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Author(s)	Miura, Takayuki; Masago, Yoshifumi; Sano, Daisuke et al.
Citation	Applied and Environmental Microbiology, 77(12), 3975-3981 https://doi.org/10.1128/AEM.02692-10
Issue Date	2011-06
Doc URL	https://hdl.handle.net/2115/47550
Type	journal article
File Information	AEM77-12_3975-3981.pdf



For Submission to Applied and Environmental Microbiology as a Long-form Paper

**Development of an effective method of viral genomic RNA recovery from
environmental silty sediments for quantitative molecular detection**

By

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Running title

Viral RNA recovery from sediments for quantitative PCR

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ABSTRACT

Nine approaches to recover viral RNA from environmental silty sediments were newly developed and compared to quantify RNA viruses in sediments using molecular methods. Four of the nine approaches employed direct procedures for extracting RNA from sediments (direct methods), and the remaining five approaches used indirect methods wherein viral particles were recovered before RNA extraction. A direct method using an SDS buffer with EDTA to lyse viral capsids in sediments, phenol:chloroform:isoamyl alcohol to extract RNA, isopropanol to concentrate RNA, and magnetic beads to purify RNA resulted in the highest recovery rate (geometric mean of 11% with a geometric standard deviation of 0.02, N=7) of poliovirus 1 (PV1) inoculated in an environmental sediment sample. The direct method exhibiting the highest PV1 recovery was applied to environmental sediment samples. One hundred eight sediment samples were collected from the Takagi River and its estuary from November 2007 to April 2009, and the genomic RNA of enterovirus and human norovirus in these samples was quantified by RT-qPCR. The human norovirus genome was detected in one sample collected at the bay although its concentration was below the quantification limit. Meanwhile, the enterovirus genome was detected in two samples at the river mouth and river at concentrations of 8.6×10^2 and 2.4×10^2 copies/g (wet), respectively. This is the first report to obtain the quantitative data of a human pathogenic virus in a river and estuarine sediments using RT-qPCR.

INTRODUCTION

Bacterial (*Mycobacterium avium* (29), *Clostridium botulinum* type E (19)), protozoan (*Cryptosporidium* (22)), and viral pathogens (enterovirus (EV) (5, 13, 15), hepatitis A virus (HAV) (6, 14), and rotavirus (6)) have been detected in environmental sediments. Whittington *et al.* reported that *M. avium* in sediments from a dam lake survived 12–26 weeks longer than it did in a water column (29). Moreover, the persistency of viral pathogens in environmental sediments has been reported. Smith *et al.* demonstrated that poliovirus 1 (PV1), coxsackieviruses B3 and A9, and echovirus 1 survived significantly longer when associated with marine sediments (23). A 3-log reduction in the infectivity of PV1 was observed in 14 days in seawater having marine sediments, whereas such a reduction was observed in four days in seawater without sediments (23). These results suggest that environmental sediments have a protective effect on the survival of pathogens (1, 20), and the association of pathogens with environmental sediments cannot be ignored while considering the fate of pathogens in water environments (22).

When storms, tides, or strong winds cause sediment resuspension, pathogens in sediments are also resuspended, resulting in high pathogen levels in the water column. Dorner *et al.* indicated the importance of resuspension of microorganisms from stream sediments rather than land-based sources according to the hydrological simulation of *Escherichia coli* during storm events (4). The quantitative detection of pathogens in environmental sediments is thus crucial for assessing the health effects of exposure to pathogen-contaminated sediments or pathogen-resuspended water. However,

quantifying pathogens in sediments using molecular methods such as quantitative PCR (qPCR) has been difficult because of the presence of inhibitory substances such as humic substances that affect the efficiency of genome extraction and enzymatic genome amplification (24, 25, 28, 30).

Particularly, the preparation of viral RNA from environmental sediments is even more difficult because viral particles are not completely different from those inhibitory substances in terms of physical characteristics such as molecular weight and isoelectric point. Furthermore, loss of viral RNA due to adsorption to soil particles and degradation by ribonuclease occurs easily.

Sample preparation methods to recover viruses from sediments, particularly molecular detection methods, are still under development. Conventional methods of preparing samples from sediments, usually consisting of the dispersion of sediments in buffer solutions to elute viral particles, centrifugation to remove sediments, and concentration of the supernatant, were developed on the premise that cell culture-based plaque assays can be used to detect viruses (5, 13, 15). Green *et al.* employed RT-PCR to detect HAV and rotavirus in concentrated samples using a conventional sample preparation method for virus detection using cell cultures (6); however, the recovery efficiency of viral RNA was not investigated. Haramoto *et al.* detected F-specific RNA phages in river sediments by combining culturing and qPCR methods (10), but to our knowledge, there is no report of quantitative data of human pathogenic enteric viral RNA in a river and estuarine sediments obtained by RT-qPCR.

In this study, nine procedures to recover viral RNA from environmental sediments were newly developed and compared in terms of efficiency and robustness of recovery. The nine

procedures employed in this study can be divided into two approaches, i.e., direct and indirect methods of viral RNA recovery. In direct methods, viral RNA was directly extracted from sediment samples, whereas viral particles were eluted from sediment samples before RNA extraction in indirect methods. The recovery rate was evaluated using PV1 inoculated into sediment samples. The procedure exhibiting the highest and most stable recovery rate was used for viral RNA recovery from sediment samples collected from the Takagi River and its estuary, and genomes of EV and human norovirus (HuNoV) were quantified by RT-qPCR.

MATERIALS AND METHODS

Environmental sediment samples. Sediment samples were collected monthly from the Takagi River, Miyagi, Japan and its estuary during ebb tide. Fig. 1 illustrates the sampling sites: St. A, B, and C were located in the bay; St. D was located at the river mouth; and St. E and F were located in the downstream river. There is a small dam to control the flow of the river just below St. F. Samples were obtained using an Ekman–Birge type bottom sampler with a square area of 15 cm × 15 cm; the top 1 cm layer was collected. All sediment samples in this study mainly consisted of silt and clay in the range of 67% to 85% and 8% to 13%, respectively (16). Water content and ignition loss (organic matter content calculated from the weight loss at 600 °C for 1 hr in an electric furnace) of the samples ranged from 62% to 81% and 2.9% to 3.8%, respectively (17). Sediment samples were transported to

the laboratory in sterile containers on ice and stored at $-20\text{ }^{\circ}\text{C}$ until analysis. One hundred eight samples were collected from the six sampling sites between November 2007 and April 2009.

Indirect methods to recover viral RNA from sediments. Sediment samples collected at St. F were used to compare viral RNA recovery rates because sediment characteristics such as particle size distribution and water content were similar among sampling sites. All indirect methods tested in this study were modified on the basis of the approach developed by Gerba *et al.* (5); this approach does not use beef extract, a possible inhibitor of viral genome detection (21). Indirect methods consist of three steps: elution of viral particles by dispersing sediments in buffer solutions and centrifugation to remove the sediments (elution step); concentration of the eluted viruses in the supernatant (concentration step); and viral RNA extraction from the virus concentrate (extraction step) using a QIAamp Viral RNA Mini Kit (Qiagen, Tokyo). The detailed elution and concentration procedures for each indirect method are described below and summarized in Fig. 2.

(i) Method A. Glycine-NaOH buffer (pH 9.0) was used as the virus elution buffer instead of the original buffer at pH 11.5, and a 15-sec vortex was employed instead of a 10-min agitation with a shake table (5). The modified procedure is as follows: Five grams of sediment sample in a wet condition were placed in a 50-mL centrifuge tube, and 15 mL of 0.25 M glycine-NaOH buffer (pH 9.0) containing 0.05 M EDTA was added. The tube was vortexed for 15 sec and centrifuged at $2,500 \times g$ for 4 min to remove the sediments. The supernatant was collected, and its pH was adjusted to 3.5 using 1 M glycine-HCl buffer (pH 2.0). Aluminum chloride (1 M) was then added to yield a final concentration of 0.06 M, and the solution was passed through an HA membrane filter (0.45- μm pore

size, 90-mm diameter, Millipore, Tokyo). Viruses were eluted from the filter by the passage of 10 mL of 0.25 M glycine-NaOH buffer (pH 11.5), and the eluate was immediately neutralized by the addition of 1 M glycine-HCl buffer (pH 2.0).

(ii) Method B. Method B was the same as Method A with one modification; the negatively charged membrane filtration method (12) was employed in the concentration step. After centrifugation at $2,500 \times g$ for 4 min in the elution step, magnesium chloride (0.25 M) was added to the supernatant at a final concentration of 0.1 M, and the solution was passed through an HA membrane filter (0.45- μm pore size, 90-mm diameter, Millipore). Subsequently, 200 mL of 0.5 mM H_2SO_4 (pH 3.0) was passed through the filter, followed by 10 mL of 1 mM NaOH (pH 11.0) to elute viral particles. The filtrate was recovered in a tube containing 100 μL of 50 mM H_2SO_4 and 100 μL of 100 \times TE buffer for neutralization.

(iii) Method C. Method C was the same as Method B with one modification: TE buffer (pH 7.2) containing Laureth-12 (Kanto Chemical Co., Tokyo) was used instead of glycine-NaOH buffer (pH 9.0) in the elution step to prevent the elution of humic substances from the sediments. The elution buffer consisted of 0.1% (wt/vol) Laureth-12, 10 mM Tris, 1 mM EDTA, and 0.015% (vol/vol) Antifoam Y-30 (Sigma-Aldrich Co., Tokyo), which is in accordance with Method 1622 of the US EPA (26). The negatively charged membrane filtration method (12) was employed in the concentration step as performed in Method B.

(iv) Method D. Method D was the same as Method B, except that the polyethylene glycol (PEG) precipitation method was applied in the concentration step instead of the membrane filtration

method. After the centrifugation at $2,500 \times g$ for 4 min in the elution step, an equal volume of a PEG solution containing 16% (wt/vol) PEG 6000 (Kanto Chemical Co.) and 4.7% (wt/vol) NaCl was added to the supernatant. The suspension was mixed vigorously and incubated overnight at 4 °C. After centrifugation at $9,000 \times g$ for 30 min at 4 °C, the supernatant was discarded. The pellet was suspended in 1 mL of deionized distilled water (DDW) with a vortex mixer and centrifuged at $9,000 \times g$ for 10 min at 4 °C. The supernatant was collected as virus concentrate.

(v) **Method E.** TE buffer (pH 7.2) containing Laureth-12 was used in the elution step instead of glycine-NaOH buffer (pH 9.0) in Method D to prevent the elution of humic substances from the sediments. The PEG precipitation method was applied in the concentration step as utilized in Method D.

Direct methods to recover viral RNA from sediments. Direct methods consisted of four steps: lysis of viral capsids in sediments (lysis step); separation of viral RNA from sediments by centrifugation (extraction step); RNA concentration from the supernatant (concentration step); and purification of the RNA concentrate (purification step). Details of each aforementioned procedure are summarized in Fig. 3 and described below.

(i) **Method F.** Five grams of wet sediment were placed in a 50-mL centrifuge tube. Five milliliters of 0.3 M sodium phosphate buffer (pH 5.8) were added to the sediment sample, and the sample was suspended using a vortex mixer. Five milliliters of lysis buffer (0.5 M Tris (pH 8.0), 0.1 M NaCl, 2% SDS, 8 mg skim milk/g sediment) were added to the suspension, and the tubes were processed in a Multi Vortex-Genie (SI-M286, Scientific Industries, New York) at 1,000 rpm for 10

min. The lysis buffer was modified from the one developed by Ikeda *et al.* (8) wherein EDTA was excluded to curb the elution of humic substances from sediments. Twelve milliliters of phenol:chloroform:isoamyl alcohol (25:24:1, v/v, molecular biology grade, Invitrogen, California) were added, and the tubes were vortexed again at 1,000 rpm for 10 min. The tubes were centrifuged at $2,300 \times g$ for 10 min at room temperature. The supernatant (upper aqueous phase of 12 mL) was collected and transferred to a clean 50-mL centrifuge tube. Twelve milliliters of isopropanol (molecular biology grade, Wako Pure Chemical Industries, Osaka) were added, and the sample was mixed vigorously with a vortex mixer. The resulting solution was incubated at $-20\text{ }^{\circ}\text{C}$ for 30 min, and the tubes were centrifuged at $2,300 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$. The supernatant was decanted, and the tubes were inverted on a paper towel for 5 min. One milliliter of DDW (DNase/RNase-free, Invitrogen) was added to the tubes, and the pellet was resuspended by placing the tubes in a heat block at $55\text{ }^{\circ}\text{C}$ for 5 min. Viral RNA was purified using a diethylaminoethyl (DEAE)-cellulose column according to the method of Ikeda *et al.* (8). Briefly, the DEAE-cellulose resin (molecular biology grade; Wako Pure Chemical Industries) was suspended in 20 volumes of TE buffer (pH 8.0) containing 0.6 M NaCl. After the resin settled, the supernatant was discarded. Twenty volumes of TE buffer (pH 8.0) containing 0.6 M NaCl were added again, and the washing step was repeated. The equilibrated resin was stored at $4\text{ }^{\circ}\text{C}$. Two milliliters of the resin were poured into the barrel of a 5-mL syringe (Terumo Co., Tokyo) plugged with a Millex syringe filter (5- μm pore size, 25-mm diameter, Millipore). The column was equilibrated with 2 mL of TE buffer (pH 8.0) containing 0.1 M NaCl (8). Two hundred microliters of TE buffer (pH 8.0) containing 0.2 M NaCl were added to an equal volume

of extracted viral RNA and loaded onto the DEAE-cellulose column. After washing the column with 2 mL of TE buffer (pH 8.0) containing 0.4 M NaCl, viral RNA was eluted with 1 mL of TE buffer (pH 8.0) containing NaCl. NaCl concentration in TE buffer for RNA elution was decided on the basis of the concentration of viral RNA in each eluted fraction (0.1–1 M), which was measured in advance.

(ii) Method G. This method was the same as Method F, except that magnetic beads were used in the RNA purification step instead of the DEAE-cellulose column. Briefly, after isopropanol precipitation in the concentration step, 200 μ L of the concentrated viral RNA was purified using FastTrack MAG micro mRNA Isolation Kits (Invitrogen) according to the manufacturer's instruction.

(iii) Method H. EDTA was added to the lysis buffer used in Methods F and G at a final concentration of 0.1 M (8). It was expected that EDTA would dissolve multivalent cations in sediments that otherwise contribute to the adsorption of viral particles onto solids (7, 21) or increase the enzymatic activity of some bacterial RNases (3). The extraction, concentration, and purification steps were the same as those in Method G.

(iv) Method I. Guanidine isothiocyanate was used in the RNA extraction buffer instead of SDS buffers. Briefly, 5 g of sediment (wet) was placed in a 50-mL centrifuge tube. Ten milliliters of TRIzol Reagent (a monophasic solution of phenol and guanidine isothiocyanate, Invitrogen) were added to the tube, and the mixture was processed in the Multi Vortex-Genie at 1,000 rpm for 5 min. The tubes were centrifuged at $2,300 \times g$ for 5 min at room temperature, and the supernatant was collected. Two hundred microliters of the supernatant containing viral RNA were purified using

FastTrack MAG micro mRNA Isolation Kits as described in Methods G and H.

Recovery of PV1 inoculated into sediments. Fig. 4 illustrates an experimental flow for evaluating the recovery rate of PV1 from sediments. PV1 was prepared using the BGM kidney cell line and following the procedures described by Sano *et al.* (21). In case of indirect methods (Methods A–E), 1 μL (1.1×10^8 copies, $\text{SD} = 5.8 \times 10^6$) of PV1 was inoculated into sediment samples, and the tubes were vortexed for 30 sec. Methods A–E were applied for the recovery of the inoculated PV1 particles from sediment samples, and total recovery rates of PV1 were calculated using experimental flow 1 (Fig. 4). On the other hand, in experimental flow 2, sediment samples were first treated with Methods A–E without PV1 inoculation, and then 1 μL (1.1×10^8 copies, $\text{SD} = 5.8 \times 10^6$) of PV1 was added to each virus concentrate (Fig. 4) to evaluate the recovery rates of the PV1 genome in the RNA extraction and RT-qPCR steps of indirect methods. Based on the recovery rates in experimental flows 1 and 2, the recovery rate in the elution and concentration steps were calculated.

In case of direct methods (Methods F–I), 1 μL (1.1×10^8 copies, $\text{SD} = 5.8 \times 10^6$) of PV1 was inoculated into sediment samples, which were processed using Methods F–I, and the total recovery rates were calculated (experimental flow 3, Fig. 4). On the other hand, for experimental flow 4 (Fig. 4), sediment samples were processed without PV1 inoculation, and 1 μL (1.7×10^6 copies, $\text{SD} = 8.1 \times 10^5$) of genomic RNA prepared from PV1 was added to the solution before the purification step to evaluate the recovery rates in the purification and RT-qPCR steps. Based on the recovery rates in experimental flows 3 and 4, the recovery rates in the lysis, extraction, and concentration steps were calculated.

Quantification of viral RNA. Complementary DNA (cDNA) was obtained from 5 μ L of extracted RNA through reverse transcription using the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Tokyo). The cDNA concentration of EV (including PV1) was determined using qPCR with the LightCycler ST300 (Roche) according to the method of Monpoeho *et al.* (18). Each 20- μ L PCR mixture contained 5 μ L of cDNA, 4 μ L of LightCycler TaqMan Master (Roche), 750 nM of each primer, and 200 nM of TaqMan probe listed in Table 1. PCR conditions consisted of a denaturing step at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 sec, annealing at 60 °C for 20 sec, and extension at 72 °C for 11 sec. The cDNA concentrations of HuNoV GI and GII were quantified using qPCR with a CFX96 Real-Time System (Bio-Rad Laboratories, Tokyo) according to the method of Kageyama *et al.* (11). Each 20- μ L PCR mixture contained 5 μ L of cDNA, 10 μ L of iQ Supermix (Bio-Rad Laboratories), 400 nM of each primer, 300 nM of RING1(a)-TP, and 100 nM of RING1(b)-TP for HuNoV GI or 300 nM of RING2-TP for HuNoV GII (Table 1). PCR conditions consisted of a denaturing step at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 sec, annealing at 56 °C for 20 sec, and extension at 72 °C for 20 sec. Based on the standard curve that was made by a 10-fold serial dilution of plasmid DNA (10^1 – 10^6 copies), the quantification limit was approximately 10 copies/PCR tube.

RESULTS AND DISCUSSION

PV1 recovery using indirect methods. Table 2 shows the recovery rates of inoculated PV1 from a sediment sample using indirect methods (Methods A–E). PV1 was not recovered from any samples tested (N = 3) using Method D. Because a high inhibitory effect on the steps of RNA extraction and RT-qPCR was observed using Method D, the combination of virus elution with glycine-NaOH buffer and virus concentration with PEG precipitation appeared ineffective in removing substances that inhibited the molecular detection of viral RNA. Meanwhile, the recovery rates of PV1 added to concentrates (experimental flow 2, Fig. 4) were greater than 100% in other methods (A–C and E), indicating that the concentrates obtained using indirect methods did not include any substances that inhibited RNA extraction and RT-qPCR. Therefore, the recovery rates in the elution and concentration steps were estimated to match the total recovery rates for all methods excluding Method D (Table 2).

The geometric mean (GM) of the total PV1 recovery rate in Method A was 1.8% (geometric standard deviation (GSD) = 0.17, N = 3). This recovery rate was much lower than that reported by Gerba *et al.* wherein 50% recovery was achieved (5). One reason for this difference in the recovery rate would be the differing sediment composition. Gerba *et al.* used sediment samples consisting of organic mud and sand (5), whereas the sediment sample used in this study (from St. F) was mainly composed of silt (67%) and clay (13%). As indicated by Johnson *et al.* the virus recovery rate from sediments could depend on the particle size distribution of the sediment, particularly the composition of silt and clay (9). Johnson *et al.* tested some elution buffers in the recovery of PV1 from a variety of sediments, and the mean recovery rates were 3.2% and 0.9% from sediments containing 4.6% and

17.4% clay, respectively (9). The latter recovery rate and clay composition reported by Johnson *et al.* are comparable to those observed in this study although they quantified PV1 using a cell culture-based plaque assay.

GM of the PV1 recovery rates in Method B was 5.4% (GSD = 0.53, N = 9), which was three-fold higher than that in Method A. This means that the negatively charged membrane filtration method (12) significantly improved PV1 recovery. However, the elution buffer of TE + Laureth-12 was ineffective even when used along with the negatively charged membrane filtration method; only 0.61% of inoculated PV1 was recovered (GSD = 0.11, N = 3, Method C in Table 2). This TE + Laureth-12 buffer also produced poor PV1 recovery when the concentration procedure was changed to PEG precipitation; only 0.18% of the inoculated PV1 was recovered (GSD = 0.11, N = 3, Method E in Table 2). Laureth-12, a surfactant, is an important component of the buffer used to elute *Cryptosporidium* oocysts and *Giardia* cysts from membrane surfaces (26, 27). This surfactant may not be effective for eluting viral particles from environmental silty sediments. Another possibility is that the interfacial activity of Laureth-12 affected the adsorption of viral particles onto the negatively charged membrane. Consequently, the combination of Glycine-NaOH buffer and the negatively charged membrane (Method B) produced the highest recovery rate (5.4%) among indirect methods.

Indirect methods have advantages in the processability of relatively larger volumes of samples because the supernatant obtained in the elution step can be processed using various concentration methods for water samples such as ultracentrifugation. Indirect methods would achieve higher recovery efficiency if viral particles can be recovered in the elution and concentration steps

without concentrating inhibitory substances.

PV1 recovery using direct methods. Table 3 shows the PV1 recovery rates in the four direct methods (Methods F–I). Naked genomic RNA of PV1 was added to the extracts from sediment samples to evaluate the inhibitory effects of coeluted substances on the purification and RT-qPCR steps. The PV1 recovery rate of Method F, wherein SDS buffer and a DEAE-cellulose column were used for viral capsid lysis and RNA purification, respectively, was extremely low (GM = 0.09%, GSD = 0.004, N = 3). Because the recovery rate in the purification step using the DEAE-cellulose column and RT-qPCR was 15% (Table 3), the recovery rate in the lysis, extraction, and concentration steps was calculated to be 0.6%. This low recovery efficiency was improved approximately 10-fold when magnetic beads were employed for RNA purification; 0.77% of genomic RNA was recovered (GSD = 0.03, N = 3, Method G in Table 3). This improvement of viral RNA recovery is owing to the increase in efficient recovery during the RNA purification and RT-qPCR steps (52%). This result indicates that magnetic beads, which can specifically capture RNA with poly(A) tails, were effective for purifying the positive-sense RNA genomes of viruses extracted from sediment samples.

However, this effective purification of viral RNA with magnetic beads was not compatible with the TRIzol-based RNA extraction method in which the recovery rate of PV1 was 0.10% (GSD = 0.24, N = 3, Method I in Table 3). The RNA extraction efficiency with TRIzol itself was 6.7%, whereas the recovery rate in the purification and RT-qPCR steps was 1.5%. This means that the eluate obtained by TRIzol-based extraction includes substances that inhibit RNA purification with magnetic beads or TRIzol itself affects the interaction of viral RNA with magnetic beads. On the other hand, the

combination of RNA extraction using an SDS buffer with EDTA and RNA purification with magnetic beads exhibited the highest and most stable recovery rate (11% with GSD of 0.02, Method H in Table 3). Because the recovery rate in the purification and RT-qPCR steps was 27% (Table 3), the PV1 recovery rate in the lysis, extraction, and concentration steps was calculated to be 41%. These results indicate that the addition of EDTA to the SDS buffer dramatically improved viral RNA recovery from sediments. Because some bacterial RNases require divalent metal ion cofactors to maintain biological activity (3), the chelation of multivalent cations with EDTA may prevent the degradation of viral RNA even after extraction from sediments.

Quantitative detection of EV and HuNoV from environmental sediment samples. One hundred eight sediment samples were collected from the Takagi River estuary from November 2007 to April 2009. Viral RNA was recovered from all samples using Method H because this method resulted in the highest and most stable recovery rate of inoculated PV1 among the nine approaches tested. A viral genome originating from HuNoV GII was detected in only one sample from St. A located at the bay although its concentration was below the quantification limit (approximately 10 copies/PCR tube). Meanwhile, two positive samples for EV were obtained from St. D in December 2008 and St. E in February 2009. The concentrations of EV genomic RNA in these positive samples were 8.6×10^2 copies/g (wet) (at St. D) and 2.4×10^2 copies/g (wet) (at St. E). To our best knowledge, this is the first report to obtain quantitative data of EVs in environmental sediment samples using RT-qPCR. In our previous study, total coliforms were detected from the sediment samples collected at St. A–F at concentrations of 7.3×10^2 – 7.5×10^4 CFU/100 g (dry) (16). In addition, human-specific

Bacteroides-Prevotella 16S rRNA genetic markers were detected (17). These results suggested that the sediment samples were contaminated by human feces. However, the detection rates of human pathogenic viruses were reasonably low. Although molecular detection methods such as PCR have been developed and widely used particularly for noncultivable viruses (e.g., HuNoV), methods of preparing samples from sediments that are compatible with molecular detection methods have not been established. It was reported that humic substances coeluted from soil and sediments inhibit nucleic acid extraction (30), hybridization (24), and Taq DNA polymerase in PCR (25, 28). The results obtained in this study suggested that the composition of multivalent cations in RNA extract from sediments is also important for stably obtaining excellent recovery rates of viral RNA.

In this study, nine approaches to recover viral RNA from environmental silty sediments were newly developed and compared to quantify human pathogenic viruses in sediments using RT-qPCR. The direct RNA extraction using an SDS buffer including EDTA to lyse viral capsids and magnetic beads to purify RNA (Method H) exhibited the best recovery rate of inoculated PV1, and Method H was effective for quantifying viral RNA in environmental sediments using the molecular method.

Further discussion regarding the inhibitory control in molecular detection will be required to acquire correctly quantified values of virus concentration in sediments. A mutant strain of mengovirus belonging to the family Picornaviridae has been successfully employed as a control for recovering HAV particles from clinical and shellfish samples (2). This mengovirus mutant could be applied as an inhibitory control in the methods developed in this study for extracting viral RNA from

environmental sediments. The inhibition of RT and qPCR steps should be also monitored, and RNA transcripts obtained by in vitro transcription can be used for this purpose (2). An appropriate setup of inhibitory controls in quantifying enteric viral RNA in environmental sediments is crucial for future studies.

ACKNOWLEDGMENT

This work was supported in part by the Japan Society for the Promotion of Science through a Grant-in-Aid for Scientific Research (S, 19106009) and Young Scientists (Start-up, 20860010) as well as JSPS Research Fellowships for Young Scientists (19-5067).

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FIGURE LEGENDS

Fig. 1. Sampling sites at the Takagi River and its estuary region.

Fig. 2. Summary of the elution and concentration steps of each indirect method.

Fig. 3. Summary of the lysis, extraction, concentration, and purification steps of each direct method.

Fig. 4. Experimental flows to evaluate the recovery rates of inoculated PV1 from a sediment sample using indirect and direct methods.

Table 1. Primer and probe sequences for the detection of EV and HuNoV.

Virus	Primer & probe	Sequence (5'-3')	Reference
Enterovirus (Poliovirus)	Ev2 (Forward)	CCCCTGAATGCGGCTAATC	18
	Ev1 (Reverse)	GATTGTCACCATAAGCAGC	
	Ev-probe	FAM-TGGGAGGGGCGATCGCAATCT-TAMRA	
Human norovirus genogroup I	COG1-F	CGYTGGATGCGNTTYCATGA	11
	COG1-R	CTTAGACGCCATCATCATTYAC	
	RING1(a)-TP	FAM-AGATYGCATCYCCTGTCCA-TAMRA	
	RING1(b)-TP	FAM-AGATCGCGGTCTCCTGTCCA-TAMRA	
Human norovirus genogroup II	COG2-F	CARGARBCNATGTTYAGRTGGATGAG	11
	COG2-R	TCGACGCCATCTTCATTCACA	
	RING2-TP	FAM-TGGGAGGGGCGATCGCAATCT-TAMRA	

Table 2. Recovery rates of inoculated PV1 from a sediment sample using indirect methods.

Method	Elution buffer	Concentration method	Total (Flow 1)		Elution & concentration ^c	Extraction & RT-qPCR (Flow 2)			N ^d	
			Detected [copies]/In oculated [copies]	Recovery rate [%]		Detected [copies]/In oculated [copies]	Recovery rate [%]			
				GM ^a	GSD ^b		GM	GSD		
A	Glycine-NaOH	Filtration method (Gerba <i>et al.</i> , 1977)	$\frac{1.9 \times 10^6}{1.1 \times 10^8}$	1.8	0.17	1.8	$\frac{1.7 \times 10^8}{1.1 \times 10^8}$	165	0.06	3
B	Glycine-NaOH	Filtration method (Katayama <i>et al.</i> , 2002)	$\frac{5.7 \times 10^6}{1.1 \times 10^8}$	5.4	0.53	5.4	$\frac{1.2 \times 10^8}{1.1 \times 10^8}$	112	0.20	9
C	TE + Laureth-12	Filtration method (Katayama <i>et al.</i> , 2002)	$\frac{6.5 \times 10^5}{1.1 \times 10^8}$	0.61	0.11	0.61	$\frac{1.4 \times 10^8}{1.1 \times 10^8}$	135	0.05	3
D	Glycine-NaOH	PEG precipitation method	$\frac{0}{1.1 \times 10^8}$	- ^e	-	Unknown	$\frac{0}{1.1 \times 10^8}$	-	-	3
E	TE + Laureth-12	PEG precipitation method	$\frac{1.9 \times 10^5}{1.1 \times 10^8}$	0.18	0.11	0.18	$\frac{1.1 \times 10^8}{1.1 \times 10^8}$	106	0.02	3

a, geometric mean; b, geometric standard deviation; c, recovery rates in the elution and concentration steps were calculated on the basis of the assumption that the recovery rate in the extraction and RT-qPCR steps was 100%; d, sample number; e, not detected.

Table 3. Recovery rates of inoculated PV1 from a sediment sample using direct methods.

Method	Lysis buffer	Purification method	Total (Flow 3)			Lysis, extraction, & concentration ^c	Purification & RT-qPCR (Flow 4)			N ^d
			Detected [copies]/In oculated [copies]	Recovery rate [%]			Recovery rate [%]	Detected [copies]/In oculated [copies]	Recovery rate [%]	
				GM ^a	GSD ^b	GM			GSD	
F	SDS without EDTA	DEAE-cellulose	$\frac{9.9 \times 10^4}{1.1 \times 10^8}$	0.09	0.004	0.6	$\frac{2.6 \times 10^5}{1.7 \times 10^6}$	15	0.18	3
G	SDS without EDTA	Magnetic beads	$\frac{8.2 \times 10^5}{1.1 \times 10^8}$	0.77	0.03	1.5	$\frac{8.9 \times 10^5}{1.7 \times 10^6}$	52	0.04	3
H	SDS with EDTA	Magnetic beads	$\frac{1.2 \times 10^7}{1.1 \times 10^8}$	11	0.02	41	$\frac{4.7 \times 10^5}{1.7 \times 10^6}$	27	0.10	7
I	TRIzol Reagent	Magnetic beads	$\frac{1.0 \times 10^5}{1.1 \times 10^8}$	0.10	0.24	6.7	$\frac{2.5 \times 10^4}{1.7 \times 10^6}$	1.5	0.11	3

a, geometric mean; b, geometric standard deviation; c, recovery rate in the lysis, extraction, and concentration steps were calculated from those of Flow 3 and Flow 4; d, sample number.

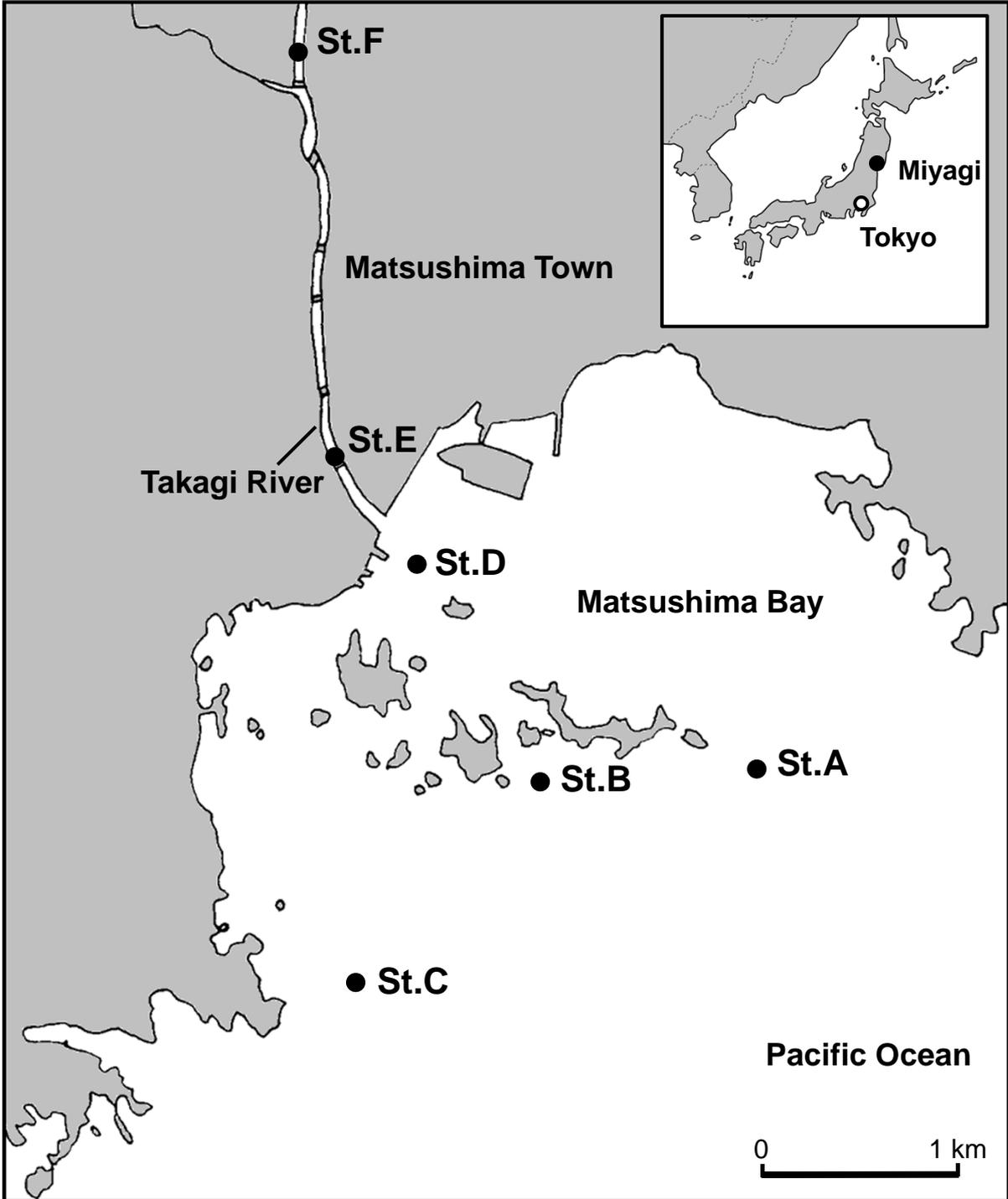


Fig. 1. Sampling sites at the Takagi River and its estuary region.

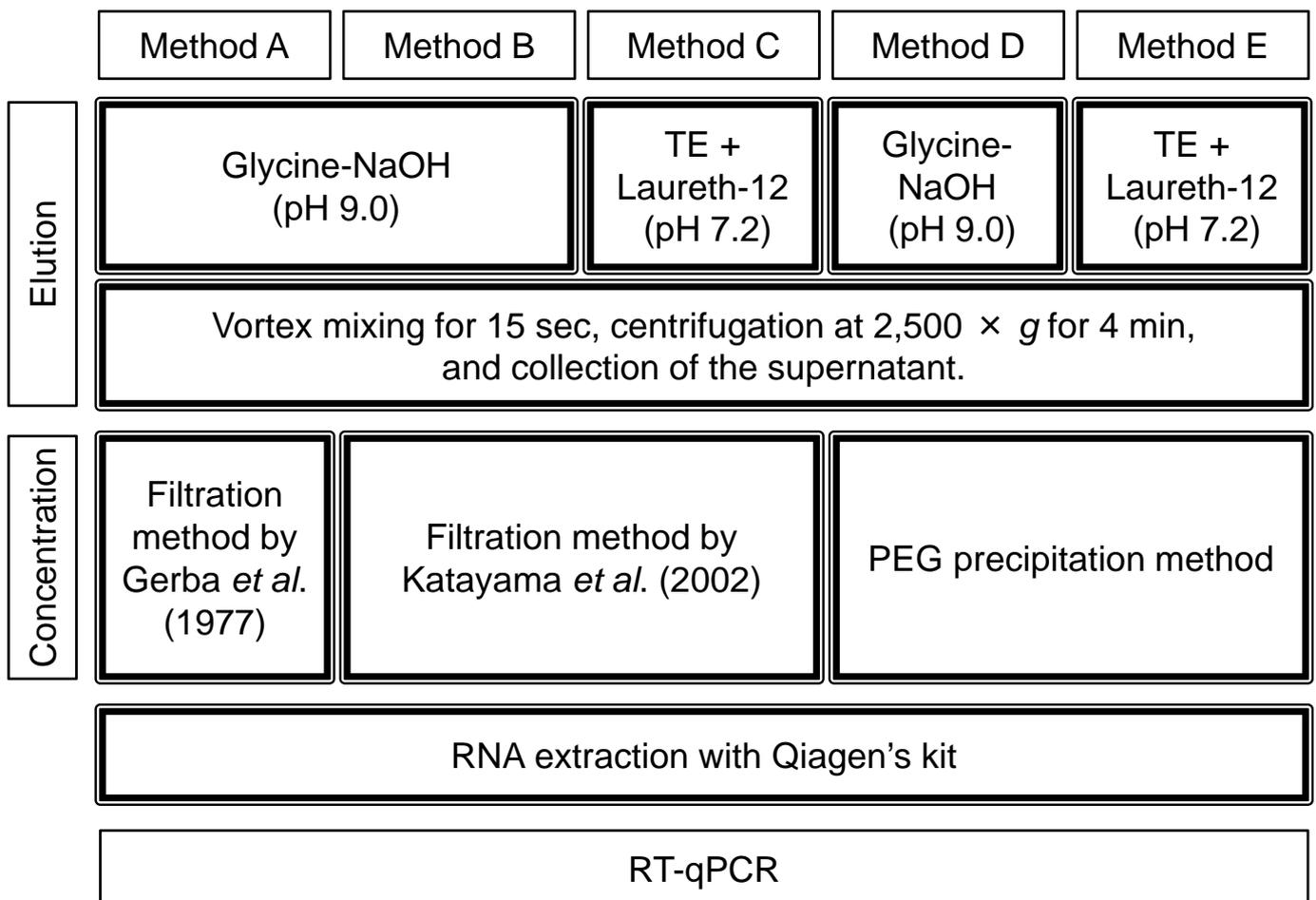


Fig. 2. Summary of the elution and concentration steps of each indirect method.

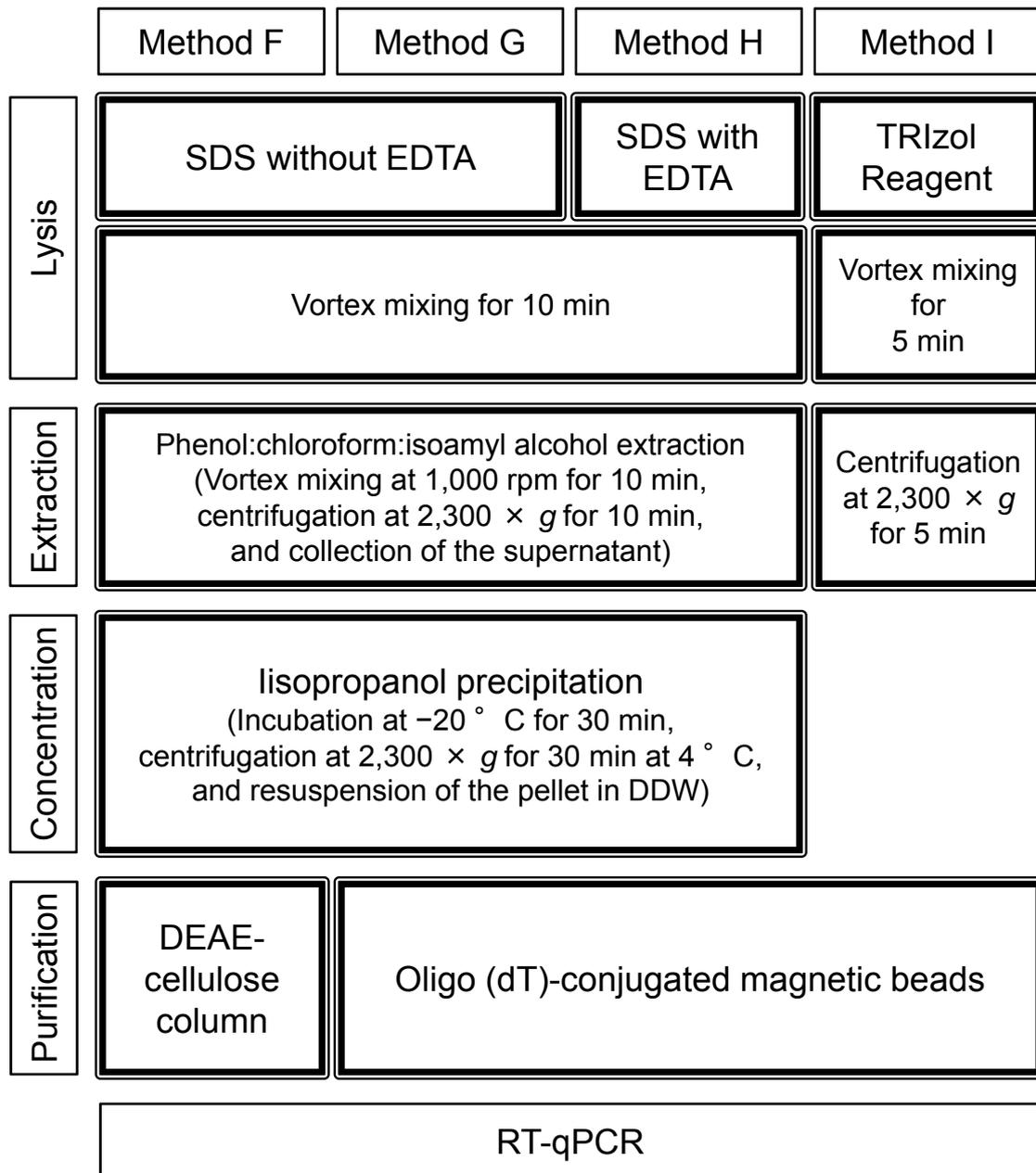


Fig. 3. Summary of the lysis, extraction, concentration and purification steps of each direct method.

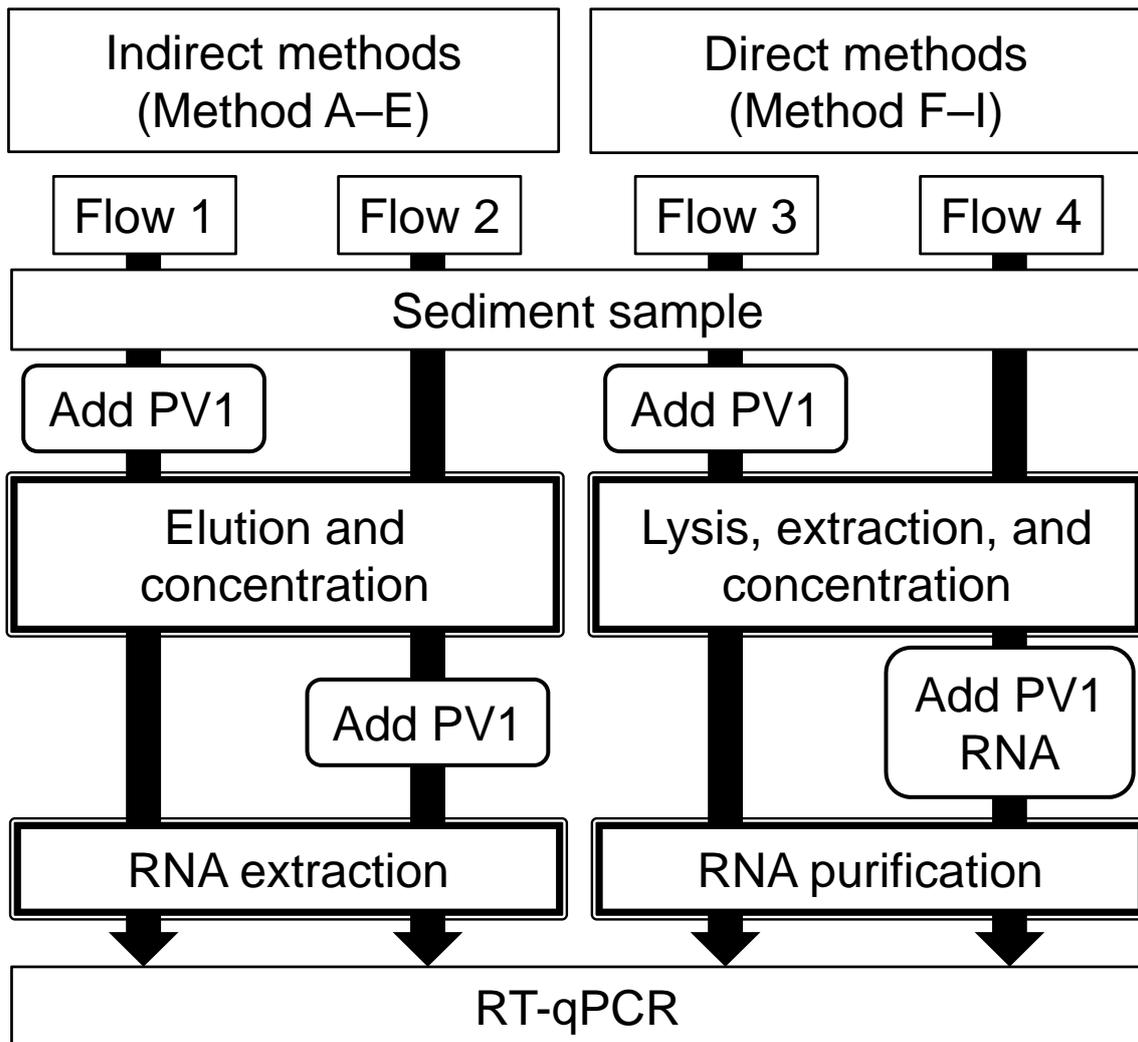


Fig. 4. Experimental flows to evaluate the recovery rates of inoculated PV1 from a sediment sample using indirect and direct methods.