Based on reports, 10–250 mL of untreated wastewater is typical for the detection of viruses [3,7]. The wastewater samples can be concentrated using one of the following approaches:

- Ultracentrifugation at 200,000 x *g* for 1 hr [12]
- Membrane filtration with a 0.45 µm filter [7,11]
- Concentration using a 10–100 kDa molecular weight cutoff (MWCO) membrane [3,7,11]
- Magnetic bead–based concentration using Invitrogen™ Dynabeads™ Intact Virus Enrichment beads (Cat. No. 10700D)

Once the samples are concentrated, viral nucleic acid is purified using either a spin column–based kit or a magnetic bead–based kit. Next, viral nucleic acid is detected by downstream quantitative PCR (qPCR), digital PCR (dPCR), loop-mediated isothermal amplification (LAMP), or next-generation sequencing (NGS).

A previous application note [13] outlines two protocols for processing small (1–2 mL) and large (>200 mL) volumes of wastewater. Those protocols enable efficient detection of SARS-CoV-2 in low-throughput settings. The protocols described here are extensions of those methods and have been optimized for processing multiple samples. They include a fully automated workflow for 10 mL samples to enable high-throughput processing; the similar workflow already has been successfully used in the field [14].

Materials and methods

Wastewater samples

Raw wastewater influents that had not undergone primary, secondary, or tertiary treatment were collected at two different wastewater reclamation facilities in north Georgia, USA, using 250 mL high-density polyethylene containers. Personal protective equipment 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 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.

Upon arrival, the wastewater samples were heat-inactivated and stored at 4°C. Inactivated SARS-CoV-2 viral particles (BEI Resources, Cat. No. NR-52287) were spiked in at a later point to analyze the efficiency of the developed protocols for wastewater concentration and molecular testing.

Direct processing of 1 mL wastewater samples without up-front concentration or mechanical homogenization

Wastewater samples spiked with inactivated SARS-CoV-2 were directly processed using 1 mL of wastewater combined with 1 mL of lysis buffer from the Applied Biosystems™ MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Cat. No. A42357). Samples were processed in 24 deep-well plates on the Thermo Scientific™ KingFisher™ Flex Purification System (Cat. No. 5400640) using a custom script (Figure 1).

It has been found that bead beating does not enhance SARS-CoV-2 viral RNA recovery, so this step can be omitted. However, mechanical disruption is necessary to obtain full microbiome profiles for wastewater samples, which contain difficult-to-lyse bacteria and fungi. Proteinase K treatment was found to be important for efficient recovery of SARS-CoV-2 RNA from the wastewater samples.

Processing 10 mL wastewater samples using beads for virus enrichment

For each 10 mL wastewater sample spiked with SARS-CoV-2, 5 mL aliquots were transferred to separate wells in two 24 deep-well plates. Samples were concentrated by adding 100 µL of Dynabeads Intact Virus Enrichment beads to only one of the plates, and the samples were processed on the KingFisher Flex Purification System using a custom script (Figure 2). This script performed sequential binding of virus from the two plates and elution into a single plate. Nanotrap™ beads (Ceres Nanosciences) were also used for a performance comparison with Dynabeads magnetic beads.

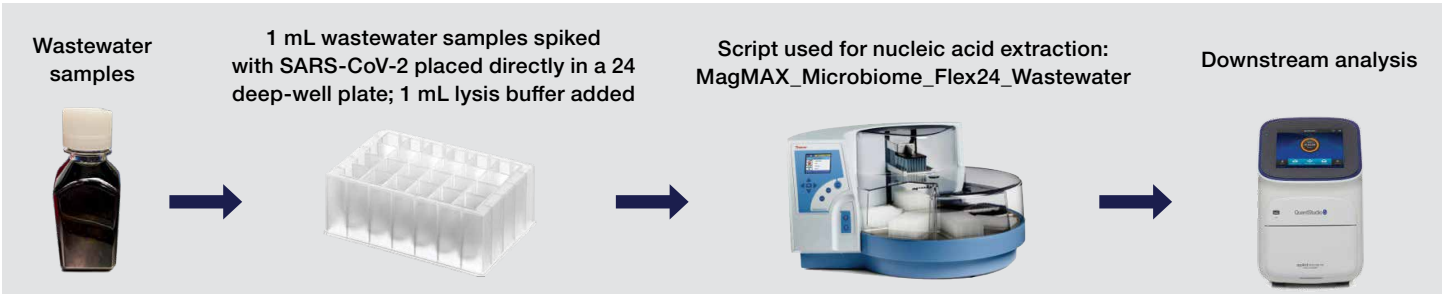

Figure 1. Direct processing of low-volume (1 mL) wastewater samples.

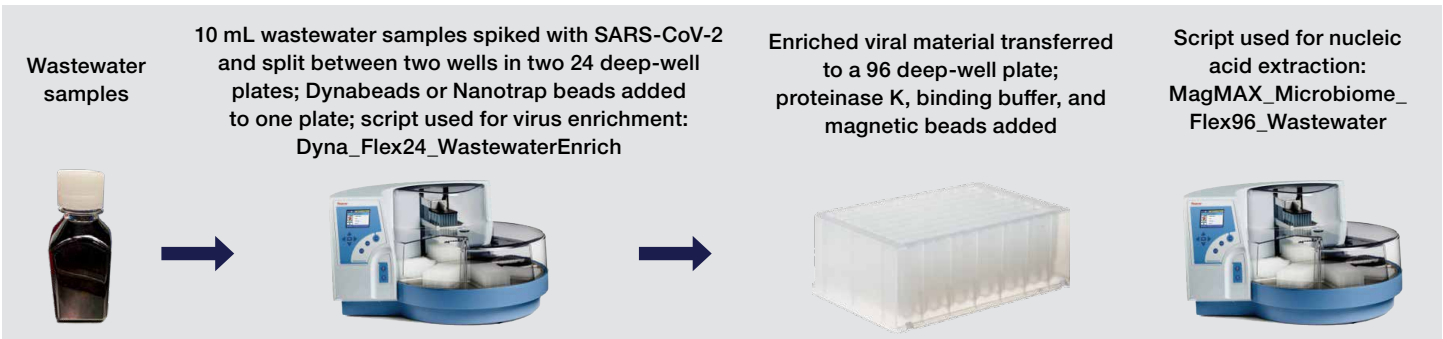

Figure 2. Processing of medium-volume (10 mL) wastewater samples.

The custom script used for the KingFisher Flex instrument (Dyna_Flex24_WastewaterEnrich) processes samples from the two sample plates and elutes the concentrated viral particles into a single elution plate containing 500 µL/well of lysis buffer from the MagMAX Microbiome Ultra kit.

From the eluate collected using the Dyna_Flex24_WastewaterEnrich script, total viral nucleic acid was purified using the MagMAX Microbiome Ultra kit on the KingFisher Flex instrument using 96 deep-well plates and the MagMAX_Microbiome_Flex96_Wastewater script. Proteinase K, binding buffer, and magnetic beads were added to the concentrated SARS-CoV-2, and the script was initiated. This protocol enables fast, easy, and reliable processing of wastewater samples in a fully automated format without inconvenient centrifugation, filtration, or bead beating steps.

Processing 50 mL wastewater samples using beads for virus enrichment

To each 50 mL wastewater sample spiked with SARS-CoV-2, 750 µL of Dynabeads Intact Virus Enrichment beads were added. The sample was vortexed briefly and incubated at room temperature on an Invitrogen™ HulaMixer™ Sample Mixer for 10 min to promote efficient virus capture in a large volume (Figure 3). Nanotrap beads were also used for a performance comparison.

The Dynabeads magnetic beads with captured virus particles were then collected using an Invitrogen™ DynaMag™ magnet. The supernatant was discarded, and the beads were resuspended in 2 mL of lysis buffer from the MagMAX Microbiome Ultra kit by briefly vortexing. The Dynabeads magnetic beads were then separated out of the suspension using a magnet, the supernatant containing enriched SARS-CoV-2 was collected, and the beads were discarded.

From the eluate containing concentrated viral particles, viral nucleic acid was purified using the MagMAX Microbiome Ultra kit on the KingFisher Flex instrument using the MagMAX_Microbiome_Flex24_Wastewater script. Samples were processed in a 24 deep-well plate after adding proteinase K, binding buffer, and magnetic beads. Manual concentration of wastewater samples using Dynabeads Intact Virus Enrichment beads can be scaled up or down depending on the input volume of wastewater being processed (5–50 mL). A summary of the automated processes used for each wastewater volume is provided in Table 1.

Downstream analysis of SARS-CoV-2 nucleic acid from processed wastewater samples

SARS-CoV-2 nucleic acid was analyzed by RT-qPCR on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System using Applied Biosystems™ TaqMan® Assays targeting SARS-CoV-2 (10 µL input).

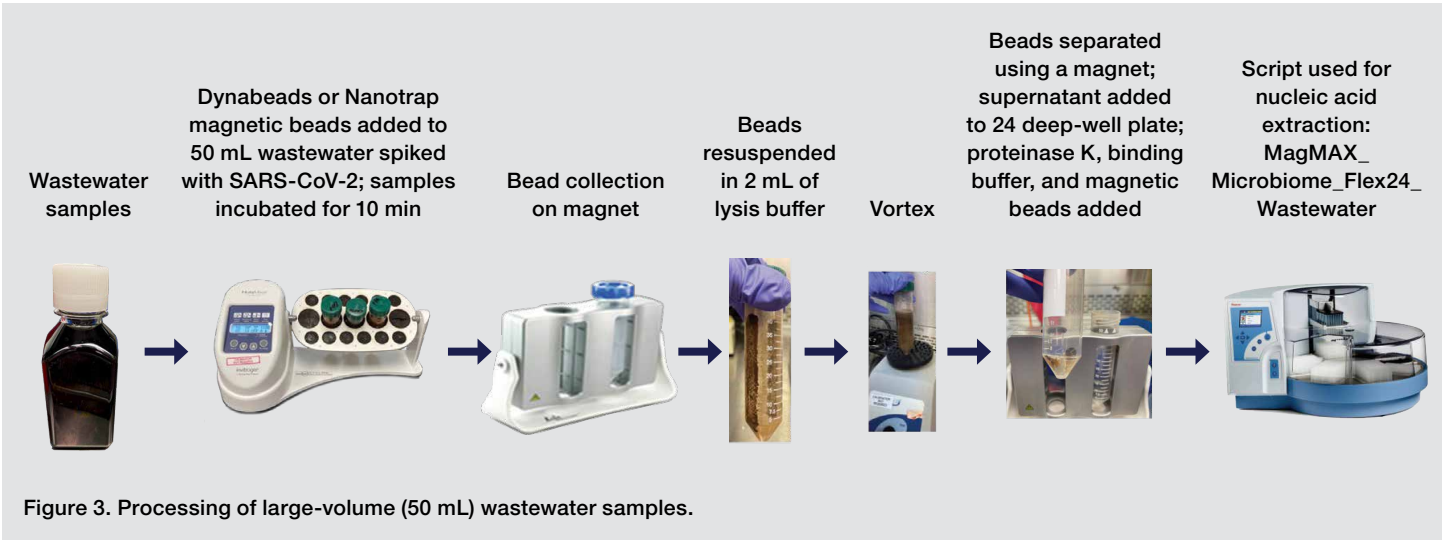

Figure 3. Processing of large-volume (50 mL) wastewater samples.

Table 1. Summary of options for processing different volumes of wastewater.

Wastewater protocol	Virus capture	Proteinase K addition	Viral RNA purification with MagMAX Microbiome Ultra kit and KingFisher Flex instrument	Total time for process
Low-volume (1 mL)	NA	1 mL wastewater combined with 1 mL lysis buffer containing 200 µL proteinase K in a 24 deep-well plate	2.5 mL of binding buffer and 100 µL of magnetic beads added for processing using the MagMAX_Microbiome_Flex24_Wastewater script	35 min
Medium-volume (10 mL)	10 mL of wastewater split into 2 wells in 24 deep-well plates Dynabeads magnetic beads (100 µL) or Nanotrap beads (150 µL) added to 24 deep-well plate Plates processed on the KingFisher Flex instrument using the Dyna_Flex24_WastewaterEnrich script Virus eluted in 500 µL of lysis buffer	Eluate from 24 deep-well plate added to 96 deep-well plate along with 40 µL proteinase K	500 µL of binding buffer and 20 µL of magnetic beads added to 96 deep-well plate for processing using the MagMAX_Microbiome_Flex96_Wastewater script	1 hr 30 min
Large-volume (50 mL)	50 mL sample transferred to 50 mL tube Dynabeads magnetic beads or Nanotrap beads (750 µL) added Sample incubated on HulaMixer Sample Mixer for 10 min Beads collected on DynaMag magnet and supernatant discarded 2 mL of lysis buffer added and supernatant with lysed virus collected	Supernatant containing lysed virus and 200 µL proteinase K added to 24 deep-well plate	2.5 mL of binding buffer and 100 µL of magnetic beads added to 24 deep-well plate for processing using the MagMAX_Microbiome_Flex24_Wastewater script	~1 hr

Results

Three robust workflow options were developed for rapid concentration and analysis of SARS-CoV-2 in different volumes of wastewater: low (1 mL), medium (10 mL), and large (50 mL).

Low-volume samples (1 mL) can be efficiently processed using the MagMAX Microbiome Ultra kit and KingFisher Flex instrument without up-front virus concentration. Results of qPCR analysis are shown in Figure 4.

Medium-volume samples (10 mL) can be rapidly concentrated using Dynabeads or Nanotrap beads, enabling robust detection by qPCR. As shown in Figure 5, 100 µL of beads was sufficient to concentrate virus in 10 mL wastewater samples. The optimal capture time was 10 min. The efficiency of virus capture was nearly 100%, compared to the PBS control without concentration. In this experiment, Dynabeads magnetic beads enabled somewhat better virus concentration than Nanotrap beads. Importantly, the workflow for 10 mL wastewater samples is fully automated on the KingFisher Flex instrument, allowing high-throughput sample processing.

Large-volume samples (50 mL) can be also concentrated using Dynabeads or Nanotrap beads. As shown in Figure 6, 750 µL of beads was optimal for virus concentration from 50 mL wastewater samples. The optimal capture time was 10 min. Dynabeads magnetic beads in this setting also yielded somewhat better virus concentrations than Nanotrap beads.

The efficiency of bead-assisted virus concentration gradually decreased with increasing wastewater volume. For 50 mL samples, the typical virus recovery efficiency was 50%. Alternative virus concentration approaches for 50 mL and larger volumes of wastewater include filtration (multiple filter materials and varied pore sizes), PEG precipitation, and ammonium sulfate precipitation.

We previously described one of the options for filtration-based concentration [13]. The main benefit of this approach is the ability to process large sample volumes (50 mL to 1 L or more). However, the process is time-consuming and inconvenient since it requires recovery of the concentrated sample from the filter, which is difficult to implement for high-throughput wastewater sample testing.

We also explored PEG 6000 and ammonium sulfate precipitation. The main benefit of precipitation is the ability to process large sample volumes (50 mL to hundreds of mL). However, the precipitation process is time-consuming (2 hr through overnight) and inconvenient. In addition, trace amounts of ammonium sulfate or other chemicals are left in the viral particle pellet, sometimes compromising the isolation of viral RNA. The typical recovery efficiency never exceeded 50% for 50–250 mL wastewater samples.

Summary

Three robust workflow options were developed for rapid concentration and qPCR analysis of SARS-CoV-2 from different volumes of wastewater: low (1 mL), medium (10 mL), and large (50 mL). Low-volume samples can be efficiently processed using the MagMAX Microbiome Ultra kit and KingFisher Flex instrument without up-front virus concentration. Medium-volume wastewater samples can be rapidly concentrated using Dynabeads or Nanotrap beads with nearly 100% efficiency, and the fully automated protocols on the KingFisher Flex instrument enable robust virus detection by qPCR. Large-volume samples can also be concentrated using Dynabeads or Nanotrap beads, but process efficiency gradually decreases as indicated by a typical recovery efficiency of 50% for 50 mL samples. Alternative virus concentration approaches for 50 mL and larger wastewater samples include filtration, PEG precipitation, and ammonium sulfate precipitation.

Note: For custom scripts, please submit a request to the technical support team at **thermofisher.com/contactus**.

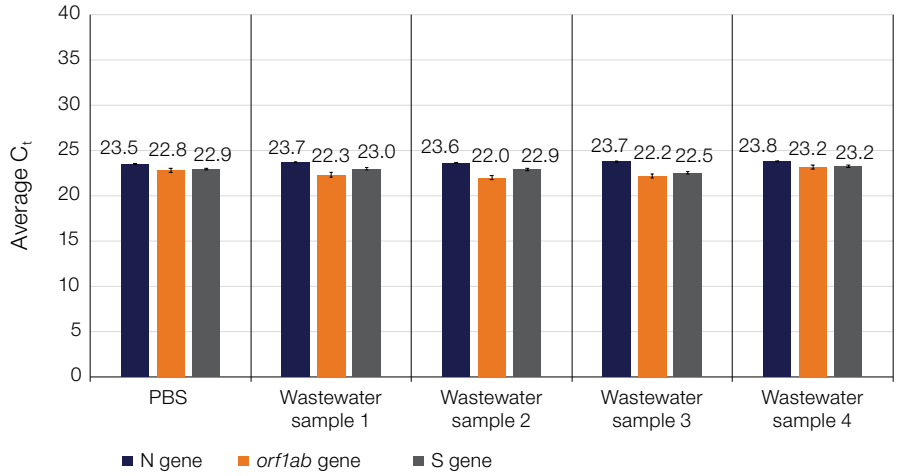

Figure 4. Low-volume direct protocol. Detection of spiked-in inactivated SARS-CoV-2 4000c was performed by qPCR after automated nucleic acid purification from 1 mL wastewater samples. PBS control: SARS-CoV-2 (4,000 copies) processed in a small volume (1 mL) without concentration.

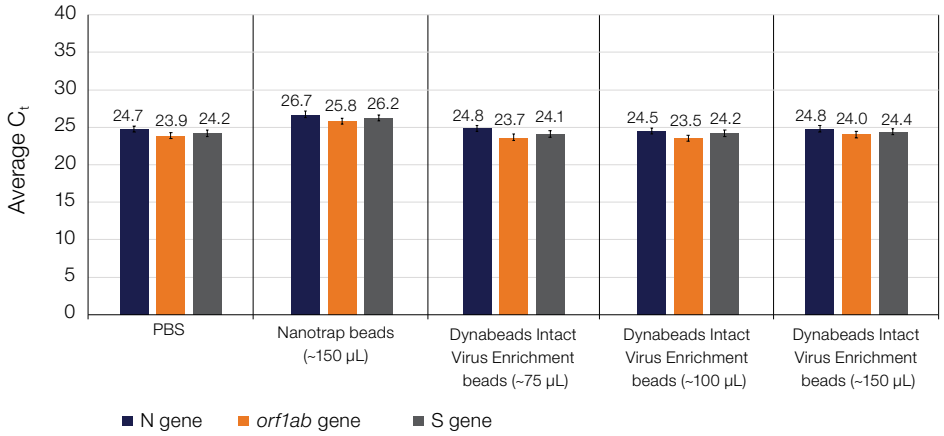

Figure 5. Medium-volume protocol. Detection of spiked-in inactivated SARS-CoV-2 (4,000 copies) was performed by qPCR after automated nucleic acid purification from 10 mL wastewater samples. Viral particles were concentrated with Dynabeads or Nanotrap beads using automation. PBS control: SARS-CoV-2 (4,000 copies) processed in a small volume (2 mL) without concentration.

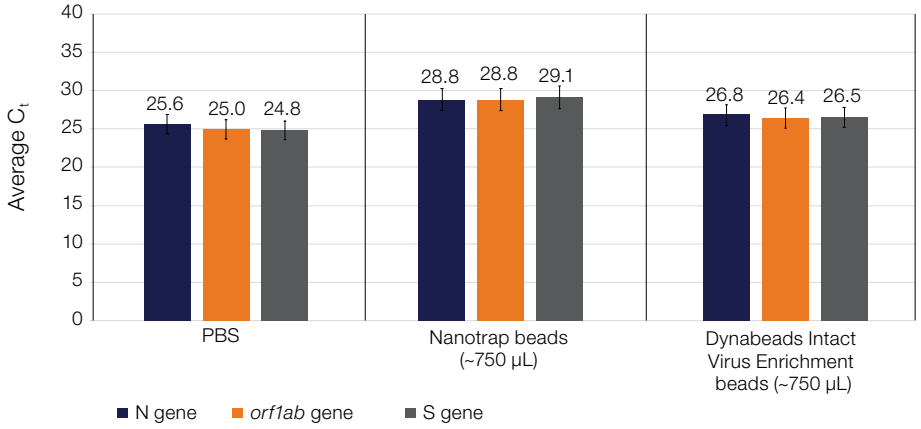

Figure 6. Large-volume protocol. Detection of spiked-in inactivated SARS-CoV-2 (2,000 copies) was performed by qPCR after automated nucleic acid purification from 50 mL wastewater samples. Viral particles were concentrated with Dynabeads or Nanotrap beads using a manual approach. PBS control: SARS-CoV-2 (4,000 copies) processed in a small volume (2 mL) without concentration.

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Ordering information

Product	Quantity	Cat. No.
Dynabeads Intact Virus Enrichment beads	100 preps	10700D
	500 preps	10701D
MagMAX Microbiome Ultra Nucleic Acid Isolation Kit, with bead plate	100 preps	A42357
MagMAX Microbiome Ultra Nucleic Acid Isolation Kit, with bead tubes	100 preps	A42358
KingFisher Flex Purification System	1 instrument	5400640
TaqMan 2019-nCoV Assay Kit v1	50 reactions	A47532
TaqMan Fast Virus 1-Step Master Mix	1 mL	4444432
QuantStudio 5 Real-Time PCR System	1 instrument	A28573

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