User Guide

Agilent
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Agilent Viral DNA/RNA Wastewater Prep Kit

Powered by Akadeum Life Sciences, part number 5610-2214

Congratulations on taking a big step in the analysis of viral DNA/RNA in wastewater samples. By purchasing the Agilent Viral RNA/DNA Wastewater extraction kit, you are one step closer to accurate, fast, and sensitive results.

This user guide provides information about each of the components included in your kit. You will also find a step-by-step protocol including tips and tricks for how to maximize your results when using this kit. At the end of the guide, you will find a simplified Benchtop Reference Protocol that can be kept in your hood or on your lab bench for easy reference.

Introduction

Two commonly practiced methods for wastewater concentration and viral processing are polyethylene glycol (PEG) precipitation and centrifugal ultrafiltration (CUF or UF). Both PEG and CUF methods use larger wastewater sample volumes and require pasteurization and the removal of suspended solids; these factors increase the amount of time spent on processing the sample. The Agilent Viral RNA/DNA Wastewater extraction kit only requires 3 mL of raw wastewater (aqueous with suspended solids and without the need to pasteurize). The kit and method provide equivalent to higher sensitivity per analyzed volume and have a quick turnaround. The results enable tracking the viral load within a community in a much more valuable, impactful time window.

The key piece of the extraction kit is the Akadeum buoyant, functionalized microbubbles. These novel, proprietary microbubbles are customized to capture nucleic acids, 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 of the sample for further processing and analysis.

Agilent provides the Viral DNA/RNA Wastewater Prep kit for the use of the microbubbles for nucleic acid extraction, allowing simple, direct collection and lysis of total wastewater samples without requiring pasteurization or filtration of solid components prior to nucleic acid purification and subsequent analysis.

Kit components

The following components are included in the Agilent Viral RNA/DNA Wastewater extraction kit (part number 5610-5514). These components are packaged and shipped separately due to their storage conditions (the microbubbles come shipped on ice). All quantities provided allow for 50 sample extractions.

Lysis buffer

Quantity = 1 bottle

The lysis buffer breaks open the viral capsids to release the nucleic acids and secures the nucleic acids for extraction.

Viral DNA/RNA microbubbles

Quantity = 1 bottle

The viral DNA/RNA microbubbles bind the nucleic acids, allowing them to be isolated from the wastewater sample.

Note: The microbubbles require refrigeration (2 to 8 °C).

Elution buffer

Quantity = 1 bottle

The elution buffer releases the captured nucleic acids from the viral DNA/RNA microbubbles.

DNA/RNA binding cup

Quantity = 50

Silica filter-based spin columns.

Collection tube, 2 mL

Quantity = 200

Microcentrifuge receptacle tubes.

Agilent viral RNA/DNA wastewater extraction protocol

The wastewater samples are concentrated and isolated by the microbubbles without the need to remove suspended solids, pasteurize, or use multiple salt wash buffers.

Before you begin

- Wipe down the work area and equipment with nuclease removal wipes or liquid.
- Prepare all necessary supplies and solutions nuclease-free.
  - 100% Ethanol (EtOH)
  - 80% Ethanol (EtOH); ethanol solutions should be prepared fresh each day as the concentration changes with evaporation.
- Preheat the elution buffer within a water bath or a heat block set to 60 °C.
- Preheat a water bath or heat block capable of holding 15 mL conical tubes to 60 °C in preparation for the wastewater lysis step.
- Prepare the sample collection tubes:
  - Add 550 µL of lysis buffer and 525 µL of 100% EtOH to a 2 mL collection tube.

CAUTION: Do not add bleach or acidic solutions directly to the sample, kit reagents, or preparation waste.

Tips are technical recommendations supplied throughout the protocol and, when followed, will improve reproducible results.

1. Resuspend raw wastewater to bring settled solids back into solution, then aliquot 3 mL of wastewater sample into a 15 mL conical tube. Add controls or spike-ins at this stage. Mix well.

Tip: If using less than 3 mL of sample, add phosphate buffered saline (PBS) or nuclease-free water to bring the total sample volume up to 3 mL.

Optional: Add 150 µL of Proteinase K. Mix well.

Note: Proteinase K is used to remove contamination from the nucleic acid preparations, which can, in turn, improve performance.

2. Add 5 mL lysis buffer and mix (invert 30 times or vortex for 3 seconds).
3. Incubate at 60 °C for 10 minutes. Following the 10-minute incubation allows samples to cool at room temperature for ~3 to 5 minutes before proceeding to the next step.
4. Add 5 mL 100% EtOH.
5. Completely resuspend viral DNA/RNA microbubbles and add 1 mL to the lysed wastewater solution.

Tip: The viral DNA/RNA microbubbles are buoyant and will form a layer at the top of the solution after sitting for extended periods of time. Ensure that the viral DNA/RNA microbubbles have been properly mixed before adding them to the sample.

It is helpful to tap the bottle on your hand to break up the microbubble layer. The solution should appear milky and homogenous when properly mixed.

6. Mix samples using an end-over-end rotator for 5 minutes at ≥ 20 rpm or invert tubes a total of 30 times.
7. Centrifuge for 30 seconds at ≥ 8,000 rpm to promote microbubble surfacing.
8. **Carefully penetrate the microbubble layer**, aspirate, and discard the subnatant and pellet with a 9" Pasteur pipette until there is < 0.5 mL of subnatant remaining below the microbubble layer.

Note: The user will need to complete several (approximately five) transfers of the subnatant volume from the sample tube to waste.

Tip: Go slowly.

Tip: For the first aspiration, limit microbubble layer disturbance by running the pipette tip down the side of the conical tube. This will create a more clarified opening following the first aspiration, allowing for subsequent aspiration of subnatant without touching the microbubble layer.

Tip: Try to limit the removal of the microbubbles, as they contain the RNA.

9. Add 2.5 mL of 80% EtOH and vortex for 3 seconds.
10. Centrifuge for 30 seconds at > 8,000 rpm to promote microbubble surfacing.
11. Add 400 µL of 60 °C elution buffer and vortex for 15 seconds; let sit to incubate at room temperature for 2 minutes.

Tip: Ensure that all the microbubbles come into contact with the elution buffer.

12. Centrifuge for 30 seconds at > 8,000 rpm to promote microbubble surfacing.

Note: The subnatant now contains the RNA.

Tip: If not immediately moving to RNA cleanup/isolation, place on ice or store at –80 °C until the sample is ready.

13. Transfer the approximately 425 µL of subnatant into the previously prepared 2 mL collection tube (harboring the lysis buffer and EtOH, refer to "Before You Begin").
14. Vortex for 10 seconds and centrifuge the samples for 30 seconds at ≥ 8,000 rpm to bring down the liquid from the lid and sides of the tube.
15. Place a DNA/RNA binding cup into a new 2 mL collection tube. Transfer ≤ 700 µL of the sample to the DNA/RNA binding cup.
16. Centrifuge for 15 seconds at ≥ 8,000 rpm.
17. Aspirate and discard flow-through and repeat steps 15 to 16 with the remaining sample as many times as needed using the same DNA/RNA binding cup.
18. Add 500 µL of 80% EtOH to the DNA/RNA binding cup.
19. Centrifuge for 2 minutes at ≥ 8,000 rpm.
20. Transfer the DNA/RNA binding cup to a new 2 mL collection tube and centrifuge for 1 minute at ≥ 8,000 rpm to remove residual EtOH.
21. Place the DNA/RNA binding cup in a new 2 mL collection tube. Add 100 µL of 60 °C elution buffer. Let the sample incubate at room temperature for at least 2 minutes before proceeding.
22. Centrifuge for 1 minute at ≥ 8,000 rpm.
23. Retain the sample eluate containing RNA and immediately place the sample on ice for subsequent analysis by qRT-PCR, or store at –80 °C until the sample is ready for qRT-PCR analysis.

CAUTION: Do not add bleach or acidic solutions directly to the sample, kit reagents, or preparation waste.

qRT-PCR Analysis

Quantitative reverse transcription PCR (qRT-PCR) is a powerful tool for gene expression analysis. The Brilliant III Ultra-Fast qRT-PCR master mix was developed for the ABI StepOnePlus and Bio-Rad CFX96 real-time PCR instruments and other fast-cycling systems (Figure 1). It performs qRT-PCR in less time without compromising target detection sensitivity, specificity, or reproducibility. The master mix includes two key components that enable it to perform optimally under fast cycling conditions:

- A mutated form of Taq DNA polymerase that has been specifically engineered for faster replication
- An improved chemical hot-start mechanism that promotes faster hot-start release to improve amplification specificity while keeping the run time of the PCR protocol to a minimum

The master mix has been successfully used with fluorescent TaqMan probes to amplify and detect a variety of high- and low-abundance RNA targets from experimental samples, including total RNA, poly(A)+ RNA, and synthetic RNA.

Note: For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system.

Agilent AriaMx

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	95°C
40	5 seconds	95°C
	10 seconds	60°C

Agilent Mx3000P and Mx3005P

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds	95°C
	20 seconds	60°C

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	12 seconds	95°C
	15 seconds	60°C

ABI 7900HT Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	15 seconds	60°C

ABI StepOnePlus

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds[c]	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	95°C
40	5 seconds	95°C
	10 seconds	60°C

QIAGEN Rotor-Gene Q

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds	95°C
	10–20 seconds	60°C

Roche LightCycler 480

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

Figure 1. RT-PCR cycling programs for the Brilliant III Ultra-Fast qRT-PCR master mix on various instrument platforms.

Recommended consumables

Each Agilent Viral RNA/DNA Wastewater extraction kit (part number 5610-5514) provides the lysis buffer, viral DNA/RNA microbubbles, elution buffer, DNA/RNA binding cups, and collection tubes for 50 sample extractions.

Agilent provides complete reagent solutions for pathogen detection in wastewater samples (Table 2).

Table 2. Suggested Agilent consumables for viral DNA/RNA analysis in wastewater samples.

Agilent Part Number	Description
600884	Brilliant III Ultra-Fast QRT-PCR master mix
401490	96-Well plates, skirted and low profile (100 µL)
401425	Optical tube strip caps (8x strip)
401492	Adhesive plate seals
401493	Tube strips (8x strip)
600886	Brilliant III Ultra-Fast SYBR Green qRT-PCR master mix
600880	Brilliant III Ultra-Fast qPCR master mix
600883	Brilliant III Ultra-Fast SYBR Green qPCR master mix
600105	AffinityScript Multiple Temperature Reverse Transcriptase for use with qPCR kits

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PR7001-1804

Printed in the USA, November 28, 2023
5994-6932EN

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Benchtop protocol for the Agilent Viral DNA/RNA wastewater prep kit

CAUTION: Do not add bleach or acidic solutions directly to the sample or preparation waste.

1. Prepare all necessary supplies and solutions nuclease-free.
2. Prepare sample collection tubes for each sample. Add 550 µL of lysis buffer and 525 µL of 100% EtOH to a 2 mL collection tube.
3. Aliquot 3 mL of resuspended wastewater sample. Add controls or spike-ins at this stage. Mix well.

   Optional: Add 150 µL of Proteinase K. Mix well.
4. Add 5 mL of lysis buffer and mix.
5. Incubate at 60 °C for 10 minutes. Allow samples to cool before proceeding.
6. Add 5 mL of 100% EtOH.
7. Add 1 mL of properly-mixed viral DNA/RNA microbubbles.
8. Mix samples using 5 minutes of end-over-end rotation at ≥ 20 rpm or invert tubes a total of 30 times.
9. Centrifuge for 30 seconds.
10. **Carefully penetrate the microbubble layer**, aspirate, and discard the subnatant and pellet with a 9" Pasteur pipette until there is < 0.5 mL of subnatant remaining below the microbubble layer.
11. Add 2.5 mL of 80% EtOH and vortex for 3 seconds.
12. Centrifuge for 30 seconds.
13. Add 400 µL of 60 °C elution buffer, vortex, and incubate for 2 minutes.
14. Centrifuge for 30 seconds. If not immediately moving to RNA cleanup/isolation, place on ice or store at –80 °C until the sample is ready.
15. Transfer approximately 425 µL of the subnatant into the previously prepared 2 mL collection tube (see step 2).
16. Vortex for 10 seconds and centrifuge for 30 seconds.
17. Place a DNA/RNA binding cup into a new 2 mL collection tube. Transfer ≤ 700 µL of the sample to the DNA/RNA binding cup.
18. Centrifuge for 15 seconds.
19. Aspirate and discard flow-through and repeat steps 17 and 18 with the remaining sample as many times as needed using the same DNA/RNA binding cup.
20. Add 500 µL of 80% EtOH to the DNA/RNA binding cup.
21. Centrifuge for 2 minutes.
22. Transfer the DNA/RNA binding cup to a new 2 mL collection tube and centrifuge for 1 minute.
23. Place the DNA/RNA binding cup in a new 2 mL collection tube.
24. Add 100 µL of 60 °C elution buffer. Incubate for 2 minutes before proceeding.
25. Centrifuge for 1 minute.
26. Retain filtrate for downstream use. Use immediately or store at –80 °C.