Title:

Two-step concentration of complex water samples for the detection of viruses

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Abstract:
The accurate detection and quantification of pathogenic viruses in water is essential to understand and reduce the risk of human infection. In this paper, we validated a two-step method for the concentration of enteric viruses in water and wastewater samples. The method involves a tangential flow ultrafiltration step that reduces the sample volume of 1 – 10 L to approx. 50 mL, followed by secondary precipitation using polyethylene glycol 6000 that reduces the volume to 1 – 4 mL. For method validation, surface water samples were spiked with different concentrations of enteric viruses and viral recoveries in the concentrates exceeded 10% in all experiments. The method is suitable for water samples with high and low salinity and turbidity, allowing the accurate comparison of viral titers in a diverse range of water types. Furthermore, the method has the potential to concentrate other pathogens, e.g. bacteria or protozoa. Hence, the use of this method can improve the holistic assessment of risks associated with wastewater contaminated environments.

- *Modified tangential flow ultrafiltration method that reduces membrane clogging.*
- *Secondary concentration with a beef extract-elution step to remove solid matter.*
- *The two-step concentration method enables at least 10% recovery for different enteric viruses in difficult matrices.*
- *Using the two-step concentration method 10,000x concentration can be achieved.*

Keywords:
tangential flow ultrafiltration; virus precipitation, qPCR; enteric viruses; norovirus; adenovirus

Specifications Table:

Subject area	Environmental Science
More specific subject area	Health-related water virology
Method name	Tangential flow ultrafiltration-based two-step water concentration
Name and reference of original method	Hill, V.R., Polaczyk, A.L., Hahn, D., Narayanan, J., Cromeans, T.L., Roberts, J.M., Amburgey, J.E., 2005. Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. Appl. Environ. Microbiol. 71, 6878–6884. doi:71/11/6878 [pii] Lewis, G.D., Metcalf, T.G., 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl. Environ. Microbiol. 54, 1983–1988. Farkas, K., Hassard, F., McDonald, J.E., Malham, S.K., Jones, D.L., 2017a. Evaluation of molecular methods for the detection and quantification of pathogen-derived nucleic acids in sediment. Front. Microbiol. 8, 53.
Resource availability	KrosFlo® Research IIi Tangential Flow Filtration System, Spectrum Labs, USA 100 kDa mPES MiniKros® hollow fibre filter module, Spectrum Labs, USA

Method details

Overview

This method describes an efficient, accurate and reproducible method for the concentration of enteric viruses in surface water and wastewater (treated and untreated) samples (Figure 1). The recommended starting volumes are 10 L for surface water and 1 L for wastewater samples. The first step of the method is a tangential flow ultrafiltration (TFUF) step using a 100 kDa cut-off modified polyethersulfone membrane. As described by others, the efficiency of the elution of the viral particles from the membrane and the cleaning of the system was enhanced using sodium polyphosphate (Hill et al., 2005). The final volume of the sample after TFUF is approx. 50 mL. In order to elute viral particles attached to solid matter in the primary concentrate, samples are further mixed with beef extract and then centrifuged. Then PEG6000 is added to the supernatant and viral particles are precipitated. These steps are based on a method for the elution and concentration of viral particles from sediment (Farkas et al., 2017; Lewis and Metcalf, 1988). The resulting pellet contains the viral particles that can be eluted in PBS and the solution can be stored at -80°C.

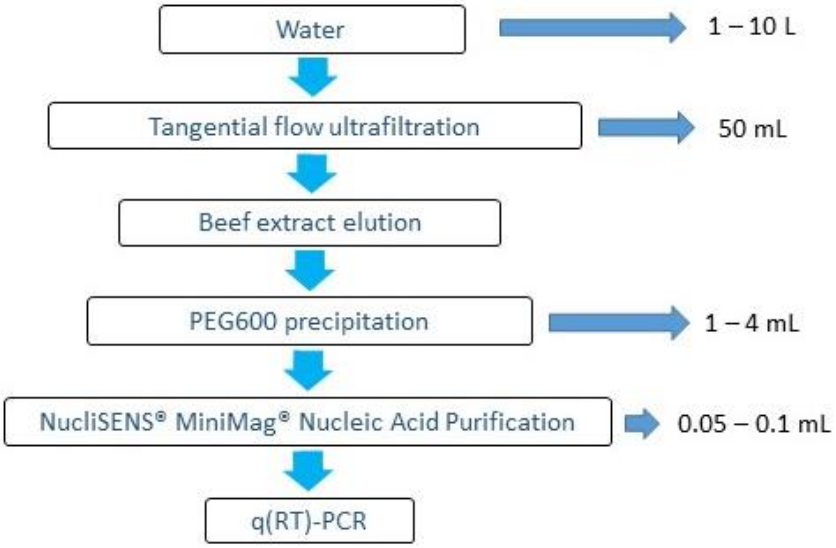

Figure 1 The stages of the tangential flow ultrafiltration-based two-step water concentration method for the detection and quantification of enteric viruses in water and wastewater.

Reagents

- Sodium-polyphosphate (NaPP) / sodium hexametaphosphate (Sigma Aldrich)
- Lab Lemco beef extract (Oxoid)
- Sodium nitrate (Sigma Aldrich)
- Polyethylene glycol (Sigma Aldrich)
- Sodium chloride (Sigma Aldrich)
- Phosphate saline buffer (PBS), pH 7.4
- Vikron ® solution

Equipment

- KrosFlo® Research IIi Tangential Flow Filtration System (Spectrum Labs, USA)
- 100 kDa mPES MiniKros® hollow fibre filter module (Spectrum Labs, USA)
- Silicone tubing #17 (Spectrum Labs, USA)
- Centrifuge (2,500xg and 10,000xg at 4°C)

Concentration of water samples

1. Tangential flow ultrafiltration (Figure 2)

   1.1 System wash

   Wash system with 1 L 0.01% NaPP solution for 5 min (permeate closed) then leave the membrane in the solution for 30-60 min. Wash the membrane with the NaPP solution (permeate open) until the solution has been removed.

   1.2 Sample filtration

   Filter 10 L of surface water or 1 L wastewater at 1-1.6 L/min flow at 5 psi (0.3 bar; 35 kPa) pressure to achieve a permeate flow of 200 – 300 mL/min. Continue filtration until approx. 5 mL sample remains in the reservoir.

   1.3 Backwash, recovery

   Set the flow to 680 mL/min with no pressure applied and circulate the concentrate for 5 min with the permeate clamp closed. Stop pump, close penetrate and retentate valves. Inject 20 mL 0.01% NaPP solution to penetrate pressure valve. Open retentate and wash with reverse flow. Collect the concentrate from the system by introducing air through the retentate port. The final volume of the concentrate is approx. 50 mL.

   1.4 Membrane wash and storage

   Wash membrane with 250 mL Virkon® solution after each sample. In order to reuse membrane, immediately wash it with 150 mL 0.01% NaPP solution. Repeat until solution is clear. Leave the membrane in the solution for at least 10 min. For long-term storage, wash the membrane with 50 mL 20% ethanol solution. Repeat until solution is clear. Dissemble system and store membrane in 20% ethanol solution at 4°C.

2. Secondary concentration

   2.1 Virus elution

   Add beef extract and $NaNO_3$ to 50 mL concentrated water sample to reach final concentration of 3% w/v and 2 mM, respectively. Adjust the pH to 5.5. Incubate at 50-90 rpm on ice for 30 min. Centrifuge at 2,500xg for 10 min, then transfer the supernatant to a new tube. Discard pellet. Adjust the pH of the solution to 7.5.

   2.2 Virus precipitation

   Add PEG6000 and NaCl to reach final concentration of 15% and 2% w/v, respectively. Mix to dissolve PEG and incubate at 4°C for 14-18 h. Centrifuge at 10,000xg for 30 min at 4°C. Discard supernatant. Dissolve pellet in 1-4 mL PBS (pH 7.4). Alternatively, viral nucleic acids can be extracted directly from the pellet.

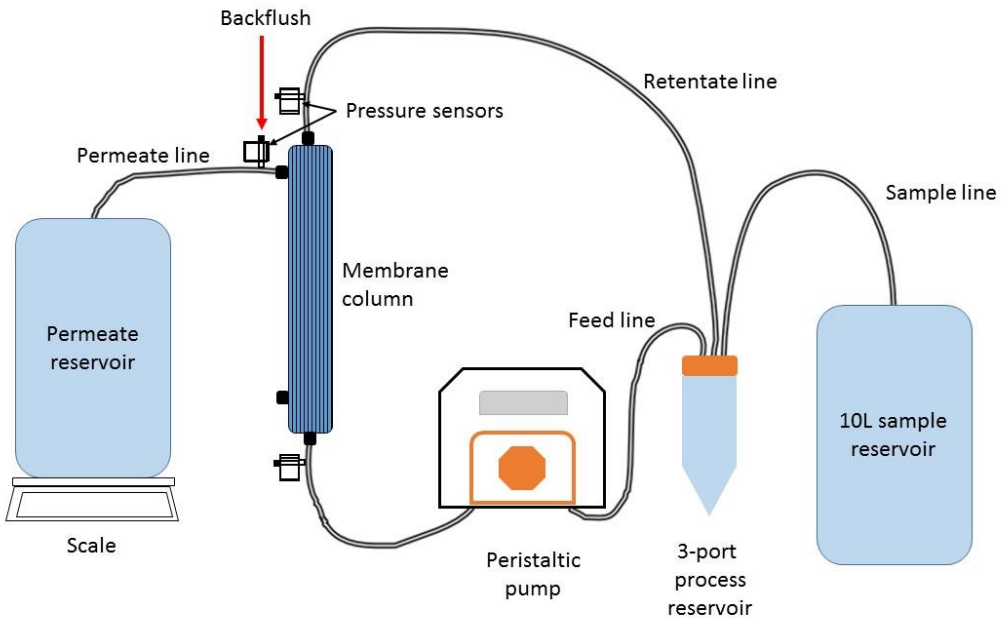

Figure 2 Schematic tangential flow ultrafiltration setup.

Method validation

For pilot validation, 2 L of deionized water was spiked with of norovirus GII to reach the final concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 genome copies (gc)/L in duplicates. Samples were concentrated using the two-step concentration method. Viral RNA was extracted from the pellet (final volume: 50 µL) and quantified using qRT-PCR (sample volume: 2 µL) as described in (Farkas et al., 2017). High recoveries were observed in all norovirus concentrations (Table 1). The high deviations between replicates were result of the limitations of the qPCR method used for quantification. The limit of quantification (LOQ) was 200 gc/L and the limit of detection (LOD) was approx. 50 gc/L (data not shown). The LOD and LOQ can be further lowered by the reduction of the RNA eluent volume and the increase of the sample volume in the qRT-PCR reaction.

Table 1 Norovirus recoveries and standard deviations (SD) in deionized water using the two-step concentration method

Norovirus concentration (gc/L)	Recovery percentile (SD)
5.28×10^6	78 (43)
4.72×10^5	121 (2)
4.31×10^4	99 (11)
3.03×10^3	91 (45)
2.0×10^2	100 (0)

For further validation, 10 L of surface water samples (river, estuarine and sea) in triplicates were spiked with known concentration of human enteric viruses (norovirus GII, sapovirus GI, hepatitis A virus and human adenovirus type 40) and with a mengovirus, which is often used as a process control for the extraction of enteric viruses from environmental matrices. The details of validation are described

elsewhere (Farkas et al, submitted to *Water Research*). In brief, the spiked samples were processed as described above and then the viral nucleic acids were extracted and quantified using a one-step qRT-PCR protocol for the RNA viruses and a SYBR Green qPCR for the adenovirus. Results showed that 10-100% recovery could be achieved in all sample types. In the same study, the usefulness of the method for wastewater samples was also investigated. Influent and effluent samples were taken in duplicates at four wastewater treatment plants. As the samples were expected to contain the target viruses, samples were processed without spiking. High viral concentrations were observed and the method showed great reproducibility as well. No cross-contamination between samples was observed.

Applications and recommendations

The method described above is suitable for the concentration of many different water samples and hence suitable for viral surveillance, contamination source tracking and to describe viral ecology. Furthermore, as the membrane used for the TFUF has a 100 kDa pore size, the method can potentially co-concentrate other microbes as well, enabling the accurate description of microbial quality of a sample. In the current study, viral nucleic acids were directly extracted from the concentrated water samples, however, the concentrates are suitable for viral infectivity and capsid integrity assays as well. For direct nucleic acid extraction, the resuspension of the PEG precipitate is not necessary; nucleic acids can be extracted from the pellet. When PCR-based approaches are used for the quantification of viruses in the concentrate, the use of robust extraction and amplification methods is recommended, as organic matter that may interfere with the enzymes used for amplification are co-concentrated with viral particles. The addition of process control (e.g. mengovirus) to each sample to determine method efficiency is also recommended.

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