Combination of paper membrane-based filtration and ultrafiltration to enhance the detection of MNV, HAV, and HCoV from soil-rich post-washing water

Zhaoqi Wang, Soontag Jung, Daseul Yeo, ..., Hyojin Kwon, Myeong-In Jeong, Changsun Choi
cchoi@cau.ac.kr

Highlights
PFC-UF outperformed others in the recovery of viruses from PWW with soil content
MNV-1 and HAV were recovered 78.8% and 44.5% from PWW with the highest soil content
Enveloped and non-enveloped viruses differed in their recovery efficiencies
PFC-UF presented a feasible approach to detecting the viruses in PWW
Combination of paper membrane-based filtration and ultrafiltration to enhance the detection of MNV, HAV, and HCoV from soil-rich post-washing water

Zhaoqi Wang,1 Soontag Jung,1 Daseul Yeo,1 Seoyoung Woo,1 Yeeun Seo,1 Md. Iqbal Hossain,1 Hyojin Kwon,2 Myeong-In Jeong,3 and Changsun Choi1,4,*

SUMMARY
Risk-assessing and controlling virus transmission from soil-rich post-washing water (PWW) are crucial during harvesting raw vegetables. However, viruses are normally difficult to concentrate because of their low concentrations and complex backgrounds. Here, ultrafiltration (UF), virus adsorption-elution (VIRADEL), and optimized paper filtration-coupled ultrafiltration (PFC-UF) methods were employed to evaluate the recovery of non-enveloped murine norovirus (MNV-1), hepatitis A virus (HAV), and enveloped human coronavirus 229E (HCoV-229E) from soil-rich PWW. Among the three methods, PFC-UF outperformed the other methods in the recovery of viruses from PWW with soil content. Under the highest soil condition with virus seeded at a titer of $10^2$ plaque-forming unit (PFU) or TCID$_{50}$, the PFC-UF method exhibited an exceedingly consistent recovery rate of 78.8 ± 13.3 (MNV-1) and 44.4 ± 25.2% (HAV). However, the recovery of enveloped HCoV-229E was inferior to non-enveloped viruses. Overall, PFC-UF provided a reliable method for recovering viruses in soil-rich PWW.

INTRODUCTION
As food safety progresses, food- and waterborne viruses have been associated with outbreaks of enteric diseases in recent years.1 Among foodborne viruses, typical non-enveloped human norovirus (HuNoV) and hepatitis A virus (HAV) infect people through contaminated raw vegetables, water, and seafood, causing serious public health safety and economic burdens in both developing and developed countries worldwide.2–6 Particularly, the risk assessment and control of foodborne viruses in raw fresh produce, root vegetables, and fruits should not be underestimated.7–10

In 2021, at the 48th session of the Codex Committee on Food Hygiene, it was determined whether the water used in fresh fruits and vegetables (FFVs) during the pre- and postharvest stages fulfilled the “fit for purpose (FFP)” criteria. The importance of quantitative microbiological risk assessment (QMRA) of FFVs in the production chain has been discussed.11 However, assessing the safety of FFP water is complex, especially during the pre- and postharvest stages, because the various factors such as weather, season, and fecal contaminants affecting irrigation water safety could contaminate the cultivated environment of FFV, which in turn increases the possibility of pathogen transmission and contamination in FFV and the following prevalence of foodborne viruses.12 Moreover, they also can persist in an aqueous environment for months, and the soil can be a favorable habitat for them.6,13

Post-washing water (PWW) is produced from harvested fresh produce, fruits, and root vegetables washed on a farm or a wash processing facility. This type of water contains complex background of contaminants including pesticide residues, high soil content, parasites, viruses, bacteria, and polymerase chain reaction (PCR) inhibitory substances from raw fresh produce, root vegetables, and fruit.14–17 In the postharvest stage, once these contaminated FFVs are processed on farms or in washing facilities, the aforementioned factors enable PWW to accumulate large amounts of pathogens. Cross-contamination of contaminated washing water with pathogens could occur in raw vegetables and fruits during the washing process, thus becoming important potential reservoirs for surveillance of viral foodborne outbreaks.17–20 In addition,
this washing processing may result in the internalization of pathogens in vegetables with lacerations. Therefore, if raw vegetables and fruits are not properly handled and controlled at all stages of production, harvesting, and processing, it will have a direct impact on food safety available through retail outlets and threaten people’s health.

To date, there is a lack of effective detection approaches for foodborne viruses in this soil-rich complex water environment. Because effective recovery of viruses from environmental water samples is still a technical challenge because of a low level of viruses in environmental water that cannot be used directly for PCR assays without concentration of viruses in the samples. Therefore, we need to develop rapid, efficient, and stable methods to detect viruses in PWW.

In previous studies, ultrafiltration (UF) and negatively charged membrane-based virus adsorption-elution (VIRADEL) methods with excellent performance in the detection of enveloped and non-enveloped viral contaminants in wastewater and highly contaminated water were widely employed. In particular, the VIRADEL method provides a solid technical support for detecting enveloped severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in wastewater. However, urban wastewater and post-washing water is totally different, and it has not been determined whether these two methods can be used for virus recovery and detection in soil-rich post-washing water conditions. In addition, we optimized a paper filtration-coupled ultrafiltration (PFC-UF) method based on UF and membrane-based VIRADEL methods to determine and compare, by seeding typical laboratory non-enveloped MNV-1 (a surrogate for human norovirus) and HAV strain HM175, and enveloped HCoV-229E (a surrogate for SARS-CoV-2) in PWW with and without soil content, the performance of the three methods and to provide a feasible approach to effectively isolate or concentrate viruses from PWW.

RESULTS
Turbidity in PWW
Turbidity (nephelometric turbidity units [NTU]) of PWW with 1%, 2%, 3%, and 5% soil content (w/v) were measured in the post-mix stage. In addition, the turbidity of filtrate from membrane-based pre- and post-filtration stages was measured for PFC-UF method as well (Figure 1). After mixing, turbidity of the PWW with 1% and 2% soil content was 147.3 ± 37.7 and 426.0 ± 20.0 NTU, respectively (Table 1). In PWW with 3% and 5% soil content, over 800 NTU was not measurable; a 10-fold dilution of the original mixtures was measured as 156.0 ± 21.0 and 340.0 ± 41.3 NTU, respectively. In PFC-UF method, before paper membrane filtration, the turbidity was 57.7 ± 21.1, 123.7 ± 35.5, 214.7 ± 33.3, and 339.3 ± 7.8 NTU in 1%,
2%, 3%, and 5% soil concentrations of PWW, respectively. After filtration, the turbidity of the filtrate was 1.6 ± 0.6, 3.9 ± 0.4, 3.7 ± 0.7, and 6.9 ± 5.4, respectively.

Background, limit of detection (LOD), inhibition assay, and performance of RT-qPCR
The RNA was extracted from PWW and tap water separately without virus seeding to determine the concentration of background viruses. Both PWW and tap water were determined to be negative for MNV-1, HAV, and HCoV-229E by RT-PCR assay. In addition, there was a LOD for each virus in the three methods. The LOD value was determined to be 2 × 10^2, 3 × 10^2, and 1 × 10^2 genome copies (GC) in the UF (50 mL), VIRADEL (500 mL), and PFC-UF (500 mL) methods, respectively. The mean Cq value for the inhibition test was 34.19 for A and 32.74 for B. The difference between the Cq values was below 2.

Recovery performance of MNV-1, HAV, and HCoV-229E in the three methods
The performance (detection and recovery rate) of each method was estimated by recovering seeded MNV-1, HAV (10^5 TCID_{50} PFU), and HCoV-229E (10^5 – 10^2 TCID_{50}) from PWW with soil (1%, 2%, 3%, and 5%; w/v) or no soil (0%, w/v) (Figures 2, 3, and 4 and Table S2). Recovered viruses were quantified to GC by RT-qPCR to determine the recovery rate (%).

In the UF method, MNV-1 and HAV were observed to be similar in detection and recovery as the recovery rate and detections decreased gradually with the decrease of virus seeded concentration and the increase of soil content. In contrast, the detection of HCoV-229E showed stable detection, and the lowest recovery rate was greater than 3.9 ± 1.9% (Figure 2 and Table S2). In the VIRADEL method, MNV-1 and HAV started with low recovery rates. As the soil content increased, the recovery rate and detection rebounded to 10^3 and 10^2 PFU seeded PWW, but extremely unstable detection and low recovery rates were seen in the 10^3 and 10^2 PFU seeded PWW with soil and no soil. In contrast, the detection of HCoV-229 in PWW with soil was stable, but the recovery rates were poor (<4.9 ± 2.2%). For the recovery of MNV-1, HAV, and HCoV-229E in PWW without soil by VIRADEL method, a consistent trend was observed, i.e., the detection and recovery rates were not as good as in PWW with soil content (Figure 3 and Table S2). In the PFC-UF method, the detection of MNV-1, HAV, and HCoV-229E seeded in PWW with soil content was the most stable among the three methods with all detected and exhibiting excellent recovery rates (Figure 4 and Table S2).

The total recovery rate of the three viruses from PWW with different soil concentrations using the three concentration methods are shown in Table 2. The PFC-UF method was the most efficient among the three methods (p < 0.05). For MNV-1, the recovery rate of the PFC-UF method was 117.4 ± 58.5% with a stable detection of 144/144, while only 2.4 ± 4.0% and 6.1 ± 10.0% recovery with an unstable detection of 136/144 and 103/144 was achieved in the UF and VIRADEL methods, respectively. Similarly, the recovery rate of HAV in the PFC-UF method was 118.2 ± 126.7% with a stable detection of 144/144. Although the recovery rate of HAV in the UF and VIRADEL methods was 6.0 ± 10.1% and 24.5 ± 19.7%, respectively, the low detection (104 and 94/144) was not optimal. Furthermore, the recovery rate of HCoV-229E using the PFC-UF method was 14.3 ± 10.9%, which was significantly higher compared with the UF and VIRADEL methods (p < 0.05). Consistent detection of 144/144 was observed among the three methods.

Recovery of virus from PWW under extreme conditions in this study
Comparison of the recovery of 10^2 PFU or TCID_{50} seeded MNV-1, HAV, and HCoV-229E from PWW with 5% soil content (over 800 NTU) using the three methods are presented in Table 3. The PFC-UF method

### Table 1. Nephelometric turbidity units (NTU) of PWW (post-washing water) with different soil content in virus recovery stages

| Soil content (w/v) | Post-mix stage | Pre-filtration stage | Post-filtration stage |
|--------------------|----------------|----------------------|-----------------------|
| 0%                 | –              | 0.1 ± 0.1            | –                     |
| 1%                 | 147.3 ± 37.7   | 57.7 ± 21.1          | 1.6 ± 0.6             |
| 2%                 | 426.0 ± 20.0   | 123.7 ± 35.5         | 3.9 ± 0.4             |
| 3%                 | 156.0 ± 21.0   | 214.7 ± 33.3         | 3.7 ± 0.7             |
| 5%                 | 340.0 ± 41.3   | 339.3 ± 7.8          | 6.9 ± 5.4             |

*Over 800 NTU, a 10-fold dilution of the mixture was measured.
exhibited highly stable and excellent recovery rates of 78.8 ± 13.3% and 44.4 ± 25.2% for MNV-1 and HAV (p < 0.05), respectively, while MNV-1 and HAV showed lower recovery rates with sporadic detection in the UF and VIRADEL methods. However, only 4.0 ± 2.7% of HCoV-229E was recovered, and there was no statistical significance compared to other UF and VIRADEL methods (p > 0.05).

**DISCUSSION**

We aimed to establish an approach for virus recovery from soil-rich PWW that would provide a rapid means of detecting viral contaminants in the post-harvest stage to monitor and manage food safety in the supply chain. Because there were limited approaches to detect viruses from PWW with soil content, UF and VIRADEL, which are frequently applied in the rapid recovery of viruses from wastewater, were used in this study. In addition, based on the previous two methods, we optimized a PFC-UF method to compare the efficiency of the three methods in recovering three viruses from PWW with and without soil.
Among the three methods, the PFC-UF method exhibited a recovery rate of 117.4 ± 58.5% and 118.2 ± 126.7% for non-enveloped MNV-1 and HAV, respectively, whereas recovery rate of 14.3 ± 10.9% was observed for the enveloped HCoV-229E. The recovery efficiency was significantly higher in comparison with the other two methods (p < 0.05) (Table 2). Moreover, under the conditions of the highest soil concentration and low virus titers, the stable recovery of virus and consistent detection indicate that the PFC-UF method provides a stable and feasible approach for the recovery and detection of the three viruses.

However, the PWW simulated in a laboratory study is not identical to the actual PWW conditions during the post-harvest stage because different types of soil are required for the cultivation of different crops. It is not clear whether the excellent recovery from the PWW with sandy loam soil observed is similar to that from PWW containing other soil types because soil organic matter (SOM) content, clay ratio, microbiota, and

Figure 3. VIRADEL (virus adsorption elution): the performance of recovery efficiency in MNV-1, HAV, and HCoV-229E seeded in PWW
X-axis: the color of the dots represents the soil content from left to right sequentially: black (genome copies seeded) indicated positive control (P.C); light blue (0% soil content); light clay to deep gray (1%, 2%, 3%, and 5% soil content; w/v). Y axis: genome copies (GC) value. The red dotted line indicates the LOD = 2 × 10^2 GC. *indicates the detections in each replicate experiment. †indicates different titer of viruses seeded in PWW. ND indicates non-detected.
the inhibitory substances from the soil can affect the virus recovery in water. \(^{31,32}\) In addition, the volume of water collected from the samples has been a debatable issue. The volume of water collected in the current study had to be consistent with the volume of water collected from raw wastewater (<1 L) as described by Rames et al. (2016). \(^{33}\) Practically, there was a limited reference about the soil concentration in PWW; we can only determine the virus contamination level based on the turbidity or total suspended solids (TSS) level in PWW. However, the correlation between the turbidity or TSS of the soil in PWW and virus contamination level requires further investigation.

**Methodology of virus recovery in PWW**

In this study, UF method as described in previous studies, using a centrifugal concentrator device to concentrate 50 mL of sample by centrifugation, was implemented. \(^{24,34}\) In UF concentration method, the sample is separated based on the size exclusion, i.e., the virion larger than membrane pore size was...
retained, and the smaller particles and PWW are filtered.\textsuperscript{35,36} According to previous studies, 10 and 30 kDa have been more frequently used to recover enveloped and non-enveloped viruses.\textsuperscript{24,32,37} The type of membrane used in this study was polyether sulphone (PES) with a 10 kDa molecular weight cutoff (MWCO). With its hydrophobicity, resistance to protein adsorption, stability in water, and high product recovery, the PES membrane has been used in virus recovery for years. These properties allow the membrane to perform well in the removal and recovery of viruses from wastewater.\textsuperscript{24,34}

For the VIRADEL method, a negatively charged membrane binding to virus by a salt (MgCl\textsubscript{2}) bridge has shown outstanding performance in the field of virus detection in wastewater.\textsuperscript{38–40} However, its performance was shown to be mediocre in this experimental design with low recoveries and unstable detection (Figure 3 and Table S2). The causes and mechanisms leading to poor performance are unclear. It is worth noting that MNV-1 and HAV started with a low recovery rate but rebounded as the soil content increased (Figure 3 and Table S2). One possible reason is that the soil matrix enhanced the virus adsorption to membrane surfaces and facilitated virus recovery.\textsuperscript{41} Similarly, the recovery rate of viruses in PWW with soil content was superior to that in PWW without soil content in this study. According to the previous recommendations, using a bead tube to shatter the membrane directly by the addition of nucleic acid lysis liquid can increase the recovery efficiency.\textsuperscript{42,43} We attempted to add garnet beads to the 2 mL bead tube to break the membrane and add lysis buffer. Eventually, the recovery rate was less than 1% by RT-qPCR assay. It may be due to the fragmented cellulose membrane that only half of the lysis buffer was obtained, as a result affecting the viral RNA recovery. Ahmed et al. (2020b)\textsuperscript{24} suggested that using 5 mL tubes may help to improve the recovery, but this was not attempted in this study. In addition, we are unsure of the capacity of the anion membrane surface during this filtration processing and whether the adsorption capacity of negative ions on the membrane surface remains constant. Referring to previous studies, we speculate that these differences may be due to several factors, such as the type of ion membrane, the type and composition of the filtrate, pH, volume, and isoelectric point of the concentrated target virus, etc., which require more consideration.\textsuperscript{44–47} Considering virus loss and analysis of membranes was not a feasible direction based on our study results; we analyzed the virus from the filtrate. Results showed that 46.1% of the total seeded virus (data not shown) was recovered. Hence, we focused further on analysis of the filtrate.

In the PFC-UF method, we adopted the same approach from the VIRADEL method but removed the soil matrix with a paper membrane without adsorption capacity and obtained a pure filtrate (Table 2). Considering its low turbidity, we performed a UF concentration method. To lower the detection limit, we considered utilizing tangential flow UF (TFUF). It has been mainly used in pharmaceutical product and antibody vaccine concentration.\textsuperscript{48,49} However, many studies have attempted to use it to recover and concentrate pathogens from the aqueous environment in recent years.\textsuperscript{36,50,51} In a preliminary experiment, TFUF may not be suitable for this study because of a few issues: water with high turbidity usually tends to clog membrane pores, affecting the permeation efficiency of viruses from the membrane\textsuperscript{52} and leading to low recovery and repeat use of membrane; costly equipment and membranes; and time-consuming

### Table 2. Comparison on the total recovery performance of three viruses from PWW with soil content (1%, 2%, 3%, and 5%; w/v) through three methods

| Concentration methods | Viruses                  | Recovery rate (%) (Mean ± SD) | Detection (detected/total) |
|-----------------------|--------------------------|-------------------------------|-----------------------------|
| **UF (ultrafiltration)** | MNV-1                    | 2.4 ± 4.0                     | 136/144                     |
|                       | HAV                      | 6.0 ± 10.1                    | 104/144                     |
|                       | HCoV-229E                | 8.5 ± 5.0                     | 144/144                     |
| **VIRADEL (virus adsorption-elution)** | MNV-1                | 6.1 ± 10.0                    | 103/144                     |
|                       | HAV                      | 24.5 ± 19.7                   | 94/144                      |
|                       | HCoV-229E                | 2.6 ± 1.8                     | 144/144                     |
| **PFC-UF (paper filtration-coupled ultrafiltration)** | MNV-1                 | 117.4 ± 58.5\textsuperscript{*} | 144/144                     |
|                       | HAV                      | 118.2 ± 126.7\textsuperscript{*} | 144/144                     |
|                       | HCoV-229E                | 14.3 ± 10.9\textsuperscript{*} | 144/144                     |

\textsuperscript{*}Indicate the significant value of p < 0.01 within three methods for each virus.
sample processing (recovery, cleaning, and preparation stages) and therefore needs to be further considered. For these reasons, TFUF may not provide an optimal, rapid approach. However, it is superior in acquisition accuracy. Eventually, the direct use of centrifugal concentrator devices uses UF for the concentration step, which is simple, fast to operate, and provides a better recovery efficiency with a lower LOD, but more expensive.

**Virus recovery performance from the three methods**

From the results, the total losses of non-enveloped MNV-1 and HAV in the UF and VIRADEL methods were relatively higher compared to those in the PFC-UF method, mainly in terms of higher SD value, lower recovery rate and detection, and recoveries below the LOD (Figures 2, 3 and Table S2). For the enveloped HCoV-229E, previous studies indicated infection of HCoV-229E of the gastrointestinal tract and considered a potential threat from contaminated food. Despite the lack of evidence that coronavirus is transmitted via food intake, this study will provide information for potential risk surveillance and control for public health in future studies.

In the UF method, the recoveries of MNV-1 and HAV were 2.4 ± 4.0% and 6.0 ± 10.1%, but the detection rates were less stable, which were 136/144 and 104/144, respectively (Table 2). The enveloped HCoV-229E outperformed the non-enveloped virus in recovery efficiency, which was 8.5 ± 5.0% with all of the detection. In comparison, a recent study used UF with 10 kDa to recover four coliphages from wastewater sludge, with recoveries as high as 50.39–88.89%. Even though the recovery was not as good as in the previous study, our results were consistent, with the recovery of the enveloped Phi6 virus being significantly higher than that of the non-enveloped virus. The results suggested that this may be related to the different structures (enveloped and non-enveloped) of the viruses. Similarly, the recovery rate of the enveloped virus murine hepatitis virus (MHV) in the wastewater was reported to be 28% using an UF device of 10 kDa size. In addition, recovery of SARS-CoV-2 was achieved using a 10 kDa UF device with recovery rates ranging from 33.0% to 42.6%. These studies indicated that there is a difference between the enveloped and non-enveloped viruses recovered from the UF device. In our study, we also considered whether the viruses lost in the suspended liquid were bound to the soil sediment. In previous studies, the adsorption of enteroviruses in sandy loam-containing soils was reported to be 51.6%–99.9% due to the interaction between virus surface and SOM charges in the soil. However, this was not verified in the present study. Therefore, the above results showed that even if the same device is used to recover different viruses in different environments, reproducibility of results remains a challenge.

MNV-1 and HAV showed similar results in the VIRADEL as in the UF method during recovery. The recovery rates were more inconsistent in replicated studies with low virus concentrations and high soil content. Most of them were non-detected (ND) (Figure 3 and Table S2). Previously, the recovery of astrovirus and

| Concentration methods | Viruses* | Seeded virus (Genome copies) (Mean ± SD) | Recovered virus (Genome copies) (Mean ± SD) | Recovery rate (%) (Mean ± SD) | Detection (detected/total) |
|-----------------------|---------|----------------------------------------|--------------------------------------------|----------------------------|---------------------------|
| UF (ultrafiltration)  | MNV-1   | 2.0 x 10^5 ± 1.9 x 10^5                | 1.4 x 10^5 ± 9.7 x 10^2                   | 2.1 ± 3.0                 | 7/9                       |
|                       | HAV     | 1.4 x 10^3 ± 5.0 x 10^2                | ND                                        | –                         | 0/9                       |
|                       | HCoV-229E | 2.5 x 10^3 ± 6.5 x 10^3               | 3.4 x 10^3 ± 9.7 x 10^2                   | 4.8 ± 1.4                | 9/9                       |
| VIRADEL (virus adsorption-elution) | MNV-1 | 9.9 x 10^4 ± 4.9 x 10^3              | 1.4 x 10^4 ± 9.5 x 10^3                   | 11.6 ± 1.5              | 2/9                       |
|                       | HAV     | 5.1 x 10^3 ± 9.6 x 10^2                | ND                                        | –                         | 0/9                       |
|                       | HCoV-229E | 6.6 x 10^3 ± 6.2 x 10^4               | 1.5 x 10^4 ± 4.3 x 10^3                   | 2.3 ± 0.7                | 9/9                       |
| PFC-UF (paper filtration-coupled ultrafiltration) | MNV-1 | 5.5 x 10^4 ± 7.7 x 10^3              | 4.1 x 10^4 ± 7.3 x 10^3                   | 78.8 ± 13.3b             | 9/9                       |
|                       | HAV     | 4.2 x 10^3 ± 4.6 x 10^2                | 1.5 x 10^3 ± 8.5 x 10^2                   | 44.4 ± 25.2b             | 9/9                       |
|                       | HCoV-229E | 7.0 x 10^5 ± 8.4 x 10^4               | 2.8 x 10^4 ± 1.9 x 10^4                   | 4.0 ± 2.7                | 9/9                       |

*Virus seeding titer at 10^2 PFU/mL for MNV-1, HAV, and 10^2 TCID50/mL for HCoV-229E. 
Indicate the significant value of p < 0.01 within three methods for each virus.
norovirus from 2 L of tap water through adsorption-elution was generally achieved at 3% with a higher SD value. In addition, in river water samples, 17.8% of norovirus and 42.7% of astrovirus were obtained with the addition of MgCl₂ at a final concentration of 5 mM compared to 25 and 50 mM. Although the results for the recovery of non-enveloped viruses in tap water were similar to those of our VIRADEL method in PWW with 0% soil content, the results in PWW with soil content were quite different because of the background of soil water type. In contrast, using a final concentration of 25 mM MgCl₂, the recovery rates of poliovirus and coliphage Qβ from river and tap water were 27.7%–86.5%, and their method has also been used to recover various non-enveloped viruses. In another comparative study for recovering bacteriophages from soil, the hemagglutinin (HA) membrane method was demonstrated to show better results compared to polyethylene glycol (PEG) and ultracentrifugation methods. For the enveloped virus HCoV-229E, the efficiency of recovery in PWW was 2.6%, which is a huge difference compared to the 65.7% recovery rate reported in the previous studies. It is concluded that diverse water types and different modified techniques lead to divergent experimental outcomes using the same experimental method. Therefore, we speculate that the reason for the poor recovery of low-concentration virus in high soil content PWW is due to the differences in enveloped and non-enveloped virus features, as well as the water type.

In the results of the PFC-UF method, the recovery of non-enveloped MNV-1 and HAV showed remarkable consistency and stability compared to HCoV-229E (Table 2). Results were significantly different compared to UF and VIRADEL, in which all were detected and average recovery rates were higher than 100% for MNV-1, HAV, and HCoV-229E (p < 0.05, Table 2). Several studies have demonstrated that recovery rates of non-enveloped viruses exceeded 100% when using whole process control (WPC). Although it cannot be more than 100%, it is worth noting that its recovery rate is close to 100%. In addition, 10² PFU seeded MNV-1 and HAV were recovered at rates of 78.8 ± 13.3% and 44.4 ± 25.2%, respectively, in the PWW with 5% soil content by RT-qPCR assay (Figure 4 and Table 3). However, recovery of 10² PFU seeded HCoV-229E in PWW was less optimal than MNV-1 and HAV in the PFC-UF method, which requires further study to optimize the recovery method of enveloped viruses in soil or water containing soil content. For instance, the use of CP (concentrating pipette) method by using InnovaPrep to track and control enveloped viruses in PWW or soil-content water can be considered. However, our study’s relevance is the finding that the paper membrane helps to remove the soil matrix and stabilizes the recovery of enveloped and non-enveloped virus in PWW with soil content. Furthermore, the corresponding removal mechanism needs to be further investigated.

Discrepancies in recovery outcomes between laboratories may be due to differences in materials, handling, and PCR procedures. There are many options for virus concentration methods, provided that the filtrate has a low turbidity level. Because of the limited literature on the recovery of viruses from soil-containing PWW, we used a PFC-UF method that was adapted from the field of wastewater studies. Its applicability would need to be determined in cases of actual detection, in the local outbreak, and in epidemiological situations. Furthermore, removal of the PCR inhibitory substances from the plant origin in PWW and improvement of methodology to recover enveloped virus need to be optimized for the PFC-UF method in future studies.

**Conclusion**

Overall, this study evaluated the feasibility of three methods to recover three different viruses (enveloped and non-enveloped) from PWW with varying soil content. The PFC-UF method performed better than the UF and VIRADEL methods in both recovery rate and detection. The results may be because of the properties of background soil and characteristics of viruses. In addition, we studied the performance of the three methods during the recovery of non-enveloped viruses and compared the recovery rate and detections between enveloped and non-enveloped viruses in the three methods. In summary, the PFC-UF method provides a rapid way to detect sources of viral contamination throughout the post-harvest stage for monitoring and managing the safety of fresh produce, root vegetables, and fruits in the supply chain in future.

**Limitation of the study**

Different soil types, such as clay ratio, SOM, microbiota, and plant-based PCR inhibitors interfere virus recovery in PWW. For practical application, the efficiency of PFC-UF should be validated with PWW collected from field setting. The correlation of TSS level with virus contamination level in PWW also should be examined in future studies.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105640.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

Z.W. and C.C. are co-inventors on related intellectual property.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| Murine norovirus 1 | Dr H. W. Virgin | – |
| Hepatitis A virus | ATCC | VR-2097 |
| Human coronavirus 229E | ATCC | VR-740 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Master Mix (2X QuantiTect Probe PCR) | QIAGEN | 204343 |
| RevertAid reverse transcriptase | Thermo Fisher | EP0442 |
| **Critical commercial assays** | | |
| QIAamp viral RNA Mini Kit | QIAGEN | 52906 |
| **Experimental models: Cell lines** | | |
| RAW 264.7 | ATCC | TIB-71 |
| FRhK-4 | ATCC | CRL-1688 |
| MRC-5 | ATCC | CCL-171 |
| **Oligonucleotides** | | |
| Primers for MNV-1, see Table S1 | Lee et al.\textsuperscript{64} | N/A |
| Primers for HAV, see Table S1 | Jothikumar et al.\textsuperscript{65} | N/A |
| Primers for HCoV-229E, see Table S1 | van Elden et al.\textsuperscript{66} | N/A |
| **Software and algorithms** | | |
| Prism- GraphPad | GraphPad | https://www.graphpad.com/ |
| **Other** | | |
| MCE membrane | Merck Millipore | HAWP04700 |
| Paper membrane | Whatman | 1004-047 |
| Vivaspin 20 | Sartorius | VS2041 |

RESOURCE AVAILABILITY

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Changsun Choi, (cchoi@cau.ac.kr).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Cells**
The murine macrophage (RAW 264.7), Macaca mulatta kidney normal (FRhK-4), and human lung fibroblast (MRC-5) cells were purchased from ATCC (Manassas, VA, US). Cells were typically maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma, US) including 10% FBS (Gibco Invitrogen) and 1% antibiotic/antimycotic (AA, Gibco Invitrogen) in culture flasks at 37°C in a 5% CO₂ incubator for virus propagation.
Viruses

Virus propagation of MNV-1, HAV strain HM175, and HCoV-229E was performed separately on confluent RAW 264.7, FRhK-4, and MRC-5 cell lines in tissue culture flasks, as described previously. First, the harvested virus solutions were pooled and homogenized to prepare the experimental virus stock. Thereafter, each virus was titrated using a plaque-forming unit (PFU) assay or a 50% tissue culture infective dose (TCID50) assay.

METHOD DETAILS

Preparation of viruses (MNV-1, HAV, and HCoV-229E) stock

To obtain a consistent titer, MNV-1 (5 x 10^6 PFU/mL), HAV (3 x 10^6 PFU/mL), and HCoV-229E (5 x 10^5 TCID50/mL) virus stocks were prepared and stored at -80°C until use. On the day of the experiment, ten-fold serial dilutions of the prepared virus stock were performed to obtain 10^5 – 10^2 PFU or TCID50/mL titers for seeding.

Post-washing water preparation (PWW)

The soil was obtained from a farmland in Icheon-si, Gyeonggi province, Republic of Korea. Large particles were removed by 8-inch mesh sieves (pore size: 2 mm) and the type of sifted soil was determined to be sandy loam by referring to the National Geography Information Institute (NGII). The collected soil was refrigerated at 4°C until further use. On the day of the experiment, soil was mixed with tap water to produce the simulated PWW with soil concentrations of 0, 1, 2, 3, and 5% (w/v), respectively. After preparing the PWW, turbidity (nephelometric turbidity unit, NTU) was measured at each virus recovery stage (turbidity meter TB-31).

Experimental design and virus concentration

Prepared 10^5 – 10^2 PFU/mL (for MNV-1 and HAV) or TCID50/mL (for HCoV-229E) were seeded in PWW containing different concentrations of soil (1, 2, 3, and 5%, w/v; n = 3) and without soil (tap water, n = 3), respectively. Meanwhile, the background presence of MNV-1, HAV, and HCoV-229E was tested in unseeded PWW and tap water to ensure that they were negative for each virus. After virus seeding, the PWW was sufficiently mixed, and the seeded virus was recovered using the following three methods as shown in Figure 1. Each virus concentration was determined separately in this study.

The UF method used a centrifugal concentrator device that was utilized to concentrate viruses from sewage water within a small volume of water (50 mL), with slight modification in this study. Briefly, the virus (1 mL) was inoculated into a Falcon tube containing 50 mL of PWW and centrifuged at 4,750 x g for 30 min at 4°C. The supernatant was transferred to a vivaspin ultrafiltration unit (10 kDa MWCO, Sartorius, Germany), and centrifuged twice at 1,500 x g for 15 min at 4°C to collect the pellet using 1 mL phosphate-buffered saline (PBS).

The VIRADEL method employed a negatively charged membrane to absorb the viruses using a salt-bridge (MgCl2) by filtering 500 mL PWW through a vacuum pump connected to a funnel device (Merck Millipore, US). Briefly, virus (1 mL) was inoculated into a centrifuge bottle containing 500 mL of PWW and centrifuged at 4,750 x g for 30 min at 4°C. Then, the supernatant was collected in a prepared clean sample bottle and 2.5 mol/L MgCl2 was added to achieve a final concentration of 25 mM. Thereafter, the viral suspensions were filtered through a negatively charged mixed cellulose ester (MCE) membrane (47 mm diameter; pore size 0.45 μm). Subsequently, the membrane was transferred to a 50 mL Falcon tube containing 10 mL of elution buffer (0.2 g/L Na4P2O7·10H2O, 0.3 g/L C10H13N2O8Na3·3H2O, and 0.1 mL/L of Tween 80) followed by sufficient vortexing with addition of a garnet bead. The final centrifugation step was performed at 2,000 x g for 10 min at 4°C to obtain the supernatant.

The PFC-UF method was aimed at removing the soil matrix using a paper membrane in PWW to obtain a purified filtrate followed by an additional concentration process. This method began with virus (1 mL) seeded, into a beaker containing 500 mL of PWW and then filtering the supernatant of PWW directly using the same filtration funnel device that was employed in the VIRADEL method, but replacing the negatively charged membrane with paper material (pore size 25 μm, Whatman, US). After filtration, one-fifth of the filtrate (~100 mL) was further concentrated using a vivaspin 20 (10 kDa MWCO, Sartorius, Germany) at
1,500 x g for 15 min at 4°C, while the last centrifugation was performed for 30 min under the same conditions to obtain the pellet which was eluted with PBS.

**Virus RNA extraction and RT-qPCR**

Viral RNA extraction of the final concentrated solution (500 µL) from each method was performed by using a QIAamp viral RNA Mini Kit according to the Quick-Start Protocol (Qiagen, Hilden, Germany). Additionally, viral RNA was extracted from the solution before concentration with each method as a control. The obtained RNA was immediately analyzed by reverse transcription-quantitative PCR (RT-qPCR) on the same day to avoid RNA degradation from freeze-thawing. The RT-qPCR (Takara TP800 Thermal Cycler Dice, Japan) was used to quantify the RNA of MNV-1, HAV, and HCoV-229E from the recovered concentrates/eluents. Primers (forward and reverse), probes, and amplification conditions of the above viruses are listed in Table S1.

Amplification was performed in a 25 µL reaction mixture, containing 12.5 µL of Master Mix, 0.25 µL of RevertAid reverse transcriptase (US), 1.25 µL of the primer-probe mixture (forward: 500 nM; probe: 250 nM), 5 µL of target (sample) RNA, and RNase-free water up to 25 µL. For quantification of RNA of each recovered virus from PWW, a quantitative synthetic standard of viral RNA of MNV-1, HAV, and HCoV-229E were purchased from ATCC (VA, US). The slope of the calibration curve for each virus was determined to be −3.591 (for MNV-1), −3.73 (for HAV), and −3.363 (for HCoV-229E), respectively. The Y-intercept value of each assay was 45.00 (for MNV-1), 39.47 (for HAV), and 37.79 (for HCoV-229E), respectively. In addition, the quantification cycle (Cq) value of each reaction was converted into genome copies (GC) and presented in the form of mean ± standard deviation (SD).

**Recovery rate and LOD value**

The virus recovery rate was determined according to the seeded and recovered genome copies (GC), for which the equation was as follows24:

\[
\text{Recovery rate(\%) = } \left( \frac{\text{GC recovered}}{\text{GC seeded}} \right) \times 100\%
\]

In this study, because the GC detected by PCR (5 µL of sample RNA) has been diluted (after calculation by standard curve), the detection limit (LOD) was calculated as follows in the three methods of reference water sample volume:

\[
\text{LOD} = D \times \left( \frac{V}{T} \right)
\]

where D = lowest GC detected in RT-qPCR assay (D = “1” GC/reaction, was considered in this study), V = target sample RNA volume (5 µL/reaction), T = total volume of the water sample (50 mL for UF; 500 mL for VIRADEL and PFC-UF).

**Inhibition test**

The PCR inhibition test was performed to assess the inhibitory substances from PWW (soil content at 5%). Briefly, solution A: 45 µL of sample RNA (virus unseeded concentrate or eluent) + 5 µL of a known concentration of MNV-1 RNA (2.59 x 10^5 GC). Solution B was prepared as a negative control: 45 µL of distilled water + 5 µL of a known concentration of MNV-1 RNA (2.59 x 10^5 GC). Solutions A and B were vortexed, then 5 µL of each solution was added to a 20 µL reaction mixture and the RT-qPCR was performed as described in Virus RNA extraction and RT-qPCR section. Additionally, a no template control (NTC) reaction was performed as well. If the difference between the Cq values of A and B was >2, PCR inhibitory substances were considered to be present in PWW (5% of soil content). Therefore, it may need an inhibitor substance removal process for improvement.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

We estimated the recovery and detection from 9 replicate trials = positive of recovered samples/replicate of seeded samples (n = 3) x replicate of PCR amplified sample (n = 3). All data are presented as means ± standard deviation (SD). Graphs were drawn using the GraphPad Prism (version 9.3.1) software for Windows. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test using the GraphPad Prism. Statistical significance was defined as \( p < 0.05 \).