Development of sample processing methods for direct molecular detection of poliovirus in non-flowing wastewater

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Abstract

Development of sample processing methods for direct molecular detection of poliovirus in non-flowing wastewater

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This study investigated method development for sample processing of non-flowing wastewater for direct molecular detection of poliovirus. The main aims were to test concentration methods using seeded septic sludge samples for effectiveness, efficiency, and plausibility, then to optimize the tested method(s). Three methods were tested, and a centrifugation method was selected for further optimization. Optimization included pH adjustment and a secondary concentration method. It was determined that the best recovery was seen after centrifugation and in the secondary concentration at an increased pH. These optimized methods are recommended for field testing to determine the recovery in a real-world setting.

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1. Introduction

1.1 Background: poliovirus

Poliovirus (PV) causes paralytic poliomyelitis, which causes flaccid paralysis, especially in children under the age of 5 (1). Flaccid paralysis occurs in only about 1 in 200 to 1 in 2000 cases, depending on the serotype. (1–4). PV is an Enterovirus with three serotypes, type 1, 2 , and 3, with minimal immunity across the different types (2). Transmission of PV is fecal-oral route (2). In 1988, the World Health Organization (WHO) created the Global Polio Eradication Initiative (GPEI) as a global effort for polio eradication (3). In 1999 wild poliovirus type 2 (WPV2) was officially declared eradicated, and in 2019 wild poliovirus type 3 (WPV3) was declared eradicated, with wild poliovirus type 1 (WPV1) circulation being limited to two countries: Pakistan and Afghanistan (5,6).

1.2 Polio surveillance and eradication efforts

Polio eradication efforts have focused on vaccination and surveillance (7). Vaccination with oral polio vaccine (OPV) and the inactivated polio vaccine (IPV) have successfully decreased transmission and cases of PV (8). The oral vaccine, which has a live attenuated virus, has led to a new problem for eradication: vaccine derived poliovirus (VDPV) (5). Because the OPV has live virus it can circulate within a populations like the wildtype virus (5). As it is transmitted, mutations can occur and be passed on to unvaccinated individuals, leading to paralytic symptoms (5). The three main types of VDPV are circulating (cVDPV), immunodeficiency-associated (iVDPV) and vaccine associated paralytic PV (VAPP). cVDPV is associated with community transmission of a strain that is greater than 1% different from the vaccine strain (for type 1 and 3) or 0.6% different than the type 2 vaccine strain (5). iVDPV is associated with VDPV in an individual with an immunodeficiency (5). VAPP is a result of a rare adverse outcome of paralytic polio from the OPV (9). As eradication has gotten closer, cVDPV has become a growing concern, as there are now more annual cases of cVDPV than WPV (5).

Active surveillance focuses on clinical symptoms, as all cases of acute flaccid paralysis (AFP) are investigated to determine if the affected individual has been infected with PV (10). This type of surveillance is important for identifying where active transmission and infections are occurring in order to deploy vaccines to vulnerable populations. Environmental surveillance is also important for tracking PV transmission, as less than 1% of infections lead to paralysis, and 3 out of 4 cases are asymptomatic (2). In order to track this asymptomatic transmission, environmental surveillance focuses on testing and processing wastewater to determine if populations have PV circulation (11). This type of surveillance is important for countries that have eradicated PV to track importations as well as tracking the spread of cVDPV without cases with clinical symptoms (11). Environmental and active AFP surveillance have both been crucial in tracking the spread of PV and working for eradication (7,11).

Current methods for environmental surveillance focus on processing flowing wastewater through sewage systems or in wastewater impacted water (12). While this is useful for areas with sewage systems or other flowing water, there is limited surveillance methods for on-site sanitation that do not have flowing wastewater (13). More than 38% of global households utilize these forms of on-site sanitation, which have also been associated with an increased risk of infections, including with enteroviruses like PV (14,15). These households may utilize pit latrines and septic tanks, which lack flowing water, and have a higher concentration of solids than wastewater previously used for surveillance (14,16). There is room for development of a method that focuses on this non-flowing wastewater for expanded environmental surveillance.

Previous methods have worked to concentrate enteroviruses like PV out of fecal sludge for the purpose of environmental surveillance (17).The Global Polio Laboratory Network (GPLN) (a part of the GPEI) has current standard methods that use tissue culture and molecular methods for environmental surveillance (18). These methods are optimized for flowing wastewater and have a long lead time due to the use of tissue culture (18). Direct molecular methods utilizing RT-qPCR are less expensive and require less time so there is an interest in methods than may be used on non-flowing wastewater and can utilize direct molecular methods for confirmation.

2. Materials and Methods

2.1 Samples (septic sludge)

Septic sludge used for this study was obtained from septic tank trucks at the South Treatment Plant in King County, WA. Sludge was stored at 4°C and mixed before use in experiments to ensure approximately equal suspended solids across multiple tests. Each sample of sludge was examined for total suspended solids (TSS) to determine if they were accurate representations of non-flowing wastewater found in pit latrines (19).

2.2 Study organism (growing up and extraction)

Sabin vaccine strain poliovirus type 1 (PV1) was used for this study. PV1 was grown in Buffalo Green Monkey Kidney (BGMK) cells and extracted through chloroform extraction (17). Aliquoted stocks were then stored at -80°C. PV1 stocks were quantified through RT-dPCR on the stock virus as well as within the sludge matrix used in the study (17,20).

2.2.1 Spike in of Virus

Using the septic sludge and PV1 as the model for method testing and optimization, PV1 stocks were first added to PBS and vortexed for about 1 minute. This step was added to decrease aggregation of the virus when added to the sludge. The mixed PBS and PV1 is then added to a chosen volume of sludge. This spiked sludge is then vortexed on high for 5 minutes, then shaken at 200RPM for 10 minutes at room temperature.

2.3 Preliminary Methods

Three methods were initially identified for testing (Figure 1). The CeresNano method utilized magnetic beads to extract RNA (21). The polyethylene glycol (PEG) method utilized PEG, NaCl, and overnight mixing for flocculation of the solids and virus particles, concentrating the virus into the solids that can be pelleted out and extracted (22). Additionally, this method included acid adsorption, elution steps prior to the application of the PEG in order to further concentrate the virus into the pellet, and secondary extractions (17). The centrifugation method used centrifugation to pull the suspended solids and virus particles down into a pellet, which could be homogenized and extracted (23).

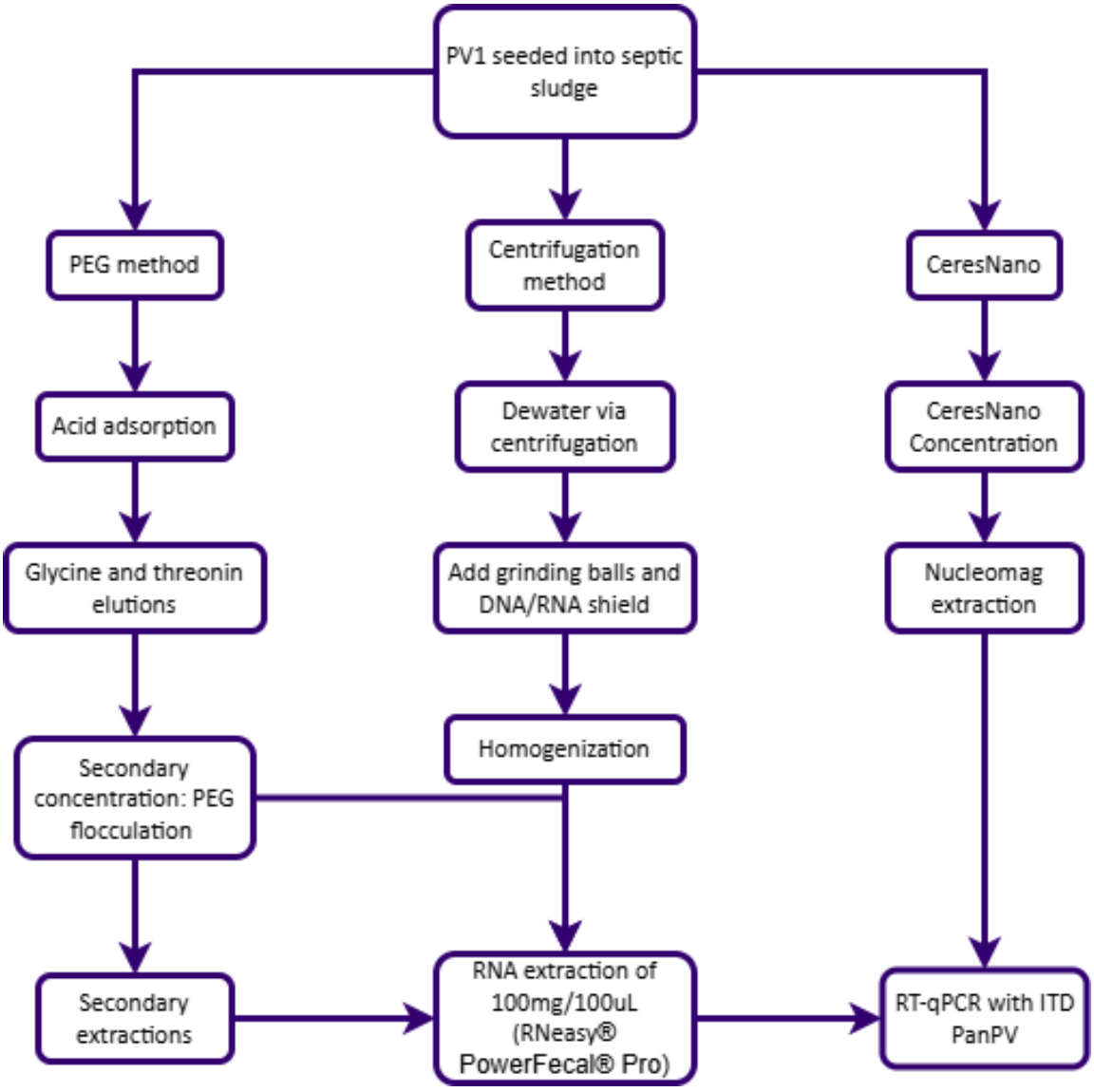

Figure 1: Flowchart showing the general steps of the preliminary tested methods with extraction and analysis.

Although the CeresNano method performed well in previous tests using liquid waste, the concentration of solids in the sludge used for this study lead to inhibition in the method that decreased the recovery and potential application of this method.

Additional steps were adjusted for the PEG method to improve recovery. These steps included the elution steps before the addition of PEG, additional extractions after PEG flocculation, and removal of the acid adsorption. The recovery was not improved enough for the initial method to continue to be assessed for application.

The centrifugation method, in which samples are centrifuged for 30 minutes at 5000xg (4°C), then the resulting pellet resuspended in DNA/RNA shield, had the highest recovery of all preliminary testing. This method was selected for further optimization to improve recovery.

2.4 Optimization (Chosen method)

2.4.1 Optimization of Centrifugation Method

The first changes to this method involved an addition of an acid adsorption step. This involved adjusting the pH to about 4.5, which is intended to encourage the PV1 virus particles to be more attached to the solids before centrifugation, increasing the yield from the pellet after centrifugation. Additionally, adjusting the pH up to 9.5 was tested. This would have the opposite effect of the acid adsorption and should elute the virus from particles in the supernatant. Previous testing had shown loss of virus in the supernatant at neutral pH, so by adjusting the pH up and increasing the concentration in the supernatant, further concentration and extraction methods may be performed. To quantify viral concentration in each fraction of the sample, RNA extraction was performed on both the pellet and the supernatant from the centrifugation method.

After initial testing of homogenization of the pellet using DNA/RNA shield and steel grinding balls, manual homogenization of the pellet was tested. This manual homogenization involved mixing the pellet with a metal spatula and yielded relatively consistent recovery between tests. This was the preferred method of homogenization as it simplified the process and eliminated the need to dilute the pellet in DNA/RNA shield prior to extraction.

2.4.2 Additional Concentration of Centrifugation Supernatant PEG

After adjusting the pH of the sludge and processing through centrifugation, the supernatant was measured and utilized for additional processing. For every 50mL of supernatant, 7.00g of PEG and 0.585g of NaCl were added and the solution mixed until they dissolved. Then samples were incubated over night at 4°C, shaking at 200rpm.

Following overnight incubation, samples were centrifuged at 4000xg for 30 minutes at 4°C. From there, the supernatant was poured off of the pellet and an extraction was taken from both.

This step was added onto the centrifugation method at 9.5 pH and testing of the centrifugation pellet at 7.5 pH and the secondary PEG pellet at 9.5 pH was done in parallel (Figure 2). This was

not utilized at 4.5 pH as this pH adjustment increased the inhibition in the molecular assay and did not improve recovery.

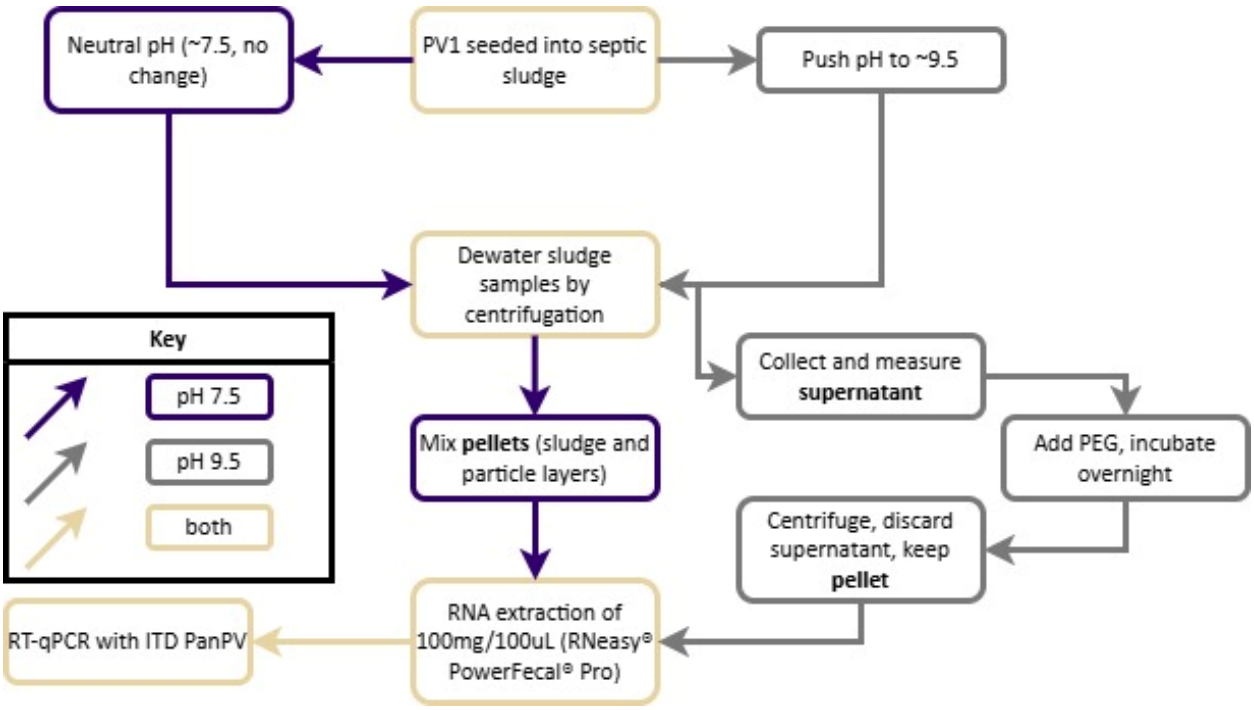

Figure 2: Final tested centrifugation method with pH adjustment and secondary processing using PEG.

2.5 RNA extraction & Quantification

Samples were extracted using the Qiagen RNeasy® PowerFecal® Pro RNA extraction kit. For liquid samples (supernatant and resuspended solids), 100uL were extracted. For solid samples (pellets) about 100mg were extracted and the exact mass extracted was noted and considered in downstream analysis. For the PEG pellet, 100uL was measured and extracted instead of weighing, as the pellets were not fully solid, and pipetting was possible.

CeresNano preliminary testing did not use the Qiagen RNeasy® PowerFecal® Pro Kit as this method included its own RNA extraction.

Quantification utilized primers and probes from the Global Polio Laboratory Network (GPLN). GLPN has developed intratypic differentiation (ITD) real-time reverse transcription PCR (RT-qPCR) assays that includes primers and probes for Sabin strains with enteroviruses, wild polioviruses, and PanPoliovirus (PanPV). Only the optimized PanPV primers were used for quantification (10,20). The primers (S1: TTG GAG TTC TTC ACI TAI TCI MGI TTY GAY ATG; 1A: GGA GCT CCG GGT GGG AYR TAC ATI ATY TGR TAI AC) and probe (FAM: TGR TTN ARI GCR TGI CCR TTR TT) were used 1nM with qScript™ XLT One-Step RT-qPCR ToughMix® (QuantaBio) (20). Primers and probe concentrations were 1uM. Cycle threshold (ct) values from this assay were used to calculate percent recovery (24).

To develop a digital PCR assay for quantification in the desired units (gene copies/uL), the Absolute Q™ 1-step RT-dPCR Master Mix (4x) was used in place of the ToughMix®. The same primers and probe were used at a concentration of 900nM and 250nM (respectively). The same temperature cycling conditions were used as well.

2.6 Data analysis

2.6.1 Standard Curve

A standard curve was run with every qPCR assay. This was a 10-fold dilution series of RNA extracted from the spiked sludge. Digital PCR on these samples was used to relate the standard curve to a concentration of virus in gene copies per microliter (gc/uL).

2.6.2 Percent Recovery

To calculate percent recovery, RT-qPCR cycle threshold (Ct) values were extrapolated from the standard curve. Given this calculated concentration, the total number of gene copies was calculated for each extracted fraction. This was calculated using the mass of the fraction, the mass or volume extracted, and the volume eluted after RNA extraction (Equation 1). Percent recovery was then calculated based on the gene copies from each sample compared to the amount spiked in.

$$\left(concentration\ \left(gene\frac{copies}{uL}\right)\right) * \frac{RNA\ extraction\ volume\ (uL)}{mass\ extracted\ (mg\ or\ uL)} * \left(fraction\ mass\ (mg)\right) = gene\ copies$$

Equation 1: Calculation used to calculate the gene copies in each fraction for percent recovery calculations.

3. Results

3.1 Total Suspended Solids

The total suspended solids was calculated for each of the collected sludge samples. For the majority of the study, sludge collected on April 1st, 2024, from the South Treatment Plant in King County, WA was used. This sludge had a TSS of 17 g/L. A second sample of sludge was collected from the same treatment plant on May 12th, 2025, and had TSS of 11 g/L. These concentrations are consistent with data on TSS in pit latrines in Kenya (16).

3.2 Preliminary results

For the preliminary testing, the PEG method was tested with and without a vertrel organic extraction step, looking at both the pellet and supernatant. Without the vertrel, the recovery in the pellet was 2.8% and 3.1% in the supernatant (Table 1). With the vertrel, the recovery in the pellet was 11% and 6.6% in the supernatant. While the vertrel did improve the recovery in this method, the highest recovery was only 11%. Because the centrifugation method showed higher recovery,

that was the method chosen for optimization instead of the PEG with the vertrel organic extraction step.

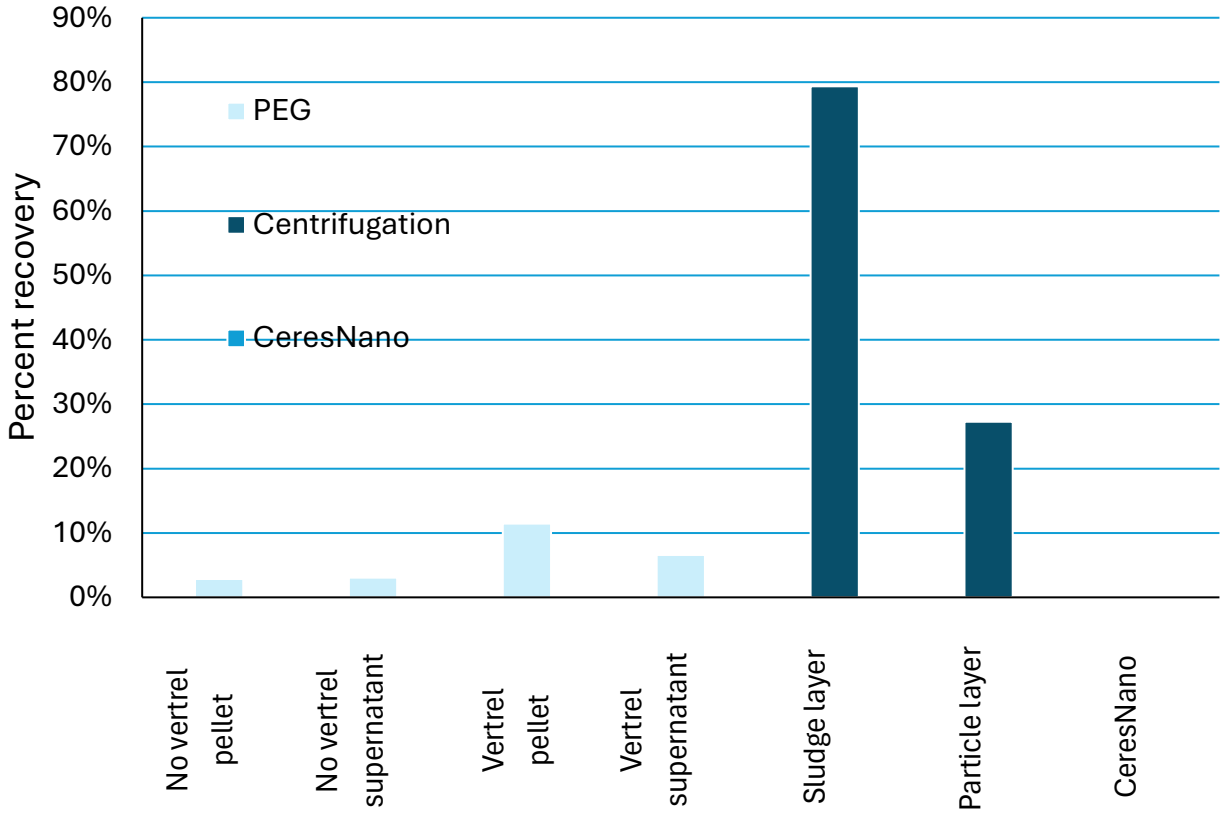

Figure 3: Percent recovery from the three preliminary methods.

Preliminary testing of the centrifugation method looked at the two layers of the pellet as separate, with a sludge and a particle layer (Supplemental Figure 1). Preliminary testing showed a higher recovery in the sludge layer than the particle layer (Table 1), but manual mixing of the layers had consistent results and was determined to be effective moving forward.

The CeresNano preliminary testing resulted in recovery of less than 1%.

3.3 One-Step RT-dPCR: Stock and Spike in Concentrations

RT-dPCR on stock PV1 virus that was spiked in had a weighted average concentration of $1.2x10^6$ (n= 3, range: $1.x1^5$ to $7.8x10^5$) gene copies per microliter. When quantifying the concentration of PV1 spiked into septic sludge, a 1:100 dilution of this stock had a weighted average concentration of $6.1x10^3$ (n=3, range: $3.4x10^3$ to $7.1x10^3$) gene copies per microliter. This is

closer to a 1:190 dilution instead of 1:100. The concentration of PV1 spiked into septic sludge at a 1:20,000 was 47 (n=1) gene copies per microliter. This is closer to a 1:24,000 dilution instead of a 1:20,000 dilution.

3.4 One-Step RT-qPCR: Sample Analysis

RT-qPCR on multiple fractions of the centrifugation method and the PEG method applied to the supernatant from that method was done to quantify the recovery across each fraction at pH 7.5 and pH 9.5 using the first sample of septic sludge collected on April 1st, 2024. The centrifugation pellet at 7.5 and the PEG pellet at 9.5 were the fractions of most interest in quantification using these methods. The centrifugation pellet at pH 7.5 had an average recovery of 136% when the septic sludge was seeded at a high concentration (Figure 4). This pellet had an average recovery of 146% when the septic sludge was seeded at a low concentration. Recovery above 100% indicates both improved recovery and potential aggregation of the virus that leads to an unequal distribution throughout the pellet that can still be recovered. The PEG pellet at pH 9.5 had an average recovery of 46% when the septic sludge was seeded at a high concentration, and an average recovery of 31% when seeded at a low concentration.

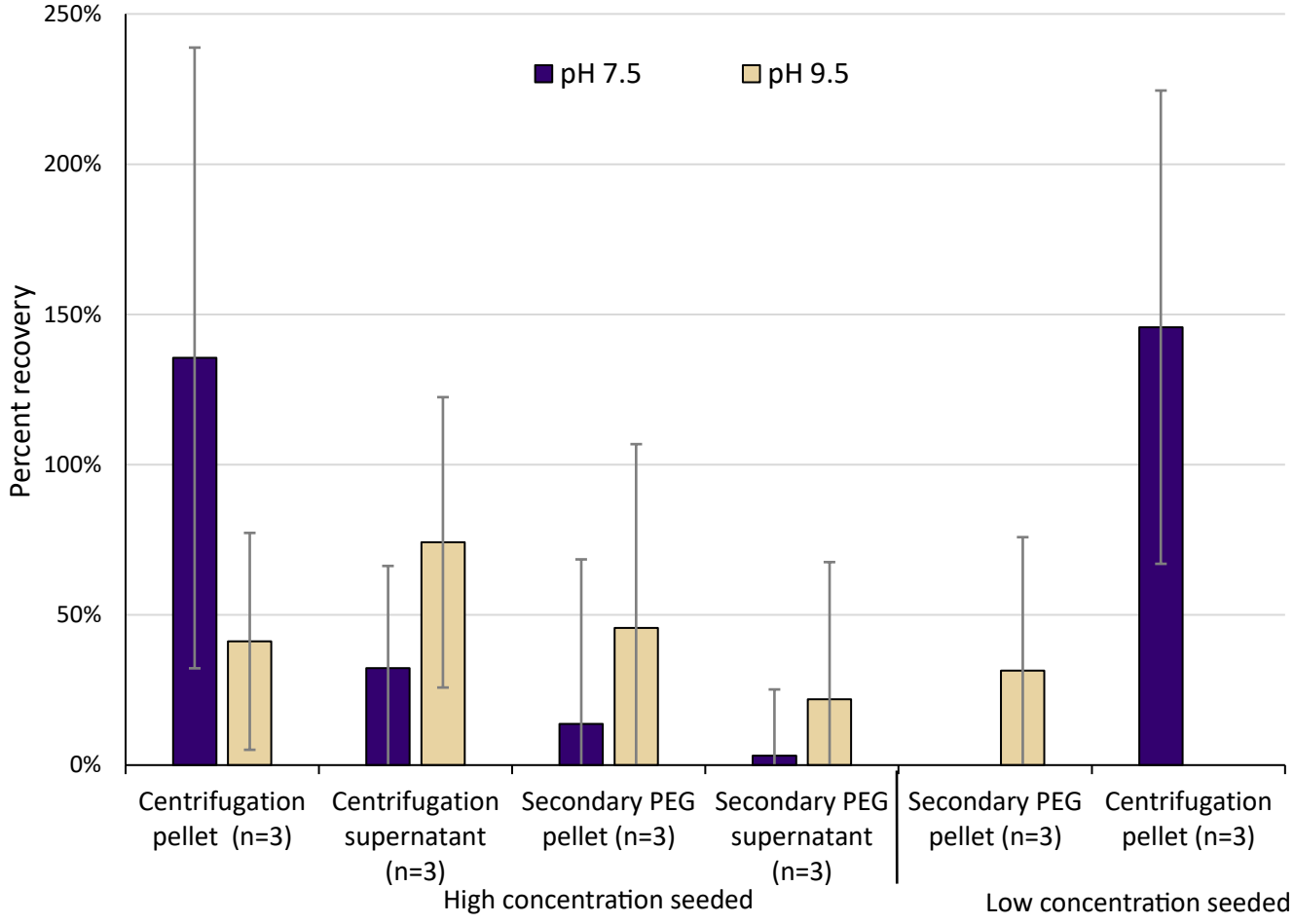

Figure 4: Percent recovery by sample fraction and pH in sludge collected on April 1st, 2024, from RT-qPCR analysis.

Additional processing and analysis were done on the septic sludge collected on May 12th, 2025. For this sample, only the centrifugation pellet at 7.5 pH and the PEG pellet from the centrifugation supernatant at 9.5 pH were analyzed. The average recovery for these pellets was 51% and 16% respectively (Figure 5).

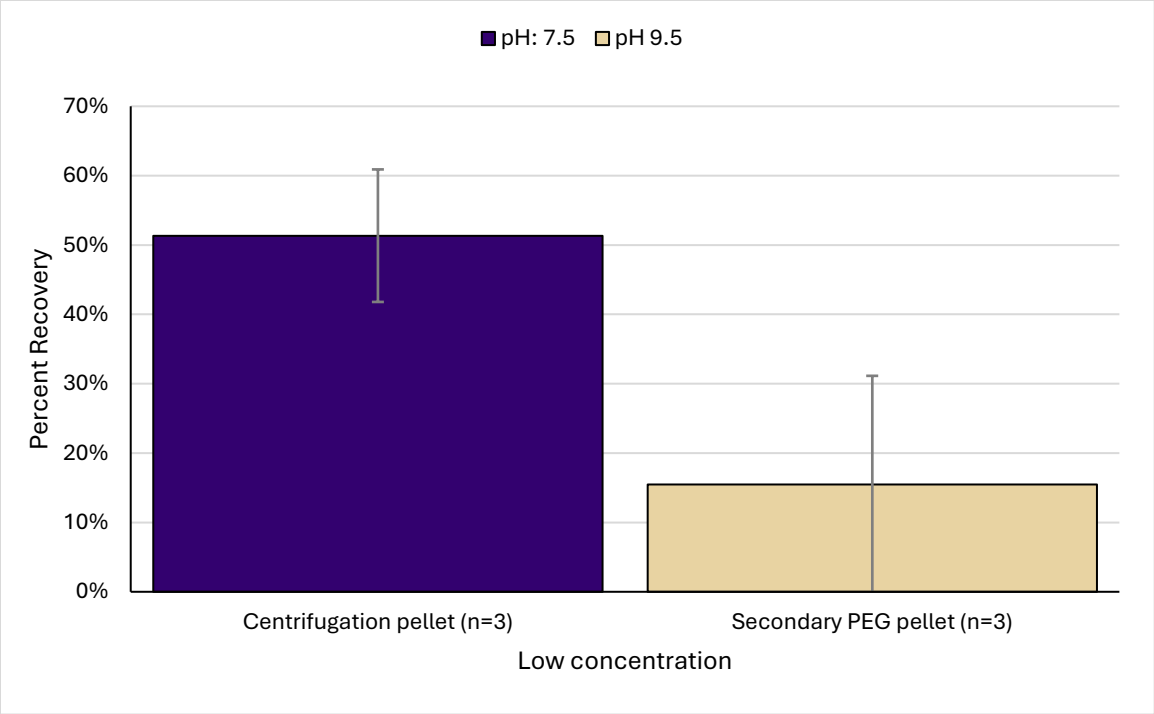

Figure 5: Percent recovery in 7.5 pH centrifugation pellet and 9.5 pH secondary PEG pellet in sludge collected on May 12th, 2025, from RT-qPCR analysis.

3.5 One-Step RT-dPCR: Sample Analysis

RT-dPCR was used to help confirm results from RT-qPCR. Samples were analyzed using the Qiagen Absolute Q digital PCR system, which outputs a concentration rather than a Ct value. This concentration was then used to recalculate the percent recovery of each sample fraction. This analysis indicated a percent recovery of 97% from the centrifugation pellet at 7.5 pH and 35% from the secondary PEG pellet at 9.5 pH, both seeded at a high concentration (Table 1).

Due to time constraints, RT-dPCR analysis was limited in application compared to the RT-qPCR. No RT-dPCR analysis was performed on the samples seeded at the low concentration.

Sample	Sample gene copies	Spike it gene copies	% recovery
pH: 7.5			
Pellet	2.9E+08	3.0E+08	97%
Supernatant	2.8E+07	3.0E+08	9.2%
PEG pellet	3.8E+07	3.0E+08	12.%
PEG supernatant	2.9E+06	3.0E+08	1.0%
pH 9.5			
Pellet	7.6E+07	3.0E+08	25%
Supernatant	5.9E+07	3.0E+08	20%
PEG pellet	1.1E+08	3.0E+08	35%

PEG supernatant	1.8E+07	3.0E+08	6.1%

Table 1: Percent recovery by sample fraction and pH in sludge collected on April 1st, 2024, from RT-dPCR analysis.

4. Discussion

4.1 pH Adjustment and Optimization

Across different spike in concentrations and different sludge samples, two of the best percent recoveries were in the centrifugation pellet at 7.5 pH and the PEG secondary pellet at 9.5 pH. The centrifugation supernatant at 9.5 pH also had a high recovery, but was not considered for further utilization due to concerns of representativeness in extractions. The supernatant was a 40-45 mL fraction and the pellets were 1-7 grams. The 100 mg or 100 uL extraction would lead to a loss of representativeness from the supernatant. Because of this, these two pellets are the target for utilizing this method in the field. The centrifugation method pellet at pH 7.5 would be the ideal candidate for a wastewater processing method for molecular detection for wastewater with high concentrations of solids, as this fraction consistently had the highest recovery. Additionally, the processing is not labor intensive and takes only a few hours. The additional PEG steps and secondary pellet at 9.5 pH may be further optimized to increase the recovery in the future. A potential next step for optimization may be a beef extract and glycine elution, which could improve the recovery at the 9.5 pH (25). Having two options available for processing and further optimization may improve the detection of cases of PV in the field.

Looking across the centrifugation pellet and supernatant and the secondary PEG pellet and supernatant at both 7.5 pH and 9.5 pH, the virus concentration increased in the supernatant and subsequent secondary PEG processing. This indicates that the pH adjustment did affect where the virus was concentrated, leading to this difference in concentration. The additional fractions were not utilized for further optimization and analysis, as the centrifugation pellet at 7.5 pH and the secondary PEG pellet at 9.5 pH were the most representative and had the highest concentrations. This method did not need to extract all of the virus out of the septic sludge. The additional processing of these fractions would not be necessary for analysis and would add extra time and resources with limited return.

4.2 Septic Sludge

Two samples of septic sludge were used in this study, the first with a higher concentration of TSS and a higher recovery percentage. In both samples, the centrifugation pellet at pH 7.5 had the highest recovery percentage.

4.3 RT-qPCR and RT-dPCR

RT-dPCR is a more precise method for quantifying the concentration of virus in samples. While it is more precise, RT-qPCR was the primary quantification used for this study. Stock virus and

spiked septic sludge was quantified using RT-dPCR and the spiked sludge was used as the standard curve for each RT-qPCR run so the concentration of samples could be extrapolated. RT-qPCR was prioritized because it is cheaper to perform, and the methods in this study are intended for use in low-income countries.

Percent recovery was calculated using both RT-qPCR and RT-dPCR. The percent recovery was similar in each of these assays, showing that while less precise, the RT-qPCR assay is still an effective molecular assay for quantifying the presence of PV in wastewater samples such as those processed using the methods described in this study.

4.4 Limitations

There were many limitations in this study. The purpose of this study was method development by testing concentration methods on non-flowing wastewater. This study did not use non-flowing wastewater samples such as pit latrines, instead it utilized wastewater from septic tanks acquired in King County, WA to simulate. There were only two sludge samples taken from King County, WA, with different recovery efficiencies. While the big picture recovery is similar, more sludge samples would have helped to fully understand the relationship between the TSS, pH, and recovery efficiency. This wastewater may be a good representation of the wastewater of interest, but further testing on samples from pit latrines in countries of interest are necessary for further assessment.

This study utilized only the PV1 strain of PV, and the method would likely need to work with all strains of PV for the most effective use. Additionally, the PCR analysis used only the PanPV primers and probe. For best use with the ITD protocol for determining the presence and strain of PV, this method should be testing using the full ITD primer and probe sets.

4.5 Future Directions/Research

A future goal of this study is to test the methods in a field study. A site should be selected where non-flowing wastewater can be sampled and where PV may be found naturally. This could be in areas with known WPV or cVDPV circulation or alternatively, in an area where OPV has been recently deployed, as this can also be successfully concentrated from wastewater.

Another goal for this study is the potential development of a passive sampling method that could be deployed at field sites for short- or long-term periods. Initial testing utilizing Millipore filters has started to determine the feasibility. Further lab testing to determine how long passive samplers can be deployed and how well they concentrate PV will continue, followed by field testing. This method can be used in conjunction with the methods described above in order to continue to expand and enhance environmental surveillance of PV.

5. Acknowledgements

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Supporting information

Sludge Seeding Method

Materials:

- Septic sludge
- PV1 stock
- Plastic bottle
- Vortex
- Shaker table
- PBS
- 15mL conical tube

Methods:

1. Thaw PV1 stock on ice for about 1 hour
2. Measure septic sludge using graduated cylinder and pour into plastic bottle
3. Pipet PBS into 15mL conical
4. Pipet PV1 into 15mL conical
5. Vortex tube for ~1 minute
6. Pipet PBS and PV1 from conical into plastic bottle
7. Vortex on high for 5 minutes
8. Shake at 200rpm at room temperature for 10 minutes

pH Adjusted Centrifugation and secondary PEG Method (optimized method)

Materials:

- Spiked sludge
- 50mL conical tubes (8x)
- Scale
- 1M NaOH
- Plastic bottles (250mL)
- Metal spatulas
- Centrifuge
- Vortex
- Graduated cylinders
- Empty bead beating tubes
- Shaker table
- Parafilm

- Waste collection bottle

Methods:

For pH adjustment to 9.5 (for pH 7.5, skip to step 4)

1. Using a pH strip, check the pH of the spiked sludge
2. If the pH is lower than 9.5, adjust to 9.5 with 1M NaOH
    a. If it is higher, adjust to 9.5 with 1M HCl
3. Shake at 200rpm at room temperature for 15 minutes
4. Label 50mL conical tubes with sample names and record the empty mass in grams
5. Invert sludge sample bottle to mix
6. In biosafety cabinet, pour 50mL of well mixed spiked sludge sample and split into two corresponding labeled 50mL conical tubes, record the mass in grams
7. Centrifuge samples at 5000xg for 30 minutes at 4°C
8. In biosafety cabinet, pour off supernatant into a graduated cylinder and record the volume
    a. For pH 9.5, take this volume and skip to step 13
9. Record the mass in grams of the pellet and 50mL conical tube
10. Use a metal spatula to mix the pellets (in both tubes)
11. Record the mass of an empty bead beating tube in grams
12. Transfer ~100mg of the mixed pellets into the empty bead beating tube, skip to step 23
13. Secondary concentration: per 50mL of supernatant, add 7g PEG 8000 and 0.585g NaCl into a sterile 250mL bottle, then add the supernatant and parafilm the bottle's lid
14. Shake the sample by hand until PEG 8000 and NaCl are dissolved
15. Shake at 200rpm at 4°C overnight (14-18 hours)
16. Label 50mL conical tubes with same names and record the empty mass in grams
17. After shaking, transfer samples to the corresponding 50mL conical tubes, weigh the tubes with the samples in grams
18. Centrifuge at 4000xg at 4°C for 30 minutes
19. Pour off supernatant into graduated cylinder and discard into a waste collection bottle
20. Record the mass in grams of the pellet and 50mL conical tube
21. Record the mass of an empty bead beating tube in grams
22. Transfer ~100mg of PEG pellet into the empty bead beating tube
23. Follow extraction protocol for Qiagen RNeasy® PowerFecal® Pro RNA extraction kit

Centrifugation Method

Initial Centrifugation Method

Materials:

- Spiked sludge
- 50mL conical tubes

- Scale
- Plastic bottles (250mL)
- Metal spatulas
- DNA/RNA shield
- Centrifuge
- Vortex
- Graduated cylinders
- Empty bead beating tubes
- Shaker table
- Parafilm
- Waste collection bottle

Methods:

Dewater Sludge Samples by Centrifugation (~50 minutes)

1. Label 50 mL conicals with the sample name
2. Record the empty conical mass in grams.
3. Invert the sludge sample bottle a few times to mix.
4. In a biosafety cabinet, pipette or pour 50 mL of well mixed primary sludge sample split into two corresponding labeled 50 mL conicals. Record the sample volume.
5. Bring the rotor to a biosafety cabinet and arrange the conicals in the rotor in a manner to ensure that it is balanced.
6. Centrifuge samples at 5000x g for 30 minutes at 4°C
7. In a biosafety cabinet, aspirate supernatant into a graduated cylinder and record the volume removed.
8. Record the mass in grams of pellet remaining in the conical.

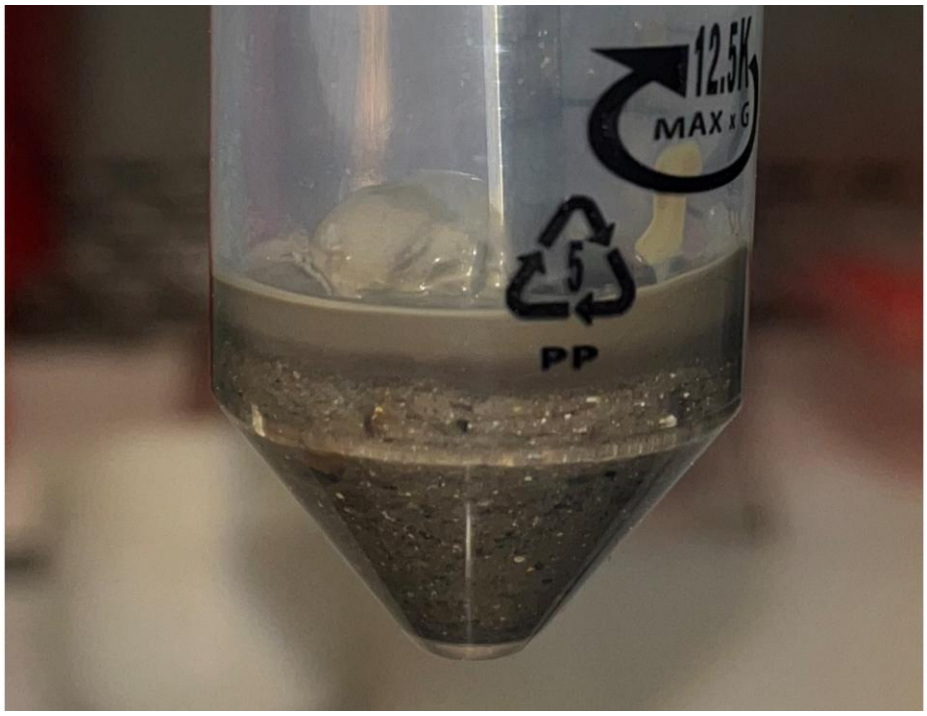
Sample Homogenization

1. Apply a label to a new 50 mL conical for each sample and record the empty conical mass in grams.
2. With a spatula (either plastic or metal), scoop a small aliquot of dewatered pellet (~0.75 g) into the new, pre-weighed 50 mL conical. Sample both the sludge layer and particle layer id present into separate conicals. Record the mass in grams of the aliquot in the conical. Set the rest of the pellet aside for dry weight measurement.
3. Add DNA/RNA shield to each conical containing solids so that the final concentration of dewatered solids resuspended in the DNA/RNA shield is ~ 75 mg/mL (10 mL DNA/RNA shield to 0.75 g pellet).
5. Add 8 grinding balls (5/32” in, stainless steel autoclaved) to each conical tube.
4. Place the conicals in the adapters on the vortex genie 2 and tightly secure the clamp.
5. Shake the conicals on the vortex genie 2 for 2 minutes at 1,000 rpm to homogenize the sample.

6. Briefly centrifuge the conicals for 30 seconds to 1 minute at 3500 x g to pull down the bubbles generated during homogenization. Sample is ready for RNA extraction.
6. Using a pipette, transfer 2 aliquots of 100 mg to bead beating tubes for extraction.

Centrifugation Method Pellet Partitions.

Throughout testing the centrifugation method, after centrifuging, the pellet appeared to have two distinct layers, a smaller sludge layer on top of a larger, grittier particle layer.



Supplemental Figure 1: Pellet from the centrifugation method. The smooth top layer was the "sludge layer" and the gritty bottom layer was the "particle layer" in preliminary testing.

CeresNano Method

Initial Ceres Nano Method

Method:

1. Prepare Sludge Samples
    - Invert the sludge bottles several times to mix.
    - Pipette 1.2 mL sludge into a 15 mL conical tube for each sample.
    - Dilute sludge 1:10 with Nanotrap Buffer 2
    - Add 3 grinding balls to diluted sludge sample, close cap firmly.
    - Vortex diluted sludge samples for 10 minutes at max speed.
    - Place the diluted sludge samples upright, leave the samples at room temperature for 10 minutes to allow the large particulates to settle out. Alternatively, you can centrifuge samples at 500 x g for 3 minutes.

- Using a serological pipette, transfer 10 mL of the supernatant carefully to a new 50 mL conical tube without disturbing the pellet.

2. Add 150 µL of Nanotrap Microbiome A Particles (Nanotrap particles) to the sample, cap the sample and then invert 2 times to mix the particles.
3. Incubate samples with Nanotrap particles at room temperature for 10 minutes.
   Note: Invert every 5 minutes or use a rotator.
4. Place the tube on a DynaMag-15 magnetic rack to separate the Nanotrap particles from the sample for 5 minutes.
5. Using a serological pipette, discard the supernatant carefully without disturbing the Nanotrap particle pellet.
   Note: Can use a P-1000 or P-200 pipette to remove any remaining supernatant from the sample (be careful to not lose any Nanotrap particles when removing supernatant).
6. Add 1 mL of Nanotrap Buffer 2 to the tube and re-suspend the Nanotrap particle pellet by pipetting on the walls of the conical tube, gently re-suspend until all Nanotrap particles have been completely collected.
7. Transfer the Nanotrap particle suspension to a new 2 mL microcentrifuge tube.
8. Place the 2 mL microcentrifuge tube on a DynaMag-2 magnetic rack to separate the Nanotrap particles from the sample for 2 minutes.
9. Using a P-1000 pipette, discard the supernatant carefully without disturbing the Nanotrap particle pellet.

Note: If any small amount of liquid is still present, use a smaller pipette to remove all the supernatant from the bottom of the tube.

*Try nucelomag protocol using the EOHML established method, in tandem with sludge protocol (below)

10. Add 500 µL of Lysis buffer MWA1 to Nanotrap particle pellet, pipette up and down until Nanotrap particles are resuspended completely. Nanotrap Microbiome A; Manual Sludge Protocol with NucleoMag DNA/RNA Water Extraction Kit 3
11. Close the tube lid, incubate the samples at room temperature for 10 minutes.
12. Place the 2 mL microcentrifuge tube on a DynaMag-2 magnetic rack to separate the Nanotrap particles from the sample for 2 minutes.

Note: May need to briefly centrifuge the tube (Mini Centrifuge at 2000 g for 2-5 seconds) to remove drops from inside the lid before magnetic separation.

13. Transfer 450 µL of supernatant/lysate to a new 1.5 mL collection tube and discard the Nanotrap particles pellet.
14. Add 475 µL of Binding Buffer MWA2 to the sample/lysate.
15. Add 25 µL of NucleoMag B-beads to the sample/lysate.

-> Protocol diverged here if doing the EOHML established Nucleomag Protocol

16. Vortex to mix, then incubate at room temperature for 10 minutes.
    Note: Invert every 5 minutes or use a rotator.
17. Place the tube on a DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes, then discard the supernatant using a pipette.
    Note: May need to briefly centrifuge the tube (Mini Centrifuge at 2000 g for 2-5 seconds) to remove drops from inside the lid before magnetic separation.
18. Add 850 µL of Wash Buffer MWA3 to sample and re-suspend the magnetic beads using a pipette.

19. Place the tube on a DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes, then discard the supernatant.
20. Repeat the pellet wash with 850 µL of Wash Buffer MWA3.
21. Place the tube on a DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes, then discard the supernatant.
22. Add 850 µL of Wash Buffer MWA4 to sample and re-suspend the magnetic beads using a pipette.
23. Place the tube on a DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes, then discard the supernatant by using a pipette.
24. Centrifuge the tube (Mini Centrifuge at 2000 g for 30 seconds).
25. Place the tube on a DynaMag-2 magnetic rack, then remove excess Wash Buffer MWA4 using a smaller pipette.
26. Take samples off magnetic rack, open caps, allow samples to air dry at room temperature for 10 minutes.
27. Add 100 µL of RNase-free Water to re-suspend the magnetic beads and then incubate at 56ºC for 5 minutes on a heat block (close caps).
28. Place the tube in the DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes.
    Note: May need to briefly centrifuge the tube (Mini Centrifuge at 2000 g for 2-5 seconds) to remove drops/condensation from inside the lid before magnetic separation.
29. Transfer the supernatant to a new tube, the sample is ready for downstream analysis or can be stored at -80ºC.

PEG Method

Initial PEG Method

Acid adsorption

1. Adjust pH to 4.8-5.0 with HCl/NaOH
2. Shake 15 min, 200 RPM, to allow adsorption
3. Centrifuge at 2,000xg for 20 min at 4C

Note: Ensure conical base is placed in centrifuge adaptor before bottle

On Pellet

Glycine elution

1. Resuspend pellet in 100 mL of 0.05 M glycine-0.14 M NaCl (pH 7.5)
2. Adjust to pH 7.5
3. Shake 15 min, 200 RPM
4. Centrifuge at 5,000xg for 20 min at 4C
5. Save supernatant for step 5 of the Threonine elution

On Supernatant:

Secondary concentration PEG:

13. Collect sample from previous step (elution/primary concentration).
14. Measure sample volume in sterile graduated cylinder.
15. Transfer sample to a sterile 275-mL conical.

Threonine elution

1. Resuspend pellet in 100 mL of 0.5 M threonine-0.14 M NaCl (pH 7.5)
2. Check pH and adjust to pH 7.5 if needed
3. Shake 15 min, 200 RPM
4. Centrifuge at 5,000xg for 20 min at 4C
5. Combine supernatant from step glycine elution with threonine elution supernatant
6. Discard pellet

Secondary concentration

PEG:

1. Collect sample from previous step (elution/primary concentration).
2. Measure sample volume in sterile graduated cylinder.
3. Transfer sample to a sterile 275-mL conical.
4. Per 100 mL sample, add 14 g PEG 8000 and 1.17 g NaCl (final concentration: 0.2 M NaCl).
5. Parafilm sample bottle.
6. Shake sample vigorously by hand until all PEG 8000 and NaCl are dissolved.
7. Shake sample on a shaker overnight (14-18 hours) at 4°C, 200 RPM.
    - If no cold chamber is available, place cold packs around sample in shaker.
8. After shaking, transfer sample to centrifuge conicals compatible with available centrifuge rotor (e.g., 150-mL bottle, (3) 50-mL conicals, etc.).
9. Spin sample at 4000x G, 4°C for 30 minutes.
10. Discard supernatant into a waste collection bottle.
11. Add 10 mL sterile PBS, pH 7.4 to pellet.
16. Per 100 mL sample, add 14 g PEG 8000 and 1.17 g NaCl (final concentration: 0.2 M NaCl).
17. Parafilm sample bottle.
18. Shake sample vigorously by hand until all PEG 8000 and NaCl are dissolved.
19. Shake sample on a shaker overnight (14-18 hours) at 4°C, 200 RPM.
    - If no cold chamber is available, place cold packs around sample in shaker.
20. After shaking, transfer sample to centrifuge conicals compatible with available centrifuge rotor (e.g., 150-mL bottle, (3) 50-mL conicals, etc.).
21. Spin sample at ~~6500~~ 4000 (5/16/24) x G, 4°C for 30 minutes (swinging bucket rotor).
22. Discard supernatant into a waste collection bottle.
23. Add 10 mL sterile PBS, pH 7.4 to pellet.
24. Vortex 5-10 minutes at maximum speed to completely resuspend pellet.
9. *Measure 100 mg/uL of the concentrate (record final volume) in extraction tube. Store in -20°C freezer.

Vertrel extract

1. Add 0.5x vol. Vertrel
2. Vortex 2 min
3. Centrifuge at 3,000xg for 15 min at 4C
4. Collect supernatant by pipetting, save for threonine-Vertrel re-extraction step 5

Threonine-Vertrel re-extraction

1. Add 0.5x vol. 0.5 M threonine-0.14 M NaCl to vertrel fractions
2. Re-extract; vortex 2 min
3. Centrifuge at 3,000xg for 15 min at 4C

5. Vortex 5-10 minutes at maximum speed to completely resuspend pellet.
6. *Measure 100 mg/uL of the concentrate (record final volume) in extraction tube. Store in -20°C freezer.

Vertrel extract

1. Add 0.5x vol. Vertrel
2. Vortex 2 min
3. Centrifuge at 3,000xg for 15 min at 4C
4. Collect supernatant by pipetting, save for threonine-Vertrel re-extraction step 5

Threonine-Vertrel re-extraction

1. Add 0.5x vol. 0.5 M threonine-0.14 M NaCl to vertrel fractions
2. Re-extract; vortex 2 min
3. Centrifuge at 3,000xg for 15 min at 4C
4. Collect supernatant by pipetting
7. Combine both supernatants from both extractions (record final volume)
8. Measure 100 mg/uL of the concentrate (record final volume) in extraction tube. Store in -20°C freezer.

4. Collect supernatant by pipetting
5. Combine both supernatants from both
6. extractions (record final volume)
7. Measure 100 mg/uL of the concentrate (record final volume) in extraction tube. Store in -20°C freezer.

Supplemental Tables

S. Table 1. Percent recovery by pH and fraction

Sample	Average Ct	Concentration (gc/uL)	Gene copies	% recovery
pH: 7.5				
Pellet 1 (-1)	24.94	1.11E+05	7.21E+08	238.51%
Pellet 2	24.62	3.84E+04	3.13E+08	103.39%
Pellet 3 (-1)	26.35	3.44E+04	1.95E+08	64.62%
Pellet 4 (-1)	34.98	3.00E+02	1.87E+06	123.70%
Pellet 5 (-1)	34.69	3.62E+02	2.43E+06	160.49%
Pellet 6 (-1)	34.69	3.61E+02	2.31E+06	153.05%
Supernatant 1	27.34	2.50E+03	1.05E+08	34.73%
Supernatant 2 (-1)	32.14	2.13E+03	9.18E+07	30.37%

Supernatant 3	27.02	2.19E+03	9.62E+07	31.82%
PEG pellet 1	24.04	1.93E+04	1.83E+07	6.07%
PEG pellet 2 (-1)	27.53	5.14E+04	8.62E+07	28.53%
PEG pellet3 (-1)	28.20	9.82E+03	2.00E+07	6.60%
PEG supernatant 1	29.98	4.87E+02	1.83E+07	6.07%
PEG supernatant 2 (-1)	35.83	1.66E+02	7.46E+06	2.47%
PEG supernatant 3	32.12	6.88E+01	2.94E+06	0.97%
pH 9.5				
Pellet 1	24.14	1.82E+04	1.72E+08	57.07%
Pellet 2 (-1)	28.46	2.71E+04	1.87E+08	62.01%
Pellet 3	26.74	2.64E+03	1.35E+07	4.46%
Supernatant 1	25.88	6.18E+03	2.66E+08	87.89%
Supernatant 2 (-1)	30.30	7.61E+03	3.20E+08	105.76%
Supernatant 3 (-1)	29.62	3.76E+03	8.69E+07	28.74%
PEG pellet 1 (-1)	25.44	8.11E+04	1.62E+08	53.69%
PEG pellet 2 (-1)	26.34	1.17E+05	2.32E+08	76.60%
PEG pellet 3 (-1)	27.86	1.24E+04	2.00E+07	6.60%
PEG pellet 4 (-1)	34.86	3.23E+02	5.49E+05	36.31%
PEG pellet 5 (-1)	34.91	3.13E+02	4.60E+05	30.43%
PEG pellet 6 (-1)	35.00	2.96E+02	4.15E+05	27.44%
PEG supernatant 1	27.08	2.94E+03	1.14E+08	37.73%
PEG supernatant 2 (-1)	32.35	1.84E+03	7.69E+07	25.46%
PEG supernatant 3 (-1)	33.20	3.31E+02	8.05E+06	2.66%