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An international inter-laboratory study to compare digital PCR with ISO standardized qPCR assays for the detection of norovirus GI and GII in oyster tissue

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Abstract :

An optimized digital RT-PCR (RT-dPCR) assay for the detection of human norovirus GI and GII RNA was compared with ISO 15216-conform quantitative real-time RT-PCR (RT-qPCR) assays in an interlaboratory study (ILS) among eight laboratories. A duplex GI/GII RT-dPCR assay, based on the ISO 15216-oligonucleotides, was used on a Bio-Rad QX200 platform by six laboratories. Adapted assays for Qiagen Qiacuity or ThermoFisher QuantStudio 3D were used by one laboratory each. The ILS comprised quantification of norovirus RNA in the absence of matrix and in oyster tissue samples. On average, results of the RT-dPCR assays were very similar to those obtained by RT-qPCR assays. The coefficient of var iation (CV%) of norovirus GI results was, however, much lower for RT-dPCR than for RT-qPCR in intra-laboratory replicates (eight runs) and between the eight laboratories. The CV% of norovirus GII results was in the same range for both detection formats. Had in-house prepared dsDNA standards been used, the CV% of norovirus GII could have been in favor of the RT-dPCR assay. The ratio between RT-dPCR and RT-qPCR results varied per laboratory, despite using the distributed RT-qPCR dsDNA standards. The study indicates that the RT-dPCR assay is likely to increase uniformity of quantitative results between laboratories.

Highlights

► A duplex RT-digital PCR assay was optimized for detection of norovirus GI and GII. ► The temperature of the reverse-transcription reaction affected GI and GII detection. ► RT-dPCR was compared to ISO-15216 real-time RT-qPCRs in an interlaboratory study. ► The ratio between results for RT-dPCR and RT-qPCR varied per participant. ► RT-dPCR is likely to increase the interlaboratory uniformity in quantitation of RNA.

Keywords : Norovirus, Oyster, Digital PCR, Food safety, duplex, quantification

1 Introduction

Noroviruses are non-enveloped, environmentally stable, positive-sense, single-stranded RNA viruses that are highly infectious and a major cause of gastroenteritis worldwide, either in sporadic cases or outbreaks. Norovirus of the genogroup I and II (GI and GII) are infectious to humans and easily transmitted from person to person or through contaminated surfaces, food or water (de Graaf et al., 2016). More precisely, norovirus outbreaks have been associated with the consumption of raw and undercooked bivalve molluscs (Bellou et al., 2013). Bivalve molluscs, such as oysters, can become contaminated with viruses during filter-feeding from surrounding waters (Lees, 2000). Norovirus has been demonstrated in different molluscan organs, such as gills, mantle and digestive tissues (Maalouf et al., 2010; Wang et al., 2008), but extraction of digestive tissues only is now common for the analyses of the presence of enteric viruses in bivalve molluscs after showing the advantages of using digestive tissues over whole animal tissues (Atmar et al., 1995). A food safety criterion for norovirus in oysters does currently not exist, but is being discussed for legislation in the European Union. Recently, the European baseline survey on the presence of norovirus in oysters (EFSA, 2019) showed an estimated prevalence of 34.5% (CI: 30.1–39.1%) in oysters in EU production areas and an estimated prevalence of 10.9% (CI: 8.2–14.4%) in oysters from EU dispatch centers. The applied method in the baseline study was the fully validated standardized method ISO 15216-1:2017 (Anonymous, 2017; Lowther et al., 2019). Herein, dissected digestive tissues of oysters are being digested by proteinase K, followed by clarification and extraction of nucleic acids from the supernatant. Subsequently, norovirus GI or GII RNA are being detected by two separate genogroup specific one-step reverse transcription quantitative real-time PCR (RT-qPCR) assays. Quantification is performed by reference to a standard curve generated from a dilution series of linearized dsDNA standards carrying the relevant target sequences. Reliable and accurate quantification methods are important for outbreak investigations and risk assessments. At the moment, certified dsDNA standards are not available. As a result, laboratories use quantification standards that are in-house prepared and quantified, which may lead to different quantification results. Moreover, whenever kits or platforms are changed, RT-qPCR assays require a check whether conditions are still optimal to achieve a good amplification efficiency (Svec et al., 2015). Despite recent efforts to harmonize and standardize methods for the analysis of viruses in food, there are still significant differences in RT-qPCR results between laboratories, as indicated by e.g. results from proficiency tests arranged by the EURL for foodborne viruses (EURL Foodborne viruses, 2022).

In recent years, digital PCR has proven to give precise quantification of nucleic acids within a wide variety of applications, including clinical (Long, 2022), environmental (Ciesielski et al., 2021) and food virology (Coudray-Meunier et al., 2015; Martin-Latil, et al., 2016), as well as in interlaboratory studies (ILS) for the detection of GMOs in soybeans (Bogožalec Košir et al., 2017), clinical genetic markers (Whale et al, 2017) or mutations (Arnolda et al., 2022), diagnostics for human *cytomegalovirus* DNA (Pavšič et al., 2017; Milavec et al., 2022) or environmental screening for SARS-CoV-2 RNA in water samples (Niu et al., 2021). The reaction mix of qPCR and dPCR assays consist both of sample nucleic acids, target specific primers and probe, and master mix with enzymes. For dPCR, however, the reaction mix is compartmentalized into thousands of partitions prior to amplification. After amplification, the fluorescence in each of the partitions is measured by the dPCR platform and the target concentration is directly calculated from the fraction of negative reactions using a Poisson statistical algorithm. In contrast, qPCR is based on measurement of fluorescence during the exponential amplification of the target PCR product. As dPCR is an end-point detection technique (Hindson et al., 2013), quantitative molecular detection by dPCR is less affected by suboptimal PCR efficiencies. Furthermore, dPCR has been

reported to be less sensitive to inhibitory substances from the matrix (Coudray-Meunier et al., 2015; Rački et al., 2014a). In addition, as no external standards are required for quantification, dPCR has the potential to facilitate harmonization across laboratories and reduce potential bias from the use of different standards.

Several RT-dPCR assays using the oligonucleotides described for RT-qPCR in Annex D of ISO 15216-1:2017 (Anonymous, 2017) have been reported (Bartsch et al., 2018; Coudray-Meunier et al., 2015; Han et al., 2021; Laroque et al., 2022; Monteiro and Santos, 2017; Nasheri et al., 2017; Persson et al., 2018; Polo et al., 2016, Sun et al., 2021; Tan et al., 2018). Quantitative results obtained by RT-dPCR are herein often compared with those obtained by these RT-qPCR, albeit to varying outcomes. Moreover, the performance of these assays, e.g., in terms of robustness and precision across multiple laboratories, is often unknown.

Here we describe a duplex norovirus GI/GII RT-dPCR that was also based on the RT-qPCR assays as described in ISO 15216-1:2017 (Anonymous, 2017), but whose performance was being tested in an ILS. The RT-dPCR assays for detection of genogroup GI and GII were first optimized separately and subsequently merged into a GI/GII duplex assay for use on a Bio-Rad QX200 platform. The protocol was also adapted for the Qiagen Qiacuity or ThermoFisher QuantStudio 3D for use in the ILS by some laboratories. The aim of the ILS was to compare quantification of norovirus GI and GII RNA in the absence of matrix as well as in oyster samples by RT-dPCR and the standardized RT-qPCR assays. For this, the quantitative agreement between the assays, the precision within runs and between laboratories, as well as the ratio of the quantified results obtained by RT-dPCR and RT-qPCR were investigated.

2 Materials and methods

2.1 Organization

The study design, optimization of the duplex RT-dPCR and data analyses for the ILS were carried out by Wageningen Food Safety Research (WFSR, WUR, the Netherlands) in collaboration with the European Union Reference Laboratory (EURL) for Foodborne Viruses (Swedish Food Agency, Sweden). The EURL was responsible for the production and characterization of the study materials and distribution of some of the reagents. Participants were National Reference Laboratories (NRLs) for Foodborne Viruses.

2.2 Development of RT-dPCR assay for the detection of norovirus GI and GII RNA

RT-dPCR assays for the detection of norovirus GI and GII RNA were set up using the primer and probe sequences as described in Annex D of ISO 15216:1-2017 (Anonymous, 2017) (Table 1). These oligonucleotides target the conserved region at the 5' end of the ORF2 of human norovirus GI and GII (Kageyama et al., 2003). Alignments of sequences of this region available in GenBank demonstrated that the respective primer and probe sets are adequate for the quantification of all GI and GII norovirus strains, whereas no positive results were obtained for six other human enteric viruses nor for seven bacteria that can be found in bivalve molluscs (Anonymous, 2017). For use in the RT-dPCR, the NVGG1p (Svraka et al., 2007) and QNIFS (Loisy et al., 2005) probes were modified with an internal ZEN quencher.

The manufacturer's kit protocol was the starting point for optimization of RT-dPCR assays. Diluted nucleic acid extracts from norovirus positive fecal samples, characterized as genotypes GI.3 and GII.4 (kindly provided by Harry Vennema, RIVM, the Netherlands) were used as input (about 50 genome copies/µl reaction mix). The optimization aimed to achieve the highest target RNA concentration and best separation of the fluorescence of the positive (P) and negative (N) droplet clusters (P/N ratio). Consecutively, the concentration of forward/reverse primers (500/500, 900/900, or 1500/1500 nM), the temperatures for reverse transcription (RT) (within a gradient of 36 – 54 ºC) and annealing (Ta) (within a gradient of 50 – 62 ºC) and the probe concentration (100, 250, 500, 750 nM) were selected. The RT-dPCR assays for norovirus GI and GII were subsequently merged into a duplex GI/GII RT-dPCR assay (as described in 2.5.3) keeping the optimal conditions as set for the single assays, including the cycling conditions.

For comparison, both singleplex and duplex RT-dPCR assays were applied to an oyster RNA extract (previously negatively tested for norovirus) that had been spiked with 50 genome copies norovirus GI and GII per µl reaction mix. In addition, the linearity of the duplex GI/GII assay in an oyster extract was determined at norovirus concentrations near the expected limit of detection. For this, a two-fold dilution series of norovirus GI and GII in oyster RNA was tested in the duplex assay (0.8 – 25 genome copies/µl reaction mix). Both tests were done in triplicate in two repeat experiments.

Subsequently, the protocol for the Bio-Rad QX200 was adapted for use on the Qiagen QIAcuity One 5-plex or ThermoFisher QuantStudio™ 3D device following the same steps as above, but using the kits associated to the respective platforms used by ILS participants (2.5.3).

2.3 Production of test materials and quantification

Study materials were prepared by the EURL for Foodborne Viruses first to be used in the proficiency test program (21EFV06). Approximately 600 European oysters (Ostrea edulis) were purchased from a producer in Sweden. A homogenous mixture was prepared by shucking the oysters, separating the digestive glands, removing adipose tissues, and finally blending and pooling the material together. The mixture was divided into 2 g aliquots per 50 ml tube, after a negative test result for norovirus GI and GII RNA with RT-qPCR assays as described in ISO 15216-1:2017 (Table 1). Each tube was spiked with norovirus positive fecal samples characterized as genotypes GI.3 and GII.4 (kindly provided by Johan Lindh, Clinical Microbiology and Health Care Hygiene, Academic Hospital, Uppsala, Sweden). Inoculation levels aimed for were 10^5 genome copies of GI.3 and 10^3 genome copies of GII.4 per gram tissue in sample A, 10^4 genome copies of GI.3 and 10^5 genome copies of GII.4 per gram tissue in sample B and 10^3 genome copies of GI.3 and 10^4 genome copies of GII.4 per gram tissue in sample C. Sufficient homogeneity was demonstrated by analyses of 10 samples of each sample code (A-C) for norovirus GI and GII using RT-qPCR assays according ISO 15216-1:2017 (EURL Foodborne viruses, 2022)(see Table 2).

2.4 Distribution of ILS test materials

Reagents and standards for the ILS were dispatched together with the contents of the regular proficiency test (21EFV06) on dry ice by courier at 26th of April 2021. Each ILS participant received three samples of 2 g blended digestive tissue from European oysters to be analyzed according to ISO 15216-1:2017. In brief, the samples were inoculated with an adequate amount of process control virus according to laboratory in-house protocols. Subsequently, 2 ml of proteinase K solution (3 U/ml) was added and the mixture was incubated for 1 h at 37 ºC (approximately 320 oscillations/min), followed by incubation at 60 ºC for 15 min, and clarification at 3000 × g for 5 min at room temperature. The volume of the supernatant was recorded, and a part of the extract was used for the proficiency test. The ILS participants were requested to store the remainder of clarified proteinase K extracts below -15 ºC until further instructions for the ILS study were provided. The intended results (Table 2) for the regular proficiency test were shared prior to the submission date of the ILS results. The ILS participants (n=10) also received *in vitro* transcribed RNA (10^4 genome copies/µl) and linearized DNA plasmids containing targets for norovirus GI and GII (10^5 genome copies/µl) (Persson et al., 2018), diluent buffer (10mM Tris, 1mM EDTA (TE)), and probes (5 µM) for detection of GI and GII by dPCR (Table 1). Not included in the dispatch were process control virus, nucleic acid extraction reagents, RT-qPCR or RT-dPCR kits, nor forward and reverse primers (Table 1), nor the RT-qPCR and RT-dPCR reaction reagents.

2.5 Study protocol

2.5.1 Samples included

Samples to be tested in RT-dPCR and RT-qPCR by the ILS participants were 1:1 (v/v) mixtures of 10-fold serial diluted *in vitro* transcribed RNA with target sequences norovirus GI or GII (10^4-10^0 genome copies/µl), dsDNA standards with target sequences norovirus GI or GII (10^3 genome copies/µl) in TE, and nucleic acids extracted from oyster samples A, B and C (undiluted and 10x diluted in molecular grade

water) (see 2.5.2). All samples as well as water and TE controls were tested in triplicates. (see supplemental protocol).

To control for inhibition, 10^4 genome copies of *in vitro* transcribed RNA were added as external amplification controls (EAC) to sample RNA and to nuclease-free water and tested in RT-qPCR and RT-dPCR. The concentration of genome copies in the sample spiked with EAC was compared to the control sample containing an equal amount of EAC RNA in nuclease-free water.

2.5.2 Nucleic acid extraction from proteinase K extracts

For the ILS, nucleic acids were extracted from stored clarified proteinase K extracts (less than 5 months below -15 ºC) of the proficiency test. Nucleic acids were extracted from 500 µl for each sample (A-C) in duplicate using the Nuclisense Magnetic Extraction Reagents kit (bioMérieux) according to the manufacturer's protocol. Nucleic acids were eluted in 100 µl elution buffer (included in the kit). Duplicate extractions of each were pooled and subsequently split for RT-dPCR (80 µl) and RT-qPCR (100 µl) to prevent multiple freeze-thaw cycles and stored below -15 ºC until analyses.

2.5.2 ILS RT-qPCR detection

Norovirus GI and GII RT-qPCR assays were performed as in-use at the laboratories (Table 3) under the condition that the assays complied with ISO 15216-1:2017, including the requirements for quality controls. For quantification, a 10-fold dilution series of the supplied linearized plasmid dsDNA standard (Persson et al., 2018) was used. The RT-qPCR assay for detection of RNA of the process control virus was according to the ILS participants' in-house protocol.

2.5.3 ILS RT-dPCR detection with optimized protocols

2.5.3.1 Bio-Rad QX200 protocol

A 5.5 µl aliquot of template RNA was added to 16.5 µl of One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad) master mix. Final concentrations of this mixture were 1x One-Step RT ddPCR mastermix, 1/10-diluted reverse-transcriptase (from master mix kit), 900 nM of each of the primers (QNIF4, NV1LCR, QNIF2 and COG2R), 500 nM of NVGG1p-ZEN and 250 nM of QNIFS-ZEN, and 15 nM dithiothreitol. After vigorously mixing, 20 µl of each sample was transferred to DG8™ Cartridges (Bio-Rad), followed by the addition of 70 µL of Droplet Generation Oil for Probes (Bio-Rad). Droplets were generated using the QX200™ Droplet Generator (Bio-Rad). Next, the droplet suspension was pipetted carefully onto 96-well plates. After sealing, RT-dPCR reactions were carried out in a deep-well thermocycler. The cycling program consisted of RT reaction at 47 °C for 60 min, followed by incubation at 95 °C for 10 min and 40 PCR cycles of denaturation at 95 °C for 30 s (ramp rate 3 ºC/s), and annealing/elongation at 53 °C for 1 min (ramp rate 3 ºC/s). A final DNA polymerase deactivation step was carried out at 98 °C for 10 min. Plates were cooled and stored at least for 30 min at 4 °C before being transferred to the QX200™ Droplet Digital™ PCR system (Bio-Rad) on the same day or the day after amplification. Results were visualized and analyzed in QuantaSoft™ version 1.7.4. Thresholds to separate the positive and negative clusters were set manually directly above the negative cluster (see e.g. Figure 1a).

2.5.3.2 Qiagen QIAcuity One 5-plex protocol

An 11 µl aliquot of template RNA was tested in a 44 µl reaction mix, including 1x QIAcuity One-Step Viral RT-PCR Master Mix and 1x Multiplex Reverse Transcription Mix, (Qiagen). Final concentrations of

oligonucleotides (Table 1) as well as cycling conditions were the same as for the Bio-Rad QX200 (2.3.5.1). Initial experiments suggested that these conditions gave comparable or better results than the manufacturer's protocol. Reaction mixes were added to wells of a Qiagen Nanoplate, which was transferred to the Qiagen QIAcuity One 5-plex machine and subject to the cycling program. Results were analyzed using Qiagen QIAcuity Software Suite. Thresholds to separate the positive and negative clusters were set automatically by the software.

2.5.3.3 ThermoFisher QuantStudio™ 3D device protocol

A 3 µl aliquot of template RNA was tested in a 15 µl reaction mix, consisting of 1x reaction AgPath-ID buffer (AgPath-ID One-step RT-PCR Reagents, Applied Biosystems), 25-fold diluted enzyme mixture (AgPath-ID One-step RT-PCR Reagents, Applied Biosystems), 15 fold-diluted enhancer (Detection enhancer for real-time PCR, Applied Biosystems), 800 nM of each of the two primers for GI (QNIF4, NV1LCR) and 200 nM of the NVGG1p probe. No reportable results for analyses for GII were obtained. After gently vortexing, the mixture was loaded on a QuantStudioTM 3D Digital PCR 20K chip (Applied Biosystems). Subsequently, the loaded chip was placed in a ProFlex PCR System Quant Studio (Applied Biosystems) thermocycler and the following thermocycling programme was run: 15 min at 55 °C, 10 min 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s 55 °C and 30 s at 65°C. After 2 min at 60 °C, the chip was read using QuantStudio™ 3D device and data uploaded at https://apps.thermofisher.com/quantstudio3d/projectHome.html for analyses. Thresholds to separate the positive and negative clusters were set manually directly above the negative cluster.

2.6 Participants equipment and reagents

Laboratories participating in the oysters proficiency testing scheme were invited for the ILS irrespective of the dPCR platform available. Ten participants subscribed to the ILS, of which eight submitted data sets were included in the study. Platforms and reagents for RT-dPCR and RT-qPCR used by these participants are summarized in Table 3. One received data set was too incomplete to include in the study, whereas another participant withdrew prior to the start of the analyses.

2.7 Calculations

2.7.1 RT-qPCR

Submitted values for Cq, slope and intercept of the dsDNA standards (Cq values versus Log_{10} copy/µl) were used to calculate the norovirus concentration in samples according to the formula: concentration C (in copy/µl) is equal to (10^(ΔCq/slope)), in which ΔCq is equal to Cq (sample) minus- Cq (intercept).

The resulting concentration of each of the 10-fold dilutions of GI and GII EAC was multiplied by two as GI and GII EAC dilutions had been mixed 1:1.

The concentration of each of the oyster samples was calculated according the formula: Copy/gram digestive tissue is equal to copy/µl RNA × total RNA (µl) × (total proteinase volume/input in RNA extraction) × 1/grams of tissue according ISO 15216-1:2017.

RT-qPCR inhibition was calculated in % according to the formula: 1-((genome $copies_{(sample+\ EAC)}$/genome $copies_{(EAC+water)}$) × 100%).

2.7.2 RT-dPCR

Results for RT-dPCR assays were accompanied with raw data files from the platforms. For both QX200 (Bio-Rad) and QIAcuity One 5-plex (Qiagen), the concentration in genome copies/µl RNA eluate is equal to 4 × genome copies/µl reaction mixture to adjust for the dilution of RNA in the reaction mixture (e.g., 5.5 µl RNA in total volume 22 µl for the QX200). For the QuantStudioTM 3D Digital (Thermo Fisher), this adjustment factor was 5 (3 µl RNA in total volume 15 µl). Back-calculations from copy/ul RNA to each of the 10-fold dilutions of EAC or oyster samples was done as described in 2.7.1. RT-dPCR inhibition was calculated as for RT-qPCR inhibition (see 2.7.1).

2.8 Variation within and between laboratories

The variation between laboratories was determined using the results reported by these laboratories, each reporting results for one set of oyster samples (A-C). In addition, the intermediate precision was determined using the results obtained for eight sets of samples (A-C) analyzed by one laboratory using RT-qPCR and RT-dPCR (Bio-Rad QX200). For the latter, each set was tested by the same person, but on separate days. The analyses of raw data were performed as described above (see 2.7).

The coefficient of variation (CV) was calculated by dividing the population standard deviation by the average value. The CV expressed in percentage was calculated for triplicate values, for values for the intermediate precision obtained between runs within one laboratory, and for values submitted by participating laboratories.

3. Results

3.1 Duplex dPCR assays for detection of norovirus GI and GII RNA in oysters

3.1.1 Optimization of the RT-dPCR assays

The assays for norovirus GI and GII were optimized separately in singleplex reactions on the Bio-Rad platform using RNA from fecal samples containing either GI.3 or GII.4 RNA. The effect of the concentration of forward and reverse primers (500, 900, 1500 nM), the temperatures for reverse transcription (RT) (gradient 36 – 54 °C) and annealing (Ta) (gradient 50 – 62 °C) and the concentration of FAM probe (100, 250, 500, 750 nM), was consecutively studied. In summary, a higher concentration of primers resulted in a higher level of fluorescence of the positive cluster, without changing the background fluorescence or target concentration (data not shown). The RT temperature did not affect the levels of fluorescence of any of the clusters, but affected the observed target concentration. Decreasing RT temperatures resulted in an increase of the number of positive droplets (Figure 1 a and c) and thereby a higher target RNA concentration until a plateau was reached at about 48 °C (Figure 1 b and d). The target RNA concentrations remained unaffected with changing Ta temperatures or probe concentrations. The ZEN internal quencher at the 9th nucleic acid of the probes improved separation between the positive and negative clusters, whereas the measured copy numbers were not affected as compared to RT-dPCR with regular probes (data not shown).

Having optimized singleplex GI and GII RT-dPCR reactions, we tested the performance of GI/GII duplex reactions (FAM/HEX channels) (see 2.5.3.1), with an RT temperature of 47 ºC. The separation between the positive and negative clusters remained distinct in the GI/GII duplex RT-dPCR assay format, with the FAM-labeled NVGG1-probe and HEX-labelled QNIFS probe. This was the case for GI and GII *in vitro* transcribed RNA (Figure 2a-b) as well as for GI and GII positive oyster RNA (Figure 2 c-f)).

The detected concentrations (genome copies per µl reaction mix) of norovirus GI RNA in the background of oyster RNA agreed very well, irrespectively of using the singleplex (49.90 ± 2.43) or duplex formats (51.28 ± 2.74). This was also true for the detection of GII between the singleplex (54.23 ± 2.17) and duplex formats (57.90 ± 3.66). In addition, linearity of the duplex GI/GII assay was determined in oyster extracts at norovirus concentrations near the expected limit of detection. For this, RNA extracted from GI and GII positive human feces samples was serially diluted in oyster RNA extracts (0.8-25 genome copies/µl reaction mix). All replicates (n=6) tested positive, with observed values that were near to the theoretically expected values with a slope of 0.99 and $R^2 > 0.99$ (Supplemental Figure 1). After affirmation of these duplex GI/GII RT-dPCR parameters, the ILS study was designed.

3.2 ILS results for the detection of norovirus GI and GII RNA in dilutions series

Initially ten laboratories subscribed and received materials for the ILS, consisting of EAC RNA for 10-fold dilutions, dilution buffers, dsDNA standards for RT-qPCR assays, the ILS protocol and a RT-dPCR platform specific protocol. The participants were asked to test 10-fold dilution series of *in vitro* transcribed norovirus GI and GII EACs as well as oyster samples belonging to a previously run proficiency test. Tests were performed by RT-dPCR as well as by ISO 15216-1:2017-conform RT-qPCR assays. Data sets were received comprising data for the Bio-Rad QX200 platform (n=6) and Qiagen QIAcuity One 5-plex (n=1). One dataset had complete results for norovirus GI only (ThermoFisher QuantStudio™ 3D device), as a technical failure occurred during the project. One laboratory withdrew before analyses and another failed to submit a complete dataset (missing RT-qPCR data).

The datasets, containing RT-dPCR and RT-qPCR results for ten-fold dilutions of *in vitro* transcribed norovirus GI or GII RNA, are presented in genome copies per µl (Figure 3), with triplicate results of each laboratory. On average, the highest EAC concentration tested positive at 3.7×10^4 genome copies/µl for norovirus GI and 1.2×10^4 genome copies/µl for norovirus GII by RT-dPCR (Figure 3 Top) and at 5.3×10^4 genome copies/µl for norovirus GI and 2.0×10^4 genome copies/µl for norovirus GII by RT-qPCR (Figure 3 Bottom). For both genogroups and with both detection formats, the concentrations decreased gradually over four ten-fold dilutions (See also Supplemental Table 1A). Linearity in RT-dPCR results was seen down to the lowest concentration tested (10^0 genome copy/µl). At this level, 79.2% of RT-dPCR reactions (19/24) resulted in values of 1 or more genome copies/µl for norovirus GI. For GII.4, this was observed for 57.1% of the reactions (12/21).

Within triplicate measurements (see also Supplemental Table 1B), the geometric mean of coefficient of variation (CV%) was low for 10^4 genome copies/µl norovirus RNA samples (3.5 – 5.1%) for all laboratories. These CV% increased at lower RNA concentrations. The same was true for the CV% of the RT-qPCR triplicates. Between laboratories, (Supplemental Table 1A), the CV% was much larger, and ranged from 24.2 to 50.6% over the tested RNA concentrations for the norovirus GI RT-dPCR. The CV% was even higher for the norovirus GI RT-qPCR (82.8 to 134.3%). For norovirus GII, however, the CV%

was more similar for the two detection formats, ranging from 47.7 to 90.2% over the test dilutions for the RT-dPCR and from 57.4 to 68.9% for the RT-qPCR. Besides RNA, linearized DNA plasmid containing the target region of GI or GII was tested at a level of 10^3 genome copies/µl. The mean concentrations over all laboratories obtained for this DNA sample was around this level by RT-qPCR, but about three-to-four times lower by RT-dPCR.

Given the different values obtained for the DNA test samples by the two detection formats, we were interested to explore the difference in results for the RNA dilutions by the two formats, expressed in RT-dPCR/RT-qPCR ratio. First, the RT-dPCR/RT-qPCR ratio was calculated for each of the five EAC dilutions tested and subsequently the ratio values over the five dilutions were averaged for each laboratory (Supplemental Table 1C). Between laboratories, the RT-dPCR/RT-qPCR ratio for norovirus GI RNA varied between 0.18 and 5.62, with a geometric mean of 1.21, whereas for norovirus GII RNA, the RT-dPCR/RT-qPCR ratio varied between 0.34 and 3.57, with a geometric mean of 0.64. The observed laboratory specific RT-dPCR/RT-PCR ratio for norovirus GII RNA detection was very much comparable between the six laboratories using Bio-Rad QX200 despite using different RT-qPCR platforms/optimizations. More variation was observed in the RT-dPCR/RT-qPCR for detection of norovirus GI RNA, even among the laboratories using the same RT-dPCR platform. No explanation was found for this observation, nor did there seem to be any connection with the choice of TM9 or NVGG1 probe in RT-qPCR for norovirus GI. The laboratory specific RT-dPCR/RT-qPCR ratio was further investigated by determining the linearity of relation between data for RT-dPCR and RT-qPCR. Differences in equations of the linearity are given for each laboratory in Supplemental Figure 2.

3.3 Intermediate precision of the detection of norovirus RNA in oysters within one laboratory

The second part of the ILS consisted of analyses of oyster tissue samples. Samples A-C, consisting of blended oyster digestive tissue with spike-in of norovirus GI.3 and GII.4, were produced for the regular proficiency test. The levels of norovirus GI and GII RNA were earlier shown to be homogeneously distributed by RT-qPCR (EURL Foodborne viruses, 2022). Eight sets of archived supernatants (proteinase K treated digested tissue) of samples A-C were re-extracted to determine the intra-laboratory repeatability of the duplex RT-dPCR assay over several days, i.e. the intermediate precision. Extracts were tested in triplicate in the duplex RT-dPCR assay (Figure 4 Top) as well as by the RT-qPCR assays (Figure 4 Bottom) in eight separate runs over several days by the same person. Spiking levels had been selected to provide a 10-fold decrease of norovirus GI from sample A to B to C and of norovirus GII from sample B to C to A. Results for both RT-dPCR as RT-qPCR followed this spiking pattern, which was also the case in the 10-fold dilution of the RNA samples (Figure 4), here presented without correction for the 10-fold dilution. The highest levels of contamination were 1.2×10^5 genome copies/g norovirus GI (sample A) and 1.5×10^5 genome copies/g norovirus GII (sample B) as determined by duplex RT-dPCR, and 6.6×10^4 and 1.2×10^5 genome copies/g, respectively, as determined by RT-qPCR (Supplemental Table 2A). The geometrical averaged CV% of triplicate measurements was comparable for RT-dPCR and RT-qPCR at this level, and increased with decreasing norovirus RNA levels (Supplemental Table 2B). With the runs performed under identical conditions, but tested over several days, the intermediate precision was determined. For norovirus GI, this precision CV% varied for all samples (both undiluted and diluted) from 4.7 to 30.5% using the duplex RT-dPCR, but varied from 25.1 to 60.0% by RT-qPCR. For norovirus GII, this precision CV% varied from 17.4 to 30.0% using the duplex RT-dPCR, and varied from 19.3 to 64.2% by RT-qPCR (Supplemental Table 2A).

3.4 ILS results for the detection of norovirus GI and GII RNA in oysters

After determination of the intermediate precision, the RT-dPCR protocol was applied on archived and re-extracted proficiency test samples set A-C by the participating laboratories. All re-extracted samples of all participants met the criteria of extraction efficiency, with a median of 4%. Data of the norovirus GI and GII results is depicted similarly as for intermediate precision dataset (Figure 5). The highest level of contamination was on average 6.7 x 10^4 genome copies of norovirus GI/g in sample A and 9.8 x 10^4 genome copies of norovirus GII/g for sample B as determined by dPCR by eight and seven laboratories, respectively (Table 4). These concentrations were lower than those obtained by RT-qPCR, 1.9 x 10^5 genome copies of norovirus GI and 1.1 x 10^5 genome copies of norovirus GII/g, respectively. Precision of triplicate measurements at this level was high, 5.1 and 5.2 % for norovirus GI and norovirus GII by RT-dPCR and 5.2 - 3.5% for norovirus GI and norovirus GII by RT-qPCR (data not shown). The differences between results of laboratories for norovirus GI was, however, much smaller with RT-dPCR than with RT-qPCR (Figure 5; Table 4). For norovirus GII, this CV% was more in the same range for RT-dPCR or RT-qPCR and, in general, lower than the CV% seen for norovirus GI.

Finally, to exclude that oyster extracts itself had an effect on the quantification results, 10^4 genome copies of supplied norovirus EAC RNA was added to oyster RNA extracts and tested. Results for norovirus GI EAC RNA in sample C and for norovirus GII EAC in sample A are depicted (Figure 5), as well as the results of EAC RNA in water samples. Absolute levels varied between laboratories, but values were very much the same for oyster RNA and water samples per laboratory (depicted in same color), indicating hardly any effect of the presence of matrix RNA on the quantification by both RT-dPCR and RT-qPCR. For all samples tested, the inhibition rate was far below the acceptability criterium (<75% according to ISO 15216-1:2017), with median values of 13% for GI RT-dPCR; 20% for GI RT-qPCR; 10% for GII RT-dPCR and 18% for GII RT-qPCR.

4. Discussion

The optimized duplex RT-dPCR assay was shown to successfully detect and quantify both norovirus GI and GII RNA in oysters in various laboratories. Compared to the standardized detection by RT-qPCR assays, quantified levels in oyster extracts by duplex RT-dPCR assays were less scattered, even though some laboratories had little to no experience with RT-dPCR. This work demonstrates the robustness of the RT-dPCR assay and strengthens the application of RT-dPCR in the field of food virology, especially when food safety criteria for norovirus in oysters will become part of future food safety EU legislation as suggested by the EU Commission.

The described norovirus GI/GII duplex RT-dPCR assay was adapted from the standardized RT-qPCR assays for norovirus GI and GII, targeting the conserved ORF1-ORF2 junction with genogroup specific oligonucleotides for the detection of widely diverse norovirus genotype strains (da Silva et al., 2007; Loisy et al., 2005; Kageyama et al., 2003; Svraka et al., 2007). The results obtained by the GI/GII duplex RT-dPCR assay are, therefore, likely to be representative for the other human GI and GII norovirus genotypes, despite that the present study was limited to the detection of norovirus GI.3 and GII.4. For the development of the duplex RT-dPCR, the singleplex RT-dPCR assays were first optimized by selection from a range of oligonucleotide concentrations as well as from temperature gradients for reverse-transcription (RT) and PCR annealing stages. In the present study, it has been shown that the RT temperature had a direct effect on the detected viral RNA quantity. For human norovirus RT-dPCR assays published to date, optimization of temperature settings was restricted to annealing temperature only (Laroque et al., 2022; Persson et al., 2018; Sun et al., 2021). Recently, root cause analyses of dispersion of quantitative results for RNA of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) among ILS participants led to the conclusion that controlling of the RT under optimum conditions is required to further improve ILS comparability of detection (Niu et al., 2021). Optimizing the RT temperature should therefore be part of the development of any new RT-dPCR assay.

After optimization of both singleplex assays, no reduction of sensitivity was observed for the duplex format RT-dPCR for norovirus GI and GII. Earlier another norovirus GI/GII duplex RT-dPCR has been described for Bio-Rad dPCR platform (Sun et al., 2021) with primers and probes as described in the informative Annex D of ISO 15216-1:2017. The separation of the positive over the negative droplet population expressed in ratio was, however, much higher in the present study (4.5 for norovirus GI and 3 for norovirus GII, Figure 2) than shown by Sun and co-workers for the optimal annealing temperature (1.2 for norovirus GI and 1.5 for norovirus GII) (Sun et al, 2021). The improved ratio achieved in the present study likely stems from the internal ZEN quencher conjugated to the probes, which reduced background fluorescence (Hirotsu et al., 2020). In earlier experiments with the NVGG1p probe without ZEN quencher the ratio was 1.3 as well (data not shown).

One of the aims of the ILS was to test the performance of the duplex RT-dPCR assay in multiple laboratories. The protocol was therefore optimized for two other platforms, the Qiagen QIAcuity One 5-plex and ThermoFisher 3D QuantStudio. The platforms in the study use different techniques to partition the mixes, i.e., separation into droplets (Bio-Rad QX200), chambers on a microfluidic digital PCR plates (Qiagen QIAcuity One 5-plex), or chambers in a single sample chip (ThermoFisher 3D QuantStudio). After adapting of the assay for these platforms, it was decided that RT-dPCR assays on the ThermoFisher 3D QuantStudio were to be performed as singleplex assays. Since the ILS study (July 2021), the platform is no longer on the market (website data retrieved March 2023). Due to the limited number of users in this study, no statistical analyses have been performed to evaluate the platforms.

Despite the limited experience in running RT-dPCR among the participants, the assay performed well in eight (norovirus GI) and seven (norovirus GII) laboratories. Variation between triplicates was small, though dependent on the target concentration, and overall linearity of quantitative results went down to an input of 10^0 EAC RNA genome copies/µl. For a better insight in the value of the RT-dPCR assay, a direct comparison was made with the ISO 15216-1:2017 RT-qPCR assays as in use in the participants' laboratories. These assays have been in use for many years, as the technical specification ISO/TS 15216 was published in 2013. Between laboratories, the CV% in norovirus GII RT-dPCR results were alike the CV% of results obtained with the standardized GII RT-qPCR assay. For norovirus GI, however, the RT-dPCR results were more uniform among the laboratories with a lower CV% than those obtained by RT-qPCR. It is important to note that quantification by RT-qPCR requires dsDNA standards, and all laboratories in the study used the dsDNA standards supplied by the EURL. Had in-house produced dsDNA standards been used instead, the difference in CV% in results for norovirus GI and GII by RT-qPCR and RT-dPCR would likely have been much bigger. Reliable, preferably certified, control DNA materials are necessary for accurate quantification by RT-PCR, as standards itself can lead to over- or underestimating results. Recently, variability in concentration between batches of commercially available RNA standards was reported, which, upon use for quantification, can introduce variability in quantitative results between laboratories (Whale et al, 2022). One of the advantages of RT-dPCR is that no standards are required.

RT-dPCR was also compared with RT-qPCR using the RT-dPCR/RT-qPCR ratio (Coudray et al., 2015, Laroque et al, 2022, Persson et al, 2018). In the present study, such ratios were calculated per laboratory for test results for diluted RNA in the absence of matrix and varied between 0.18 - 5.62 (see Supplemental Table 1C). For norovirus GI, this ratio seemed unrelated to the probe (TM9 or NVGG1p) used in the RT-qPCR. Variations were seen, but no trend was observed between the temperatures for reverse transcription and/or annealing, the concentrations of the primers and probes, or the detection kits used in the RT-qPCR assays that could explain the variation in the RT-dPCR/RT-qPCR ratios. In addition, the quality of the dilution of standards, or the RT-qPCR baseline interpretation may have contributed to the observed variation in ratios. The highest RT-dPCR/RT-qPCR ratios were observed for the laboratory using the Qiagen QIAcuity One 5-plex platform, but as it is a single observation no conclusions can be drawn. In general, many factors may contribute to a RT-dPCR/RT-qPCR ratio, therefore caution on direct comparison of results between RT-dPCR and RT-qPCR is warranted.

The detection of norovirus by the GI/GII duplex RT-dPCR assay was very sensitive and linear at the lower end of the assay using a single batch of oysters. For future practical applications, it will be important to investigate the limit of detection or limit of quantification using market-ready bivalve molluscs. The norovirus RNA levels in the ILS samples were higher than the levels observed in retail samples (Dirks et al., 2021; EFSA 2019; Lowther et al., 2012; Lowther et al., 2018; Suffredini et al., 2014). Monitoring data obtained by RT-dPCR in such samples are scarce (Polo et al., 2016; Tan et al., 2018). So far no microbiological criteria have been set for norovirus RNA in oysters. Official laboratories may prefer, or are only allowed, to use the validated standardized RT-qPCR assays until a RT-dPCR assay for testing of bivalve molluscs for norovirus RNA has been validated against the ISO 15216-1:2017. The uniform quantitative RT-dPCR results among laboratories in the present study encourage validation against RT-qPCR assays and possible addition of RT-dPCR assays in ISO 15216 in the future, especially for use in routine laboratories.

Besides the independence of DNA standards, RT-dPCR has other advantages, such as more resistance to inhibitory substances (Rački et al, 2014b) that are often co-extracted from food. RT-dPCR detection

proved to be more tolerant to inhibitors for detection of virus in unfiltered RNA from berries (Sun et al., 2019), virus detection in lettuce samples (Coudray et al., 2015) and soft fruits (Fraisse et al., 2017). It is therefore anticipated that the advantage of RT-dPCR will be demonstrated for the analyses of these type of matrices in either multi-centered ILS or proficiency tests. RT-dPCR was also developed to detect other foodborne viruses like hepatitis E virus (HEV) in raw pig livers (Wang et al., 2023) or pork products (Martil-Latil et al., 2016), sapovirus (Varela et al., 2018) or rotavirus in water (Racki et al, 2014a).

The duplex RT-dPCR, as in the present study, was less expensive (roughly 6 Euros) than two separate RT-qPCR assays for one sample (roughly 8 Euros). Especially in small series of samples, the dsDNA dilution series for quantification curve adds to the total costs. Some studies went beyond the duplex, and developed triplex dPCR assays on the Bio-Rad ddPCR (Han et al., 2022; Fang et al., 2023). We experienced that different wild type norovirus strains resulted in fluorescence at different levels (data not shown), probably due to different binding affinities to the primers or probe. Triplex assays on the Bio-Rad ddPCR with two channels only could become complex to interpret in the case of mixed norovirus strain contamination that often occur in filter-feeding bivalve molluscs.

Taken together, to our knowledge this is the first ILS study to apply RT-dPCR for the detection of viral RNA in food. We present an optimized duplex RT-dPCR that performed robustly in all participating laboratories for detection of human norovirus GI and GII in both the absence and presence of oyster matrix. The study indicates that virus nucleic acid measurements using RT-dPCR are likely to increase uniformity of quantitative results between laboratories, compared to the current situation where laboratories apply an RT-qPCR assay in conjunction within-house prepared dsDNA standards, and supports implementation of RT-dPCR in routine analyses.

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Declaration of Generative AI and AI-assisted technologies in the writing process.

During the preparation of this work the author(s) have not used AI technologies in writing.

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Legends to figures

Figure 1 Effect of the RT temperature on the detection of norovirus RNA by RT-dPCR.

Detection of norovirus GI (a, b) and GII (c, d) RNA using a singleplex GI or GII RT-dPCR assay was performed at a range of RT temperatures (46 – 54 ºC). Separation of the positive (blue) and negative (black) droplet populations are shown in panels a and c, with the threshold set manually (pink). The corresponding obtained average target concentrations, measured in duplicate wells, are depicted in panels b and d.

Figure 2 Duplex RT-dPCR assay for norovirus GI and GII RNA.

Norovirus GI (a, c, e) and GII (b, d, f) RNA was detected using the duplex RT-dPCR in an RNA extract from oysters (sample B) (a, b) and in a 1:1 mixture of GI and GII EAC (10^3 genome copies/µl) (c-f). The fluorescence amplitude is depicted in blue for GI RNA positive (NVGG1p-FAM), in green for GII RNA (QNIFS-HEX) and in black for GI and GII RNA negative droplets (a-d). The frequency of droplets at different fluorescence levels as observed in figures c and d are depicted in histograms (e, f), showing the separation between positive and negative droplet populations.

Figure 3 ILS results for norovirus GI and GII RNA dilutions tested by RT-dPCR and RT-qPCR.

Norovirus GI (panels at the left) and GII (panels at the right) RNA was detected in a dilution series of RNA (10^0-10^4 genome copies/µl) and a sample of 10^3 genome copies/µl dsDNA standard (D10+03) by eight (norovirus GI) and seven (norovirus GII) ILS participants using RT-dPCR (top panels) and the respective RT-qPCR assays (bottom panels). Triplicate values for one set of RNAs are depicted in the same color. Levels are expressed as genome copies per µl.

Figure 4 Intermediate precision of the RT-dPCR and RT-qPCR assays in oyster RNA extracts.

Norovirus GI (left) and GII (right) RNA was detected in undiluted (A-C) or diluted (A 1/10, B 1/10, C1/10) RNA from eight sets of samples (A-C) using the duplex RT-dPCR (top panels) and the respective RT-qPCR assays (bottom panels). RNA levels are expressed as genome copies per gram digestive tissue. Triplicates of one set of RNAs (one run) are depicted in the same color. Notably results have not been corrected for the RNA dilution factor.

Figure 5 ILS results of the RT-dPCR and RT-qPCR assays in oyster RNA extracts.

Norovirus GI (left) and norovirus GII (right) RNA was detected in undiluted (A-C) or diluted (A 1/10, B 1/10, C1/10) RNA by eight (norovirus GI) or seven (norovirus GII) ILS laboratories using RT-dPCR (top panels) and the respective RT-qPCR assays (bottom panels). Triplicate values of one set of RNAs are depicted in the same color. RNA levels are expressed as genome copies per gram digestive tissue. Results for EAC (1×10^4 genome copies) added to sample A (panels at the left) and C (panels at the right) or water are presented in genome copies/µl (single measurements).

Table 1 Oligonucleotides for norovirus GI/GII duplex RT-dPCR[a]

Target	Type	Name	Sequences	References
GI	F	QNIF4	CGC TGG ATG CGN TTC CAT	Da Silva et al., 2007
	R	NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC	Svraka et al., 2007
	P	NVGG1p	FAM-TGG ACA GGA-**ZEN**-GAY CGC RAT CT-IBFQ	Svraka et al., 2007
GII	F	QNIF2	ATG TTC AGR TGG ATG AGR TTC TCW GA	Loisy et al., 2005
	R	COG2R	TCG ACG CCA TCT TCA TTC ACA	Kageyama et al., 2003
	P	QNIFS	HEX-AGC ACG TGG-**ZEN**- GAG GGC GAT CG-IBFQ	Loisy et al., 2005

[a] Primers and probes are as mentioned in ISO 15216-1:2017. In present study the probes were modified with extra internal ZEN quencher for use in RT-dPCR only.

Table 2 Quantitative results of the homogeneity test for the 21EFV06 proficiency test samples

Sample	Range of genome copies per gram of digestive oyster tissue [a]	
	norovirus GI	norovirus GII
A	$1.03 \times 10^5 - 1.72 \times 10^5$	$3.13 \times 10^2 - 1.20 \times 10^3$
B	$9.89 \times 10^3 - 1.52 \times 10^4$	$4.44 \times 10^4 - 1.03 \times 10^5$
C	$6.95 \times 10^2 - 2.20 \times 10^3$	$5.06 \times 10^3 - 8.80 \times 10^3$

[a] Ranges based on a 95% confidence interval determined as two geometric standard deviations above and below the geometric mean for ten reference samples.

Table 3 Platforms, detection kits and oligonucleotides for RT-dPCR and RT-qPCR

Lab ID	RT-dPCR Platform and kit [a]	RT-qPCR Platform	Kit [b]	Oligonucleotides [c]
1	Q	Mx3005P, Stratagene	U	TM9
2	B	CFX96™, Bio-Rad	QT	TM9
3	B	QuantStudio, ThermoFisher	U	TM9
4	B	AriaMx, Agilent	U	NVGG1P
5	B	Rotor-Gene Q, Qiagen	G	TM9
6	B	LightCycler 96, Roche	U	TM9
8	B	CFX96™, Bio-Rad	U	NVGG1P
9	T	AriaMx, Agilent	U	NVGG1P

[a] Q= QIAcuity One 5-plex and QIAcuity one-step viral RT-PCR kit (both Qiagen); B= QX200 and One-step RT-ddPCR Advanced kit for Probes (both Bio-Rad); T= QuantStudio 3D Digital PCR and AgPath-ID One-Step RT-PCR Reagents + enhancer (both ThermoFisher); Oligonucleotides as in Table 1

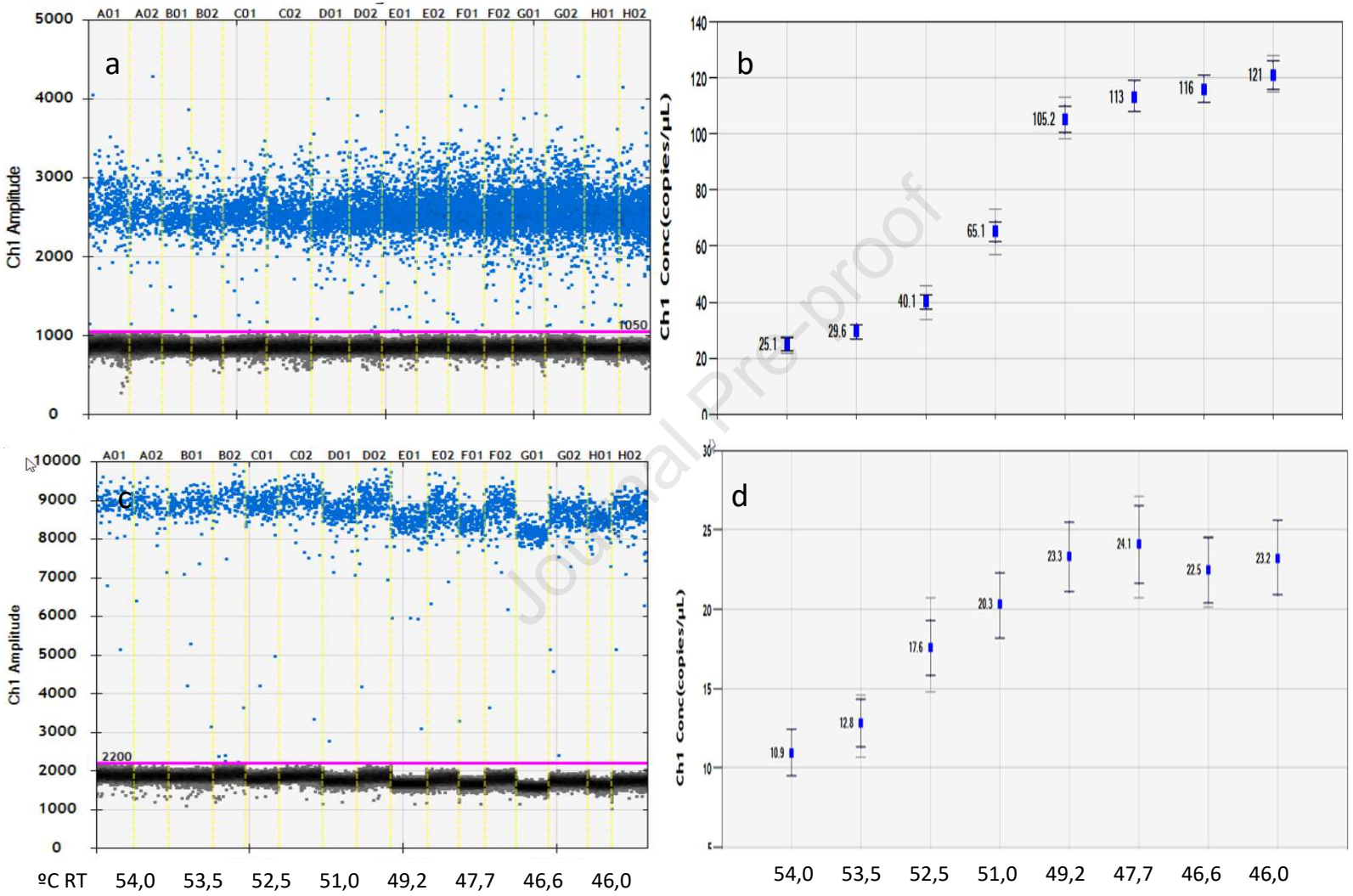
[b] U= RNA Ultrasense One-step quantitative RT-PCR System, ThermoFisher; QT = QuantiTect® Probe RT-PCR kit (Qiagen); G= GoTaq® Probe 1-Step RT-qPCR System (Promega)

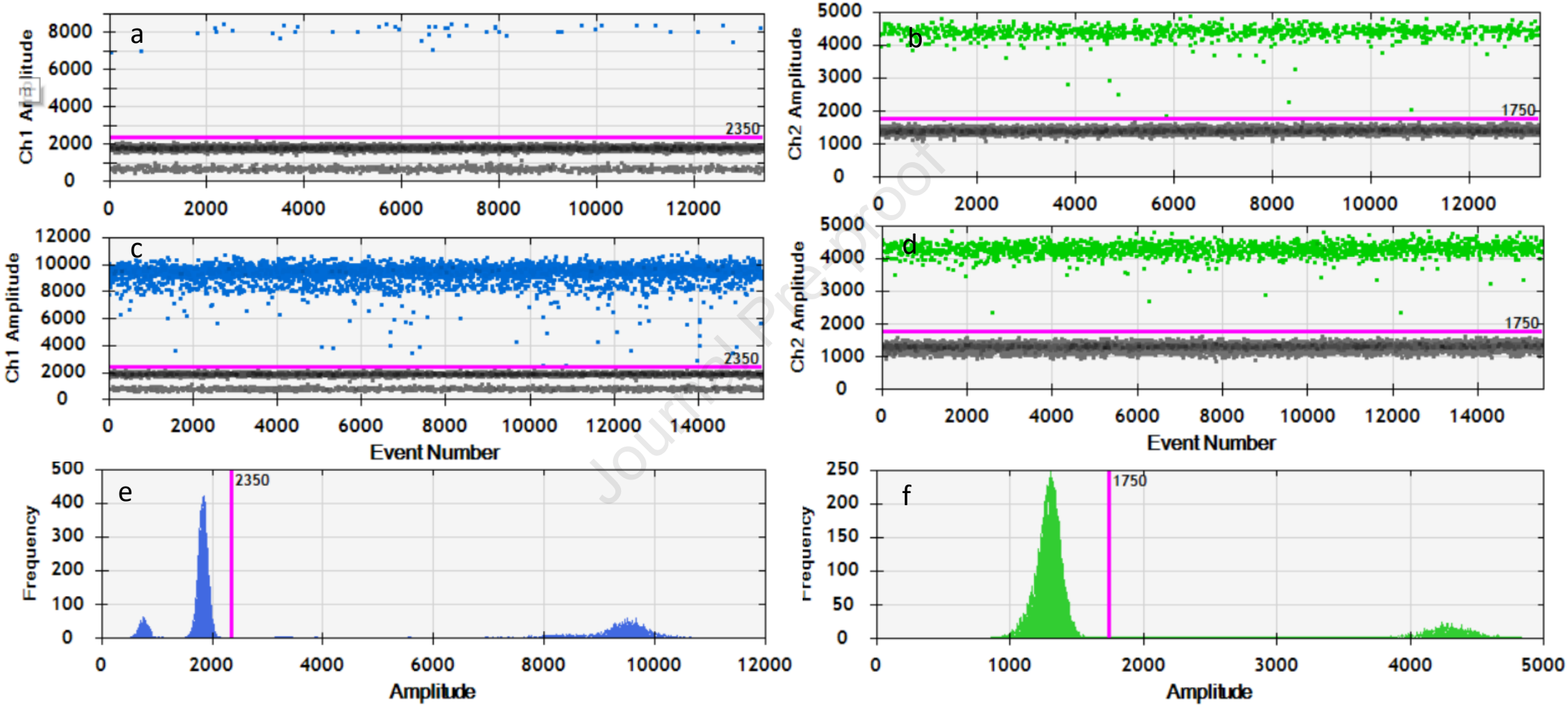
[c] Oligonucleotides for norovirus GI RT-qPCR: QNIF4, NV1LCR, NVGG1p (without ZEN quencher) or TM9 probe (FAM-TGG ACA GGA GAT CGC-MGB-NFQ) (Hoehne and Schreier, 2006). Oligonucleotides for norovirus GII RT-qPCR: QNIF2, COG2R and QNIFs (without ZEN quencer) (for references see Table 1)

Table 4

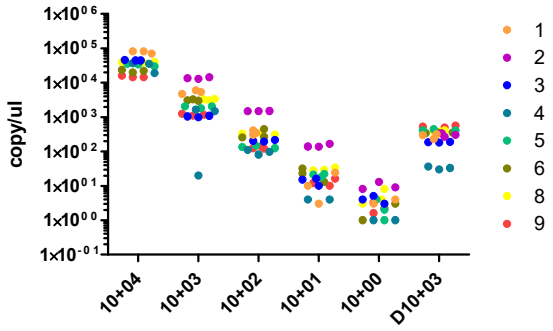
Detection of norovirus RNA in oyster tissue using RT-dPCR and RT-qPCR

	Norovirus GI dPCR (8 laboratories)				Norovirus GII dPCR (7 laboratories)				
	RT-dPCR		RT-qPCR			RT-dPCR		RT-qPCR	
Oyster	avg copies/g	CV%	avg copies/g	CV%	Oyster	avg copies/g	CV%	avg copies/g	CV%
A	6.7×10^4	58.0	1.9×10^5	143.0	B	9.8×10^4	5.2	1.1×10^5	13.2
B	7.0×10^3	54.2	1.9×10^4	123.0	C	8.7×10^3	8.1	1.1×10^4	13.9
C	7.1×10^2	60.0	1.5×10^3	115.2	A	9.1×10^2	38.2	2.0×10^3	42.5
A 1/10	6.9×10^3	53.4	1.9×10^4	144.9	B 1/10	9.4×10^3	11.3	1.4×10^4	12.0
B 1/10	7.9×10^2	71.4	3.0×10^3	124.4	C 1/10	9.5×10^2	33.2	1.4×10^3	20.9
C 1/10	1.4×10^2	66.5	3.6×10^2	116.0	A 1/10	1.6×10^2	71.1	1.6×10^3	61.8





GI RT-dPCR



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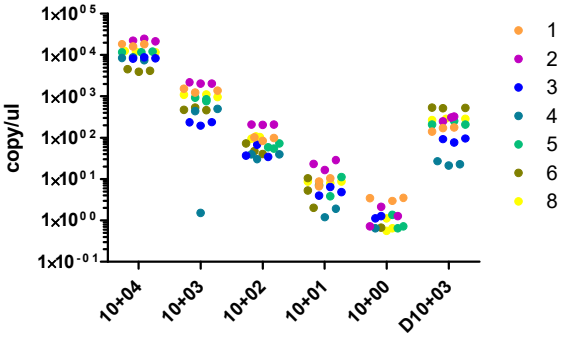

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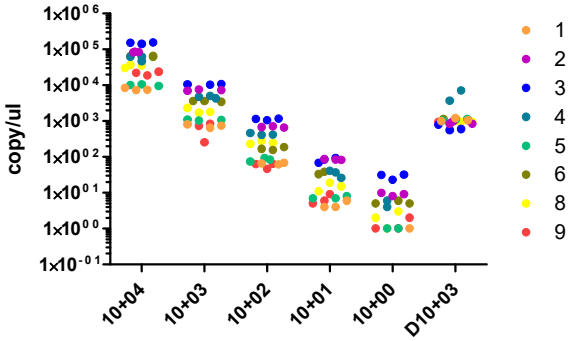

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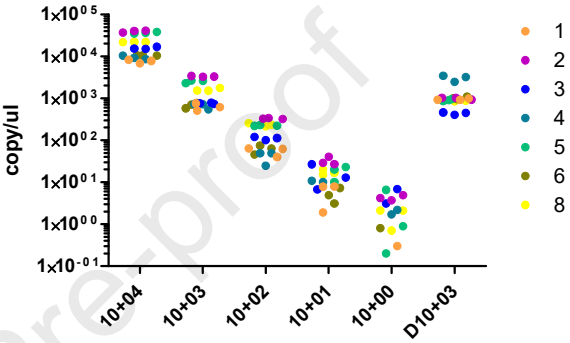

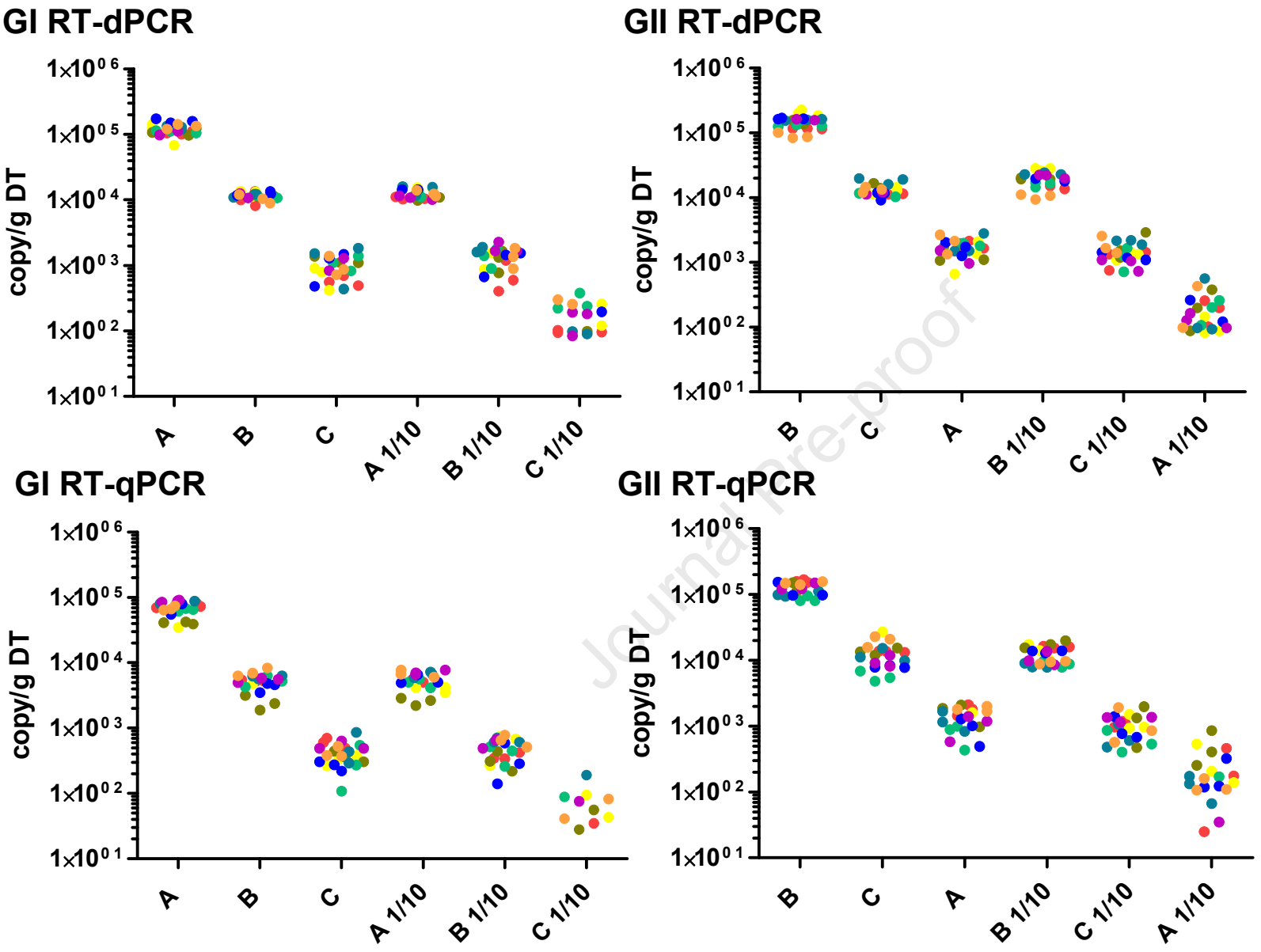

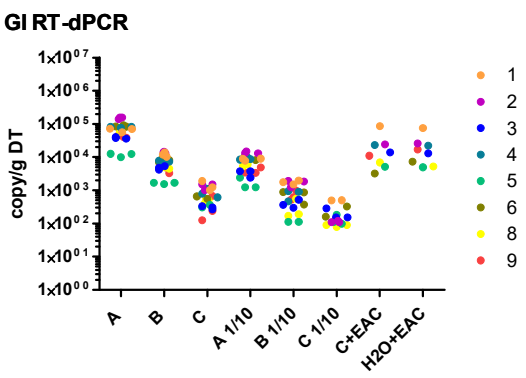

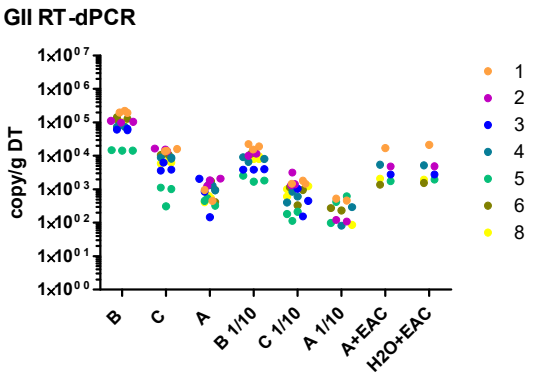

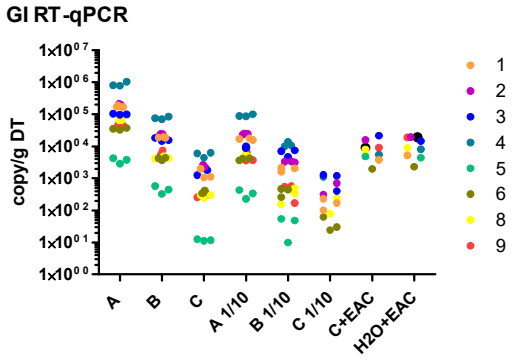

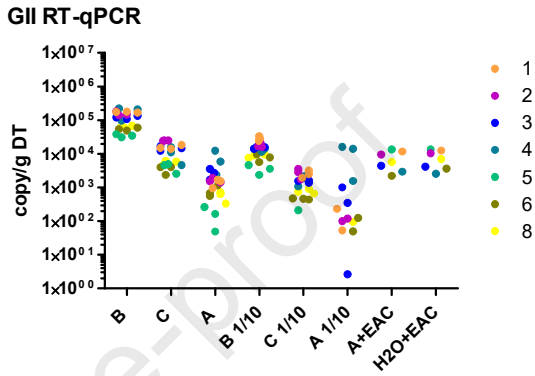

Highlights

- A duplex RT-digital PCR assay was optimized for detection of norovirus GI and GII
- The temperature of the reverse-transcription reaction affected GI and GII detection
- RT-dPCR was compared to ISO-15216 real-time RT-qPCRs in an interlaboratory study
- The ratio between results for RT-dPCR and RT-qPCR varied per participant
- RT-dPCR is likely to increase the interlaboratory uniformity in quantitation of RNA

Editor-in-Chief of Food Microbiology
Prof. Frédéric F. Carlin,
National Research Institute for Agriculture Food and Environment Provence-Alpes-Côte d'Azur, Avignon, France

Wageningen, 9th of October 2023

Dear Prof. F. Carlin,

Please find enclosed our manuscript entitled 'An international inter-laboratory study to compare digital PCR with ISO standardized qPCR assays for the detection of norovirus in oysters' by Boxman and co-workers, which we would like to submit as a Research Article for publication in Food Microbiology.

All authors confirm not to have any conflict of interest with the submitted work and all agree with the final version. This work was funded by the Netherlands Food and Consumer Product Safety Authority (NVWA), the Netherlands, and by the European Union.

Thank you in advance for considering our manuscript for publication in Food Microbiology. We look forward to hearing from you.

Yours sincerely,

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