Application Note

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The Development of a Sensitive and Reliable qRT-PCR-Based SARS-CoV-2 Wastewater Analysis Protocol

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Weck Laboratories (located in Southern California) is an environmental-certified analytical testing laboratory. In a recent webinar, Dr. Agustin Pierri (Technical Director, Weck Laboratories) demonstrated how they developed a sensitive and reliable qRT-PCR-based SARS-CoV-2 wastewater analysis workflow.

Abstract

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. Researchers at Weck Laboratories developed a qRT-PCR-based SARS-CoV-2 test for wastewater using Agilent RNA purification kits as well as qRT-PCR reagents and instrumentation. The assay was able to reliably determine SARS-CoV-2 viral RNA levels in a variety of raw wastewater samples with high sensitivity (observed as low as 8,000 viral genome copies per liter of wastewater). Finally, it has been demonstrated that there is a correlation between SARS-CoV-2 RNA levels in wastewater and clinical cases[1], potentially providing predictive models for the community and healthcare institutions.

Introduction

The SARS-CoV-2 virus was initially identified in December 2019 and, in the months since, rapidly expanded into a global pandemic. Methods to track viral spread within the community, such as individual testing and contact tracing, were quickly deployed.

While these methods are key to managing pandemic responses, additional information can help capture a more complete picture. One such source of information can come from monitoring wastewater reservoirs as a potential indicator of imminent viral spread in the larger community. As pathogenic microbes (including SARS-CoV-2[1–3]) pass through the gastrointestinal tract, wastewater represents an aggregate sample of the selected population. With strategic placement of monitoring stations, samples can be analyzed from targeted communities including schools, dormitories, and retirement homes. Additionally, since wastewater monitoring is proactive rather than reactive, it can provide information on the presence of SARS-CoV-2 in the community roughly five days earlier than clinical testing[1,4,5].

In a recent webinar, Agustin Pierri, Ph.D., Technical Director, Weck Laboratories, demonstrated the development of a sensitive and reliable qRT-PCR-based SARS-CoV-2 wastewater analysis workflow. This study provides further details of the Weck Laboratories protocol and demonstrates real-world applications for monitoring SARS-CoV-2 levels in wastewater.

Materials and Methods

Collection, processing, and RNA extraction from wastewater

Raw wastewater (50 mL) was collected into a conical tube, pasteurized at 75 °C for 90 minutes, and pulled through a 0.22 µm filter to remove larger suspended solids. The 50 mL filtrate was then concentrated by passage through a 10 kilodalton (kDa) molecular weight cut-off (MWCO) polyethersulfone (PES) filter, resulting in the concentration of the 50 mL wastewater sample to approximately 400 µL.

RNA was then isolated from the 400 µL concentrated wastewater using the Agilent Absolutely RNA Miniprep kit (Part Number 400800) following Appendix II, "*Purifying RNA Following an Enzymatic Reaction*" as outlined in the Absolutely RNA Miniprep kit manual. The protocol was modified to include one additional wash step each for both the High-Salt and Low-Salt Wash Buffers to reduce the presence of PCR-inhibitors. RNA was eluted into a final volume of 100 µL.

qRT-PCR and quality control considerations

Following RNA purification, 2 µL of RNA was used for each qRT-PCR reaction using Agilent Brilliant III Ultra-Fast qRT-PCR Master Mix (Part Number 600884)[6] following manufacturer's instructions. Two separate qRT-PCR plates were run, one using the CDC-EUA SARS-CoV-2 N1 primer/probe set and the other using the N2 primer/probe set[7], on an Agilent AriaMx Real-Time PCR system.

SARS-CoV-2 N1 and N2 targets were analyzed for each wastewater sample in duplicate. Given the impurity of wastewater samples, stringent quality controls were included on each plate. These included:

- A calibration curve using Armored RNA Quant SARS-CoV-2 positive control (Asuragen) spanning five orders of magnitude [1 x 105 – 10 genomic copies/qRT-PCR) run in triplicate]
- Batch QCs, all of which were run in triplicate, included:
  - A no-template control (nuclease-free water)
  - A negative template control (human coronavirus OC43, a coronavirus not detected by the CDC-EUA SARS-CoV-2 N1 or N2 primer sets)
  - A second preparation of the Armored RNA Quant SARS-CoV-2 positive control synthetic RNA) as a secondary confirmation of N1 and N2 primer set assay positivity
- A process control in the form of a matrix spike, in which a non-infectious replication-deficient SARS-CoV-2 viral mimic (SeraCare AccuPlex SARS-CoV-2 Reference Material) is added to a representative incoming wastewater sample that is processed through the entire procedure including concentration, RNA extraction, and qRT-PCR (extracted RNA run in triplicate).
- Wastewater sample PCR-inhibition control, in which extracted RNA from each wastewater sample is spiked with the same quantity as the second source preparation of the Armored RNA Quant SARS-CoV-2 positive control. An increase in Cq value relative to the second-source SARS-CoV-2 positive control indicates the presence of a PCR-inhibitor within the RNA extracted from the specific wastewater sample. Note, for wastewater samples with endogenous SARS-CoV-2, a decrease in the Cq value relative to its associated non-spike-in wells may be observed.

Many wastewater concentration and extraction methods were evaluated and proved unsuccessful, either due to technical issues (such as particulate matter clogging the 10 kDa MWCO filter) or insufficient removal of salt and/or heavy metals from the extracted samples as indicated by PCR inhibition. Following extensive testing, the researchers finalized the method described in the workflow below (Figure 1).

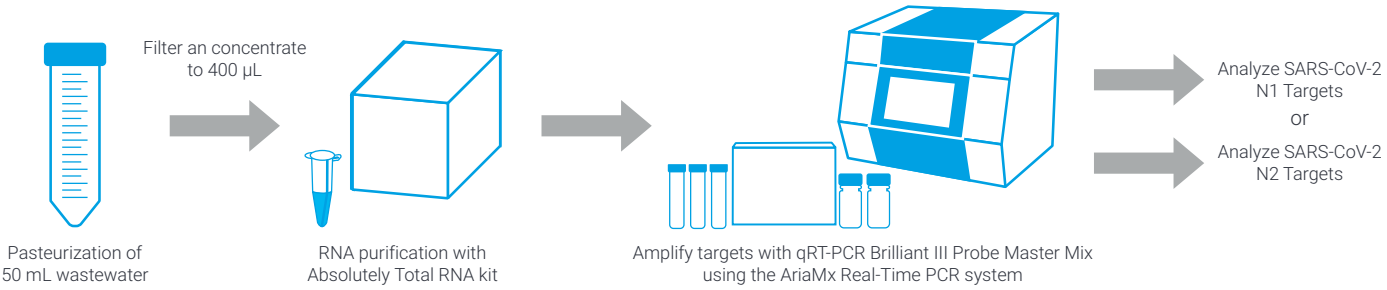

Figure 1. Overview of Weck Laboratories' workflow for extracting and detecting SARS-CoV-2 RNA in wastewater.

Optimization of wastewater pretreatment and RNA extraction

One of the primary challenges of using wastewater as a starting sample is the complexity and variability of constituent organic and inorganic molecules and microorganisms, including (but not limited to) industrial and local runoff, rainfall, and other contaminants. To address this, researchers investigated several concentration and RNA extraction methodologies which included:

- Polyethylene glycol (PEG)/NaCl precipitation and centrifugation
- Initial filtration through a 0.22 µm filter, followed by PEG/NaCl precipitation and centrifugation
- Raw wastewater water filtration and concentration through use of a 10 kDa MWCO filter (i.e., no initial filtration through a 0.22 µm filter).
- Use of lysis and stabilizing buffer
- Magnetic silica-based bead RNA purification
- Spin column-based RNA purification (Agilent Absolutely RNA Miniprep kit)
- Initial filtration through a 0.22 µm filter, followed by ultrafiltration using a 10 kDa MWCO PES filter

PEG/NaCl precipitation and centrifugation concentration methods provided low sample recovery regardless of whether the samples received an initial 0.22 µM filtration. Without an initial filtration of the raw wastewater through a 0.22 µM filter, the 10 kDa MWCO device clogged, leading to ineffective sample concentration. RNA extraction was negatively impacted when samples were processed with lysing and stabilizing buffers, especially with wastewater samples containing higher total suspended solids (TSS). Both magnetic silica-based beads and spin column-based strategies were able to provide sufficiently pure RNA.

After assessing the various concentration and extraction methods outlined above, the best method was determined to be an initial filtration of the raw wastewater sample through a 0.22 µm filter to remove larger suspended solids, passing it through a 10 kDa MWCO ultrafiltration device, and RNA extraction from the retained concentrate using spin column-based RNA purification. The data presented in this application note were generated using this method.

Results

Successful detection of SARS-CoV-2 in wastewater

When analyzing purified wastewater specimens by qRT-PCR, SARS-CoV-2 RNA levels were detectable across five 10-fold serial dilutions, representing a broad dynamic range for both CDC-EUA primer sets for N1 (Figure 2A and N2 (data not shown). Log input viral RNA copy numbers were highly concordant with cycle threshold values (Cq; r^2=0.99621, Figure 2B), demonstrating the linearity and high sensitivity of the qRT-PCR. The assay demonstrated a mean detection limit of approximately 8,000 viral genome copies per liter of raw wastewater, with the N1 primer set offering slightly greater sensitivity than the N2 primer set (data not shown).

Testing wastewater SARS-CoV-2 levels reveal infection trends within a residential community*

In another example of how this assay can provide important information, Weck Laboratories monitored wastewater SARS-CoV-2 levels downstream of a student residence hall (Figure 3). When monitoring began, minimal staff were onsite. On day 5, students moved in and monitoring continued for 7 days. Viral load levels started at 10,000 copies per liter for the first four days of monitoring, but levels began to rapidly rise beginning at day 5 when an unspecified number of students were reported by the residence hall management team to have moved into the residence hall. SARS-CoV-2 levels plateaued at 22,500 copies/liter, indicating that the viral load had stabilized and was not continuing to rise.

Measuring SARS-CoV-2 RNA levels in wastewater was also able to show a rise in viral load within this community roughly five days ahead of a rise in SARS-CoV-2 cases based on individual testing (as reported by the residence hall management).

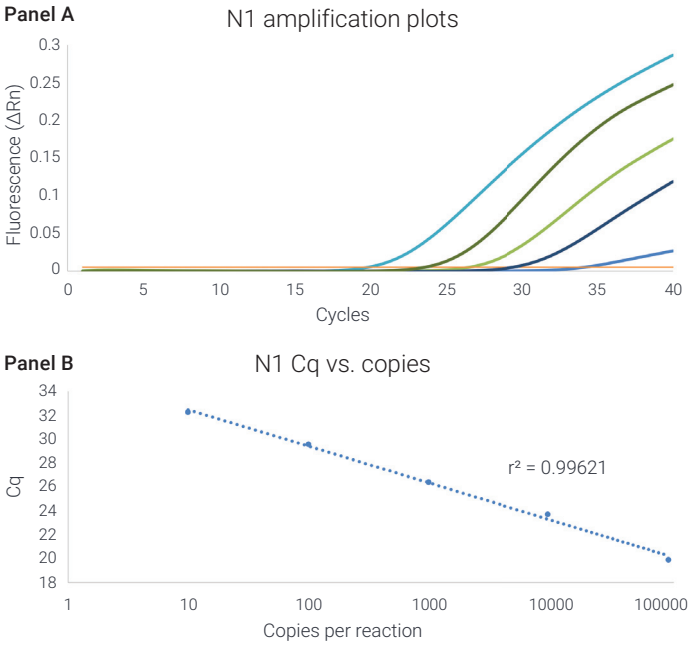

Figure 2. Sensitive qRT-PCR detection of SARS-CoV-2 RNA from wastewater samples. Amplification plots (panel A) demonstrate detection of SARS-CoV-2 mRNA across five orders of magnitude (undiluted sample, 10-, 100-, 1,000, and 10,000-fold dilutions). Cycle threshold values reveal extremely high correlation with viral copy number (panel B). Brilliant III qRT-PCR kit used on the AriaMx real-time PCR instrument.

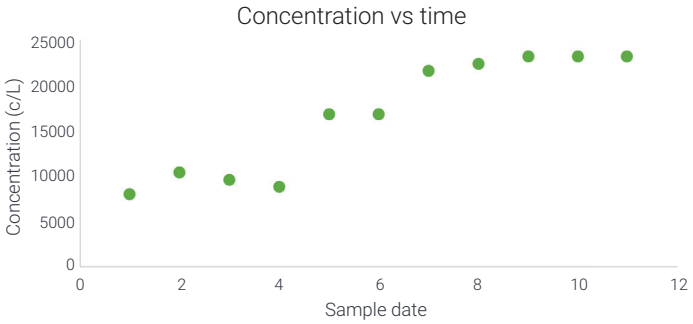

Figure 3. Analysis of SARS-CoV-2 levels in wastewater in a student residence hall. Only staff were onsite days 0-4. With the arrival of students, an increase in the concentration of SARS-CoV-2 viral RNA was observed. Student resident move-in occurred on approximately day 5, blue vertical bar. This was followed by a detectable spike in SARS-CoV-2 levels.

* *Data on file at Weck Laboratories*

Conclusions

As the COVID-19 pandemic continues, researchers are striving to develop novel technologies that provide a clearer picture of SARS-CoV-2 spread. The method described here uses wastewater as an aggregate sample to provide a 'snapshot' of SARS-CoV-2 in a given community. With ongoing wastewater testing, researchers and public health officials are able to identify trends in viral load within communities to detect an increase in SARS-CoV-2 viral RNA levels which may indicate a potential increase in SARS-CoV-2 cases.

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Published in the USA, September 15, 2021
5994-2875EN

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