Application Note

Genomics

Agilent

Trusted Answers

Detection of SARS-CoV-2 RNA in Wastewater Samples Using Quantitative PCR

Author

Christabel Monteiro,
Rubaiya Jesmin,
Nadiar Yakob, and
Johnny Mitchell
Pace Analytical, Mt. Juliet, TN

Jessica Westland
Agilent Technologies, Inc.

Abstract

With viral infections, it often takes days for symptoms to present, if symptoms appear at all. This delay means that the traditional approach of contact tracing and individual testing are trailing indicators of viral spread. Today, wastewater testing can track the aggregate viral load within a community, including asymptomatic cases. It is sensitive, and can often detect a handful of cases in a population of hundreds of thousands.[1] In general, where wastewater testing has been implemented, it leads community case load predictions by four to five days.[2] This is a valuable window of time for making decisions. This application note compares the use and sensitivity of polyethylene glycol (PEG) precipitation/purification to the Agilent Viral DNA/RNA Wastewater Prep Kit (powered by Akadeum Life Sciences) in wastewater testing for the detection of SARS-CoV-2 RNA.

Introduction

SARS-CoV-2, the virus that causes COVID-19, is a single-stranded RNA virus and part of a group of viruses referred to as coronaviruses. Infected individuals, even those who are asymptomatic, shed RNA fragments from the virus in their waste. Analyzing wastewater samples for these viral fragments can help officials monitor infection rate trends and identify the variants present in a community.[3] As voluntary individual testing wanes in communities across the country, wastewater monitoring is even more vital. Because wastewater monitoring does not require the individual to do anything other than go about their daily routine, it helps to fill in some of the knowledge gaps left by COVID-weary communities. Additional tests to determine which variants are present in a sample can also help community and health officials further understand the transmissibility and threat from these new variants. This information allows officials to make data-driven decisions about how to respond.[3]

One of the most common methods for wastewater concentration and viral processing is PEG precipitation. The PEG protocol processes 40 mL of wastewater sample and requires the removal of suspended solids, which increases the amount of time spent on processing the sample. The Agilent Viral DNA/RNA Wastewater Prep Kit only requires 3 mL of raw wastewater (aqueous along with suspended solids) and provides equivalent or higher sensitivity with a quicker turnaround.

The key piece of the 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 can simply be mixed into the sample, where they grab onto the target (in this case, the viral RNA) and float it to the top for further processing and analysis.[4]

Experimental

Sample collection

Wastewater samples with a sample volume of 250 mL were collected in 125 microbiological containers, chemically preserved with sodium thiosulfate (Na-thiosulfate), and stored at 0 to 6 °C until sample preparation. Samples were prepared within 2 weeks of collection and analyzed within 24 hours after sample preparation was completed.

Sample preparation

The sample preparation methods followed and described in the following protocol were performed by the Pace Analytical National Center for Testing and Innovation in Mt. Juliet, Tennessee.

PEG precipitation and RNA extraction protocol (Figure 1)

1. Filter the sample using a vacuum filter by transferring 50 mL of wastewater sample into the 1.5 and 0.2 µm FlipMate sample cups, respectively, and label the FlipMate unit with the sample ID.
2. Transfer 40 mL of filtered sample to a clean 50 mL centrifuge tube labeled with the same sample ID.
3. Add 10 mL of 5x PEG/NaCl solution to each sample.
4. Add 450 + 50 mg of BSA (1/4 tsp) to 40 mL of 0.2 µM filtered wastewater containing 10 mL of 5x PEG/NaCl. Mix well to dissolve BSA by placing tubes on a rotator.
5. Add 50 µL of a BRSV spike (accuracy and precision are important) to each tube with filtered wastewater and PEG/NaCl using a repeater pipette.
6. For every run of samples, a filtration positive and negative and concentration positive and negative are set up and prepared with DI or RNase-free water. For every 20 samples, a matrix spike and duplicate are set up.
7. Mix all tubes well by inverting several times. Incubate at 2 to 8 °C overnight.
8. Centrifuge at 12,000 g for 30 minutes at 4 °C.
9. Pour the supernatant carefully from the side of the tube opposite from the pellet.
10. Centrifuge at 12,000 g for 3 minutes at 4 °C, then remove the supernatant completely with a 1 mL pipette. Suspend the pellet in 200 µL of RNA-free water.
11. Flash spin at 1,000 g for 15 seconds at 4 °C.
12. During 30 minutes of centrifugation, label and arrange the microcentrifuge tubes according to sample and control numbers.
13. Add 25 µL of Proteinase K (included with the Invitrogen Pure Link Viral RNA/DNA Mini Kit) into a sterile microcentrifuge tube according to sample and control numbers.
14. Add 200 µL of cell-free sample (equilibrated to room temperature) to the microcentrifuge tube after flash spinning.
15. Add 200 µL of lysis buffer (containing 5.6 µg of carrier RNA). Close the lid and mix by vortexing for 15 seconds. Incubate at 56 °C for 15 minutes.

16. Add 250 µL of 96 to 100% ethanol to the lysate tube to obtain a final concentration of 37%; close the lid and mix by vortexing for 15 seconds.
17. Incubate the lysate with ethanol for 5 minutes at room temperature.
18. Briefly centrifuge the tube to remove any drops from the inside of the lid.
19. Transfer the lysate with ethanol onto the viral spin column.
20. Close the lid and centrifuge the column at approximately 6,800 g for 1 minute. Discard the collection tube with flow-through to waste.
21. Place the spin column in the clean wash tube (2 mL) included with the kit and add 500 µL of wash buffer with ethanol to the spin column.
22. Centrifuge the column at approximately 6,800 g for 1 minute. Discard the flow-through and place the spin column back into the wash tube.
23. Wash the spin column twice.
24. Place the spin column in another clean tube and centrifuge the column at maximum speed in a microcentrifuge for 1 minute to dry the membrane completely. Discard the wash tube with flow-through to waste.
25. Place the viral spin column in a clean 1.5 mL recovery tube.
26. Add 50 µL of sterile RNA-free water to the center of the column. Close the lid. Incubate at room temperature for 1 minute.
27. Centrifuge the column at maximum speed for 1 minute to recover purified viral RNA. Discard the spin column.
28. Store reconstituted RNA at 2 to 4 °C if using within 4 hours, or at –80 °C for long-term storage. Load qPCR plates as soon as possible.

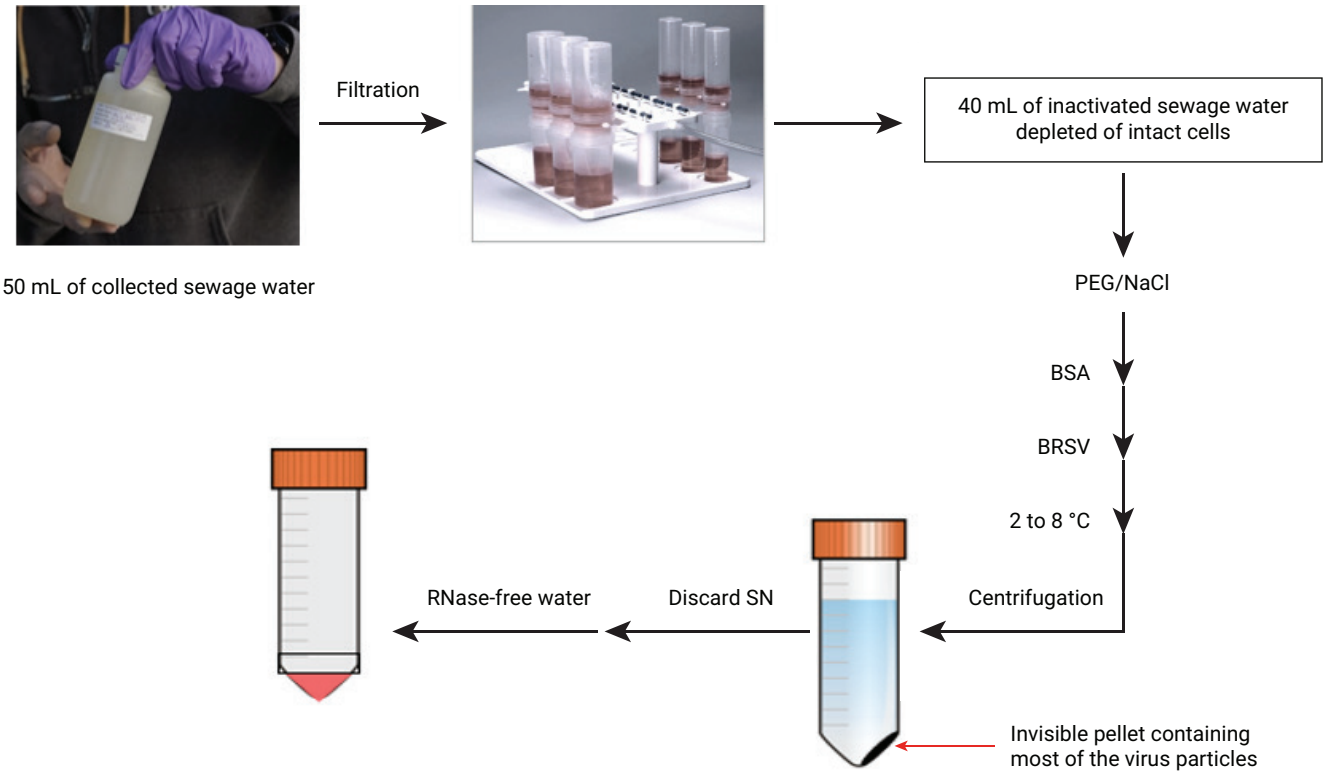

Figure 1. Pace Analytical National workflow for PEG precipitation and RNA extraction protocol.

The Agilent Viral DNA/RNA Wastewater Prep Kit protocol[5]

1. After equilibration, aliquot 3 mL of wastewater sample into a prelabeled 15 mL conical tube.
2. Add 5 mL of buffer lysis solution (LS) and 150 µL of Proteinase K to each sample tube. Vortex for 10 seconds to mix, then incubate in a 60 °C water bath for 10 minutes.
3. Allow the samples to cool at room temperature for ~3 to 5 minutes.
4. To each cooled sample, add 5 mL of 100% EtOH and 1 mL of microbubbles to each sample tube. 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).
5. Centrifuge the samples at 3,000 x g for 30 seconds to promote the surfacing of the microbubbles.
6. Carefully remove the subnatant (liquid below the bubbles) using a 2 mL Pasteur pipette.
7. Try to limit the removal of microbubbles, as they retain the RNA.
8. Add 2.5 mL of 80% ethanol wash to the remaining microbubbles, vortex for 3 seconds, and centrifuge the samples at 3,000 x g for 30 seconds.
9. Carefully remove as much of the 80% ethanol wash subnatant as possible using a 2 mL Pasteur pipette.
10. Try to limit the removal of microbubbles, as they retain the RNA.
11. Add 400 µL of elution buffer (prewarmed to 60 °C prior to use) to the microbubbles, vortex for 15 seconds, and let sit to incubate at room temperature for 2 minutes.
12. Centrifuge the samples at 3,000 x g for 30 seconds
13. Transfer the subnatant/eluate (which contains RNA) into a prelabeled 2 mL microcentrifuge tube.
14. 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.
15. Sample contents: 425 µL sample + 400 µL lysis buffer + 400 µL 100% ethanol.
16. Vortex for 10 seconds and quickly centrifuge the samples to bring down the liquid from lid and sides of the tube. Place on ice or store at –80 °C until the sample is ready for RNA isolation.
17. Place RNA binding cups into prelabeled 2 mL microcentrifuge tubes for each sample.
18. Transfer ≤ 700 µL of sample [eluate: LS: ethanol] into its respective RNA binding cup.
19. Centrifuge at maximum speed for 15 seconds, discard the filtrate, and place the RNA binding cup back into the 2 mL microcentrifuge tube.
20. 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.
21. Add 500 µL of 80% ethanol to the RNA binding cup, and centrifuge for 2 minutes at maximum speed.
22. Transfer the RNA binding cup to new, prelabeled 2 mL microcentrifuge tubes. Discard the 80% ethanol flow-through and the previous 2 mL microcentrifuge tube.
23. Add 100 µL of elution buffer (prewarmed to 60 °C prior to use) directly to the RNA binding cup matrix; avoid touching matrix surface. Incubate at room temperature for 2 minutes.
24. Centrifuge at maximum speed for 30 seconds to 1 minute; retain the sample eluate (contains the RNA) and place the sample on ice or store at –80 °C until the sample is ready for qRT-PCR analysis.

qRT-PCR analysis

Pace uses real-time, quantitative polymerase chain reaction (RT-qPCR) to test wastewater samples for SARS-CoV-2. The qPCR test detects RNA, the genetic signature of SARS-CoV-2, in wastewater. The cycle threshold (CT) values obtained are used to quantify the copies of RNA from all strains of SARS-CoV-2 present in the samples. The results are reported as copies of viral RNA/mL. When implementing a COVID-19 wastewater monitoring program, it is important to understand that every community is likely to have SARS-CoV-2 viral fragments in its wastewater (Table 2).

Table 1. Primers/probe sets for qRT-PCR analysis.

Name	5' Modification	Sequence (5'-3')	3' Modification
nCoV_N1 Forward Primer	None	GAC CCC AAA ATC AGC GAA AT	None
nCoV_N1 Reverse Primer	None	TCT GGT TAC TGC CAG TTG AAT CTG	None
nCOV_N1 Probe	FAM	ACC CCG CAT TAC GTT TGG TGG ACC	3IABkFQ
nCoV_N2 Forward Primer	None	TTA CAA ACA TTG GCC GCA AA	None
nCoV_N2 Reverse Primer	None	GCG CGA CAT TCC GAA GAA	None
nCOV_N2 Probe	VIC (SUN)	ACA ATT TGC CCC CAG CGC TTC AG	MGBNFQ
BRSV Forward Primer	None	GCAATGCTGCAGGACTAGGTATAAT	None
BRSV Reverse Primer	None	ACACTGTAATTGATGACCCCATTCT	None
BRSV Probe	VIC (SUN)	ACCAAGACTTGTATGATGCTGCCAAAGCA	None
PMMoV Forward Primer	None	TTGTCGGTTGCAATGCAAGT	None
PMMoV Reverse Primer	None	TTGTCGGTTGCAATGCAAGT	None
PMMoV Probe	FAM	CCTACCGAAGCAAATG	MGB-NFQ
2019-nCoV_N2-F	2019-nCoV_N2-R	2019-nCoV_N2-P	CoV-2 N2
PMMoV-Zhang-mod-For	PMMoV-Zhang-Rev	PMMoV-Zhang-mod-Probe - FAM	PMMoV

Results and discussion

The PEG precipitation requires 40 mL of water to test, and the microbubble kit only requires 3 mL, so the initial data result will be a lot higher for the PEG method because it shows the amount of virus in 40 mL of water versus 3 mL for the microbubbles. Therefore, normalize the results to measure how much viral RNA is present in the same sample for both methods. After analyzing multiple batches of samples, the Viral DNA/RNA Wastewater Prep Kit produced consistent results, which are comparable to and, in most cases, proved to be more sensitive than the PEG precipitation method (Table 2).

Table 2. Comparison of RNA copies/mL of SARS-CoV-2 primer quantitated from each method in multiple wastewater samples.

Sample ID	RNA Copies/mL		Sensitivity Comparison
	Microbubbles (MB)	PEG	
L1581785-01	7.258	8.048	PEG > MB
L1583336-01	3.383	0.846	MB > PEG
L1584794-01	17.433	3.843	MB > PEG
L1584459-01	20.875	11.732	MB > PEG
L1584461-01	5.683	3.297	MB > PEG
L1584462-01	6.758	9.917	PEG > MB
L1585187-01	8.100	2.594	MB > PEG
L1585297-01	5.200	0.320	MB > PEG
L1585665-01	18.300	0.944	MB > PEG
L158749-02	3.658	0.130	MB > PEG
L1585749-01	2.008	0.683	MB > PEG
L1586258-01	6.967	7.816	PEG > MB
L1586660-01	41.825	8.136	MB > PEG
L1586684-01	4.217	2.537	MB > PEG
L1587199-01	139.942	16.531	MB > PEG

Sample ID	RNA Copies/mL		Sensitivity Comparison
	Microbubbles (MB)	PEG	
L1587973-01	4.333	0.821	MB > PEG
L1588020-01	1.900	0.442	MB > PEG
L1588484-01	2.567	1.954	MB > PEG
L1593556-01	39.550	0.033	MB > PEG
L1594002-01	10.392	4.702	MB > PEG
L1594567-01	4.758	0.000	MB > PEG
L1594600-01	23.442	0.133	MB > PEG
L1594848-01	7.642	3.029	MB > PEG
L1594868-01	2.000	0.273	MB > PEG
L1595294-01	4.083	1.022	MB > PEG
AKAA220813I	6.98E-04	3.93E-04	MB > PEG
AKFL220813I	1.08E-03	9.97E-04	MB > PEG
AKTM220814I	9.29E-04	6.22E-04	MB > PEG
AKYC220811I	1.59E-03	8.78E-04	MB > PEG

Conclusion

Through ongoing sampling and monitoring of results, wastewater treatment data becomes a resource for tracking the prevalence of infections and where there may be potential spikes in COVID-19 cases. This information could prove valuable in mitigating the spread of the virus and/or to ensure communities and healthcare facilities have their resources ready to respond. Using nucleic acid binding, buoyant, functionalized microbubbles to extract nucleic acids from 3 mL of wastewater samples, the Agilent Viral DNA/RNA Wastewater Prep Kit yielded high sensitivity. The kit was also able to process less wastewater sample volume and had a shorter processing time compared to the PEG precipitation method for wastewater analysis.

References

1. Hart, O. E.; Halden, R. U. *Science of the Total Environment* **2020**, *730*, 138875.
2. 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
3. Monitoring Wastewater for SARS-CoV-2 and Its Variants. *Pace Analytical eBook*, March **2022**.
4. Microbubble Optimization for SARS-CoV-2 RNA Extraction in Saliva Samples Enables Advanced Diagnostic Opportunities; Akadeum Life Sciences, April **2021**.
5. Viral DNA/RNA Wastewater prep kit, *Agilent Technologies user guide*, publication number 5994-6932EN, **2023**.

www.agilent.com

This information is subject to change without notice.

PR7001-1805

© Agilent Technologies, Inc. 2024

Printed in the USA, January 11, 2024
5994-6939EN

Agilent
Trusted Answers