Supplementary Information

Suitability of pepper mild mottle virus as a human enteric virus surrogate for assessing the efficacy of thermal or free-chlorine disinfection processes by using infectivity assays and enhanced viability PCR

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

Viruses		Oligonucleotide sequences (5' -> 3')[a]	Positions[b]	References
Adenovirus	Forward primer	AAC TTT CTC TCT TAA TAG ACG CC	30372-30394	Ko *et al.* , 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	
Coxsackievirus	Forward primer	CCT CCG GCC CCT GAA TG	449-465	Shieh *et al.* , 1995
	Reverse primer	ACC GGA TGG CCA ATC CAA	643-626	
	TaqMan probe	FAM-CCG ACT ACT TTG GGT GTC CGT GTT TC-TAMRA	542-567	Katayama *et al.* , 2002
Pepper mild mottle virus	Forward primer	GAG TGG TTT GAC CTT AAC GTT TGA	1878-1901	Haramoto *et al.* , 2013
	Reverse primer	TTG TCG GTT GCA ATG CAA GT	1945-1926	Zhang *et al.* , 2006
	TaqMan probe	FAM-CCT ACC GAA GCA AAT G-TAMRA	1906-1921	

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

[b] GeneBank accession numbers for sequence positions are NC_001454 for adenovirus, AF114383 for coxsackievirus and AB254821 for pepper mild mottle virus.

Table S2. Estimated parameters for the fitted efficiency factor Hom (EFH) model of the chlorine treatment experiments.

Viruses	Initial free chlorine concentration (mg-Cl_2/L)	Parameter				$R^{2\,a}$
		k' (min^{-1})	k	n	m	
CV	0.1	0.041	886.7	3.693	1.802	1.00
	0.5	0.043	0.0002	-13.28	0.758	1.00
PMMoV	0.5	0.003	0.114	-1.494	0.448	0.87

[a] R^2 is the coefficient of determination between the predicted and observed values of virus inactivation curves.

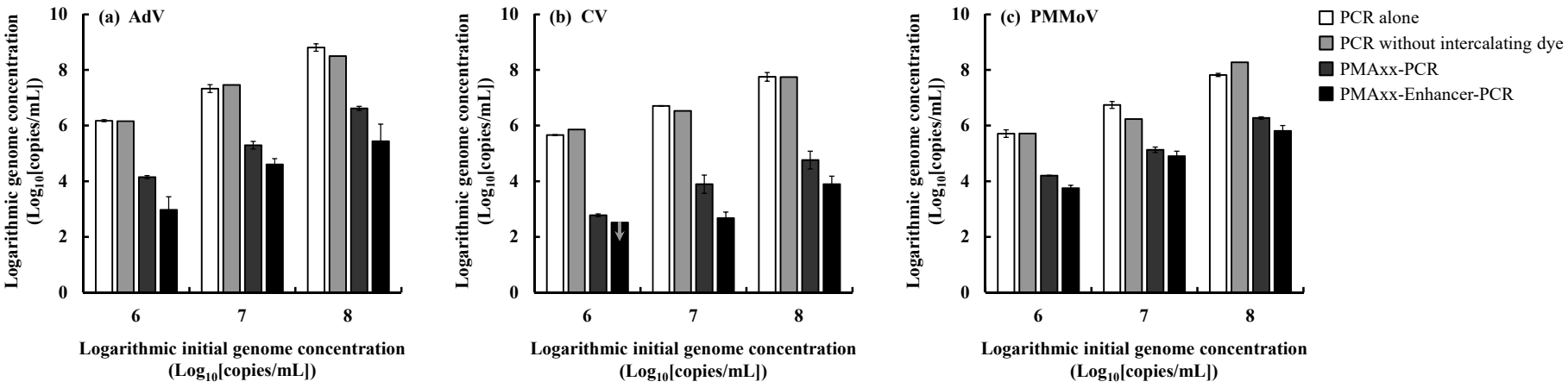

Figure S1. Effect of the initial concentration of plasmid DNA containing the PCR target sequence for adenovirus type 40 (AdV) (a), coxsackievirus B5 (CV) (b), and pepper mild mottle virus (PMMoV) (c) on the performances of PMAxx-PCR and PMAxx-Enhancer-PCR. PMAxx concentration, 200 μM. Values were determined from a single experiment or are the means of duplicate experiments; error bars indicate standard deviations. The arrow indicates that the plasmid DNA concentration was below the limit of quantification of the PCR.

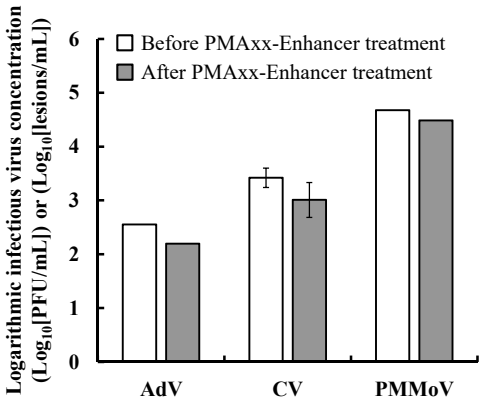

Figure S2. Effect of PMAxx-Enhancer treatment on virus infectivity of adenovirus type 40 (AdV), coxsackievirus B5 (CV), or pepper mild mottle virus (PMMoV). Values were determined from a single experiment or are the means of duplicate experiments; error bars indicate standard deviations.

Preparation of naked DNA and RNA

The DNA of adenovirus type 40 Dugan strain (AdV; linear, double-stranded DNA, 34214 bp, GenBank accession number NC_001454) or the RNA of coxsackievirus B5 Faulkner strain (CV; linear, positive-sense, single-stranded RNA, 7400 nt, AF114383) or pepper mild mottle virus pepIwateHachiman1 strain (PMMoV; linear, positive-sense, single-stranded RNA, 6356 nt, AB254821.1) was extracted from a purified solution of AdV or CV, or a stock solution of PMMoV, by using a QIAamp MinElute Virus Spin Kit (Qiagen, Tokyo, Japan). The extracted viral DNA or RNA was then added to phosphate-buffered saline (PBS) at an initial concentration of $10^{6–7}$ copies/mL for AdV, $10^{7–8}$ copies/mL for CV, or $10^{9–10}$ copies/mL for PMMoV. Plasmid DNA (total 2433–2575 bp; GeneArt Gene Synthesis; Thermo Fisher Scientific Inc., Waltham, MA, USA) containing the polymerase chain reaction (PCR) target sequence for AdV (118 bp, GenBank accession number NC_001454), CV (195 bp, AF114383), or PMMoV (68 bp, AB254821) was added to PBS at an initial concentration of approximately 10^6, 10^7, and 10^8 copies/mL for AdV, CV, and PMMoV, respectively.

Quantitative real-time PCR and quantitative real-time reverse-transcription PCR

Viral DNA or RNA was extracted from 200 μL of sample by using a QIAamp MinElute Virus Spin Kit to obtain a final volume of DNA or RNA of 20 μL. 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 (RT) reaction, which was conducted at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s with subsequent cooling to 4 °C in a thermal cycler (Thermal Cycler Dice Model TP600; Takara Bio Inc., Otsu, Japan). Extracted DNA solution, the cDNA solution resulting from RT of the extracted RNA, or plasmid DNA solution was then amplified with TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems Japan) or TaqMan Universal Master Mix II, no UNG (Applied Biosystems Japan) together with 400 nM of primers

(HQ-SEQ grade; Takara Bio Inc.), 250 nM TaqMan probe (Applied Biosystems Japan) and DNase/RNase-free distilled water. The oligonucleotide sequences of the primers and the probes used for the quantification of AdV, CV, and PMMoV are shown in Table S1. Amplification was conducted at 50 °C for 2 min, 95 °C for 10 min, and then 50 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).

For each PCR analysis, each sample alongside a dilution series of plasmid DNA containing the PCR target sequence of AdV, CV, or PMMoV (see previous section) and negative controls were analyzed. The genome copy concentration of the samples was determined from the log–linear relationship of quantification cycle (C_q value) and concentration, which was constructed using a 1-$\log_{10}$-fold (i.e., 10-fold) or 0.5-$\log_{10}$-fold serial dilution of the plasmid DNA at a concentration of 10^9–$10^{2.5}$ copies/mL. The resulting standard curves showed PCR efficiency ranging from 73% to 95% and R^2 values ranging from 0.99 to 1.00 (note: the PCR efficiency of <90% may lead to an overestimation of virus concentrations and an underestimation of the magnitude of the PCR signal reduction). The quantification limit of the PCR assay was between $10^{2.5}$ and 10^3 copies/mL, which was equivalent to between 2 and 5 copies/reaction.

Efficiency factor Hom model and CT value calculation

The Solver function in Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA) was used to minimize the sum of squares of the difference between the observed residual free-chlorine concentration and the concentration calculated by the first-order kinetic equation (equation 1: Haas and Joffe, 1994) to determine the first-order free-chlorine decay rate constant, k' (min^{-1}), for each experiment:

$$C = C_0 \exp(-k't) \qquad (1)$$

where t is the contact time (min), and C and C_0 are the residual free-chlorine concentration (mg-Cl_2/L) at contact time t and time 0 (initial free-chlorine concentration), respectively.

The natural logarithm of the survival ratios (infectious virus concentration after contact with free chlorine divided by the initial infectious virus concentration) determined by plaque assay for AdV or CV, or by local lesion count assay for PMMoV, for each chlorine treatment experiment were fit to the efficiency factor Hom (EFH) model. Survival ratios that were below the detection limit were excluded from the calculation.

The Solver function in Microsoft Excel 2013 was also used to minimize the sum of squares of the difference between the observed survival ratios and the survival ratios calculated with the EFH model (equation 2: Haas and Joffe, 1994) to determine the virus inactivation rate constant, k (dimensionless); the coefficient of dilution, which represents the average number of molecules combined with the virus necessary to cause inactivation, n (dimensionless); and Hom's exponent, m (dimensionless), for each experiment:

$$\ln\frac{N_c}{N_0} = -k{C_0}^n t^m \left[\frac{1-\exp\left(-\frac{nk't}{m}\right)}{\left(\frac{nk't}{m}\right)}\right]^m \qquad (2)$$

where t is contact time (min), and N_c and N_0 are the infectious virus concentrations (PFU/mL or lesions/mL) at contact time t and time 0 (initial infectious concentration), respectively. The estimated EFH model parameters are shown in Table S2.

To evaluate virus sensitivity to chlorine treatment, CT values (mg-Cl_2·min/L) were calculated for 2-, 3-, and 4-$\log_{10}$ inactivation (99%, 99.9%, and 99.99%, respectively) of each virus by the chlorine treatment by using the determined parameters of the EFH model.

References

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