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## **Supplementary Information**

### **Investigation of enteric adenovirus and poliovirus removal by coagulation processes and suitability of bacteriophages MS2 and $\phi$ X174 as surrogates for those viruses**

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**Table S1. Oligonucleotide sequences of primers and probes used in the present study.**

<b>Viruses</b>		<b>Oligonucleotide sequences (5' -&gt; 3')<sup>a</sup></b>	<b>Positions<sup>b</sup></b>	<b>References</b>
Adenovirus	Forward primer	AAC TTT CTC TCT TAA TAG ACG CC	30372-30394	Ko <i>et al.</i> , 2005
	Reverse primer	AGG GGG CTA GAA AAC AAA A	30489-30471	
	TaqMan probe	FAM-CTG ACA CGG GCA CTC TTC GC-TAMRA	30405-30424	
Poliovirus	Forward primer	CCT CCG GCC CCT GAA TG	444-460	Shieh <i>et al.</i> , 1995
	Reverse primer	ACC GGA TGG CCA ATC CAA	638-621	
	TaqMan probe	FAM-CCG ACT ACT TTG GGT GTC CGT GTT TC-TAMRA	537-562	Katayama <i>et al.</i> , 2002
MS2	Forward primer	GTC GCG GTA ATT GGC GC	632-648	O'Connell <i>et al.</i> , 2006
	Reverse primer	GGC CAC GTG TTT TGA TCG A	708-690	
	TaqMan probe	FAM-AGG CGC TCC GCT ACC TTG CCC T-TAMRA	650-671	
φX174	Forward primer	CCT TGC GCA GCT CGA GAA	152-169	This study
	Reverse primer	ATC CAA CGC GTC AGT TTT TGA	231-211	
	TaqMan probe	FAM-CTC TTA CTT TGC GAC CTT TCG CCA TCA AC-TAMRA	171-199	

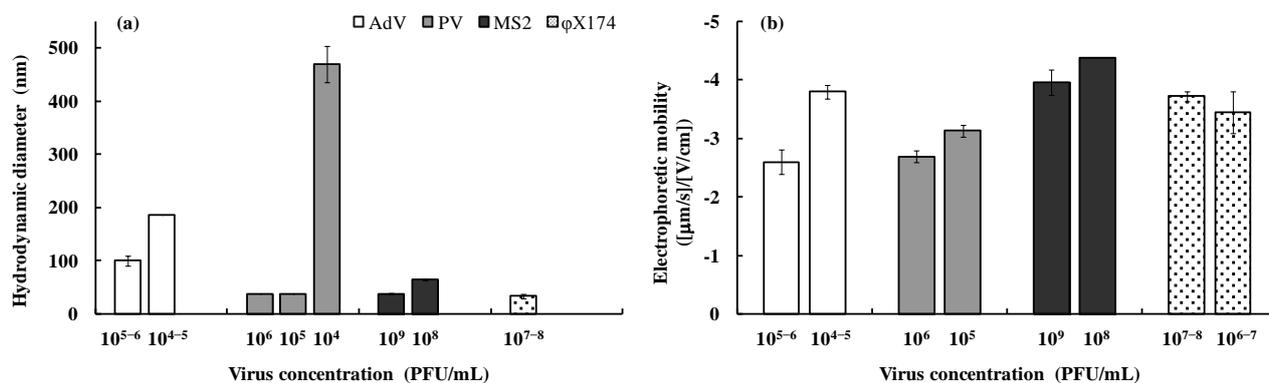
<sup>a</sup> FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

<sup>b</sup> GenBank accession numbers for sequence positions are NC\_001454 for adenovirus, V01150 for poliovirus, NC\_001417 for MS2 and J02482 for φX174.

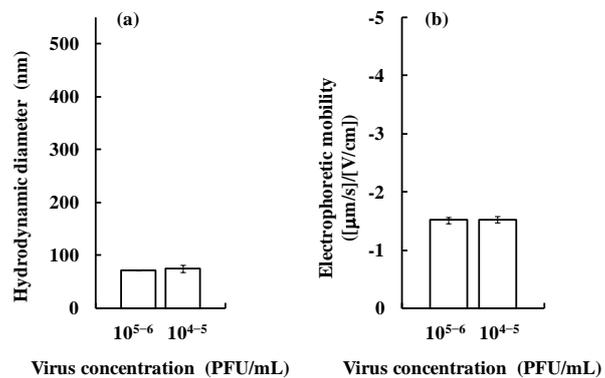
**Table S2. Differences in relationships between enteric virus and bacteriophage removal ratios, determined by analysis of covariance.<sup>a</sup>**

Data sets	<i>P</i> -value			
	AdV and MS2	PV and MS2	AdV and $\phi$ X174	PV and $\phi$ X174
PACl (settling) vs. alum (settling)	0.65	0.94	1.00	1.00
PACl (settling) vs. FeCl <sub>3</sub> (settling)	0.75	0.62	0.25	0.87
alum (settling) vs. FeCl <sub>3</sub> (settling)	1.00	0.43	0.56	0.97
PACl (filtration) vs. alum (filtration)	0.76	0.55	0.10	1.00
PACl (filtration) vs. FeCl <sub>3</sub> (filtration)	0.99	ND	0.80	ND
alum (filtration) vs. FeCl <sub>3</sub> (filtration)	0.64	ND	0.07	ND
PACl (settling) vs. PACl (filtration)	0.00	0.00	0.00	0.00
alum (settling) vs. alum (filtration)	0.00	0.06	0.00	0.01
FeCl <sub>3</sub> (settling) vs. FeCl <sub>3</sub> (filtration)	0.01	ND	0.12	ND

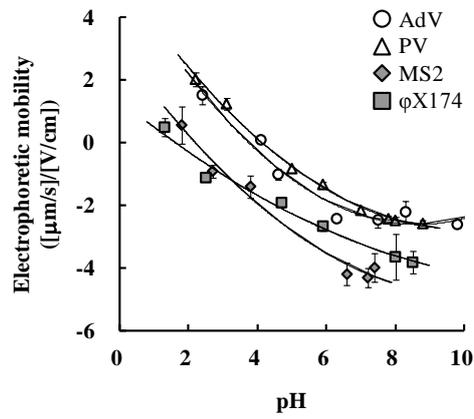
<sup>a</sup> ND, not determined. Because the PV removal ratios by coagulation with FeCl<sub>3</sub> and subsequent filtration were below the detection limit, we could not conduct an analysis of covariance for those data sets.



**Figure S1. Hydrodynamic diameters (a) and electrophoretic mobilities (b) of AdV, PV, MS2, and φX174 in prepared Milli-Q water at pH 7. Values were determined from one measurement or are means, and error bars indicate standard deviations ( $n = 2-9$ ).**

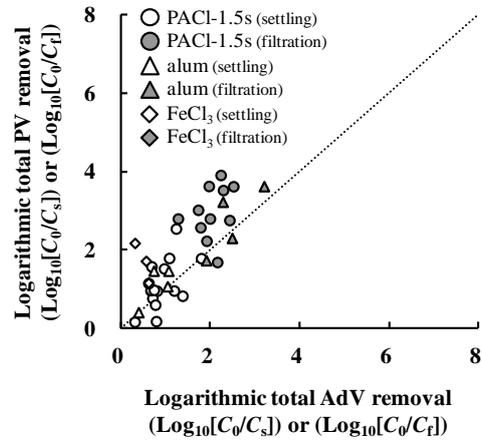


**Figure S2. Hydrodynamic diameters (a) and electrophoretic mobilities (b) of AdV in filtered source water sample C at pH 7. Values are means, and error bars indicate standard deviations ( $n = 4-9$ ).**



**Figure S3. Electrophoretic mobilities of AdV, PV, MS2, and  $\phi\text{X174}$  in prepared Milli-Q water.**

Values are means, and error bars indicate standard deviations ( $n = 9$ ).



**Figure S4. Relationship between total PV and AdV removal ratios during coagulation.**

## Propagation and purification of AdV and PV

AdV stock solution, prepared as described below, was diluted 10-fold with 1X Dulbecco's modified Eagle's medium (DMEM) containing 100 units/mL penicillin and 100 µg/mL streptomycin. A monolayer of approximately 90% confluent A549 cells in a 75-cm<sup>2</sup> flask was inoculated with the 1 mL of the diluted virus solution, and the flask was incubated in a humidified incubator at 37 °C in 5% CO<sub>2</sub> for 60 min with rotation every 15 min. After incubation, 20 mL of 1X DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin was added to the flask, which was then incubated at 37 °C in 5% CO<sub>2</sub> until 100% cytopathic effect of A549 cells was confirmed (11–12 days). Fresh cell culture medium (20 mL) was added to the flask after 7 days of incubation. At the end of the incubation period, viruses were released from the infected A549 cells by three cycles of freezing at –83 °C and thawing at 37 °C. The AdV culture solution was centrifuged (2000 × *g*, 10 min) and then passed through a hydrophilic cellulose acetate membrane filter (nominal pore size 0.2 µm; Dismic-25cs, Toyo Roshi Kaisha, Tokyo, Japan) to prepare the AdV stock solution. The filtrate was further purified by using a tangential-flow filtration cassette (nominal molecular weight cutoff 1000 kDa, regenerated cellulose [RC] membrane; Pellicon XL, Millipore Corp.) to ensure that only minimal amounts of dissolved organic carbon components from the culture medium were introduced into the virus-spiked source water (see section 2.4). The AdV concentration in the purified solution was approximately 10<sup>5–6</sup> PFU/mL.

PV stock solution, prepared as described below, was diluted 10-fold with Dulbecco's phosphate-buffered saline (PBS, without Ca and Mg ions, Nissui Pharmaceutical Co.). A monolayer of approximately 90% confluent buffalo green monkey kidney epithelial cells (BGM cells) in 75-cm<sup>2</sup> flask was inoculated with 1 mL of the diluted stock solution, and the flask was incubated in a humidified incubator at 37 °C in 5% CO<sub>2</sub> for 30 min. Then 20 mL of 1X Eagle's minimum essential medium (EMEM) was added to the flask, which was subsequently incubated at 37 °C in 5% CO<sub>2</sub> until cytopathic effects were confirmed in 100% of the BGM cells (2–3 days), at which point viruses were released from the infected cells by three cycles of freezing at –83 °C and thawing at 37 °C. The PV stock solution was prepared by passing PV culture solution through a hydrophilic cellulose acetate membrane filter (nominal pore size 0.2 µm; Dismic-25cs). The filtrate was further purified by means of a tangential-flow filtration cassette (nominal molecular weight cutoff 1000 kDa, RC membrane; Pellicon XL) and two uses of a centrifugal filter device (nominal molecular weight cutoff 100 kDa, RC membrane; Amicon Ultra-15, Millipore Corp.) to ensure that only minimal amounts of dissolved organic carbon components from the culture medium were introduced into the virus-spiked source water (see section 2.4). The concentration of PV in the purified solution was approximately 10<sup>6</sup> PFU/mL.

Propagation and purification of bacteriophages

Bacteriophage MS2 was propagated for 22–24 h at 37 °C in bacterial host *E. coli* (NBRC 13965), and bacteriophage  $\phi$ X174 was propagated for 18 h at 37 °C in bacterial host *E. coli* (NBRC 13898). The bacteriophage culture solution was centrifuged ( $2000 \times g$ , 10 min) and then passed through a hydrophilic cellulose acetate membrane filter (nominal pore size 0.45  $\mu\text{m}$ ; Dismic-25cs, Toyo Roshi Kaisha) to prepare the bacteriophage stock solution. The filtrate was further purified twice with a centrifugal filter device (nominal molecular weight cutoff 100 kDa, RC membrane; Amicon Ultra-15) to ensure that only minimal amounts of dissolved organic carbon components from the culture medium were introduced into the virus-spiked source water (see section 2.4). The concentrations of MS2 and  $\phi$ X174 in the purified solutions were approximately  $10^{10}$  and  $10^{7-8}$  PFU/mL, respectively.

#### Plaque assay for quantification of AdV and PV

Approximately 90% confluent A549 cells in a 75-cm<sup>2</sup> flask were seeded in 6-well (8.96 cm<sup>2</sup>/well) tissue culture plates. One milliliter of a sample serially diluted 10-fold with 1X DMEM containing 100 units/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin or 1 mL of a sample diluted 2-fold with 2X DMEM (Life Technologies) containing 200 units/mL penicillin and 200  $\mu\text{g}/\text{mL}$  streptomycin, and 7.4 g/L sodium hydrogen carbonate was inoculated onto a monolayer of 100% confluent A549 cells in a 6-well plate, and the plate was then incubated in a humidified incubator at 37 °C in 5% CO<sub>2</sub> for 90 min. After incubation, the inoculum was removed by inverting the plate, and 3 mL of agar

overlay prepared by combining 2X DMEM supplemented with 20% heat-inactivated fetal bovine serum, 200 units/mL penicillin, 200 µg/mL streptomycin, and 7.4 g/L sodium hydrogen carbonate with an equal volume of 1% (w/v) agarose (SeaKem ME, Lonza Group, Rockland, ME, USA) was applied to the cell monolayer. After the addition of the agar overlay, the plate was incubated at 37 °C in 5% CO<sub>2</sub> until plaques were produced on the monolayer (11–12 days). Fresh agar overlay (3 mL) was added to the plate after 7 days of incubation. At the end of incubation, the cell monolayer was stained with 1 mL of 0.6 g/L neutral red at 37 °C in 5% CO<sub>2</sub> for 6 h, and then excess stain was discarded by inversion of the plate. The plaques of each well were counted until no new plaques appeared, which generally took 2 days from the time that the neutral red was discarded. The average plaque count of triplicate wells or 12 wells prepared from one sample was considered as the infectious AdV concentration for that sample. The detection limit of the plaque assay for quantification of AdV was 1/6 PFU/mL when 12 wells were prepared.

Approximately 90% confluent BGM cells in a 75-cm<sup>2</sup> flask were seeded in 6-well (8.96 cm<sup>2</sup>/well) tissue culture plates. A monolayer of 100% confluent BGM cells in a 6-well plate was inoculated with 1 mL of a sample serially diluted 10-fold with 1X EMEM supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1.125 g/L sodium hydrogen carbonate or 1 mL of a sample diluted 2-fold with 2X EMEM containing 4 mM L-glutamine, 200 units/mL penicillin, 200 µg/mL streptomycin, and 2.25 g/L sodium hydrogen carbonate, and then the plate was incubated in a humidified incubator at 37 °C in 5% CO<sub>2</sub> for 90 min. After incubation,

inoculum was removed by inverting the plate, and then 3 mL of agar overlay prepared by combining 2X EMEM (without phenol red, Nissui Pharmaceutical Co.) supplemented with 2% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 200 units/mL penicillin, 200 µg/mL streptomycin, and 2.25 g/L sodium hydrogen carbonate with an equal volume of 2.5% (w/v) agarose (Agar-EPI, Nacalai Tesque, Inc., Kyoto, Japan) was applied to the cell monolayer. After addition of the overlay, the plate was incubated at 37 °C in 5% CO<sub>2</sub> for 1 day; the cell monolayer was stained with 1 mL of 0.15 g/L neutral red at 37 °C in 5% CO<sub>2</sub> for 3 h; and then excess stain was discarded by inverting the plate. The plaques of each well were counted until no new plaques appeared, with generally took 2 days from the time that the neutral red was discarded. The average of the plaque counts of triplicate wells or 12 wells prepared from one sample was considered as the infectious PV concentration for that sample. The detection limit of the plaque assay for quantification of PV was 1/6 PFU/mL when 12 wells were prepared.

#### Quantification of AdV and PV by real-time PCR or real-time RT-PCR

Viral DNA or RNA was extracted from 200 µL of sample with a QIAamp MinElute Virus Spin Kit (Qiagen, Tokyo, Japan) to obtain a final volume of 30 µL for AdV and 20 µL for PV. Extracted RNA solution was added to a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan, Tokyo, Japan) for the reverse-transcription reaction, which was conducted at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s and subsequent cooling to 4 °C

in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Otsu, Japan). The extracted DNA solution, or the resulting cDNA solution, was then amplified with a TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems Japan) with 400 nM primers (HQ-SEQ grade, Takara Bio Inc.) and 250 nM TaqMan probe (Applied Biosystems Japan). The oligonucleotide sequences of the primers and the probes used to quantify AdV and PV are shown in Table S1. Amplification was conducted at 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan). The standard curve for the real-time PCR method was based on the relationship between the plaque-assay-measured concentration of infectious AdV or PV in a freshly prepared purified AdV or PV solution and the number of PCR amplification cycles (the  $C_t$  value). The detection limit of the PCR assay for quantification of AdV or PV was approximately 0.1 PFU/mL.

#### Quantification of bacteriophages by real-time PCR or real-time RT-PCR

Following extraction of viral RNA or DNA with a QIAamp MinElute Virus Spin Kit and reverse transcription with a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor for the extracted RNA solution, the cDNA solution or the extracted DNA solution was then amplified and quantified by the procedure used for quantification of AdV and PV. The oligonucleotide sequences of the primers and the probes used for MS2 and  $\phi$ X174 quantification are shown in Table S1. The

primers and probe specific to  $\phi$ X174 were designed by means of Primer Express Software (ver. 3.0, Applied Biosystems Japan). The standard curve for the real-time PCR method was based on the relationship between the plaque-assay-measured concentration of infectious MS2 or  $\phi$ X174 in a freshly prepared purified MS2 or  $\phi$ X174 solution and the  $C_t$  value. The detection limit of the PCR assay for quantification of MS2 or  $\phi$ X174 was approximately 10 PFU/mL.

#### Measurement of electrophoretic mobility

The alkalinity of Milli-Q water was brought to 20 mg-CaCO<sub>3</sub>/L by the addition of 0.4 mM sodium hydrogen carbonate, and the pH was adjusted to 2–10 with HCl or NaOH. Source water samples were filtered through a stirred ultrafiltration cell (Model 8050, Millipore Corp.) with an ultrafiltration membrane (molecular weight cutoff 100 kDa, RC membrane; Ultrafiltration Disks, PLHK, Millipore Corp.) to exclude large particles; and the pH of the filtrate was adjusted to 6 or 7 with HCl or NaOH. The Milli-Q water and the source water samples were allowed to stand for 1 day at 20 °C to stabilize the pH. Just before the measurement of the electrophoretic mobility, purified virus solution was suspended in the water at approximately 10<sup>5–6</sup> PFU/mL for AdV, 10<sup>6</sup> PFU/mL for PV, 10<sup>9</sup> PFU/mL for MS2, and 10<sup>7–8</sup> PFU/mL for  $\phi$ X174, and then the water was filtered through a polytetrafluoroethylene membrane filter (nominal pore size 0.2  $\mu$ m, Dismic-25HP, Toyo Roshi Kaisha) to exclude dust. The electrophoretic mobilities of AdV, PV, MS2, and  $\phi$ X174 were measured with a Zetasizer Nano ZS (50 mW 532-nm green laser, Malvern Instruments,

Malvern, Worcestershire, UK) at 25 °C and at a 17° measurement angle.

The sensitivity of the apparatus for the detection of the virus particles (i.e., measurement of particle size) was determined by using samples containing suspended viruses at various concentrations in Milli-Q water (prepared as described above) at pH 7 for each virus (Figure S1). The hydrodynamic diameters of AdV, PV, MS2, and φX174 were measured with the apparatus used for the measurement of the electrophoretic mobility at 25 °C and at a 173° measurement angle. The intensity-based hydrodynamic diameter was obtained by means of dynamic light scattering. The hydrodynamic diameter of AdV at approximately  $10^{5-6}$  PFU/mL was approximately 100 nm (Figure S1a), which is close to the previously reported AdV diameter of 80–100 nm (Jiang, 2006; Bosch, 2007). Wong *et al.* (2012) also reported that the hydrodynamic diameter of AdV determined with a Zetasizer Nano series was approximately 100 nm in 1 mM NaCl solution at pH 7. In contrast, the hydrodynamic diameter of AdV was determined to be approximately 200 nm at a virus concentration of approximately  $10^{4-5}$  PFU/mL, owing to the uncertainty caused by the low concentration of virus particles in the sample. This result suggests that an AdV concentration of approximately  $10^{4-5}$  PFU/mL is too low to measure particle movement, which is the basis for determination of particle size and electrophoretic mobility. Therefore, an AdV concentration of approximately  $10^{5-6}$  PFU/mL is required for measurement of the electrophoretic mobility of AdV in the prepared Milli-Q water. In fact, the electrophoretic mobility of AdV in the prepared Milli-Q water depended strongly on the virus concentrations in the water samples containing suspended

viruses, and the electrophoretic mobility value for the sample with the lower AdV concentration exhibited a large level of uncertainty (Figure S1b). These results indicate that we were able to measure the electrophoretic mobility of AdV when the virus concentration in the prepared Milli-Q water was approximately  $10^{5-6}$  PFU/mL.

The hydrodynamic diameters of PV, MS2, and  $\phi$ X174 at approximately  $10^6$  and  $10^5$  PFU/mL for PV,  $10^9$  PFU/mL for MS2, and  $10^{7-8}$  PFU/mL for  $\phi$ X174 were approximately 30 nm (Figure S1a), and this value is almost equal to the value of 20–30 nm previously reported for PV, MS2, and  $\phi$ X174 (Fong and Lipp, 2005; Mesquita *et al.*, 2010); in contrast, the hydrodynamic diameters of PV and MS2 were determined to be approximately 500 and 70 nm, respectively, at virus concentrations of approximately  $10^4$  PFU/mL for PV and  $10^8$  PFU/mL for MS2, owing to the high level of uncertainty due to the low concentration of virus particles in the sample. In addition, the electrophoretic mobilities of PV and MS2 in the prepared Milli-Q water depended on the virus concentrations in the water samples containing suspended viruses (Figure S1b), as was the case for AdV. Accordingly, we were able to measure the electrophoretic mobilities of PV, MS2, and  $\phi$ X174 when the virus concentrations in the prepared Milli-Q water were approximately  $10^6$  PFU/mL for PV,  $10^9$  PFU/mL for MS2, and  $10^{7-8}$  PFU/mL for  $\phi$ X174.

The sensitivity of the apparatus for the detection of the virus particles in the filtered source water sample, prepared as described above, was also determined at pH 7 for AdV (Figure S2). The hydrodynamic diameter of AdV was approximately 80 nm, and the electrophoretic mobility of AdV

did not vary markedly in the concentration range of approximately  $10^{4-6}$  PFU/mL (Figure S2a,b). These results indicate that an AdV concentration of approximately  $10^{5-6}$  PFU/mL was enough for measurement of the electrophoretic mobility in the filtered source water samples. Accordingly, the virus concentrations in the water samples containing suspended viruses prepared in the present study (i.e., approximately  $10^{5-6}$  PFU/mL for AdV,  $10^6$  PFU/mL for PV,  $10^9$  PFU/mL for MS2, and  $10^{7-8}$  PFU/mL for  $\phi$ X174) were suitable for the measurement of electrophoretic mobility not only in the prepared Milli-Q water but also in the filtered source water samples.

#### Measurement of hydrophobicity

Three hydrocarbon solvents—*n*-hexadecane ( $\log K_{ow} = 8.25$ ), *n*-octane (5.18), and *p*-xylene (3.15)—and toluene (2.69) were used for hydrophobicity measurements, as follows. Viruses were simultaneously suspended at approximately  $10^{5-6}$  PFU/mL for AdV,  $10^6$  PFU/mL for PV,  $10^9$  PFU/mL for MS2, and  $10^{7-8}$  PFU/mL for  $\phi$ X174 in 2 mL of PBS. The solution was supplemented separately with 2 mL of each of the four solvents. After 10 min of preincubation at 30 °C, each mixture was intensely vortexed for 2 min and then allowed to rest for 15 min at 20 °C to allow the water and solvent to separate. The concentrations of AdV, PV, MS2, and  $\phi$ X174 in the water phase were quantified by real-time PCR or real-time RT-PCR. A decrease in virus concentration in the water phase after mixing with solvent was used as a measure of the surface hydrophobicity of the virus.

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