



HOKKAIDO UNIVERSITY

Title	Environmental Detection of Genogroup I, II, and IV Noroviruses by Using a Generic Real-Time Reverse Transcription-PCR Assay
Author(s)	Miura, Takayuki; Parnaudeau, Sylvain; Grodzki, Marco et al.
Citation	Applied and environmental microbiology, 79(21), 6585-6592 https://doi.org/10.1128/AEM.02112-13
Issue Date	2013-11
Doc URL	https://hdl.handle.net/2115/56454
Type	journal article
File Information	52835_2_merged_1376288771.pdf



Environmental detection of GI, GII and GIV norovirus using a generic real-time RT-PCR assay.

Short title: Norovirus generic real-time RT-PCR

Takayuki Miura^{1,2}, Sylvain Parnaudeau¹, Marco Grodzki¹, Satoshi Okabe², Robert L. Atmar³ and
Françoise S. Le Guyader^{1*}

1: Laboratoire de Microbiologie, IFREMER, Nantes, France

2: Division of Environmental Engineering, Faculty of Engineering, Hokkaido University,
Sapporo, Japan

3: Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston,
Texas, USA.

*Corresponding author

Françoise S. Le Guyader,

Laboratoire de Microbiologie, LSEM-SG2M, IFREMER, BP 21105, 44311 Nantes Cedex 03,
France

Phone: (33) 2 40 37 40 52, fax: (33) 2 40 37 40 73, email: sleguyad@ifremer.fr

24

25 ABSTRACT

26 Norovirus is the most common agent implicated in foodborne outbreaks and is frequently
27 detected in environmental samples. These viruses are highly diverse and three genogroups infect
28 humans (GI, GII, and GIV). Being non-cultivable viruses, real-time RT-PCR is the only sensitive
29 method available for their detection in food or environmental samples. Selection of consensus
30 sequences for the design of sensitive assay has been challenging due to sequence diversity and
31 led to the development of specific real-time RT-PCR assays for each genogroup. Thus, sample
32 screening can require several replicates for amplification of each genogroup (without considering
33 positive and negative controls or standard curves). This study reports the development of a
34 generic assay that detects all three human norovirus genogroups using a one-step real-time RT-
35 PCR assay on a qualitative basis. The generic assay achieved good specificity and sensitivity for
36 all three genogroups, detected separately or in combination. At variance with multiplex assays,
37 the choice of the same fluorescent dye for all three probes specific to each genogroup allows the
38 fluorescence to be added and may increase assay sensitivity when multiple strains from different
39 genogroups are present. Applied to sewage sample extracts, this generic assay successfully
40 detects all positive samples compared to genogroup-specific RT-PCR. The generic assay also
41 identified all norovirus-positive samples among 157 archived nucleic acid shellfish extracts,
42 including samples contaminated by all three genogroups.

43

44 **Keywords:** human norovirus, genogroups I, II and IV, real-time RT-PCR, generic RT-PCR,
45 shellfish, sewage analysis.

Viral contamination of water samples and foodstuffs is increasingly recognized through outbreak investigations, epidemiological surveys, and sample analysis. Among the large diversity of human enteric viruses discharged into the environment, norovirus (NoV) is the most common pathogen. Belonging to the *Caliciviridae* family, the *Norovirus* genus is divided into six genogroups, and three of these (GI, GII, and GIV) infect humans (1, 2). NoVs cause gastroenteritis characterized by vomiting and diarrhea in persons of all ages, and a predominance of GII strains are reported in clinical cases. Infection with many strains is dependent on histo-blood group antigen (HBGA) expression, as HBGAs serve as an attachment factor necessary to initiate virus infection (3). NoVs are the major cause of non-bacterial gastroenteritis worldwide and have been identified as the predominant cause of foodborne outbreaks (4). The large amount of virus shed by infected persons and the high level of resistance to inactivation in the environment are likely factors associated with virus prevalence in environmental waters (5-7). Although food handlers have been implicated as the source of food contamination in some outbreaks, it is clear that foods such as berries, green vegetables and shellfish can be contaminated during production (8-10).

Screening of food or environmental waters such as raw or treated sewage is one approach that can be considered as a strategy to prevent virus-associated outbreaks. The achievement of sensitive methods and in real-time reverse transcription PCR (*r*RT-PCR) allows controls on food or environmental samples. The aim of this study was to develop a generic assay that can detect all three human NoV genogroups (GI, GII, and GIV) using a one-step *r*RT-PCR assay on a qualitative basis. The generic assay, developed and optimized based on previously reported primers and probes, showed a sensitivity comparable to genogroup-specific assays. The newly developed assay was used to analyze naturally contaminated samples such as raw and treated sewage and shellfish samples, and compared to genogroup specific-real-time RT-PCR.

MATERIALS AND METHODS

73 Virus strains, stool samples and reference materials.

74 To validate the NoV assays in this study, we used human fecal samples containing GI.1 and GII.3,
 75 as well as a reference NoV RNA panel containing *in vitro* transcribed RNA from nine GI, nine
 76 GII, and one GIV genotypes (provided by H. Vennema and M. Koopmans, RIVM, Bilthoven, the
 77 Netherlands). Other human enteric viruses were obtained from cell cultures: Aichi virus (AiV)
 78 genotype A (P. Pothier, CHU Dijon), astrovirus (AstV) type 4, poliovirus (PV) type 3 (both from
 79 B-M. Marcillé, CHU Nantes), hepatitis A virus (HAV) HM175, mengovirus (MgV) strain vMC₀
 80 (both from A. Bosch, University of Barcelona, Spain), and rotavirus (RV) RF (, D. Poncet,
 81 INRA-CNRS, Gif sur Yvette). For hepatitis E virus (HEV), a porcine stool characterized as a
 82 genotype 3 was included (N. Pavio, ANSES, Paris, France).

83 Nucleic acids (NAs) were extracted from 10% stool suspension using NucliSens kit
 84 (bioMerieux, Lyon, France) following the manufacturer's instructions (11) or by 5-min boiling
 85 for cell culture strains.

87 Archived nucleic acid samples.

88 Archived NAs from 157 naturally contaminated shellfish samples (oyster, mussel and clam) (12-
 89 14) (and unpublished data), eight bioaccumulated oyster samples (11), and 16 untreated and 12
 90 treated wastewater samples (7) were included in the study.

92 Primers and probes.

93 For the genogroup-specific real-time RT-PCR (spe-*r*RT-PCR) detecting NoV GI, GII and GIV
 94 separately, previously described primers and probes were used (*i.e.* QNIF4, NV1LCR, and
 95 NV1LCpr for GI, QNIF2d, COG2R, and QNIFS for GII, and Mon4F, COG2R, and Ring4 for
 96 GIV) (15-18). These primers and probes were modified for the generic *r*RT-PCR (gen-*r*RT-PCR)
 97 detecting the three genogroups on a qualitative basis (NV1LCR, COG2R, NIFG1F, NIFG2F,
 98 NIFG4F, NIFG1P, QNIFS, NIFG4P) (Figure 1). For AiV, AstV, HAV, HEV, MgV, PV, RV, and
 99 SaV, primers and probes described previously were used (19-27).

Real-time RT-PCR.

All amplifications were performed with an Mx3000P QPCR System (Agilent Technologies, France) using RNA UltraSense One-Step Quantitative RT-PCR System (Life technologies, France), and 5- μ L of extracted sample per well (final volume of 25 μ L). The thermal conditions consisted of RT reaction for 15 min at 55 °C, inactivation of RT and activation of *Taq* polymerase for 5 min at 95 °C, followed by 45 cycles of denaturing for 15 sec at 95 °C, annealing for 1 min at 55 °C, and extension for 1 min at 65 °C. For the *spe-rRT-PCR*, conditions were as previously described, final concentration of 900 nM for the reverse primer (GI: NV1LCR or GII/GIV: COG2R), 500 nM for the forward primer (GI: QNIF4 or GII: QNIF2d or GIV: Mon4F), and 250 nM for the probe (GI: NV1LCpr or GII: QNIFS or GIV: Ring4) (22). For the *gen-rRT-PCR*, the same conditions were used except that each well contained a mixture of two reverse primers (NV1LCR and COG2R), three forward primers (NIFG1F, NIFG2F, and NIFG4F), and three probes (NIFG1P, QNIFS, and NIFG4P) (Figure 1) at a final concentration of 400 nM for each primer and 200 nM for each probe. All probes were labeled using the same fluorescent dye and quencher (FAM, BHQ). These conditions were applied in preliminary studies (Table1) with the primers and probes currently used in our laboratory (*i.e.* QNIF4, NV1LCR, NV1LCpr, QNIF2d, COG2R, QNIFS, Mon4F, COG2R, Ring4 mixed together). For later experiments (Table 2), newly designed primer and probe sets for GI (NIFG1F, NV1LCR, and NIFG1P), GII (NIFG2F, COG2R, and QNIFS), and GIV (NIFG4F, COG2R, and NIFG4P) were used separately in conditions used for the *spe-rRT-PCR* (see above).

The cycle threshold (C_T) was defined as the cycle at which a significant increase in fluorescence occurred. For assays of reference strains and bioaccumulated samples, results are expressed as the mean $C_T \pm$ standard deviation calculated using triplicate wells. For naturally contaminated samples, screening was first performed using a single well to determine C_T for all 3 *spe-rRT-PCR* and *gen-rRT-PCR*, as limited amount of extracts was available. When a discordant result was obtained for one genogroup, triplicate wells were used to determine whether the

sample is positive in both assays for this specific genogroup. The *r*RT-PCR assays for AiV, AstV, HAV, HEV, MgV, PV, RV, and SaV were performed as described previously (19-27).

Specificity and reactivity of the gen-*r*RT-PCR.

NA extracts from AiV, AstV, HAV, HEV, MgV, PV, RV, and SaV, and the reference NoV RNA panel were diluted and adjusted to get C_T values of ~30 by *r*RT-PCR using the respective pathogen-specific primers and probe, and amplified by the gen-*r*RT-PCR.

Sensitivity of the gen-*r*RT-PCR.

Reference GI.1, GII.3, and GIV.1 NA extracts were 10-fold serially diluted (upon a range of C_T values from 24 to 38 as determined by spe-*r*RT-PCR), and amplified by gen-*r*RT-PCR. To mimic multiple contaminations, these NA extracts adjusted to C_T values of ~36 were mixed (2 μ L each) in different combinations (2 or 3 genogroups) and amplified.

RESULTS

Primer and probe design.

In preliminary studies, the previously developed GI-, GII-, and GIV-specific primers and probes were mixed to amplify high, medium, and low concentrations of three NoV strains (GI.1, GII.3, or GIV.1). An increase of at least 4 C_T units was observed compared to the spe-*r*RT-PCR (*i.e.* using primers and probes separately), with low concentrations not being detected (Table 1). The spe-*r*RT-PCR assays target a short conserved region from the junction between open reading frames 1 (ORF1) and 2 (ORF2) that allows efficient detection of strains from the different genogroups. This area was also selected for development of the gen-*r*RT-PCR assay. Based on reference strain sequence analysis, a number of different primers and probes were evaluated (data not shown), and the best combination is described in Figure 1. For GI NoV, the forward primer (NIFG1F: atgttcgctggatgcg) was moved 6 bases upstream from QNIF4, the probe (NIFGIP: tgtggacaggagaycgcratct with y: c or t and r: a or g) was made longer than NV1LCpr by adding 2

bases to the 5' end, and the reverse primer (NV1LCR: ccttagccatcatcatttac) was not modified. The reverse primer (COG2R: tcgacgccatcttcattcaca) and probe (QNIFS: agcacgtgggagggcgatcg) for GII were not changed but the forward primer QNIF2d was shortened 3 bases at the 3' end (NIFG2F: atgttcagrtggatgagrttctc with r: a or g). For GIV NoV, the GII reverse primer COG2R, which matches perfectly with available GIV NoV sequences, was selected, the forward primer (NIFG4F: atgtacaagtggatgcgrttc with r: a or g) was moved 9 bases downstream, and the probe (NIFG4P: agcacttgggaggggggatcg) 6 bases upstream from Mon4F and Ring4, respectively. The amplified fragments for each of the three genogroups are approximately 90 nucleotides in length (92 for GI, 89 for GII, and 89 for GIV). The absence of hairpin-dimer formation and probe-probe, primer-primer and probe-primer interactions were verified (data not shown). The T_m for all reverse primers is 56°C while for the forward primers T_m are ~55°C (with a maximum of 58°C for NIFG2F). All three probes are positive sense and with T_m ~70 °C. As a consequence, the extracts amplified with the new primers and probes combinations were detected at comparable C_T values compared to the spe-*r*RT-PCR, with a better sensitivity for the GIV strain (Table 1).

Gen-*r*RT-PCR specificity.

The modified sequences were checked for non-specific annealing by Blast, and none was found. Next, NA extracts of other human enteric viruses that can be detected in food or environmental samples were adjusted to a comparable level as estimated by pathogen-specific *r*RT-PCR assays, and these samples were amplified by the gen-*r*RT-PCR assay in triplicate. None of them [AiV (C_T 30.6 ± 0.2), AsV (C_T 31.1 ± 0.6), HAV (C_T 30.3 ± 0.7), HEV (C_T 29.4 ± 0.1), PV (C_T 31.7 ± 0.9), RV (C_T 29.0 ± 0.2), SaV (C_T 30.1 ± 0.1)] was detected by the gen-*r*RT-PCR. The gen-*r*RT-PCR did not amplify MgV (C_T 31.8 ± 0.2) used in our laboratory as an extraction control.

Gen-*r*RT-PCR reactivity.

The reactivity of the gen-*r*RT-PCR was evaluated using the reference NoV RNA from nine GI, nine GII, and one GIV genotypes, all NAs being adjusted to the same C_T values (Table 2) (the C_T

value for GI.5 was adjusted using NIFG1F-NVILCR and NIFG1P since it was not detected by the *spe-rRT-PCR* assay). Using primers and probes for *gen-rRT-PCR* separately, no differences in C_T values were observed except for two strains (GI.5 and GIV.1), for which detection sensitivity was improved compared to *spe-rRT-PCR*. Using the *gen-rRT-PCR* among the nine GI strains analyzed, the GI.7 and GI.8 were less efficiently amplified (loss of 2.8 and 1.8 C_T units, respectively). C_T values obtained with the NIFG1F-NV1LCR and NIFG1P alone were identical to the *spe-rRT-PCR*, showing that the loss of sensitivity was due to the presence of the other primers and probes rather than sequence mismatches. The GI.5 strain that was not detected by the *spe-rRT-PCR* assay (three mismatches with the forward primer QNIF4) was successfully detected by the *gen-rRT-PCR*. The C_T values for the GII strains were very similar for the two assays (less than 1 C_T unit difference). Another major improvement of this assay was for the detection of GIV.1, the new primers and probe increasing the sensitivity by more than 3 C_T units. Based on GIV.1 sequences available in GenBank and the better fit of primers and probe, similar findings would be anticipated for other GIV.1 strains.

Gen-*rRT-PCR* sensitivity.

The GI.1 and GII.3 NoV NA extracts, and GIV.1 RNA fragment were 10-fold serially diluted and amplified by the *gen-rRT-PCR*. The sensitivity limits achieved were quite similar to those obtained with the *spe-rRT-PCR* assays (Figure 2). The variability among replicates was less than 1 C_T unit for high C_T values (near the limit of detection). For the GIV detection, the new set of primers and probe lowered the C_T values (Figure 2).

As contamination with multiple NoV strains can occur in food or environmental samples, NoVs from different genogroups were mixed in different combinations and amplified. When two virus strains were mixed together, the mean C_T values were lower than when a comparable amount of the single strain was assayed. When GI.1, GII.3 and GIV.1 were amplified separately, C_T values were 37.1 ± 1.1 , 36.7 ± 0.4 and 35.7 ± 0.4 , respectively. When two genogroups were mixed, the resulting C_T values were 35.1 ± 0.2 for GI.1 and GII.3, 34.9 ± 0.3 for GII.3 and GIV.1,

and 34.7 ± 0.2 for GI.I and GIV.1. The decrease observed C_T units demonstrates that fluorescence increased following the mixture of two strains. Similarly, when all three genogroups were mixed, the C_T value decreased further (34.1 ± 0.5). The shift of magnitude for this assay was almost 2 C_T units, confirming that all strains were amplified, considering *r*RT-PCR variability. If strains were mixed at different ratios, the C_T variations were less demonstrative as C_T values follow the most concentrated strain (data not shown).

Gen-*r*RT-PCR applied to sewage sample extracts.

NA extracts of 16 untreated and 12 treated wastewater samples known to be positive for at least one NoV genogroup were selected (Figure 3). The C_T values obtained with the gen-*r*RT-PCR were lower than or similar to those obtained with the spe-*r*RT-PCR, with one exception (arrow 1 Figure 3B), with the GI NoV spe-*r*RT-PCR giving a lower C_T value. On the other hand, arrow 2 showed a sample containing only a GI NoV for which the gen *r*RT-PCR was more sensitive than the spe-*r*RT-PCR.

Gen-*r*RT-PCR applied to shellfish sample extracts.

Initial studies on shellfish samples were performed using oyster extracts contaminated in bioaccumulation experiments. NA extracts obtained from different experiments were amplified by the spe-*r*RT-PCR and gen-*r*RT-PCR in triplicate (Table 3). C_T values were similar but consistently higher in the gen-*r*RT-PCR assay than the spe-*r*RT-PCR, with a difference of 0.4 to 1.7 C_T units between the two assays. When the new primers and probes for the gen-*r*RT-PCR were tested separately and compared to the primers and probes used for spe-*r*RT-PCR, the maximum difference in C_T values was 0.5 C_T units (observed for a GII sample). For the GI samples the differences observed ranged from 0.1 to 0.3 C_T units. These results confirmed the earlier observation that mixing primers and probes can lead to a decrease in sensitivity as measured by C_T detection level.

To further evaluate the applicability of the gen-*r*RT-PCR assay, 157 archived oyster,

mussel and clam NA extracts were analyzed using the *spe-rRT-PCR* and the *gen-rRT-PCR*. Sixty samples were negative and 97 samples were positive by both the *spe-rRT-PCR* and *gen-rRT-PCR* (Figure 4). The greatest variability was observed among the 15 GI positive samples with one sample showing a difference of more than 3 C_T units in favor of the *gen-rRT-PCR* (arrow 1 Figure 4 A). Surprisingly the *gen-rRT-PCR* assay improved the C_T values compared to the *spe-rRT-PCR*, at odds with the results obtained in the strain validation studies (Table 2). Two samples considered as negative by the *spe-rRT-PCR* assay (C_T values of 41) gave C_T values under 40 in the *gen-rRT-PCR*, and thus were interpreted as positive, these samples being negative for the other NoV genogroups. Among the 38 GII NoV positive samples similar C_T values were obtained with both assays (Figure 4 A). Additionally, 39 samples positive both for GI and GII NoV were also detected by the *gen-rRT-PCR*. Measured C_T values resemble the C_T obtained for NoV GII, which was usually the lowest C_T value. In only two samples (arrows 2 and 3 Figure 4 B), NoVs were detected more efficiently by the *spe-rRT-PCR*, but these samples were still positive by the generic assay. Five samples contaminated by all three NoV genogroups were successfully detected by the *gen-rRT-PCR*.

DISCUSSION

Development of *rRT-PCR* allows sensitive and specific detection of NoV for environmental monitoring, food analysis or clinical diagnosis. As there is no cell line or animal model to detect human NoVs (28), *rRT-PCR* is the standard detection method and has been recognized as an ISO technical specification for NoV detection in food samples (ISO/TS 15216 -1 and 2). The work described here is the development of a *gen-rRT-PCR* assay able to detect a wide diversity of NoV strains belonging to the three human genogroups over a large concentration range, compatible with environmental analysis including shellfish. By combining detection of all three genogroups in one assay, the cost per reaction is lowered to a third of the original cost and allows the sensitive screening between positive or negative samples.

Since the first demonstration of a conserved area among genetic diversity of these viruses

(18), advantages of probe-based *r*RT-PCR compared to conventional RT-PCR have been demonstrated in many occasions both in terms of sensitivity, specificity, and simplicity of application. This method is now the most widely used in environmental studies such as sewage or water sample analysis (5-7), food (29-31) or shellfish samples (22, 32-35). Almost all *r*RT-PCR assays target the ORF1-ORF2 junction region, originally targeted 10 years ago (18). However some primer or probe modifications have been made to increase sensitivity or to adapt to newly described NoV strains (15, 17, 36-43). It is important to adapt the assay to strain evolution and the assay described here was checked against the sequence of the newly described strains such as the GII.4 2012 variant, (Hu/GII.4/Sydney/NSW0514/2012/AU, JX459908). However, although the developed assay was able to detect all tested genotypes, the ongoing emergence of new variants and the high genetic diversity of NoV may make it necessary to continue to evaluate the performance of these primers or probes in the future.

The recognition that NoVs are highly prevalent and that food plays an important role in their transmission is now clear (9, 44). NoVs have been detected on berries, tomatoes, and shellfish from the European, Canadian, American and Japanese markets with prevalence frequencies ranging from less than 4% to more than 76% (31, 35, 45, 46, 47). A review performed by an expert panel on the biology, epidemiology, diagnosis and public health importance of foodborne viruses identified NoVs (as well as HAV) as the most frequent causes of foodborne illness among all virus/food commodity combinations (8). One recommendation of this expert panel was to obtain more data on NoV food contamination to develop a risk assessment and to evaluate the impact of food on NoV epidemiology. For oysters, for which more data are available, one recommendation is that NoV testing should be considered for food business operators (48).

Screening environmental and food samples requires attention be given to the presence of potential inhibitors of the RT or PCR enzymes. Strategies to assess the presence of inhibitors and to evaluate extraction efficiency include testing undiluted and diluted NA extracts with and without internal controls (20, 22, 45, 46). This can lead to the need for at least two wells per genogroup and eventually one well per genogroup with internal control so at least 6 to 9 separate

amplifications, without considering positive and negative controls or standard curves. The time spent for analysis and the costs are an issue, especially if other viral or bacterial pathogens need to be screened and the frequency of positive results is <10% (45). The development of generic assays for virus identification in clinical samples is one strategy to address this problem (49, 50). To date, such an approach for NoVs has not been feasible due to their genetic diversity. This study describes the successful development of a generic RT-PCR assay allowing a sensitive detection of GI, GII, and GIV NoVs, based on a model previously developed for sapovirus (27). One characteristic of NoV epidemiology, besides high genetic diversity, is the co-circulation of strains in the human population (1). NoV GII strains are more frequently detected in clinical cases, but GI strains are relatively more frequently found associated with foodborne outbreaks (10, 51), making their detection important for environmental or food samples screening. It may also be important to look for GIV NoVs as they have been detected in sewage (7, 52, 53) and shellfish samples (unpublished data)(14, 54). Improving primer and probe design for GIV strains may help to determine their circulation in the environment and to prevent further distribution among human population.

Multiplex *r*RT-PCR assays for GI and GII NoVs, based upon mixtures of previously published primers and probes, have been successfully applied in the analysis of clinical samples (55-57). However, the ratio of concentrations of the 2 genogroups can have an impact on assay sensitivity, as noted in the current studies. When different concentrations were mixed together, a mutually competitive effect was observed compared to individual GI and GII reactions (58). The loss in sensitivity was not considered to be a major disadvantage in clinical diagnosis using fecal samples, as viral loads in feces are high (59). A concern is that this may not be the case when analyzing environmental samples that contain lower virus concentrations. A recently described duplex assay had no loss of sensitivity compared to GI and GII monoplex assays, and when applied to surface and ground water samples this assay was more efficient than conventional RT-PCR (60). Only one other study targeting all three human NoV genogroups in a multiplex assay based on GI and GII primers/probes described previously and newly designed primers and probe

for GIV has been described (18, 61). As noted by these authors, the sensitivity of the developed multiplex real-time assay was lower than those of its corresponding monoplex assays due to interactions of primers and probes, confirmed by the failure of NoV detection in three of seven food or environmental samples. In contrast, although our generic assay amplified two GI genotypes less efficiently than monoplex assays, all water and shellfish samples were still positive when evaluated by the new developed assay. Use of the same fluorescent dye for all three TaqMan probes allows the fluorescence to be added. This may increase assay sensitivity when several genogroups are mixed, but presents the disadvantage of no discrimination between genogroups. However, the sensitivity achieved is quite similar to the *spe-rRT-PCR* on NA extracts from stool or bioaccumulated shellfish samples, when only qualitative determination are made. This study also demonstrates the importance of validation using naturally-contaminated samples as the GI detection is efficient using the *gen-rRT-PCR*, despite the lost of sensitivity observed on the reference NoV RNA panel. Even if precautions are taken to purify NA from environmental samples, various inhibitors may be present depending on the sample matrix. Additionally, other NAs may be present in extracts, possibly interfering with the amplification. The other challenge for environmental samples is to achieve adequate sensitivity (62). This is critical as low concentrations of NoV may constitute a health risk (63, 64). The validation of this assay on sewage or shellfish extracts, two types of challenging samples based on inhibitors or low contamination, demonstrates that it is efficient. Two shellfish samples negative in the *spe-rRT-PCR* ($C_T \sim 41$) detected positive with the *gen-rRT-PCR* could raise a specificity question although it is plausible that these detections were due to improved assay sensitivity. No false negative or positive results were observed and differences in C_T values were always minimal, making us confident in its application for shellfish sample analysis based on our experience.

Food screening for NoV is likely to become more frequent in coming years or even mandatory. Depending on samples analyzed, season (summer compared to winter), or climatic event, a large number of samples may be negative. Additionally, in some situations, the qualitative information, as provided by the assay developed in this work, may be sufficient to

take sanitary decision like recall from the market or prevent trading of contaminated foods, at least until molecular assays are developed that allow an assessment of virus infectivity (65,66). For samples needing more precise data such as genogroup identification and virus quantification, genogroup specific RT-qPCR then can be performed, including controls to allow virus quantification.

Acknowledgments:

This work was supported by the French government (DGAI), 2012-2100762726, programme 206-sous action 37, by grant 2010 CESA 010 ‘ HEVECODYN’ from the Agence Nationale pour la Recherche (ANR), by the Japan Society for the Promotion of Science (JSPS) through JSPS Research Fellowships for Young Scientists (23-4434), and by Public Health Service grant NIH P01 AI057788, Agriculture and Food Research Initiative Competitive grant 2011-68003-30395 from the USDA National Institute of Food and Agriculture, and the John S. Dunn Research Foundation.

REFERENCES

1. **Atmar RL**. 2010. Noroviruses: state of the art. *Food Environ. Virol.* **2**:117-126.
2. **Mesquita JR, Barclay L, Nascimento MSJ, Vinje J**. 2010. Novel norovirus in dogs with diarrhea. *Emerg. Infect. Dis.* **16**:980-982.
3. **Tan M, Jiang X**. 2011. Norovirus–host interaction: multi-selections by human histo-blood group antigens. *Trends Microbiol.* **19**:382-388.
4. **Scallan E, Chockstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones J L, Griffin PM**. 2011. Foodborne illness acquired in the United States-major pathogens. *Emerg. Infect. Dis.* **17**:7-12.
5. **Yang N, Qi H, Wong MML, Wu RSS, Kong RYC**. 2012. Prevalence and diversity of norovirus genogroups I and II in Hong Kong marine waters and detection by real-time PCR. *Mar. Poll. Bull.* **64**:164-168.
6. **Perez-Sautu U, Sano D, Guix S, Kasimir G, Pinto RM, Bosch A**. 2012. Human norovirus occurrence and diversity in the Llobregat river catchment, Spain. *Environ. Microbiol.* **14**:494-502.
7. **Sima LC, Schaeffer J, Le Saux J-C, Parnaudeau S, Elimelech M, Le Guyader FS**. 2011. Calicivirus removal in a membrane bioreactor wastewater treatment plant. *Appl. Environ. Microbiol.* **77**:5170-5177.
8. **EFSA panel of Biological Hazards (BIOHAZ)**. 2011. Scientific opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. *EFSA J.* **9**:2190 (doi:10.2903/j.efsa.2011.2190).
9. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM**. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerg. Infect. Dis.* **19**:407-415.
10. **Bitler EJ, Matthews JE, Dickey BW, Eisenberg JNS, Leon JS**. 2013. Norovirus outbreaks: a systematic review of commonly implicated transmission routes and vehicles. *Epidemiol. Infect.* ahead of print
11. **Maalouf H, Schaeffer J, Parnaudeau S, Le Pendu J, Atmar RL, Crawford SE, Le Guyader FS**. 2011. Strain-dependent norovirus bioaccumulation in oysters. *Appl. Environ. Microbiol.* **77**:3189-3196.
12. **Le Guyader FS, Kroll J, Ambert-Balay K, Ruvoen-Clouet N, Desaubliaux B, Parnaudeau S, Le Saux J-C, Ponge A, Pothier P, Atmar RL, Le Pendu J**. 2010. Comprehensive analysis of a norovirus-associated gastroenteritis outbreak, from the environment to the consumer. *J. Clin. Microbiol.* **48**:915-920.
13. **Grodzki M, Ollivier J, Le Saux J-C, Piquet J-C, Noyer M, Le Guyader FS**. 2012. Impact

- of *Xynthia tempest* on viral contamination of shellfish. *Appl. Environ. Microbiol.* **78**:3508-3511.
14. **Benabbes L, Ollivier J, Schaeffer J, Parnaudeau S, Rhaissi H, Nourlil J, Le Guyader FS.** 2013. Norovirus and other human enteric viruses in Moroccan shellfish. *Food Environ. Virol.* **5**:35-40.
15. **Trujillo AA, McCaustland KA, Zheng D-P, Hadley LA, Vaughn G, Adams SM, Ando T, Glass RI, Monroe SS.** 2006. Use of Taq-Man real-time reverse transcription PCR for rapid detection quantification and typing of norovirus. *J. Clin. Microbiol.* **44**:1405-1412.
16. **Svraka S, Duizer E, Vennema H, de Bruin E, van der Veer B, Dorredteijn B, Koopmans M.** 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in the Netherlands from 1994 through 2005. *J. Clin. Microbiol.* **45**:1389-1394.
17. **Loisy F, Atmar RL, Guillon P, Le Cann P, Pommepuy M, Le Guyader SF.** 2005. Real-time RT-PCR for norovirus screening in shellfish. *J. Virol. Meth.* **123**:1-7.
18. **Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K.** 2003. Broadly ractive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J. Clin. Microbiol.* **41**:154-157.
19. **Monpocho S, Deh  e A, Mignotte B, Schwartzbrod L, Marechal V, Nicolas J-C, Billaudel S, Ferr   V.** 2000. Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *BioTechniques* **29**:88-92.
20. **Costafreda MI, Bosch A, Pinto RM.** 2006. Development, evaluation, and standardization of a real-time taqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Appl. Environ. Microbiol.* **72**:3846- 3855.
21. **Jothikumar N, Cromeans TL, Roberstib BH, Meng XJ, Hill VR.** 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J. Virol. Meth.* **131**:65-71.
22. **Le Guyader FS, Parnaudeau S, Schaeffer J, Bosch A, Loisy F, Pommepuy M, Atmar RL.** 2009. Detection and quantification of noroviruses in shellfish. *Appl. Environ. Microbiol.* **74**:618-624.
23. **Le Guyader FS, Le Saux J-C, Ambert-Balay K, Krol J, Serais O, Parnaudeau S, Giraudon H, Delmas G, Pommepuy M, Pothier P, Atmar RL.** 2008. Aichi virus, norovirus, astrovirus, enterovirus and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *J. Clin. Microbiol.* **46**:4011-4017.
24. **Le Cann P, Ranarijaona S, Monpocho S, Le Guyader FS, Ferr   V.** 2004. Quantification of human astroviruses in sewage using real-time RT-PCR. *Res. Microbiol.* **155**:11-15.
25. **Pinto RM, Costafreda MI, Bosch A.** 2009. Risk assessment in shellfish-borne outbreaks of

- hepatitis A. *Appl. Environ. Microbiol.* **75**:7350-7355.
26. **Pang X, Cao M, Zhang M, Lee B.** 2011. Increased sensitivity for various rotavirus genotypes in stool specimens by amending three mismatched nucleotides in the forward primer of a real-time RT-PCR assay. *J. Virol. Meth.* **172**:85-87.
27. **Oka T, Katayama K, Hansman GS, Kageyama T, Ogawa S, Wu F-T, Takeda N.** 2006. Detection of human sapovirus by real-time reverse transcription polymerase chain reaction. *J. Med. Virol.* **78**:1347-1353.
28. **Herbst-Kralovetz M, Radtke AE, Lay MK, Hjelm BE, Bolick AN, Sarker SS, Atmar RL, Kingsley DH, Arntzen CJ, Estes MK, Nickerson CA.** 2013. Lack of norovirus replication and histo-blood group antigen expression in 3-dimensional intestinal epithelial cells. *Emerg. Infect. Dis.* **19**:431-438.
29. **Brassard J, Gagne M-J, Genereux M, Côté C.** 2012. Detection of human food borne and zoonotic viruses on irrigated field grown strawberries. *Appl. Environ. Microbiol.* **78**:3763-3766.
30. **Morales-Rayas R, Wolffs PFC, Griffiths MW.** 2010. Simultaneous separation and detection of hepatitis A virus and norovirus in produce. *Int. J. Food Microbiol.* **139**:48-55.
31. **Baert L, Mattison K, Loisy-Hamon F, Harlow J, Martyres A, Lebeau B, Stals A, van Coillie E, Herman L, Uyttendaele M.** 2011. Norovirus prevalence in Belgian, Canadian and French produce: a threat to human health? *Int. J. Food Microbiol.* **151**:261-269.
32. **Woods JW, Burkhardt III W.** 2010. Occurrence of norovirus and hepatitis A virus in US oysters. *Food Environ. Virol.* **2**:176-182.
33. **Diez-Valcarce M, Kokkinos P, Soderberg K, Bouwknecht M, Willems K, de Roda-Husman A-M, von Bonsdorff C-H, Bellou M, Hernandez M, Maunula L, Vantarakis A, Rodriguez-Lazaro D.** 2012. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. *Food Environ. Virol.* **4**:73-80.
34. **Flannery J, Keaveney S, Rajko-Nenow P, O'Flaherty V, Dore W.** 2012. Concentration of norovirus during wastewater treatment and its impact on oyster contamination. *Appl. Environ. Microbiol.* **78**:3400-3406.
35. **Lowther JA, Gustar NE, Powell AL, Hartnell RE, Lees DN.** 2012. Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Appl. Environ. Microbiol.* **78**:5812-5817.
36. **Jothikumar N, Lowther JA, Henshilwood K, Lees DN, Hill VR, Vinje J.** 2005. Rapid and sensitive detection of noroviruses by using taqMan based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.* **71**:1870-1875.

37. **Van Stelten A, Kreman TM, Hall N, DesJardin LE.** 2011. Optimization of a real-time RT-PCR assay reveals an increase of genogroup I norovirus in the clinical setting. *J. Virol. Meth.* **175**:80-84.
38. **Schultz A-C, Vega E, Dalsgaard A, Christensen LS, Norrung B, Hoorfar J, Vinje J.** 2010. Development and evaluation of novel one-step TaqMan realtime RT-PCR assays for the detection and direct genotyping of genogroup I and II noroviruses. *J. Clin. Virol.* **50**:230-234.
39. **Svraka S, van der Veer B, Duizer E, Dekkers J, Koopmans M, Vennema H.** 2009. Novel approach for detection of enteric viruses to enable syndrome surveillance of acute viral gastroenteritis. *J. Clin. Microbiol.* **47**:1674-1679.
40. **Dreier J, Stormer M, Made D, Burkhardt S, Kleesiek K.** 2006. Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. *J. Clin. Microbiol.* **44**:2714-2720.
41. **Henke-Gendo C, Harste G, Juergens-Saathoff B, Mattner F, Deppe H, Heim A.** 2009. New real time PCR detects prolonged norovirus excretion in highly immunosuppressed patients and children. *J. Clin. Microbiol.* **47**:2855-2862.
42. **Nordgren J, Bucardo F, Dienus O, Svensson L, Lindgren P-E.** 2008. Novel light upon extension real-time PCR assays for detection and quantification of genogroups I and II noroviruses in clinical specimens. *J. Clin. Microbiol.* **46**:164-170.
43. **Butot S, Le Guyader FS, Krol J, Putallaz T, Amoroso R, Sanchez G.** 2010. Evaluation of various real-time RT-PCR assays for the detection and quantitation of human norovirus. *J. Virol. Meth.* **167**:90-94.
44. **Wobus C, Nguyen TH.** 2012. Viruses are everywhere-what do we do? *Cur. Op. Virol.* **2**:60-62.
45. **dePaola A, Jones JL, Woods J, Burkhardt III W, Calci KC, Krantz JA, Bowers JC, Kasturi K, Byars RH, Jacobs E, Williams-Hill D, Nabe K.** 2010. Bacterial and viral pathogens in live oysters: 2007 United States market survey. *Appl. Environ. Microbiol.* **76**:2754-2768.
46. **Nishida T, Nishio O, Kato M, Chuma T, Kato H, Iwata H, Kimura H.** 2007. Genotyping and quantitation of norovirus in oysters from two distinct sea areas in Japan. *Microbiol. Immunol.* **51**:177-184.
47. **Schaeffer J, Le Saux J-C, Lora M, Atamr RL, Le Guyader FS.** 2013. Norovirus contamination on French marketed oysters. *Int. J. Food Microbiol.* doi: [10.1016/j.ijfoodmicro.2013.07.022](https://doi.org/10.1016/j.ijfoodmicro.2013.07.022)
48. **EFSA Panel on Biological Hazards (BIOHAZ).** 2012. Scientific Opinion on Norovirus (NoV) in oysters: methods, limits and control options. *EFSA J.* **10**:2500,

doi:10:2903/j.efsa.2012.2500..

49. **Etienne L, Eymard-Duvernay S, Aghokeng A, Butel C, Monleau M, Peeters M.** 2013. Single real-time reverse transcription-PCR assay for detection and quantification of genetically diverse HIV-1, SIVcpz, and SIVgor strains. *J. Clin. Microbiol.* **51**:787-798.
50. **Lu X, Trujillo-Lopez E, Lott L, Erdman DD.** 2013. Quantitative real-time PCR assay panel for detection and type-specific identification of epidemic respiratory human adenoviruses. *J. Clin. Microbiol.* **51**:1089-1093.
51. **Le Guyader FS, Atmar RL, Le Pendu J.** 2012. Transmission of viruses through shellfish: when specific ligands come into play. *Cur. Op. Virol.* **2**:103-110.
52. **La Rosa G, Iaconelli M, Pourshaban M, Fratini M, Muscillo M.** 2010. Molecular detection and genetic diversity of norovirus genogroup IV: a year long monitoring of sewage throughout Italy. *Arch. Virol.* **155**:589-593.
53. **Kitajima M, Oka T, Haramoto E, Phanuwat C, Takeda N, Katayama K, Katayama H.** 2010. Genetic diversity of genogroup IV noroviruses in wastewater in Japan. *Lett. Appl. Microbiol.* **52**:181-184.
54. **La Rosa G, Fratini M, Vennarucci VS, Guercio A, Purpari G, Muscillo M.** 2012. GIV noroviruses and other enteric viruses in bivalves: a preliminary study. *New Microbiol.* **35**:27-34.
55. **Rolfe KJ, Parmar S, Mururi D, Wreghitt TG, Jalal H, Zhang H, Curran MD.** 2007. An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. *J. Clin. Virol.* **39**:318-321.
56. **Dung TTN, Phat VV, Nga TVT, My PVT, Duy PT, Campbell JI, Thuy CT, Mi Hoang NV, Minh PV, Phuc HL, Tuyet PTN, H Vinh, Kien DTH, Huy HLA, Vinh NT, Nga TTT, Hau NTT, Chinh NT, Thuong TC, Tuan HM, Simmons C, Farrar JJ, S Baker.** 2013. The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting Rotavirus A and Norovirus. *J. Virol. Meth.* **187**:138-143.
57. **Wolf S, Williamson WM, Hewit J, Rivera-Aban M, Lin S, Ball A, Scholes P, Greening GE.** 2007. Sensitive multiplex real-time reverse transcription PCR assay for the detection of human and animal noroviruses in clinical and environmental samples. *Appl. Environ. Microbiol.* **73**:5464-5470.
58. **Stals A, Baert L, Botteldoorn N, Werbrouck H, Herman L, Uyttendaele M, Van Coillie E.** 2009. Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1. *J. Virol. Meth.* **161**:247-253.
59. **van Maarseveen N, Wessels E, de Brouwer CS, Vossen ACTM, Claas ECJ.** 2010. Diagnosis of viral gastroenteritis by simultaneous detection of Adenovirus groupF, Astrovirus,

- Rotavirus group A, Norovirus genogroups I and II, and Sapovirus into two internally controlled multiplex real-time PCR assays. *J. Clin. Virol.* **49**:205-210.
60. **Park Y, Cho Y-H, Ko GP.** 2011. A duplex real-time RT-PCR assay for the simultaneous genogroup-specific detection of noroviruses in both clinical and environmental specimens. *Virus genes* **43**:192-200.
61. **Yan Y, Wang H-H, Gao L, Ji J-M, Ge Z-J, Zhu X-Q, He P-Y, Chen Z-W.** 2013. A one-step multiplex real-time RT-PCR assay for rapid and simultaneous detection of human norovirus genogroup I, II and IV. *J. Virol. Meth.* **189**:277-282.
62. **Julian TR, Schwab KJ.** 2012. Challenges in environmental detection of human viral pathogens. *Cur. Op. Virol.* **2**:78-83.
63. **Teunis PFM, Moe CL, Liu P, Miller SE, Lindesmith L, Baris RS, Le Pendu J, Calderon RL.** 2008. Norwalk virus: how infectious is it? *J. Med. Virol.* **80**:1468-1476.
64. **Thebault A, Teunis PFM, Le Pendu J, Le Guyader FS, Denis J-B.** 2013. Infectivity of GI and GII noroviruses established from oyster related outbreaks. *Epidemics.* **5**:98-110.
65. **Bae S, Wuertz S.** 2012. Survival of host-associated bacteroidales cells and their relationship with *Enterococcus* spp., *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium, and adenovirus in freshwater microcosms as measured by propidium monoazide-quantitative PCR. *Appl. Environ. Microbiol.* **78**:922-932.
66. **Kim SY, Ko G.** 2012. Using propidium monoazide to distinguish between viable and nonviable bacteria, MS2 and murine norovirus. *Let. Appl. Microbiol.* **55**:182-188.

FIGURE LEGENDS

Figure 1: Oligonucleotide primers and probes.

The sequences of reference strains GI.1 Norwalk virus (GenBank accession n° M87661, nt 5271 to 5380), GII.4 Lordsdale virus (GenBank accession n° X86557, nt 4998 to 5107), and GIV.1 Saint-Cloud virus (GenBank accession n° AF414427, nt 681-790) are represented. The primers and probes used in the developed generic real-time RT-PCR assay are represented below the reference sequences, and the original ones are above. Stars represent un-modified bases and letters mixed bases (n: any; y: c or t; r: a or g), the arrows indicate the primer orientation, with all the probes being a positive sense.

Figure 2: Comparison of C_T values obtained with the gen-*r*RT-PCR and the spe-*r*RT-PCR assays. Serial dilutions of the three reference strains were amplified using both assays, and the respective C_T values are reported. Black diamond: GI.1, grey square: GII.3, and white triangle: GIV.1. The error bars represent the standard deviations of triplicate measurements.

Figure 3: Analysis of sewage sample extracts.

Archived nucleic acids from raw sewage (A) and treated sewage (B) samples were analyzed by both assays. Grey: spe-*r*RT-PCR (diamond: GI, square: GII, and triangle: GIV). Black circle: gen-*r*RT-PCR. Dashed line: limit of acceptable C_T value. Arrows indicate samples discussed in the text.

Figure 4: Analysis of shellfish sample extracts.

Archived nucleic acids from naturally contaminated shellfish samples were analyzed by both

583 assays. Grey: spe-*r*RT-PCR (diamond: GI, square: GII, and triangle: GIV). Black circle: gen-*r*RT-
584 PCR. Dashed line: limit of acceptable C_T value. Arrows indicate samples discussed in the text.
585

Figure 1

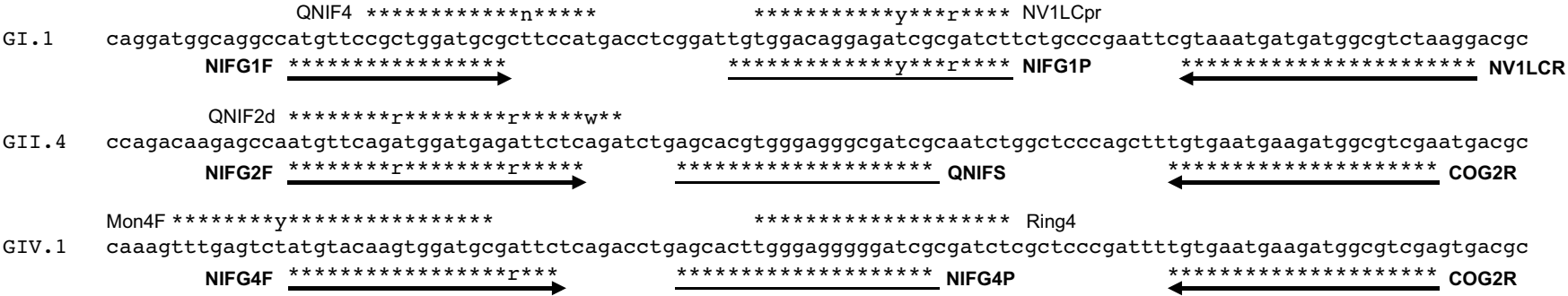


Figure 2

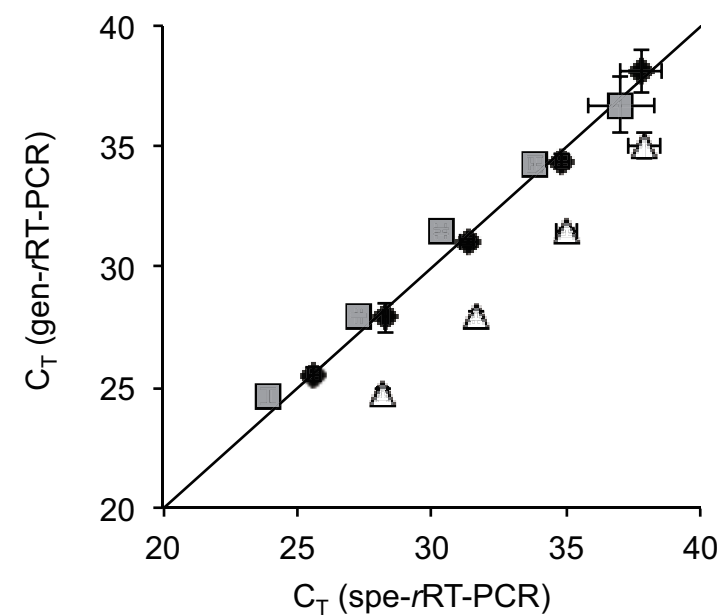


Figure 3

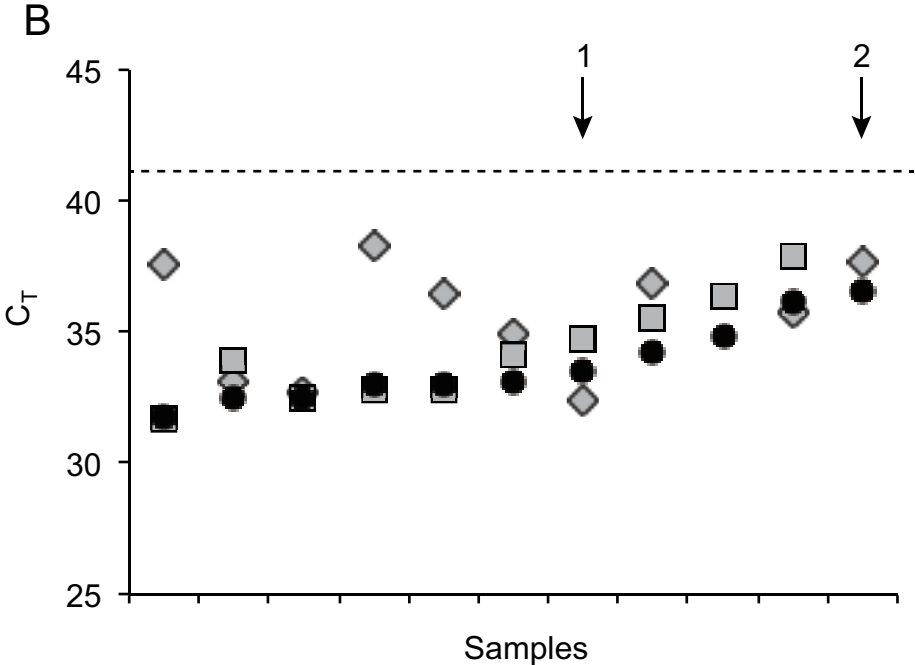
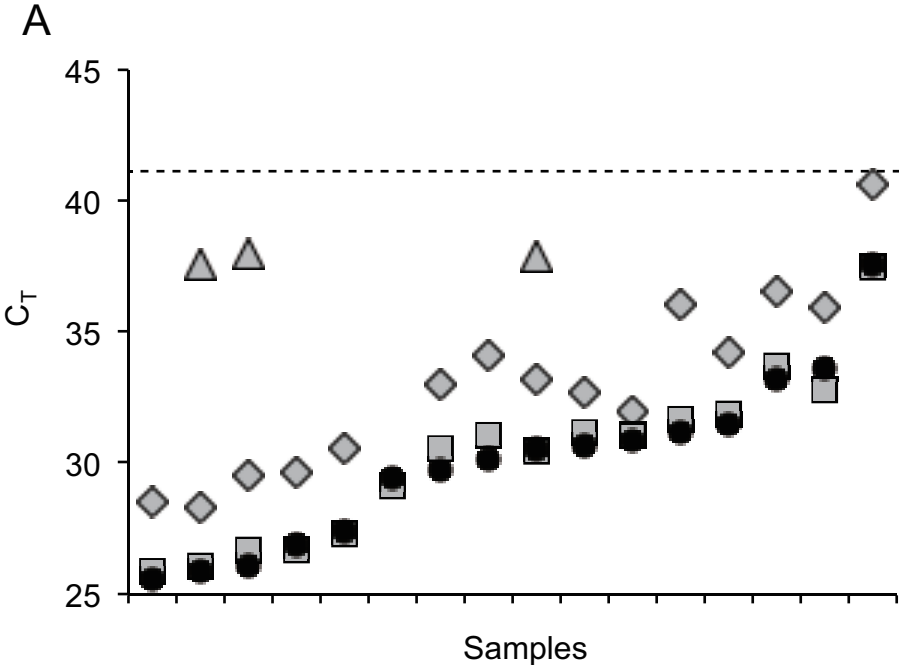


Figure 4

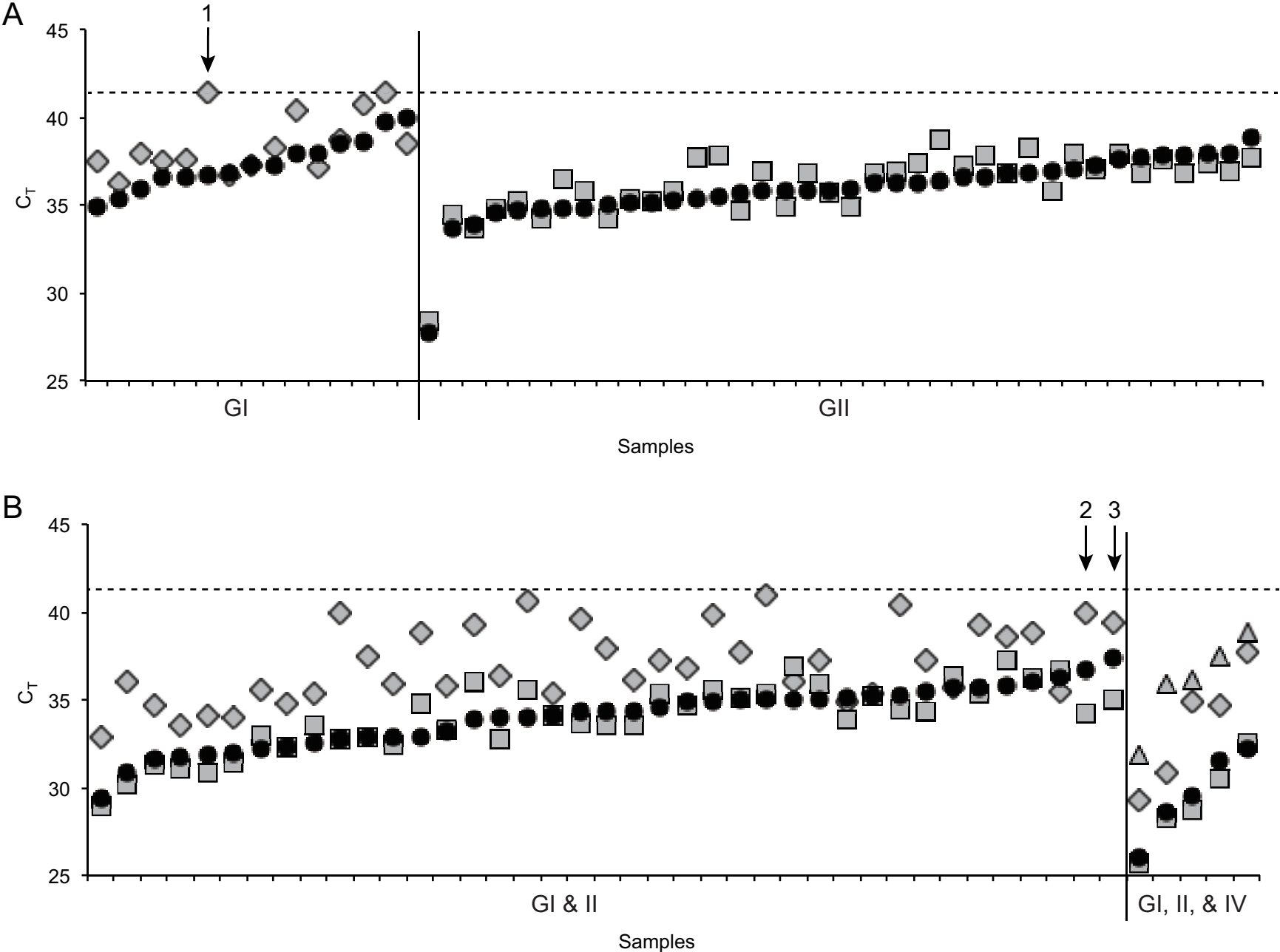


TABLE 1. Comparison of *spe-rRT-PCR* and *gen-rRT-PCR* on three NoV reference strains.

NoV	Concentration	Spe- <i>rRT-PCR</i>		Gen- <i>rRT-PCR</i>
		Separate	Mix	Mix
GI.1	High	25.6 ± 0.2	29.7 ± 0.2	25.6 ± 0.3
	Medium	31.3 ± 0.3	35.2 ± 1.3	31.0 ± 0.1
	Low	37.8 ± 0.8	no C _T	38.2 ± 0.9
GII.3	High	23.9 ± 0.1	28.5 ± 0.3	24.7 ± 0.3
	Medium	30.3 ± 0.2	35.4 ± 0.1	31.5 ± 0.1
	Low	37.0 ± 1.3	no C _T	36.7 ± 1.2
GIV.1	High	28.2 ± 0.2	28.2 ± 0.1	24.7 ± 0.2
	Medium	35.0 ± 0.4	34.7 ± 0.1	31.4 ± 0.2
	Low	no C _T	no C _T	36.7 ± 0.3

Each NA was amplified at three levels of concentrations by the *spe-rRT-PCR* using primers and probes separately (GI, GII, or GIV) or mix together, and by the *gen-rRT-PCR*. The results are expressed as mean C_T value ± standard deviation.

TABLE 2. *Gen-rRT-PCR* reactivity among different NoV strains.

NoV	Spe- <i>rRT-PCR</i>	Gen- <i>rRT-PCR</i>	
	Separate	Separate	Mix
GI.1	28.3 ± 0.1	29.1 ± 0.1	27.9 ± 0.6
.2	30.0 ± 0.1	29.1 ± 0.2	30.5 ± 0.3
.2	31.3 ± 0.3	30.4 ± 0.4	31.3 ± 0.5
.3	30.3 ± 0.1	29.2 ± 0.1	30.3 ± 0.3
.4	29.4 ± 0.4	28.6 ± 0.4	30.4 ± 0.8
.5	no C _T	28.3 ± 0.4	30.9 ± 0.6
.6	28.5 ± 0.1	27.3 ± 0.4	29.1 ± 0.1
.7	31.3 ± 0.3	31.1 ± 0.1	34.1 ± 0.7
.8	30.8 ± 0.2	30.8 ± 0.2	32.6 ± 0.7
GII.1	30.5 ± 0.2	30.4 ± 0.3	30.7 ± 0.2
.2	29.2 ± 0.5	29.4 ± 0.4	29.4 ± 0.6
.3	31.4 ± 0.2	31.6 ± 0.8	31.8 ± 0.1
.4	29.3 ± 0.2	29.3 ± 0.1	29.4 ± 0.1
.6	30.1 ± 0.1	30.2 ± 0.1	30.2 ± 0.2
.7	30.8 ± 0.1	30.4 ± 0.5	30.2 ± 0.1
.10	31.3 ± 0.1	31.4 ± 0.2	31.9 ± 0.2
.b	31.8 ± 0.3	31.7 ± 0.2	32.2 ± 0.4
.c	30.2 ± 0.2	29.9 ± 0.1	30.6 ± 0.3
GIV.1	34.1 ± 0.1	30.5 ± 0.5	30.7 ± 0.3

NoV RNA fragments were diluted to obtain a C_T ~30 using *spe-rRT-PCR* assay (except for GI.5 RNA adjusted with the modified GI primers and probe) and amplified by the *gen-rRT-PCR* using primers and probes separately (GI, GII, or GIV) or by the mixture as described in the method section. The results are expressed as mean C_T value ± standard deviation.

TABLE 3. Sensitivity of the gen-*r*RT-PCR assay to NoV RNA extracted from oyster samples contaminated in bioaccumulation experiments.

NoV	Concentration*	Spe- <i>r</i> RT-PCR	Gen- <i>r</i> RT-PCR
GI.1	Medium	28.2 ± 0.2	29.4 ± 0.1
		30.2 ± 0.1	31.4 ± 0.1
	Low	33.9 ± 0.4	35.3 ± 0.4
		34.1 ± 0.2	35.8 ± 1.0
GII.3	Medium	28.2 ± 0.1	28.7 ± 0.2
		28.4 ± 0.2	28.8 ± 0.2
	Low	33.8 ± 0.1	34.6 ± 0.6
		35.6 ± 1.0	36.9 ± 0.1

Bioaccumulated oyster NA extracts were amplified using the two *r*RT-PCR assays and results are expressed as the mean C_T value ± standard deviation, * for each concentration two different samples were analyzed.