Application Note

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Microbubble Extraction and RNA Cleanup for SARS-CoV-2 in Wastewater Samples

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Abstract

The use of wastewater-based epidemiology has increased dramatically in recent years due to the publication of COVID-19 online trackers and the media's focus on the pandemic. Yet the analysis of viromes in wastewater has widely been applied for several decades in conjunction with traditional chemical analysis approaches. Real-time quantitative polymerase chain reaction (qRT-qPCR)-based molecular detection methods are mainstream in large and small labs. However, the procedures to extract nucleic acids from wastewater samples are not yet standardized or optimized to enable routine and robust analysis and results interpretation. The Agilent Viral DNA/RNA Wastewater Prep Kit (powered by Akadeum Life Sciences) uses Akadeum microbubbles. These microbubbles allow for a simple, direct collection and lysis of total wastewater samples without requiring pasteurization or filtration of solid components prior to analysis.[1]

Introduction

Wastewater surveillance is a useful tool to monitor viral infections[2], and testing predicts community spread by four to five days.[3] From an analytical perspective, wastewater is an atypical matrix to process and analyze. Starting from EPA method 1615[4], many wastewater testing labs have protocols that perform reasonably well but are labor-intensive and require large volumes of wastewater to achieve target sensitivity levels. Filtration and precipitation-based workflows are often slow with time-consuming pasteurization and centrifugation processes.

As the COVID-19 pandemic continues to cause illness around the world, there is hope on the horizon as improved testing, treatments, and vaccines become available to the global population. With the ongoing emergence of new SARS-CoV-2 variants and mutations, it is critical that testing becomes even more widely available, as early diagnosis of infected individuals plays a critical role in stopping further escalation of this deadly disease.[5]

Two commonly practiced methods for wastewater concentration and viral processing are polyethylene glycol (PEG) precipitation and centrifugal ultrafiltration (CUF or UF). Both PEG and UF process 45 mL of wastewater sample and require the removal of suspended solids, which increases the amount of time spent on processing the sample. The Agilent Viral DNA/RNA Wastewater Prep Kit requires only 3 mL of raw wastewater (i.e., entire sample, both aqueous and suspended solids). The kit provides equivalent or higher sensitivity on a viral copy per analyzed volume equivalence, along with a quicker turnaround. Therefore, the process enables tracking of a viral load within a community in a much more valuable time window. It should also be noted that working with a smaller sample volume means that there is less contact with infectious agents within the laboratory.

The key piece of the wastewater extraction kit is the Akadeum buoyant, functionalized microbubbles. These novel, proprietary microbubbles are specifically functionalized to capture nucleic acid, including SARS-CoV-2 viral RNA. Unlike the inherent limitations of magnetic bead-based separation, microbubbles do not have the same volume and equipment restrictions. The microbubbles are mixed into the sample to isolate the targeted nucleic acid and then float to the top for further processing and analysis.[5]

This work demonstrates the use of microbubbles for nucleic acid extraction, allowing simple, direct collection and lysis of total wastewater samples. The process does not require pasteurization, centrifugation, or filtration of solid components prior to nucleic acid purification and subsequent analysis.

Experimental

Sample collection

Wastewater influent grab samples were obtained from the Walnut Creek Wastewater Treatment Plant (Austin, TX) on three separate dates. Before wastewater testing, all three wastewater samples were mixed to bring the settled solids back into solution, then aliquoted for processing. Specified wastewater samples were spiked with attenuated SARS-CoV-2 (Heat Inactivated 2019 Novel Coronavirus, ATCC, VR-1986HK). To allow enveloped virus equilibration between wastewater solid and aqueous phases, viral spiked wastewater samples and concurrent negative process control (NPC, autoclaved filtered (18 Ω) water) were incubated at 4 °C for 1.5 hours with periodic mixing.

Sample processing and RNA isolation

Two commonly practiced methods for wastewater concentration and viral processing, PEG and CUF, were compared to the Viral DNA/RNA Wastewater Prep Kit protocol.

PEG precipitation

This wastewater concentration method uses PEG/NaCl with an overnight precipitation to isolate (pellet) organic material such as viral particles. This procedure requires the removal of larger suspended solids prior to PEG/NaCl overnight precipitation.

1. After viral equilibration, pasteurize samples at 60 °C for 1.5 hours.
2. To pellet/remove larger solids in the pasteurized wastewater sample: add 45 mL of each pasteurized wastewater sample into a 50 mL conical tube and centrifuge at 3,000 × g (4 °C) for 15 minutes.
   - The break was "off" to prevent dislodging of the pellet during the slowdown of the centrifuge.
3. During centrifugation, add 4.5 g of PEG 8000 and 0.79 g of NaCl to prelabeled 50 mL centrifuge tubes (10% PEG 8000 + 300 mM NaCl in 45 mL).
4. Carefully decant the wastewater supernatant into a prelabeled 50 mL conical tube containing the PEG 8000 and NaCl.
5. Close lids tightly and wrap with Parafilm M. Manually mix/shake samples until the PEG/NaCl dissolves into solution.
6. Place samples on a rocker for overnight mixing at 4 °C.

7. The following morning, remove the Parafilm M from the tubes and centrifuge samples at 3,200 × g (4 °C) for 60 minutes.
   - The viral pellet will be slightly opaque to invisible. Establish a consistent tube orientation within the centrifuge to know where the viral pellet is located relative to the centrifugal force (i.e., the viral pellet will be located opposite the centrifuge center).
8. Carefully decant the supernatant into the appropriate waste receptacle. Re-centrifuge samples at 3,200 × g (4 °C) for 1 to 2 minutes to bring the remaining solution to the bottom of the tube. Carefully remove the remaining liquid with a pipette (avoid touching the pellet).
9. Add 400 µL of phosphate buffered saline (PBS) to the pellet, washing the viral pellet from the side of the 50 mL conical tube to ensure complete resuspension. Vortex (10 seconds) and centrifuge samples at 3,200 × g (4 °C) for 1 minute to bring the pellet to the bottom of the tube.
10. Transfer the resuspended pellet into a prelabeled 2 mL microcentrifuge tube and place on ice or store at –80 °C until the sample is ready for RNA isolation.

CUF Method

This wastewater concentration method uses an ultrafiltration device to selectively separate molecules based on molecular weight (size). To use this concentration method, larger suspended solids first need to be removed (e.g., via a vacuum filtration unit) to prevent ultrafiltration device clogging.

1. After viral equilibration, pasteurize samples at 60 °C for 1.5 hours.
2. To remove larger solids from the pasteurized wastewater samples: pull 45 mL of each pasteurized wastewater sample through a 0.22 µM vacuum filtration device and retain the filtrate.
3. Add 15 mL of the 45 mL sample to a prelabeled centrifugal filter unit (Amicon Ultra-15, part number UFC903008). Process the sample through the filter by centrifugation at 3,000 × g (4 °C) for 1 to 3 minutes until the entire 45 mL sample has been processed, discarding the flow-through after each centrifugation.
4. Transfer the concentrated sample (≤ 400 µL) into a prelabeled 2 mL microcentrifuge tube and place on ice or store at –80 °C until the sample is ready for RNA isolation.
   - If the concentrate is < 400 µL, add PBS (or nuclease-free water) to bring the total volume to 400 µL.

RNA isolation (PEG and CUF only)

RNA is isolated from wastewater concentrates (after PEG and CUF methods) using the Agilent Absolutely RNA miniprep kit (part number 400800).

To each resuspended viral pellet (PEG) or concentrated wastewater sample (CUF):

1. Prewarm 100 µL of elution buffer to 60 °C for each sample to be processed.
2. Add 400 µL of lysis buffer (without β-mercaptoethanol). Vortex (15 to 30 seconds) and briefly centrifuge samples.
3. Add 400 µL of 100% ethanol to the centrifuged sample (step 1). Vortex (15 to 30 seconds) and briefly centrifuge the samples.
   - Sample contents: 400 µL 100% ethanol + 400 µL wastewater sample + 400 µL lysis buffer.
4. Add ≤ 700 µL of the sample (step 2) to an RNA binding spin cup seated in a 2 mL microcentrifuge tube. Centrifuge samples at maximum speed for 30 to 60 seconds.
5. Remove (and retain) the spin cup, discard the filtrate, and replace the spin cup back into the 2 mL microcentrifuge tube. Repeat until the entire sample is processed through the same RNA binding spin cup (1,200 µL total volume = 2 × 600 µL processes)
6. Add 500 µL of high-salt wash buffer to the RNA binding spin cup and centrifuge at maximum speed for 30 to 60 seconds.
7. Remove (and retain) the spin cup, discard the filtrate, and replace the spin cup back into the 2 mL microcentrifuge tube. Repeat the high-salt wash buffer step an additional time for a total of two 500 µL washes.
8. Add 500 µL of low-salt wash buffer to the RNA binding spin cup and centrifuge at maximum speed for 30 to 60 seconds.
9. Remove (and retain) the spin cup, discard the filtrate, and replace the spin cup back into the 2 mL microcentrifuge tube.
10. Add 300 µL of low-salt wash buffer to the RNA binding spin cup and centrifuge at maximum speed for 2 minutes.
11. Remove (and retain) the spin cup, discard the filtrate, and replace the spin cup back into the 2 mL microcentrifuge tube. Repeat the low-salt buffer wash step an additional time for a total of 2 × 300 µL washes.
12. Transfer the spin cup into a fresh prelabeled 2 mL microcentrifuge tube and discard the filtrate and 2 mL microcentrifuge tube.

13. Add 50 µL of elution buffer (prewarmed to 60 °C prior to use) directly to the spin cup matrix, being careful not to touch the matrix surface with the pipette tip. Incubate the sample at room temperature for 2 minutes.
14. Centrifuge samples at maximum speed for 30 to 60 seconds, and retain the filtrate (RNA). Repeat the elution buffer (60 °C) step an additional time for a total filtrate volume of 100 µL (2 × 50 µL).
15. Place the sample on ice or store at –80 °C until the sample is ready for qRT-PCR analysis.

The Agilent Viral DNA/RNA Wastewater Prep Kit protocol[6]

The wastewater sample is concentrated and isolated by the microbubbles without the need to remove suspended solids or utilize multiple salt wash buffers. The Viral DNA/RNA Wastewater prep kit (part number 5610-2214) is powered by Akadeum Life Sciences.

1. Prewarm 500 µL of elution buffer to 60 °C for each sample to be processed.
2. After equilibration, aliquot 3 mL of the entire wastewater sample into a prelabeled 15 mL conical tube.
3. Add 5 mL of buffer lysis solution (LS) and 150 µL of proteinase K into each sample tube. Vortex (10 seconds) to mix and incubate in a 60 °C water bath for 10 minutes.
   - **Note:** Directly lysing the raw wastewater as opposed to after the laborious removal of solids and sample concentration steps in the PEG and CUF methods lowers laboratory personnel to the exposure to infectious agents.
4. Remove the samples from the 60 °C water bath and allow them to cool at room temperature for ~3 to 5 minutes.
5. To each room-temperature sample, add 5 mL of 100% EtOH and 1 mL of microbubbles. Mix the samples by mechanical end-over-end rotation for 5 minutes at ≥ 20 rpm (or manually invert the tubes a total of 30 times).
6. Centrifuge the samples at 3,000 × g for 30 seconds to promote the surfacing of the microbubbles.
7. Carefully remove the subnatant (liquid below the bubbles) using a 2 mL Pasteur pipette.
   - Try to limit removal of the microbubbles, as they retain the RNA.
8. Add 2.5 mL of 80% ethanol wash to microbubbles, vortex for 3 seconds, and centrifuge the samples at 3,000 × g for 30 seconds to promote microbubble surfacing.
9. Carefully remove as much of the 80% ethanol wash subnatant as possible using a 2 mL Pasteur pipette.
   - Try to limit removal of the microbubbles, as they retain the RNA.
10. Add 400 µL of elution buffer (prewarmed to 60 °C prior to use) to the microbubbles, vortex for 15 seconds, and incubate at room temperature for 2 minutes.
11. Centrifuge the samples at 3,000 × g for 30 seconds.
12. Transfer the subnatant/eluate (which contains RNA) into a prelabeled 2 mL microcentrifuge tube. Place on ice or store at –80 °C until the sample is ready for RNA isolation.
13. Transfer ~425 µL of the subnatant/eluate (previous step) into a separate prelabeled 2 mL microcentrifuge tube containing 400 µL of LS and 400 µL of 100% ethanol.
   - Sample contents: ~425 µL sample + 400 µL lysis buffer + 400 µL 100% ethanol
14. Vortex for 10 seconds and quickly centrifuge the samples to bring down the liquid from the lid and sides of the tube.
15. Place RNA binding cups into prelabeled 2 mL microcentrifuge tubes for each sample.
16. Transfer ≤ 700 µL of sample [eluate: LS: ethanol] into its respective RNA binding cup.
17. Centrifuge at maximum speed for 15 seconds, discard the filtrate, and place the RNA binding cup back into the 2 mL microcentrifuge tube.
18. Transfer the remaining ≤ 700 µL of sample into their respective RNA binding cups, centrifuge at maximum speed for 15 seconds, discard the filtrate, and place the RNA binding cup back into the 2 mL microcentrifuge tube.
19. Add 500 µL of 80% ethanol to the RNA binding cup, and centrifuge for 2 minutes at maximum speed.
20. Transfer the RNA binding cup to a new prelabeled 1.5 mL microcentrifuge tube. Discard the 80% ethanol flow-through and 2 mL microcentrifuge tube.
21. Add 100 µL of elution buffer (prewarmed to 60 °C prior to use) directly to the RNA binding cup matrix, avoiding contact with the matrix surface. Incubate at room temperature for 2 minutes.
22. Centrifuge at maximum speed for 30 to 60 seconds, retain the eluted RNA, and place the sample on ice or store at –80 °C until the sample is ready for qRT-PCR analysis.

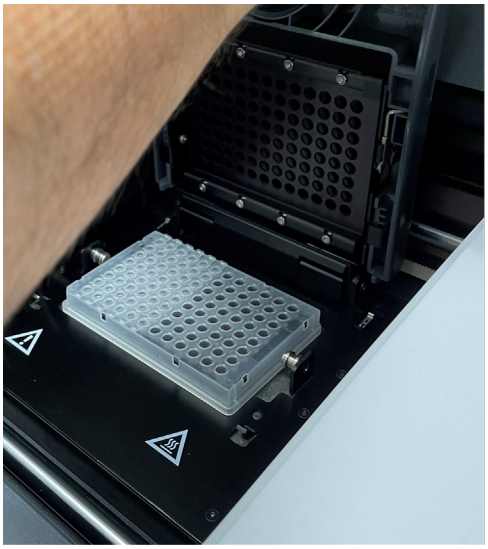
qRT-PCR analysis

qRT-PCR was performed using the Agilent Brilliant III Ultra-Fast qRT-PCR master mix (part number 600884) in a 100 µL Agilent AriaMx 96-well plate (part number 401490) with optical tube strip caps (part number 401425) on an Agilent AriaMx Real-Time PCR system (part number G8830A).

Figure 1. Agilent AriaMx Real-Time PCR system.

Extracted RNA is first reverse transcribed at 50 °C for 10 minutes, followed by a 95 °C denaturation for 3 minutes, 45 cycles of 95 °C denaturation for 5 seconds, and annealing/extension at 60 °C for 30 seconds (Figure 2). No template controls (NTCs) were analyzed concurrently to assess contamination within the qRT-PCR. The quantification cycle (Cq) values were obtained from the Agilent AriaMx software (version 1.6).

A 2 µL volume of each sample was analyzed using Agilent Brilliant III Ultra-Fast qRT-PCR Master Mix[7] and the AriaMx instrument per the manufacturer instructions. Samples were analyzed in technical replicates using three sets of primers/probe, two sets for SARS-CoV-2 (CoV-2 N1, CoV-2 N2), and one set for the Pepper mild mottle virus (PMMoV) (Figure 3). SARS-CoV-2 N1 and N2 primers/probes are the design of the Center for Disease Control and Prevention Emergency Use Authorization (CDC EUA), and the PMMoV primers/probe are an appended version of that described in Zhang *et al.*[8]



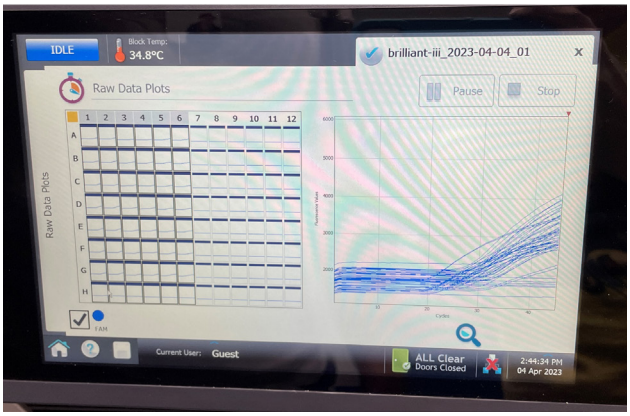


Figure 2. Agilent AriaMx setup.

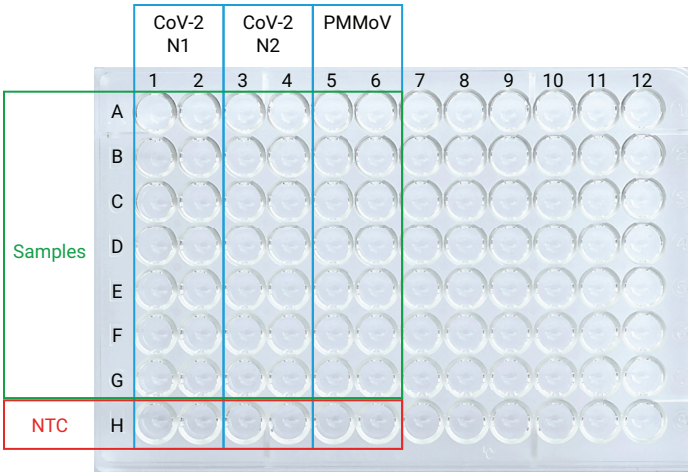


Figure 3. Example of 96-well plate setup for qRT-PCR analysis.

Results and discussion

With over 100 measurements taken for sample extraction through RNA isolation/cleanup, the microbubbles sample preparation protocol performed 79% faster than PEG precipitation and 38% faster than CUF (Table 2) methods.

The use of 3 mL of raw wastewater samples provided consistent results that were equivalent or, in most cases, proved to be more sensitive than PEG precipitation on a wastewater volumetric equivalence (Figures 4 to 6). The PEG and CUF methods both process 45 mL of wastewater, whereas the microbubbles protocol only processes 3 mL (93% smaller sample size). When calculating the RNA copies/L, the microbubbles were found to provide a more sensitive measurement than the PEG and CUF methods. The sensitivity captured from this analysis can lead to earlier identification of a spike in the virus when analyzing trends within a population.

SARS-CoV-2 copies/L processed wastewater was calculated from a standard line created through the analysis of 10-fold serially diluted single stranded RNA (ssRNA) fragments of SARS-CoV-2 (Sigma-Aldrich, EURM019), analyzing 500,000 to 50 copies/qRT-PCR (Table 3).

Table 1. Primers/Probe Sets for qRT-PCR Analysis.

Working Stock Name	Forward	Reverse	Probe
CoV-2 N1	Cov-2 N1-F GACCCCAAAATCAGCGAAAT	Cov-2 N1-R TCTGGTTACTGCCAGTTGAATCTG	Cov-2 N1-P FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
CoV-2 N2	Cov-2 N2-F TTACAAACATTGGCCGCAAA	Cov-2 N2-R GCGCGACATTCCGAAGAA	Cov-2 N2-P FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1
PMMoV	PMMoV-For GAGTGGTTTGACCTTAACG	PMMoV-Rev TTGTCGGTTGCAATGCAAGT	MMoV-Probe 6-FAM-CCTACCGAAGCAAATGTCG-BHQ-1

Table 2. Wastewater processing method comparison.

Material/Process	Microbubbles	PEG	CUF
Wastewater Volume	3 mL	45 mL	45 mL
Spike-in Incubation	4 °C for 90 min	4 °C for 90 min	4 °C for 90 min
Pasteurization	60 °C for 90 min	60 °C for 90 min	60 °C for 90 min
Removal of Suspended Solids	Not applicable	3,000 × g (4 °C) for 15 minutes	0.22 mM centrifugal filter unit
Concentration	Agilent Viral DNA/RNA Wastewater Prep Kit	10% PEG/0.3 M NaCl (overnight at 4 °C)	30 kDa NMWL ultrafiltration device
RNA Isolation		Agilent Absolutely RNA miniprep kit	Agilent Absolutely RNA miniprep kit
RNA Elution Reagents	Elution buffer	Elution buffer	Elution buffer
RNA Elution Volume	100 µL	100 µL	100 µL
Wastewater (mL) Equivalents/qRT-PCR	3 mL × (0.005 mL/0.1 mL) = 0.15 mL 10 mL × (0.005 mL/0.1 mL) = 0.50 mL	45 mL × (0.005 mL/0.1 mL) = 2.25 mL	45 mL × (0.005 mL/0.1 mL) = 2.25 mL
Elapsed Time from Sample to RNA (~30 samples)	5.8 hours	9.4 hours	23.2 hours

NMWL = Nominal molecular weight limit
Elution buffer = 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA

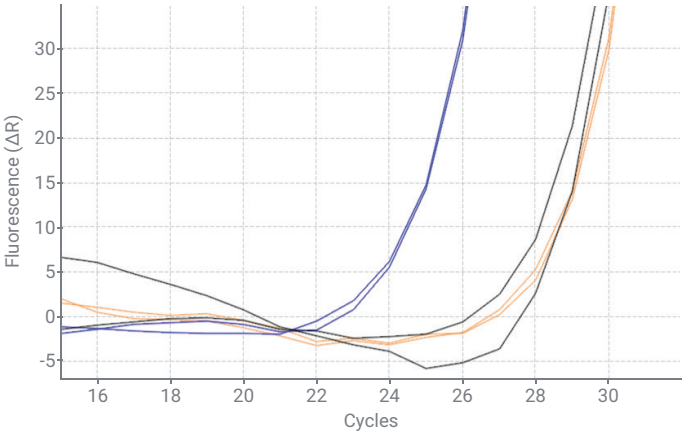


Figure 4. Graphical displays of wastewater sample #13 (CoV-2 N1); PEG = blue (Cq = 25.68); CUF = orange (Cq = 29.72); AKD3 mL = black (Cq = 29.12).

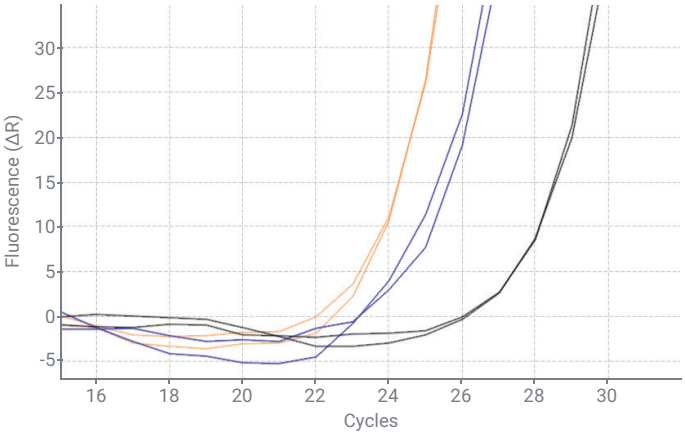


Figure 5. Graphical displays of wastewater sample #20 (CoV-2 N1); PEG = blue (Cq = 26.15); CUF = orange (Cq = 24.94); AKD3 mL = black (Cq = 29.11).

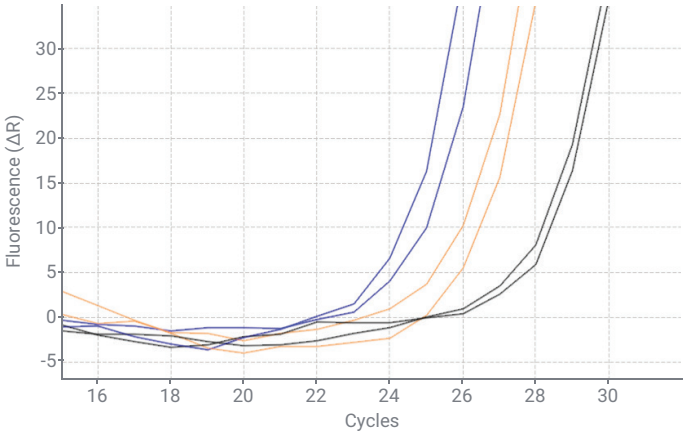


Figure 6. Graphical displays of wastewater sample #26 (Cov-2 N1); PEG = blue (Cq = 25.54); CUF = orange (Cq = 27.17); AKD3 mL = black (Cq = 29.26).

Table 3. Estimated SARS-CoV-2 copies/L of processed wastewater (WW) by each method. Primer technical replicates were provided for three different spiked wastewater samples.

	RNA Copies/L Wastewater				
Sample ID	Primer	Microbubbles (MB)	PEG	CUF	Sensitivity Comparison
WW#13	Cov-2 N1	869,048,673	742,104,040	42,687,253	MB > PEG > CUF
WW#13	Cov-2 N1	1,098,623,339	752,722,294	42,385,100	MB > PEG > CUF
WW#13	Cov-2 N2	716,571,538	673,307,209	37,748,290	MB > PEG > CUF
WW#13	Cov-2 N2	550,752,721	668,659,922	36,972,036	MB > PEG > CUF
WW#20	Cov-2 N1	966,760,669	429,457,755	1,291,499,179	CUF > MB > PEG
WW#20	Cov-2 N1	994,624,012	467,670,900	1,300,705,947	CUF > MB > PEG
WW#20	Cov-2 N2	817,355,531	489,605,844	1,070,896,082	CUF > MB > PEG
WW#20	Cov-2 N2	778,673,165	566,257,525	978,686,742	CUF > MB > PEG
WW#26	Cov-2 N1	875,243,899	621,355,302	294,724,269	MB > PEG > CUF
WW#26	Cov-2 N1	764,740,787	951,567,278	220,257,775	PEG > MB > CUF
WW#26	Cov-2 N2	521,066,176	325,369,093	80,309,353	MB > PEG > CUF
WW#26	Cov-2 N2	539,427,072	384,209,062	74,418,078	PEG > MB > CUF

Conclusion

Wastewater testing offers valuable insight on community-level data for various analytes, including microbes that are associated with infectious diseases. Quantitative reverse transcriptase PCR (qRT-PCR) is well suited for wastewater analyses of microbes due to its sensitivity, scalability, and cost-effectiveness.[5] The sensitivity captured with the silica functionalized microbubbles can lead to the identification of an increase in virus levels within a population. This application note presents a workflow enabling the analysis of wastewater without the need for preprocessing samples. The described workflow allows the analysis of nucleic acids extracted from 3 mL raw wastewater using the Agilent Viral DNA/RNA Wastewater Prep Kit. The Viral DNA/RNA Wastewater Prep Kit yielded higher sensitivity processing per wastewater sample volume, and required less processing time than commonly used wastewater analysis methods.

References

1. Wegner, C.; Roussey, J.; Carey, M.; Macintyre, B.; Snow, T.; Sieglaff, D.; Bridi, A.; Battiste, S. Microbubbles for Improved Nucleic Acid Extraction in Wastewater Samples for Viral RNA Detection, *medRxiv preprint*. doi: https://doi.org/10.1101/2022.07.10.22277342
2. Hart, O. E.; Halden, R. U. *Science of the Total Environment* **2020**, *730*, 138875.
3. Wu, F.; *et al.* Titers in Wastewater Foreshadow dynamics and Clinical Presentation of New COVID 19 Cases. *medRxiv* **2020**. https://doi.org/10.1101/2020.06.15.20117747
4. Fout, G.; Cashdollar, J.; Griffen, S.; Brinkman, N.; Varughese, E.; Parshionikar, S. UPA Method 1615. Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. *J. Vis. Exp*. **2016**, *107*, 52646. doi: 10.3791/53646
5. Akadeum Life Sciences, Inc. Microbubble Optimization for SARS-CoV-2 RNA Extraction in Saliva Samples Enables Advanced Diagnostic Opportunities, April **2021**.
6. Viral DNA/RNA Wastewater Prep Kit, *Agilent Technologies user guide*, publication number 5994-6932EN, **2023**.
7. The Development of a Sensitive and Reliable qRT-PCR-Based SARS-CoV-2 Wastewater Analysis Protocol, *Agilent Technologies*, publication number 5994-2875EN, **2021**.
8. Zhang, T.; Breitbart, M.; Lee, W. H.; Run, J. Q.; Wei, C. L.; Soh, S. W. L.; Hibberd, M. L.; Liu, E. T.; Rohwer, F.; Ruan, Y. J. RNA Viral Community in Human Feces: Prevalence of Plant Pathogenic Viruses. *PLoS Biol.* **2006**, *4(1)*, 108–118.

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