Multiple workflow options for detection of SARS-CoV-2 in wastewater samples

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 pathogen detection and surveillance are outlined here with a focus on SARS-CoV-2.

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 the typical volume for 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]
- PEG or ammonium sulfate precipitation
- Magnetic bead–based concentration using Applied Biosystems™ Dynabeads™ Wastewater Virus Enrichment beads (included in Cat. No. A52610)

Once the samples are concentrated, viral nucleic acid is purified using the Applied Biosystems™ MagMAX™ Wastewater Ultra Nucleic Acid Isolation 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).

The protocols described here have been optimized for processing multiple samples prepared using different up-front concentration methods. They include a fully automated workflow for 10 mL samples to enable high-throughput processing; a similar workflow already has been successfully used in the field [13,14]. Also described is a procedure for direct isolation of nucleic acids from 1 mL wastewater samples without concentration.

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 (BEI Resources, Cat. No. NR-52287) was spiked in at a later point to analyze the efficiency of the developed protocols for wastewater concentration and molecular testing.

Processing of 10 mL wastewater samples using beads for virus enrichment

For processing of 10 mL samples, wastewater samples spiked with SARS-CoV-2 were vortexed at high speed and centrifuged for 10–15 min at 10,000 x *g*. Clean supernatant was transferred into two 24 deep-well plates with 5 mL added to each plate. Dynabeads Wastewater Virus Enrichment beads (100 µL) were added to only one of the plates for sample concentration. The samples were processed on the Thermo Scientific™ KingFisher™ Flex, Apex, or Duo Prime Purification System using a custom script (Figure 1).

The custom script used for the KingFisher Flex, Apex, and Duo Prime instruments 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 Wastewater Ultra kit. From the eluate collected using the custom enrichment script, total viral nucleic acid was purified using the MagMAX Wastewater Ultra kit on the KingFisher Flex, Apex, or Duo Prime instrument using 96 deep-well plates and a custom script (Figure 1). 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 an automated format without inconvenient filtration, precipitation, or bead beating steps.

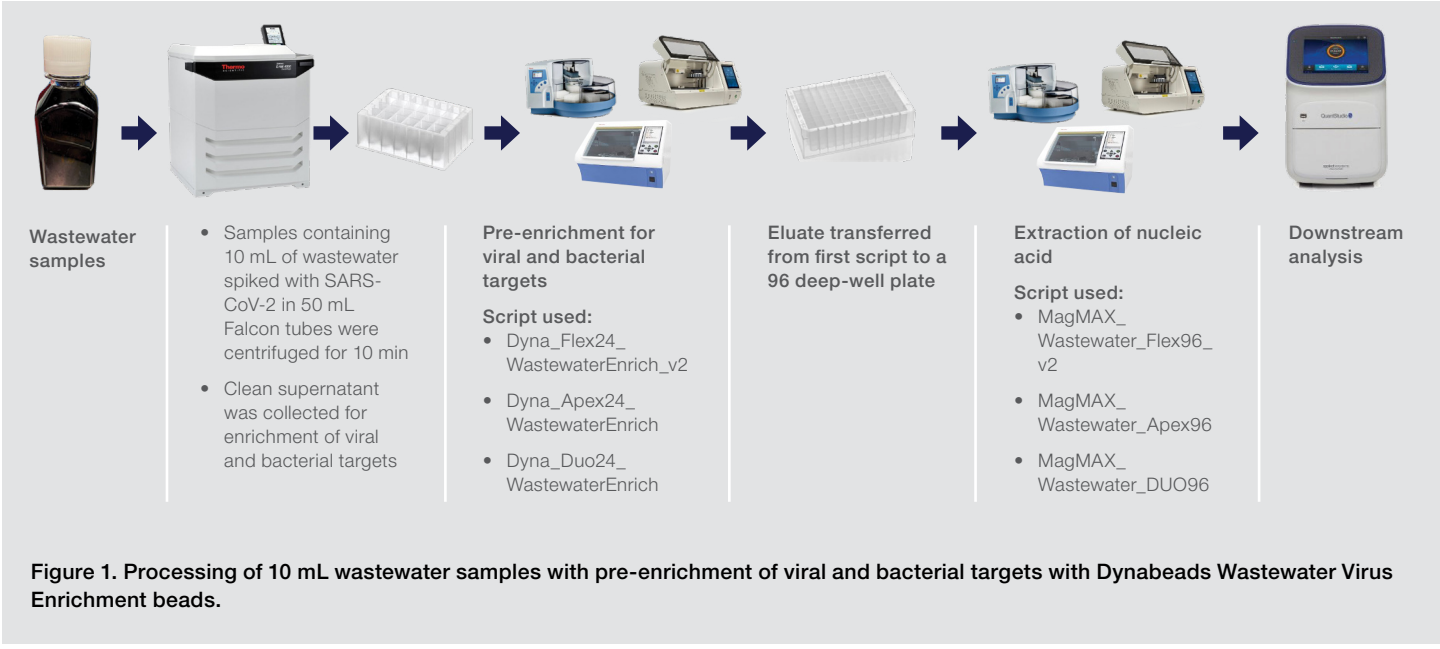

Figure 1. Processing of 10 mL wastewater samples with pre-enrichment of viral and bacterial targets with Dynabeads Wastewater Virus Enrichment beads.

Processing of 200 µL pre-concentrated samples

Note: Wastewater samples should be pre-concentrated to a volume of 200 µL using ultracentrifugation or precipitation prior to starting this protocol.

Wastewater samples spiked with inactivated SARS-CoV-2 were processed using 200 µL of wastewater combined with 200 µL of lysis buffer from the MagMAX Wastewater Ultra kit. Samples were vortexed and centrifuged at 10,000 x *g* for 2 min to collect the clean supernatant for isolation of nucleic acid. These clean supernatants of ~400 µL were processed in 96 deep-well plates on the KingFisher Flex, Apex, or Duo Prime Purification System using a custom script (Figure 2).

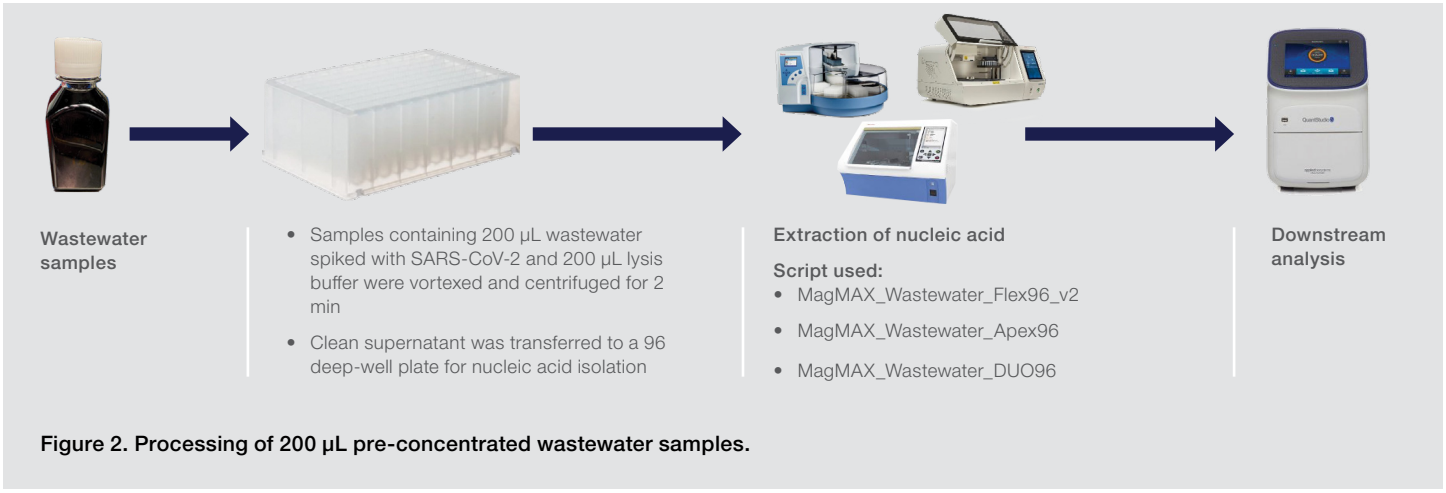

Figure 2. Processing of 200 µL pre-concentrated wastewater samples.

Processing of 1 mL direct or pre-concentrated wastewater samples

Wastewater samples (1 mL) spiked with inactivated SARS-CoV-2 were mixed with 1 mL of lysis buffer from the MagMAX Wastewater Ultra kit. Samples were vortexed at high speed and centrifuged at 10,000 x *g* for 2 min to collect the clean supernatant for isolation of nucleic acid. Samples were processed in 24 deep-well plates on the KingFisher Flex, Apex, or Duo Prime Purification System using a custom script (Figure 3). The same protocol can be used for wastewater samples that have been pre-concentrated to a final volume of 1 mL using ultracentrifugation or precipitation.

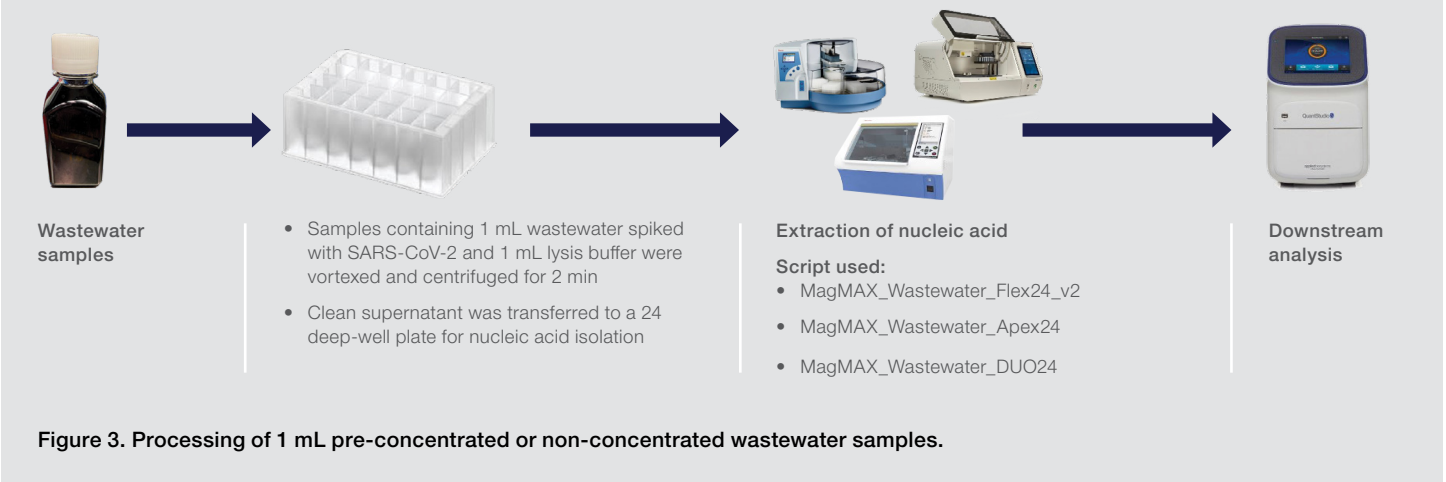

Figure 3. Processing of 1 mL pre-concentrated or non-concentrated wastewater samples.

Processing of 50–500 mL wastewater concentrated via filtration

Wastewater samples (50–500 mL) spiked with inactivated SARS-CoV-2 were centrifuged at 10,000 x *g* for 10 min with the deceleration speed set at 5, and the supernatant was filtered using Thermo Scientific™ Nalgene™ Rapid-Flow™ 0.45 µm nylon filters (Cat. No. 150-0045). The filter membrane was taken out onto the filter lid using forceps after a few cuts were made on the membrane with a razor blade. The membrane was cut into small pieces with a razor blade, and pieces were added to a 15 mL Falcon™ tube. Lysis buffer (2 mL) from the MagMAX Wastewater Ultra kit was added to the tube holding the membrane pieces, and the tube was vortexed at high speed first with the bottom of the tube making contact with the vortex and then with the cap of the tube contacting the vortex. Proteinase K was added to the tube, and the tube was incubated at 65°C for 20 min. After incubation, tubes were centrifuged at 4,000 x *g* for 3 min to collect the supernatant. The collected supernatants were processed in 24 deep-well plates on the KingFisher Flex, Apex, or Duo Prime Purification System using a custom script (Figure 4).

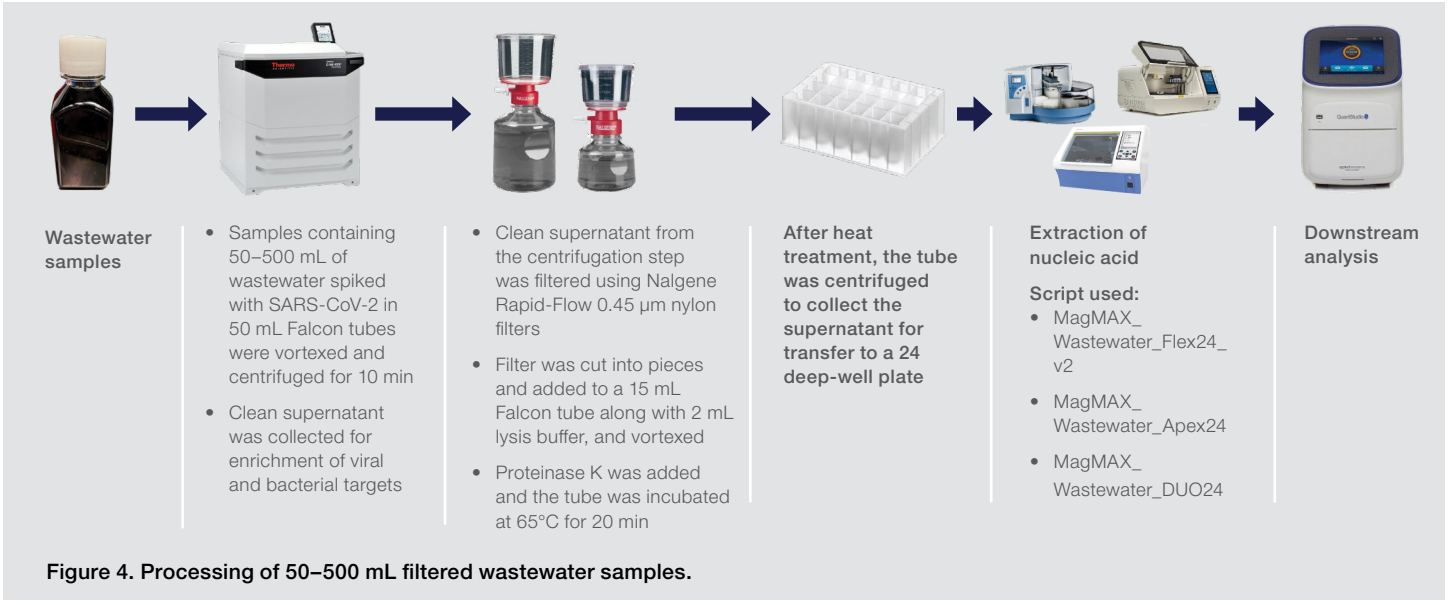

Figure 4. Processing of 50–500 mL filtered wastewater samples.

Processing of sludge samples

Sludge samples (200 mg) spiked with inactivated SARS-CoV-2 were processed using 2 mL of lysis buffer from the MagMAX Wastewater Ultra kit. Samples were vortexed at high speed and centrifuged at 10,000 x *g* for 2 min. Clean supernatant was processed in 24 deep-well plates on the KingFisher Flex, Apex, or Duo Prime Purification System using a custom script (Figure 5).

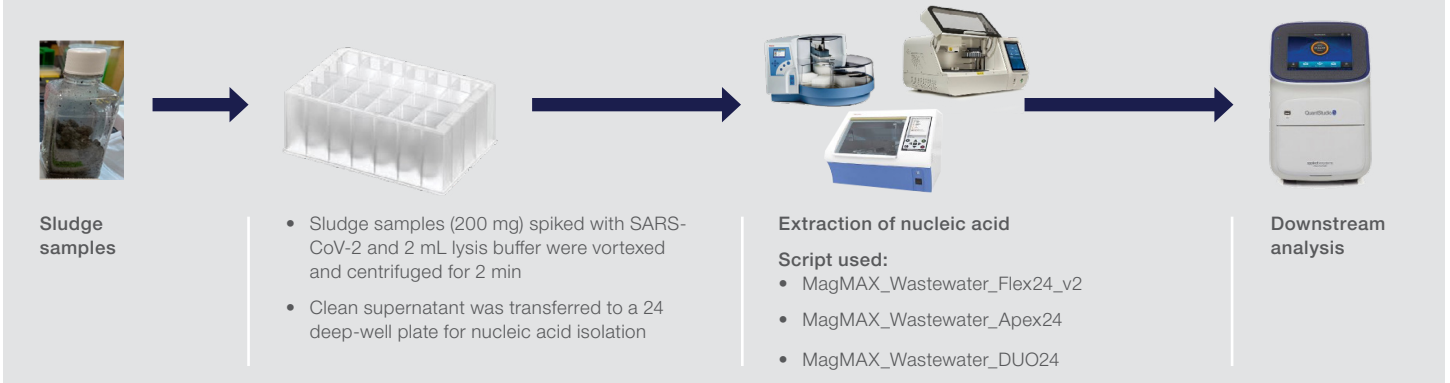

Figure 5. Processing of 200 mg sludge samples.

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

The protocols used for processing of wastewater samples are summarized in Table 1. Following nucleic acid isolation, SARS-CoV-2 RNA was analyzed by qPCR on the Applied Biosystems™ QuantStudio™ 5 or 7 Real-Time PCR System using Applied Biosystems™ TaqMan® Assays targeting SARS-CoV-2 (10–14 µL elution input).

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

Protocol	Wastewater starting amount	Sample details	Number of preps per kit	Instrument scripts available	Protocol time	Required kit	Cat. No.
1	10 mL	Concentration of wastewater using Dynabeads magnetic beads, followed by nucleic acid isolation	100	KingFisher Flex, Apex, Duo Prime	90 min	MagMAX Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment	A52610
2	200 µL	Wastewater pre-concentrated using a preferred method (e.g., ultracentrifugation)	100	KingFisher Flex, Apex, Duo Prime	45 min	MagMAX Wastewater Ultra Nucleic Acid Isolation Kit	A52606
3	1 mL	Non-concentrated wastewater or wastewater concentrated using a preferred method (e.g., precipitation)	20	KingFisher Flex, Apex, Duo Prime	45 min		
4	50–150 mL	Concentration of wastewater using filtration, followed by nucleic acid isolation	20	KingFisher Flex, Apex, Duo Prime	< 2 hr		
5	200 mg	Unprocessed sludge	20	KingFisher Flex, Apex, Duo Prime	45 min		

Results

Processing of 10 mL wastewater samples

Samples of 10 mL volume can be rapidly concentrated using Dynabeads magnetic beads, enabling robust detection of SARS-CoV-2 RNA by qPCR. As shown in Figure 6, 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. Other viral and bacterial targets (Zeptometrix Respiratory Panel Controls) were successfully tested for all five methods, but data is shown only for the 10 mL protocol (Figure 7).

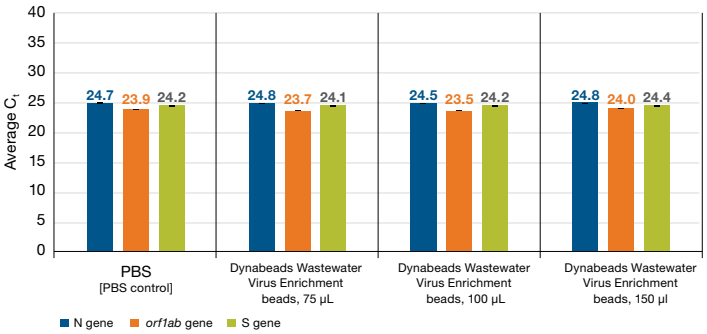

Figure 6. Processing of 10 mL wastewater for detection of SARS-CoV-2 RNA. Detection of spiked-in inactivated SARS-CoV-2 (4,000 copies) was performed by qPCR after automated nucleic acid purification from 10 mL wastewater samples. Virus was concentrated with Dynabeads Wastewater Virus Enrichment beads. PBS control: SARS-CoV-2 (4,000 copies) processed in a small volume (2 mL) without concentration.

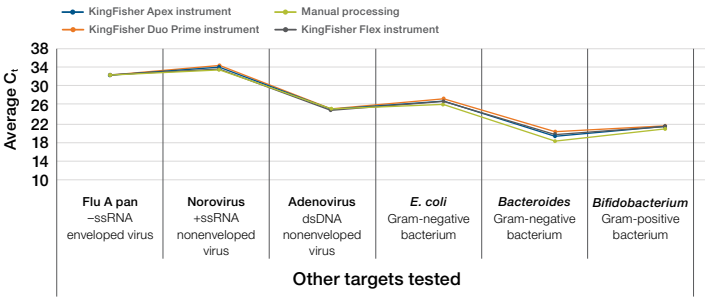

Figure 7. Processing of 10 mL wastewater for detection of other viral and bacterial targets. Detection of spiked-in ZeptoMetrix™ Respiratory Panel Controls was performed by qPCR after automated nucleic acid purification from 10 mL wastewater samples. Virus was concentrated with Dynabeads Wastewater Virus Enrichment beads.

Processing of 200 µL (pre-concentrated) wastewater samples

Pre-concentrated 200 µL samples can be efficiently processed using the MagMAX Wastewater Ultra kit and KingFisher Flex, Apex, or Duo Prime instrument. Results of qPCR analysis are shown in Figure 8.

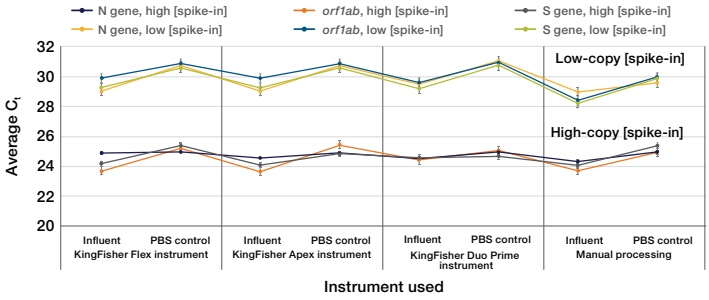

Figure 8. Processing of 200 µL wastewater for detection of SARS-CoV-2 RNA. Detection of spiked-in inactivated SARS-CoV-2 (2,800 copies for high-copy spike-in, 140 copies for low-copy spike-in) was performed by qPCR after automated nucleic acid purification from 200 µL wastewater samples. PBS control: SARS-CoV-2 processed in a small volume (200 µL) without concentration.

Processing of 1 mL direct or pre-concentrated wastewater samples

Samples of 1 mL volume can be efficiently processed using the MagMAX Wastewater Ultra kit and KingFisher Flex, Apex, or Duo Prime instrument with or without up-front virus concentration. Results of qPCR analysis for non-concentrated samples are shown in Figure 9.

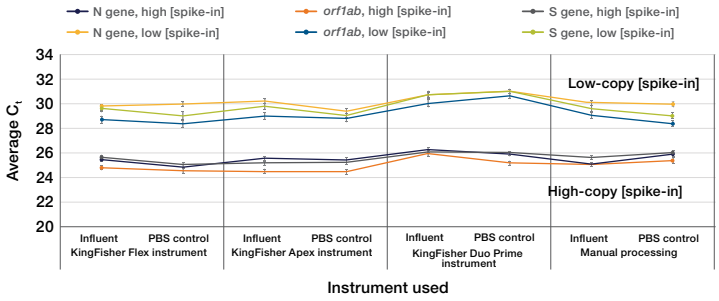

Figure 9. Processing of 1 mL direct or pre-concentrated wastewater for detection of SARS-CoV-2 RNA. Detection of spiked-in inactivated SARS-CoV-2 (2,800 copies for high-copy spike-in, 140 copies for low-copy spike-in) was performed by qPCR after automated nucleic acid purification from 1 mL wastewater samples. PBS control: SARS-CoV-2 processed in a small volume (1 mL) without concentration.

Processing of 50–500 mL wastewater concentrated via filtration

The efficiency of bead-assisted virus concentration gradually decreased with increasing wastewater volume. For 50 mL samples, the typical virus recovery efficiency was 50%. Thus, an alternative virus concentration approach was developed for 50–500 mL volumes of wastewater. As shown in Figure 10, filtration with a Nalgene Rapid-Flow 0.45 µm nylon filter is an efficient method to concentrate large volumes of wastewater.

Processing of sludge samples for detection of SARS-CoV-2

Sludge (200 mg) samples can be efficiently processed using the MagMAX Wastewater Ultra Kit. Results of qPCR for sludge samples are shown in Figure 11.

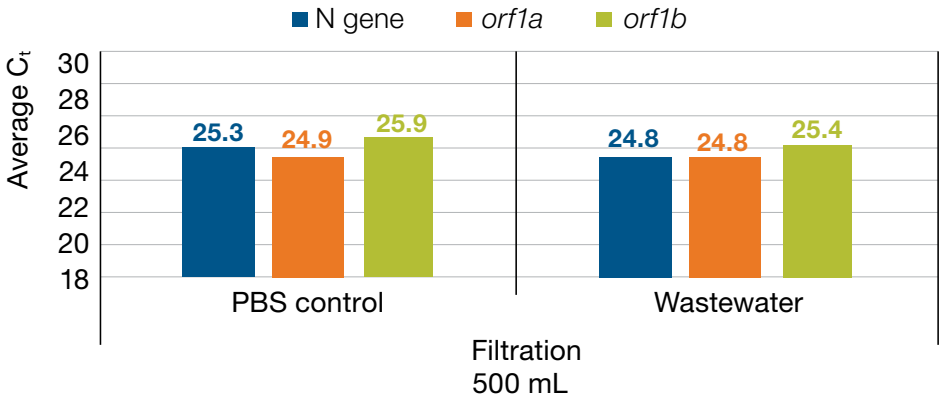

Figure 10. Processing of 50–500 mL wastewater samples concentrated via filtration for detection of SARS-CoV-2 RNA. Detection of spiked-in inactivated SARS-CoV-2 (2,800 copies) was performed by qPCR after automated nucleic acid purification from 500 mL wastewater samples that had been filtered and preprocessed. PBS control: SARS-CoV-2 (2,800 copies).

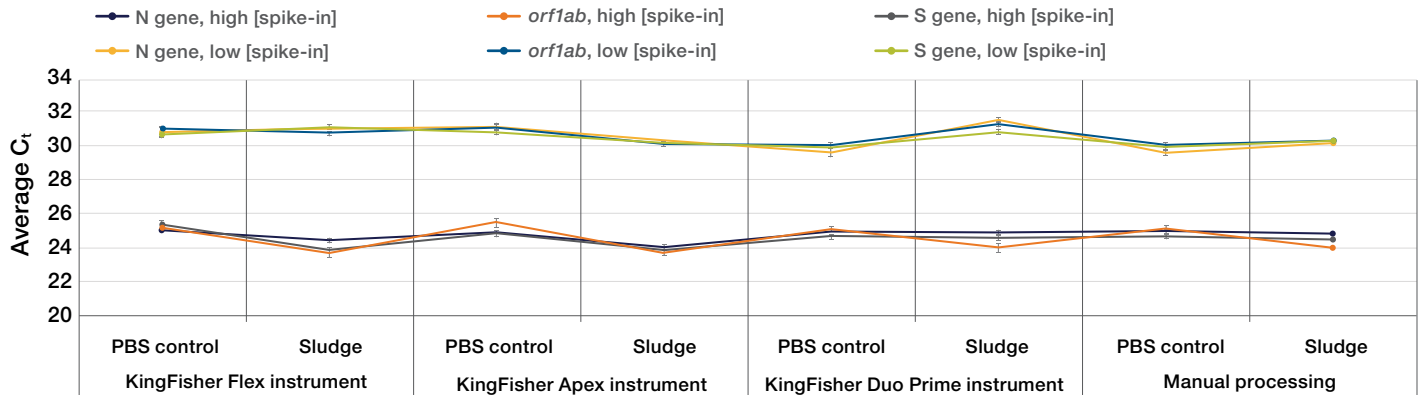

Figure 11. Processing of sludge samples for detection of SARS-CoV-2 RNA. Detection of spiked-in inactivated SARS-CoV-2 (2,800 copies for high-copy spike-in, 140 copies for low-copy spike-in) was performed by qPCR after automated nucleic acid purification from 200 mg samples that had been preprocessed. PBS control: SARS-CoV-2 processed in a small volume (2 mL).

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Summary

Five robust workflow options were developed for rapid concentration and qPCR analysis of SARS-CoV-2 from different volumes of wastewater ranging from 200 µL to 500 mL. Samples of 1 mL volume can be efficiently processed using the MagMAX Wastewater Ultra kit and KingFisher Flex instrument with or without up-front virus concentration. Wastewater samples of 10 mL volume can be rapidly concentrated using Dynabeads magnetic beads with nearly 100% efficiency, and the fully automated protocols on the KingFisher Flex instrument enable robust virus detection by qPCR. Large volumes of wastewater samples can be efficiently concentrated using a filtration method as shown in the filtration workflow.

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

Ordering information

Product	Quantity	Cat. No.
MagMAX Wastewater Ultra Nucleic Acid Isolation Kit	100 preps	A52606
MagMAX Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment	100 preps	A52610
KingFisher Flex Purification System	1 instrument	Go to **thermofisher.com/kingfisher**
KingFisher Apex Purification System		
KingFisher Duo Prime Purification System		
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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