Evaluation of viability PCR performance for assessing norovirus infectivity in fresh-cut vegetables and irrigation water

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Abstract

Norovirus (NoV) detection in food and water is mainly carried out by quantitative RT-PCR (RT-qPCR). The inability to differentiate between infectious and inactivated viruses and the resulting overestimation of viral targets is considered a major disadvantage of RT-qPCR. Initially, conventional photoactivatable dyes (i.e. propidium monoazide, PMA and ethidium monoazide, EMA) and newly developed ones (i.e. PMAxx and PEMAX) were evaluated for the discrimination between infectious and thermally inactivated NoV genogroup I (GI) and II (GII) suspensions. Results showed that PMAxx was the best photoactivatable dye to assess NoV infectivity. This procedure was further optimized in artificially inoculated lettuce. Pretreatment with 50 µM PMAxx and 0.5% Triton X-100 (Triton) for 10 min reduced the signal of thermally inactivated NoV by ca. 1.8 logs for both genogroups in lettuce concentrates. Additionally, this pretreatment reduced the signal of thermally inactivated NoV GI between 1.4 and 1.9 logs in spinach and romaine and lamb's lettuces and by more than 2 logs for NoV GII in romaine and lamb's lettuce samples. Moreover this pretreatment was satisfactorily applied to naturally-contaminated water samples with NoV GI and GII. Based on the obtained results this pretreatment has the potential to be integrated in routine diagnoses to improve the interpretation of positive NoV results obtained by RT-qPCR.

Keywords: Norovirus, Quantitative RT-PCR, photoactivatable dyes, propidium monoazide, irrigation water

Introduction

Gastroenteritis caused by human noroviruses (NoVs) is the leading cause of acute viral gastroenteritis throughout the world and is mainly transmitted via the fecal-oral route. In 2013, the CDC identified viruses as the causative agent of 36% of illnesses due to food consumption in outbreaks with a single confirmed etiologic agent. NoVs were the most common cause, being responsible for 154 outbreaks, while *Salmonella* was next, accounting for 149 (34%) outbreaks (CDC, 2015). Within the European Union, *Salmonella* remained the most commonly confirmed causative agent in the foodborne outbreaks reported (22.5 %), followed by NoV which accounted for 18 % (EFSA, 2015).

The current knowledge of NoV has been hampered by the lack of a cultivation system for their *in vitro* propagation. Recently the use of B lymphocytes combined with the presence of HBGA-expressing enteric bacteria showed the effective growth of a GII.4-Sydney NoV strain isolated from a stool sample (Jones et al., 2014). However, until issues are resolved regarding cell-culture method complexity, cost effectiveness, and validity for the detection of a broad spectrum of NoV genotypes, infectivity is not yet a useful method for detecting NoV in water and food samples. Thus, current methodologies for the detection of NoV naturally present in water and foods are based on molecular techniques (reviewed by Bosch et al., 2011).

Despite advances in the development of standardized molecular techniques, for example the technical specification norm for NoV and hepatitis A virus (HAV) detection in foodstuffs (ISO/TS 15216), the food and environmental virology field still presents many difficulties at the analytical level. For instance, molecular detection methods still require approaches to better assess the infectivity of the samples (reviewed by Knight et al., 2013). In this sense, the use of photoactivatable dyes has received special attention due to its compatibility with RT-qPCR assays, and the potential to be used in food and food processing facilities (reviewed by Elizaquível et al., 2014). The use of photoactivatable dyes on enteric viruses was first introduced by Parshionikar in 2010 by applying a propidium monoazide (PMA) pretreatment (Parshionikar et al., 2010). Theoretically, these photoactivatable dyes cannot enter intact capsids but are able to penetrate destroyed or damaged capsids. Once penetrated, the photoactivatable dye intercalates covalently into RNA/DNA after exposure to strong visible light, interfering with PCR and RT-PCR amplification.

Until now, photoactivatable dyes combined with qPCR (viability PCR) have successfully been applied to discriminate between infectious and thermally-inactivated poliovirus, coxsackievirus, echovirus, HAV and murine norovirus suspensions (Lee et al., 2015; Kim et al., 2011; Parshionikar et al., 2010; Sánchez et al., 2012a). However, reports on the application of this procedure in environmental and food samples are somewhat limited. To date, only Parshionikar

et al. (2010) and Moreno et al. (2015) have successfully applied PMA pretreatment in water and food samples for infectious poliovirus and HAV detection.

For NoV, the performance of viability PCR is still under discussion. Karim et al. (2015) reported that PMA-RT-PCR and PMA-RT-qPCR could not differentiate selectively between infectious and thermally (5 min at 72°C), chlorine (0.5 mg/l) or UV light (187 mJ/cm2) treated NoV suspensions while Parshionikar et al. (2010) reported that PMA-RT-PCR was able to discriminate between infectious and thermally-treated NoV (1 min at 72°C) suspensions. Additionally, Escudero et al. (2014) have recently reported that a SYBR Green PMA-RT-qPCR assay, but not a Taqman RT-qPCR, distinguished between infectious and thermally-treated NoV GI when applied to a monodispersed NoV suspension.

The purpose of this work was to further evaluate the potential of photoactivatables dyes to discern between infectious and thermally-inactivated NoV suspensions using two NoV genogroups and the RT-qPCR assays proposed in the framework of the ISO/TS 15216, and to assess its applicability in water and food samples.

Materials and methods

2.1. NoV samples

NoV genogroup I genotype 4 (GI.P4) and a genogroup II genotype 4 (GII.4 variant Den Haag 2006b) from stool specimens of patients with gastroenteritis (kindly provided by Dr. Javier Buesa, University of Valencia, Spain) were used as NoV reference material. NoV stool sample were suspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2M $NaNO_3$, 1% beef extract, and 0.1% Triton X-100 (Sigma-Aldrich) (pH 7.2) and pelleted at 1,000×g for 5 min. The supernatant was stored at -80 ºC in aliquots.

2.2. Optimization of photoactivatable dye treatments on NoV suspensions

PMA and ethidium monoazide reagents (EMA; GenIUL) were dissolved in 20% dimethylsulfoxide (DMSO) at 20 mM while PEMAX™ (a double dye technology developed by GenIUL) and PMAxx™ (a new and improved version of PMA developed by Biotinum) reagents were dissolved in water at 4 mM. All reagents were stored at −20°C protected from light. Photoactivatable dyes were added to 3-4 log PCRU of infectious and thermally-treated NoV suspensions (99 ºC for 5 min) diluted in PBS, PMA enhancer for gram-negative bacteria buffer 1X (buffer designed to improve PMA mediated discrimination developed by Biotium), standard buffer 1X or reaction buffer plus 1X (buffers for combining with PMA or EMA developed by GenIUL) to obtain a final concentration of 50 µM (PMA, PEMAX™ and PMAxx) or 20 µM (EMA). After the addition of the photoactivatable dye, incubation in the dark at room

temperature was performed for 10 min at 150 rpm to allow reagent penetration, unless otherwise indicated. Immediately, samples were exposed to light for 15 min using a photo-activation system (Led-Active Blue, GenIUL). After photoactivatable dye pretreatments, RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. Three types of controls were always included in the experiments; infectious viruses treated with photoactivatable dyes and infectious and thermally-inactivated viruses without photoactivatable dye treatment. Experiments were carried out in triplicate. In the present study, all experiments that include photoactivatable dyes were performed in DNA LoBind 1.5 ml tubes (Eppendorf) to avoid photoactivatable dye interaction with the plastic surface of the tubes.

2.3. Detection and quantification of viral RNA

The set of primers and probes used in this study are targeted to the junction of open reading frame 1 and 2 (ORF1/ORF2) of NoVs (ISO/TS 15216, 2013). RNA samples were analyzed in duplicate by RT-qPCR using the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen SA) and the LightCycler 480 instrument (Roche Diagnostics). For each RT-qPCR, serial dilutions of standard curve were run in quintuplicates and the numbers of PCRU were calculated.

2.4. Performance of photoactivatable dye treatments to discriminate infectious from thermally-inactivated NoV in vegetables samples

Initial experiments were performed with romaine lettuce (*Lactuca sativa*) obtained from a local supplier that was used to prepare lettuce concentrates as previously described (Sánchez et al., 2012b). Briefly, lettuce was washed with Buffered Peptone Water (BPW) using the Pulsifier equipment (Microgen Bioproducts) and concentrated by polyethylene glycol (PEG) precipitation. The pellet was immediately resuspended in 500 µl of PBS. Aliquots of 100 µl of lettuce concentrate were inoculated with 3-4 log PCRU of infectious or thermally-inactivated (99°C for 5 min) NoV GI or NoV GII suspensions. Thereafter, samples were added to 50 µM PMAxx (based on results from section 2.2) with or without 0.5% Triton and incubated in the dark at room temperature for 10 or 30 min at 150 rpm. Finally, samples were exposed to light for 15 min using a photoactivation system (Led-Active Blue). After photo-induced cross-linking, samples were pretreated with the Plant RNA Isolation Aid product (Ambion) to remove plant PCR inhibitors such as polyphenolics and polysaccharides (Sánchez et al., 2012b). For this purpose, 100 µl of the concentrated sample was mixed with 25 µl of the Plant RNA Isolation Aid and 600 µl of lysis buffer from the NucleoSpin® RNA virus kit and subjected to pulse-vortexing for 1 min. Afterwards, the homogenate was centrifuged for 5 min at 10,000×g to remove the debris. The supernatant was subsequently processed using the NucleoSpin® RNA

virus kit according to the manufacturer's instructions. All the experiments were performed in triplicate.

In the second part of the study, spinach (*Spinacia oleracea*) and romaine and lamb's lettuce (*Valerianella locusta*) concentrates were prepared as described above. One-hundred microliters aliquots of vegetable concentrates were inoculated with 10^2 or 10^3 PCRU of thermally-inactivated (99°C for 5 min) NoV GI or NoV GII suspensions and added to 50 µM PMAxx and 0.5 % Triton. Photoactivation, RNA extraction and RT-qPCR were performed as described above. All the experiments were performed in triplicate.

2.5. Naturally contaminated irrigation water samples

Three types of irrigation water obtained from an experimental growing field located in Murcia (Spain) were used: tertiary treatment effluent from the urban wastewater treatment plant of Roldán-Balsicas (tertiary), secondary treatment effluent from the same treatment plant (secondary), and surface water from an irrigation community (surface). Secondary treatment consisted in activated sludge systems followed by coagulation-flocculation. Tertiary treatment effluent was obtained after the secondary reclaimed water was sand-filtered followed by UV disinfection. Recovery of NoV from water was performed as described by Helmi et al. (2011). Briefly, $MgCl_2$ was added to 200 ml of water samples to a concentration of 0.05 M, adjusting the pH to 3.5. Then water samples were filtered through 0.45 µm cellulose nitrate filters (Sartorius). Filters were then transferred to sterile tubes and 5 ml of elution buffer (1% beef extract, 3% Tween-80 and 0.5 M NaCl) were added and pH adjusted to 9.5. Tubes were shaken for 1 min in a vortex, kept for 4 min in an ultrasonic bath and shaken again in a horizontal orbital shaker at 250 rpm for 10 min and then, pH was adjusted to 7. Samples were kept at -70 °C until analysis. Thereafter, 100 µl of concentrated samples were added with 50 µM PMAxx and 0.5 % Triton (PMAxx-Triton). Photoactivation, RNA extraction and RT-qPCR were performed as described in section 2.3 and 2.4. As a control, 100 µl of concentrated samples were processed without performing the PMAxx-Triton pretreatment. All the experiments were performed in duplicate.

2.6. Statistical analyses

Results were statistically analyzed and significance of differences was determined on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. In all cases, a value of $p<0.05$ was considered to be significant.

3. Results

3.1. Evaluation of photoactivatable dyes and buffers on NoV suspensions

As a first step in exploring the potential of photoactivatable dyes to distinguish between infectious and thermally-inactivated NoV suspensions using the Taqman RT-qPCR assay proposed in the framework of the ISO/TS 15216, NoV GI and GII suspensions were inactivated by incubating at 99 °C for 5 min and treated with four different photoactivatable dyes for 10 min. Results showed that all of them rendered statistically significant ($p<0.05$) signal reductions for NoV GI suspensions, while PMAxx reduced the signal of thermally-inactivated NoV GI by 1.5 logs as compared to the non-treated inactivated viruses (Table 1). For thermally-inactivated NoV GII suspensions, statistically significant values ($p<0.05$) were only achieved after PMAxx pretreatment with 1.1 log reduction.

Performance of PMA and PMAxx was further evaluated using different buffers recommended by the manufacturers. While enhancer buffer is recommended by Biotium to improve PMAxx performance, standard and reaction buffer plus are recommended by GenIUL for PMA. Results showed that performance of both photoactivatable dyes, PMA and PMAxx, was improved by the use of recommended buffers (Table 2). For example, PMA pretreatment in PBS reduced the signal of thermally-inactivated NoV GI by 0.88 logs whereas PMA pretreatment in standard buffer and reaction buffer plus reduced the signal by 1.16 and 1.80 logs, respectively. Similar results were obtained for PMAxx, while PMAxx pretreatment in PBS reduced the signal of thermally-inactivated NoV GI by 2.35 logs, in enhancer buffer the RT-qPCR signal was reduced by 3.73 logs (Table 2). Surprisingly, RT-qPCR signal of thermally-inactivated NoV GII diluted in enhancer buffer were completely eliminated without the addition of PMAxx. In order to rule out the presence of inhibitors in the enhancer buffer, infectious NoV GII suspension was diluted in enhancer buffer and RNA was further extracted. No differences were observed between RT-qPCR results from samples diluted with the enhancer buffer or the PBS (data not shown).

3.2. Application of PMAxx for the discrimination of infectious and thermally-inactivated NoV in leafy vegetables

In order to adapt the procedure to the routine analysis of NoV in the agri-food industry and particularly for leafy greens, the PMAxx was combined with Triton 0.5% as recommended by Moreno et al. (2015). Thermally-inactivated NoV GI and GII suspensions were inoculated in romaine lettuce concentrates at 10^3 PCRU and treated with 50 μM PMAxx for 10 min, with or without 0.5% Triton. Results showed that PMAxx–Triton pretreatment reduced the signal of thermally-inactivated NoV GI by 1.79 logs whereas PMAxx alone reduced the signal by 1.62 logs. For NoV GII, signal was completely eliminated in 2 out of 3 lettuce samples treated with PMAxx–Triton (Table 3). Pretreatment incubation time was extended to 30 min in order to optimize the viability PCR, however 30-min pretreatment rendered similar results ($p >0.05$) than 10-min pretreatment (Table 3).

The 10-min pretreatment with PMAxx- Triton was further evaluated in spinach and romaine and lamb's lettuces artificially inoculated with 5×10^3 and 5×10^2 PCRU of thermally-inactivated NoV GI suspensions. Results showed that the PMAxx-Triton pretreatment was partially efficient in reducing the RT-qPCR signal of thermally-inactivated NoV GI suspensions between 1.4 and 1.9 logs (Table 4). The quantification levels of thermally-inactivated NoV GII in vegetable samples with or without pretreatment with PMAxx–Triton are shown in Table 5. Results showed that the pretreatment was partially efficient in reducing the RT-qPCR signal between 1 and more than 3 logs when inoculated at 2×10^3 PCRU. The RT-qPCR signal was completely eliminated by the PMAxx-Triton pretreatment in vegetable concentrates inoculated at 2×10^2 PCRU of NoV GII.

3.3. Application of the PMAxx-Triton pretreatment for the NoV quantification in naturally contaminated surface and reclaimed irrigation water

Additionally, naturally contaminated water samples used as a source of irrigation water in the area of Murcia were comparatively quantified using the PMAxx-Triton pretreatment. For NoV GI, results showed that the PMAxx-Triton pretreatment removed completely the RT-qPCR signal only in one undiluted sample (Table 6). In order to assess the effect of the matrix, water concentrates were ten-fold diluted and further pretreated with PMAxx and Triton, results for NoV GI showed that diluted surface-1 water became negative after pretreatment with PMAxx–Triton. Only 4 positive undiluted samples for NoV GII were detected, but after PMAxx-Triton pretreatment no detection of NoV GII was observed in 2 out of them (Table 6).

Discussion

Although it has been technically challenging to detect NoV contamination in environmental samples, recent standardized RT-qPCR methods has allowed the monitoring of enteric viruses in food and water samples (e.g. ISO TS 15216-1 and ISO TS 15216-2). Despite these improvements, there is still a need to better understand the relationship between RT-qPCR signals and infectivity. To illustrate the importance of this, Baert et al. (2011) detected NoV by RT-qPCR in 28.2%, 33.3% and 50% of leafy greens tested in Canada, Belgium and France, respectively. However sequence confirmation was not successful for the majority of the samples tested, increasing concern about the interpretation of positive NoV results by RT-qPCR.

In this sense, the use of conventional photoactivatable dyes (i.e. PMA and EMA) has been shown as an innovative and promising technology to selectively detect infectious enteric viruses by RT-qPCR (Coudray-Meunier et al., 2013; Escudero et al., 2014; Kim et al., 2011; Lee et al., 2015; Moreno et al., 2015; Parshionikar et al., 2010; Sánchez et al., 2012a). In this study, we attempted to provide some insights to the use of viability PCR for discrimination of infectious

and thermally-inactivated NoV suspensions and evaluate its application in routine food and water analysis using the RT-qPCR assays proposed in the CEN/ISO TS 15216-1 and CEN/ISO TS 15216-2 standards. First, the pretreatment for the discrimination of infectious and thermally-inactivated NoV GI and GII was evaluated by using conventional photoactivatable dyes (i.e. PMA and EMA) and newly developed ones (i.e. PMAxx and PEMAX) designed to improve the detection of viable bacteria. When applied in virus suspensions, all photoactivatable dyes statistically ($p<0.05$) decreased the RT-qPCR signal of thermally-inactivated NoV GI suspensions, being PMAxx the most effective. The efficacy of PMA has also been assessed on NoV GI suspensions by other researchers. Parshionikar et al. (2010) reported that RT-qPCR using two different sets of primers and probes could not distinguish between infectious and thermally-treated (at 72°C) Norwalk virus (NoV GI) after PMA treatment. Deviations from the results reported here may be due to heat inactivation treatment, photoactivatable dye incubation conditions, light source, presence of virus aggregates and the length of PCR product. This latter point is supported by results from Escudero et al. (2014) who reported that only a SYBR Green PMA-RT-qPCR assay (213 bp), but not a Taqman assay (98 pb), was able to distinguish between infectious and thermally-inactivated Norwalk virus suspensions. In the present study, the use of PMA combined with a Taqman RT-qPCR assay slightly reduced detection (ca. 0.5 logs) of thermally-inactivated NoV GI. This maybe partially explained by the use of a standardized commercial LED-based instrument instead of high-power halogen lamps (Fittipaldi et al., 2012), the dye incubation conditions and the use DNA LoBind tubes (to avoid PMA interaction with the plastic surface of the tubes) (Moreno et al., 2015).

Interestingly, PMAxx was the only photoactivatable dye able of significantly reduced the RT-qPCR signal for NoV GII. Moreover, RT-qPCR signal was completely removed for thermally-inactivated NoV GII diluted in enhancer buffer and without PMAxx pretreatment. These results indicate, on the one hand, that photoactivatable dye pretreatments behave differently according to different NoV genotypes. On the other hand, disclosure of the composition of the commercially available enhancer buffer would be desirable in order to better understand the mechanism of eliminating the RT-qPCR signal of thermally-inactivated NoV GII suspensions.

An important improvement has been made by Coudray-Meunier and collaborators, by introducing the use of surfactants to improve the viability PCR for HAV and rotavirus detection (Coudray-Meunier et al., 2013). These compounds may disrupt viral capsids that have not accumulated sufficient damage. Moreover, Triton X-100 has recently been found to be effective for the selective detection of infectious HAV in lettuce wash water and food samples (Moreno et al., 2015). Following, procedure improvement was approached by testing the addition of Triton and extending the incubation time of the PMAxx. The combination of 50 µM PMAxx and 0.5% Triton for 10 min was highly useful to reduce the signal of thermally-inactivated NoV

in lettuce concentrates although no significantly differences were observed when extending the incubation time or the presence of Triton.

Since one of the main difficulties of viability PCR is the application in water and food samples (reviewed by Elizaquível et al., 2014), performance of this pretreatment was evaluated on different leafy vegetables. As already reported for HAV (Moreno et al. 2015), signal reductions of thermally-inactivated NoV were influenced by food matrix and virus concentration. Overall, the application of PMAxx–Triton pretreatment for the detection of NoV GI and GII in leafy vegetables greatly helps generating more meaningful data, since 1 to more than 3 logs of the RT-qPCR signal can be eliminated by a simple pretreatment and can be easily incorporated to the ISO standard for virus detection in food (CEN/ISO TS 15216-1 and CEN/ISO TS 15216-2). Additionally, this pretreatment can be applied straightforward for routine analyses, since it lasts 25 min only.

In line to the results obtained for HAV (Moreno et al., 2015), this study shows that viability RT-qPCR cannot completely prevent PCR amplification from thermally-inactivated NoV in food samples, leading to an overestimation of infectious NoV. Additional strategies to improve PMAxx-Triton treatment efficiency include repeated dye exposure, modification of the incubation temperature, incubation with other surfactants or extend the photoactivation step (Desneux et al., 2015; Nkuipou-Kenfack et al., 2013).

Due to the difficulties to get leafy vegetable samples positive for NoV, we further evaluated the PMAxx-Triton pretreatment in irrigation water samples previously tested as positive for NoV (López-Gálvez, under revision). Keeping in mind the unknown-status (infectious or inactivated) of the NoV present in water samples, this study shows that some of the water samples contained only inactivated viruses. In addition, RT-qPCR signal from NoV GII was easier to remove than signal of NoV GI by PMAxx-Triton pretreatment in water samples, indicating greater environmental persistence of NoV GI. This is in line with the greater resistance of NoV GI to some common food manufacturing processes (Butot et al., 2007; 2009; da Silva et al., 2007) and the unexpected high prevalence of NoV GI in the environment (reviewed by Le Guyader et al., 2012) considering that most of strains circulating in humans belong to GII.

Despite the fact that the PMAxx-Triton pretreatment is a step forward to better interpret quantitative data, still this pretreatment faces some challenges that need to be tackled in the future. One of the most evident challenges is the fact that this pretreatment has only been evaluated in thermally-inactivated NoV in food concentrates. As different inactivation processes (e.g. high pressure processing, UV treatment, electric pulsed field, chlorine, etc.) may have different mechanisms of inactivation, thus, this pretreatment must be evaluated for each treatment separately (Leifels et al., 2015) and applied in the food matrix instead of the vegetable concentrates.

Overall, adopting viability RT-qPCR in routine monitoring will allow for more accurate quantification of potentially infectious NoV, leading to improve our knowledge on the impact of these emerging pathogens on the public health.

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Table 1. Quantification by RT-qPCR of infectious and thermally-inactivated NoV suspensions after viability dye treatment[a]

	NoV[b,c]			
	GI (log PCRU)		GII (log PCRU)	
	Quantification	Reduction	Quantification	Reduction
Infectious	3.64 ± 0.35		2.95 ± 0.14	
Inactivated	3.90 ± 0.18A		3.46 ± 0.35A	
Inactivated + PMA (50 µM)	3.43 ± 0.06B	0.47	3.03 ± 0.18A	0.43
Inactivated + PMAxx (50 µM)	2.40 ± 0.60B	1.50	2.33 ± 0.07B	1.13
Inactivated + PEMAX (50 µM)	3.49 ± 0.03B	0.41	3.23 ± 0.06A	0.23
Inactivated + EMA (20 µM)	3.21 ± 0.04B	0.69	2.97 ± 0.14A	0.49

[a]Each condition was replicated three times, and NoV titers were obtained by RT-qPCR using a standard curve made by means of NoV PCRU

[b]Reduction in titers obtained between inactivated viruses before and after viability dye treatment

[c]Mean values with different letters in the same column denote significant differences between treatments ($P < 0.05$).

Table 2. Log scale reduction of RT-qPCR titers of thermally-inactivated NoV suspensions diluted in different buffers by photoactivatable dye treatments [a]

	Photoactivatable dyes (log PCRU reduction)			
	PMA (50 µM)		PMAxx (50 µM)	
	NoV GI	NoV GII	NoV GI	NoV GII
PBS	0.88 ± 0.04	0.63 ± 0.16	2.35 ± 0.02	1.09 ± 0.07
Enhancer buffer	NT	NT	3.73 ± 0.00	**
Standard buffer	1.16 ± 0.07	0.78 ± 0.50	NT	NT
Reaction buffer plus	1.80 ± 0.31	1.06 ± 0.08	NT	NT

[a]Reduction in titers obtained between thermally-inactivated NoV before and after viability dye treatment in different buffers

NT: non tested

**Thermally-inactivated NoV GII diluted in enhancer buffer was not detected by RT-qPCR

Table 3. Log scale reduction of RT-qPCR titers of thermally-inactivated NoV suspensions inoculated in romaine lettuce concentrates by PMAxx pretreatment [a]

Pretreatment			NoV[b,c]			
			GI (log PCRU)		GII (log PCRU)	
Pre-treatment time (min)	**PMAxx (50 µ M)**	**Triton (0.5%)**	Quantification	Reduction	Quantification	Reduction
10	-	-	3.89 ± 0.10A		3.09 ± 0.02A	
	+	-	2.27 ± 0.66B	1.62	0.72 ± 0.19B	2.37
	+	+	2.10 ± 0.10B	1.79	1.25[c]	>1.84
30	-	-	3.84 ± 0.03A		3.08 ± 0.01A	
	+	-	2.26 ± 0.07B	1.58	1.98[d]	>1.1
	+	+	1.84 ± 0.02B	2.00	1.27 ± 0.33B	1.81

[a]Each condition was replicated three times, and NoV titers were obtained by RT-qPCR using a standard curve made by means of NoV PCRU

[b]Reduction in titers between thermally-inactivated viruses before and after pretreatment

[c]Mean values with different letters in the same column and pretreatment time denote significant differences between treatments ($P < 0.05$).

[d]Two negative samples out of three

Table 4. Quantification of thermally-inactivated NoV GI suspensions inoculated in leafy vegetable concentrates by PMAxx-Triton pretreatment and RT-qPCR [a]

		Levels of NoV GI in vegetable concentrates (PCRU/100 µl)			
		5×10^3		5×10^2	
PMAxx-Triton pretreatment		Quantification[b] (log PCRU)	Reduction[c]	Quantification[b] (log PCRU)	Reduction[c]
Lettuce	-	3.82 ± 0.12A		2.59 ± 0.11	
	+	2.20 ± 0.17B	1.62	1.79[d]	0.8
Spinach	-	3.78 ± 0.08A		2.68 ± 0.12A	
	+	1.92 ± 0.06B	1.86	1.30 ± 0.20B	1.38
Lamb´s lettuce	-	3.74 ± 0.01A		2.87 ± 0.07A	
	+	1.85 ± 0.14B	1.89	1.00 ± 0.09B	1.87

[a]Each condition was replicated three times, and NoV titers were obtained by RT-qPCR using a standard curve made by means of NoV PCRU

[b]Mean values with different letters in the same column and same matrix denote significant differences between treatments ($P < 0.05$).

[c]Reduction in titers between thermally inactivated viruses before and after pretreatment

[d]Two out of three concentrated samples were negative

Table 5. Quantification of thermally inactivated NoV GII suspensions inoculated in leafy vegetable concentrates by PMAxx-Triton pretreatment and RT-qPCR[a]

		Levels of NoV GII in vegetable concentrates (PCRU/100 µl)			
		4×10^3		4×10^2	
	PMAxx-Triton pretreatment	Quantification[b] (log PCRU)	Reduction[c]	Quantification[b] (log PCRU)	Reduction[c]
Lettuce	-	**3.08 ± 0.01A**		**2.27 ± 0.04**	
	+	**1.01 ± 0.01B**	**2.07**	**ND**	**>2.27**
Spinach	-	**3.11 ± 0.31A**		**2.17 ± 0.11**	
	+	**2.09 ± 0.08B**	**1.02**	**ND**	**>2.17**
Lamb´s lettuce	-	**2.99 ± 0.03**		**2.41 ± 0.25**	
	+	**ND**	**>2.99**	**ND**	**>2.41**

ND: Non detected

[a]Each condition was replicated three times, and NoV titers were obtained by RT-qPCR using a standard curve made by means of NoV PCRU

[b]Mean values with different letters in the same column and same matrix denote significant differences between treatments ($P < 0.05$).

[c]Reduction in titers between thermally inactivated viruses before and after pretreatment

Table 6. RT-qPCR detection of NoV GI and NoV GII in naturally contaminated irrigation water samples (undiluted and tenfold diluted) compared with the values obtained using the PMAxx–Triton pretreatment[a]

Irrigation waters		PMAxx-Triton pretreatment	NoV GI Quantification[b] (log PCRU)	NoV GI Reduction[c]	NoV GII Quantification[b] (log PCRU)	NoV GII Reduction[c]
Surface-1	Undiluted	-	1.41 ± 0.10A		ND	
		+	1.06 ± 0.21B	0.35	ND	
	Diluted	-	0.90[d]		ND	
		+	ND	>0.90	ND	
Secondary-1	Undiluted	-	1.94 ± 0.16A		1.56 ± 0.32A	
		+	2.03 ± 0.03A	-	1.49 ± 0.33A	0.07
	Diluted	-	1.45 ± 0.00A		ND	
		+	1.25 ± 0.17A	0.20	ND	
Secondary-2	Undiluted	-	2.75 ± 0.07A		2.28 ± 0.30	
		+	1.92[d]	>0.83	ND	>2.28
	Diluted	-	1.87 ± 0.45A		1.50 ± 0.07	
		+	1.17[d]	>0.70	ND	>1.50
Secondary-3	Undiluted	-	2.08 ± 0.31A		1.87 ± 0.05	
		+	1.63 ± 0.19A	0.45	ND	>1.87
	Diluted	-	ND		ND	
		+	ND		ND	
Tertiary-1	Undiluted	-	1.90 ± 0.10A		1.70 ± 0.01	
		+	2.07± 0.27A	-	1.41[d]	>0.29
	Diluted	-	1.77 ± 0.40A		1.53 ± 0.36	
		+	1.66 ± 0.12A	0.11	ND	>1.53
Tertiary-2	Undiluted	-	1.87 ± 0.29		ND	
		+	ND	>1.87	ND	

Diluted	-	ND	ND
	+	ND	ND

[a]Each condition was replicated two times, and NoV titers were obtained by RT-qPCR using a standard curve made by means of NoV PCRU

[b]Mean values with different letters in the same column and same water denote significant differences between treatments ($P < 0.05$).

[c]Reduction in titers between samples with or without PMAxx-Triton pretreatment

[d]One out of two samples were negative

ND: Non detected