FS 101040

Work Package 2 Interim Report

Determining the origins of human norovirus RT-qPCR signal

16 December 2014 – Angus Knight

Summary

The European Committee for Standardisation (CEN) have developed and published an ISO standard method for the detection of human noroviruses (hNoVs) in foods (ISO/TS 15216). Unfortunately these methods only specifically detect part of the hNoV genome using signals obtained using the reverse transcription quantitative polymerase chain reaction (RT-qPCR) and cannot determine hNoV infectivity; in addition hNoVs cannot at present be grown in culture. This interim report provides results and a description of progress to date in the development and application of molecular infectivity assays for determining the origins and significance of human NoV RT-PCR signals, their application to the methods already developed in ISO/TS 15216, and the contribution of food and catering premises to hNoV infections in the UK The first of these assays is based upon the measurement of the integrity of the virus coat or capsid and is termed the “capsid integrity assay” or CIA. The second assay is based upon a novel approach termed “VPg immuno-capture RT-qPCR” that attempts to measure both the integrity of the norovirus capsid and its genome simultaneously.

Results obtained using the CIA showed excellent agreement in a collaborative blind trial between three different laboratories using six different hNoV positive stool samples belonging to different hNoV genogroups and genotypes. Although most samples showed extensive exposure of capsid protected RNA following heat treatment, indicative of intact infectious particles, one of the hNoV samples (GII.1) was more resistant requiring increased heat treatment to achieve significant RNA exposure. The hNoV capsid was significantly more stable in water (pH 5.2) than seawater (pH 8.0) or phosphate buffered saline (pH 7.2).The GII.4 hNoV capsid was stable following three freeze thaw cycles at -80^{0}C and at 37^{0}C for 48h showing that the freezing of food and environmental samples is not anticipated to influence infectivity and that the GII.4 virus capsid is persistent *in vitro*.

The application of the CIA to shellfish extract resulted in protein precipitation and reduced extraction efficiency. RNA exposure was not obtained for hNoV probably owing to the low pH of the shellfish extract which appeared to stabilise the hNoV capsid,

although bacteriophage MS2 showed greater exposure in shellfish extracts. Further studies are required in order to optimise the CIA method. In contrast the application of the CIA to hNoV was shown to be unaffected by the chloroform butanol extraction used in the ISO method for hNoV spiked soft fruit.

Results for VPg immuno-capture PCR are at a preliminary stage. Rabbit polyclonal antibodies have been raised and purified against three conserved VPg peptide sequences present in human and murine noroviruses. Pooled antisera from all three peptide domains has been bound to protein A magnetic beads. The resulting VPg immuno-magnetic affinity beads have been directly used in RT-qPCR reactions and shown to capture RNA preferentially from heated norovirus particles (VPg exposed) compared with unheated samples (VPg not exposed). Furthermore since VPg is covalently linked to the genomic RNA the resulting RT-qPCR signal must be derived from largely intact genomic RNA (>5000 nucleotides). This preliminary data shows that this novel method can be applied to simultaneously measure capsid and genomic integrity for the first time.

Table of contents

Summary ..... **I**

Table of contents ..... **III**

List of figures V

List of tables VI

Part 1 Application of the Capsid Integrity Assay (CIA) to CEN methods **1**

Introduction 1

Methods 4

Results 5

Inter-laboratory comparison of the ISO/TS 15216 RT-qPCR method 5

Inter-laboratory comparison of the CIA method 5

RNase activity in hNoV samples 8

Thermal Stability of hNoV GII.1 9

Capsid Stability 12

Freeze-thaw stability of GII.4 hNoV 14

Application of the CIA to Shellfish 15

Effect of the Proteinase K step on extraction efficiency of hNoV from shellfish using the ISO/TS 15216 method 17

Application of the CIA to hNoV in shellfish extracts 17

Application of the CIA to berries and leafy greens 22

Application of the CIA to raspberries 23

Application of the CIA after freezing of fresh raspberries spiked with hNoV strains 24

Discussion 27
Capsid Integrity Assay 27
Application of the CIA to Shellfish 28
Application of the CIA to berries and leafy greens 29
Part 2 Application of a combined capsid and genomic integrity assay to CEN methods 30
Introduction 30
Methods 33
Results 34
Identification of conserved sequences 34
Development of VPg immuno-capture 34
Testing of VPg immuno-capture PCR 34
Discussion 36
References 37
Appendix 1 39
Protocol for determining the exposure of capsid RNA using RT-QPCR and RNase ONE 39
RNA extraction 41
Qiagen Method 41
RT-qPCR 42

List of figures

Figure 1. Cq values obtained for the different hNoV samples obtained in the different laboratories at the commencement of this study 5

Figure 2. ΔCq values obtained from a blind inter-laboratory comparison of the CIA using the six different 0.1% (v/v) hNoV samples comparing values obtained following heat treatment at 80^{0}C for 2 minutes with control values obtained after heating to 80^{0}C for 2 minutes without added RNase. * denotes a ΔCq > 8 6

Figure 3. ΔCq values obtained from a blind inter-laboratory comparison of the CIA using the six different hNoV 0.1% (v/v) stool samples and comparing results obtained following heat treatment at 80^{0}C for 2 minutes with added RNase compared with unheated (4^{0}C) controls without added RNase. * denotes a ΔCq > 8. 8

Figure 4. Endogenous RNase activity present in the six diluted (0.1% v/v) hNoV stool samples before [■] and after heating [■] to 80^{0}C for 2 minutes. 9

Figure 5. Cq values obtained using the CIA and 0.1 – 0.001 % (v/v) dilutions of GII.1 stool sample before [■] and after heating [■] to 80^{0}C for 2 minutes. 10

Figure 6. LOC capillary electrophoresis of RT-qPCR products derived from GII.1, GII.4 and GII.5 samples. 11

Figure 7. ΔCq values obtained using the CIA following heating at different temperature for 2 minutes for the GII.1 sample [■] in comparison to the GII.4 sample [■]. 12

Figure 8. Accelerated stability testing of GII.4 hNoV at 37^{0}C, with RNase treatment [■] and without RNase treatment [■] after heating at 80^{0}C for 2 minutes and at 4^{0}C [■]. . 13

Figure 9. Comparison of hNoV capsid stability in the CIA assay following heat treatment at 80^{0}C for 2 minutes for 0.1% (v/v) hNoV stool samples diluted in PBS (pH 7.2), artificial seawater (pH 8.0) and water (pH 5.2), heated with RNase treatment [■] heated without RNase treatment [■] and at 4^{0}C [■]..... 14

Figure 10. ΔCq values obtained using the CIA following three freeze thaw cycles at -80ºC in PBS or water. 15

Figure 11. Reductions in infectivity of MS2 FRNA bacteriophage in a norovirus/MS2 mix following a time-course of germicidal UV exposure..... 16

Figure 12. CIA data obtained using A) hNoV GII and B) MS2 RT-qPCR assays from a norovirus/MS2 mix following a time-course of germicidal UV exposure. For each data set results are shown following incubation; heated with RNase treatment [■] heated without RNase treatment [■] and at 4^{0}C [■]..... 17

Figure 13. Endogenous RNase activity and added RNase I activity in PBS, shellfish extract, shellfish extract in 10X PBS, and in GII.4 hNoV (data supplied by LFR) 22

Figure 14. ΔCqs obtained from the CIA on 6 hNoV strains 3 months after the interlaboratory comparison 23

Figure 15. MNV-1 VPg and NV VPg share significant amino acid sequence identity. Sequences encoding MNV-1 VPg and NV VPg were aligned with ClustalW. Asterisks indicate identical residues. Semicolons are conservative substitutions. Amino acid numbers correspond to the position in the ORF1 polyprotein. Reproduced from Daughenbaugh *et al.,* 2006 31

List of tables

Table 1. Cq or copy number measurements A, B, C and D required for measuring capsid integrity 2

Table 2. hNoV stool samples provided by PHE 4

Table 3. Application of the CIA to shellfish extracts. NC = ΔCq not calculated (one or other result negative), NQ = ΔCq not quantifiable (one or other result less than the limit of quantification), x = no reference ΔCq available (naturally contaminated samples). .. 21

Table 4. Cq values obtained from RT-qPCR amplification of hNoV GII.4 sequences extracted from spiked-raspberry extracts subjected to the capsid integrity assay. 24

Table 5. Cq values obtained from RT-qPCR amplification of hNoV sequences following the CIA using fresh or frozen raspberries. 25

Table 6. hNoV stool samples provided by PHE 33

Table 7. Results of VPg magnetic immuno-capture PCR using dilute 0.1% hNoV stool samples and purified GII.4 RNA. 35

Part 1 Application of the Capsid Integrity Assay (CIA) to CEN methods

Introduction

To comply with developing food safety legislation the European Committee for Standardisation (CEN) have developed standardised methods for the detection of human NoVs (and also Hepatitis A virus) in food and environmental samples; these methods were published as an ISO in 2013 (ISO/TS 15216). These detection methods are based upon quantitative reverse transcription polymerase chain reaction (RT-qPCR) or qualitative RT-PCR. This study reports data from RT-qPCR studies. Unfortunately these methods cannot determine if the signals obtained originate from infectious or non-infectious virus particles. There is therefore a potential problem in assessing the risk to human health from samples detected as positive in both RT-qPCR and RT-PCR assays and it would be beneficial to determine if these RT-qPCR signals originate from infectious particles (Knight *et al*., 2012). The objective of Work Package 2 (WP2) of this project is to investigate if it is possible to differentiate between infectious and non-infectious norovirus in food matrices using molecular approaches and to develop a standard operating procedure for with minimal modifications to ISO/TS 15216methods. The first of these is based upon measuring the integrity of the virus capsid using a capsid integrity assay (CIA) (reported in Part 1) and the second (reported in Part 2) is based upon a combined measurement of capsid and genomic integrity (VPg immuno-capture). It is anticipated that this research will allow the development of standard operating procedure to be used in surveys of fresh produce and shellfish in WP3 and WP4 of this project in parallel to testing using the unmodified ISO methods.

The loss of capsid integrity and infectivity following high heat treatment (typically 70-80^{0}C for 2 minutes) in RNA viruses is accompanied by exposure of virus RNA from intact virus particles resulting in particles with a decreased sedimentation coefficient as determined by ultra centrifugation (termed 80S empty capsids). This exposure of RNA

from intact and infectious virus particles can be measured using a combination of RNase treatment and RT-qPCR in a capsid integrity assay (CIA). This observation can be applied in order to investigate the origins of RT-qPCR signals from intact presumed infectious particles.

RNA within intact particles is protected from RNase but is exposed and digestible by RNase following heat treatment. RNase digestion is never complete owing to residual protein RNA complexes, termed ribonucleoprotein or RNP. Free “naked” RNA is digested by RNase, and RNPs are resistant to both RNase and heat treatment, reviewed by (Knight, *et al*., 2012).

A typical CIA experiment e.g. to determine capsid integrity after heating to 80^0C for 2 minutes, measures RT-qPCR signals before and after heat treatment, with or without added RNase. The RNase is added to ensure that the maximum digestion of any RNA exposed from intact virus following heat treatment is achieved. The CIA requires four different measurements resulting in copy number/Cq determinations and results A, B, C, and D for each sample as shown in Table 1. Absolute copy number determination is not actually required since results are compared within the same experiment resulting in ΔCq values, this minimises errors in RT-qPCR associated with comparisons using separate determinations.

Table 1. Cq or copy number measurements A, B, C and D required for measuring capsid integrity

	Unheated control	**Heat treatment (e.g. 80^0C 2min)**
- RNase digestion	A	C
+ RNase digestion	B	D

Results from A measure the starting copy number. Results from B show that free “naked RNA” is or is not present in the sample when compared with results from A. Results from C show that the heat treatment does or does not affect the starting copy number when compared with results from A that may result from the effect of heat

treatment on extraction efficiency or endogenous RNase activity. Finally, results from D when compared with results from A show that the heat treatment does or does not result in the exposure of RNase sensitive RNA.

In practice control B is not required and results are identical to A since “naked” RNase sensitive RNA is not present in clinical sample or tissue culture virus lysates owing to endogenous RNase activity. Additionally, all published literature (based upon RNA transfection studies and RT-qPCR) shows that heat treatment < 90 ^{0}C affects the virus capsid and does not degrade RNA sufficiently to influence small fragment RT-qPCR assays; consequently control C is also not required absolutely. This means in practice that the three results from A, B and C are the same. Results from D in comparison to any of the controls A, B or C shows if exposure of RNase sensitive RNA from the intact virus capsid has occurred following heat treatment (indicative of intact virus particles). The purpose of adding RNase is to ensure as far as possible that any endogenous RNase activity goes to completion. Typical results for D obtained from infectious virus particles using surrogate feline calicivirus (FCV) show a Δ 5-6 Cq increase when compared with A, equivalent to a 98% reduction in the starting copy number and corresponding to a predicted > 4 log reduction in infectivity following heat treatment at 62^{0}C for 2 minutes. Similar reductions were observed for three epidemiologically unlinked GII.4 NoVs, (Topping *et al*., 2009) resulting in predicted hNoV inactivation following heat treatment at 76^{0}C for 2 minutes. Based upon published studies, the residual 2 % RT-qPCR signal that remains following heat treatment is resistant to RNase digestion and protected by protein and corresponds to ribonucleoprotein complex (RNP). The persistence and occurrence of RT-qPCR signals derived from RNPs in food and environmental samples is unknown. This study attempts to apply the CIA assay to the methods in ISO/TS 15216 with minimal method modification.

Methods

Following initial assessment by participants, a standard operating procedure (SOP) for the CIA assay was provided by LFR (Appendix 1). Unless otherwise stated the CIA used a heat treatment at 80^0C for two minutes. This temperature and time combination was selected based upon LFR published data showing that maximal exposure of capsid RNA required a minimum heat treatment of 76 ^{0}C for 2 minutes for GII.4 hNoVs (Topping *et al*., 2009). RT-qPCR and RNA extraction from shellfish and berries was according to ISO/TS 15216. Unless otherwise stated all experiments were performed at least in duplicate with triplicate RT-qPCR. RNase activity was measured using a commercially available kit (RnaseAlert Life Technologies) as relative fluorescence units (RFU) at 525nm. Lab on a Chip (LOC) capillary electrophoresis (Agilent) was in accordance with manufacturer's instructions and used 1 µl RT-qPCR product. hNoV positive stool samples were kindly supplied by D. Allen, Public Health England (PHE) and are listed in Table 2. Viable FRNA bacteriophage was enumerated using a double overlay plaque assay method as described in ISO 10705-1.

Table 2. hNoV stool samples provided by PHE.

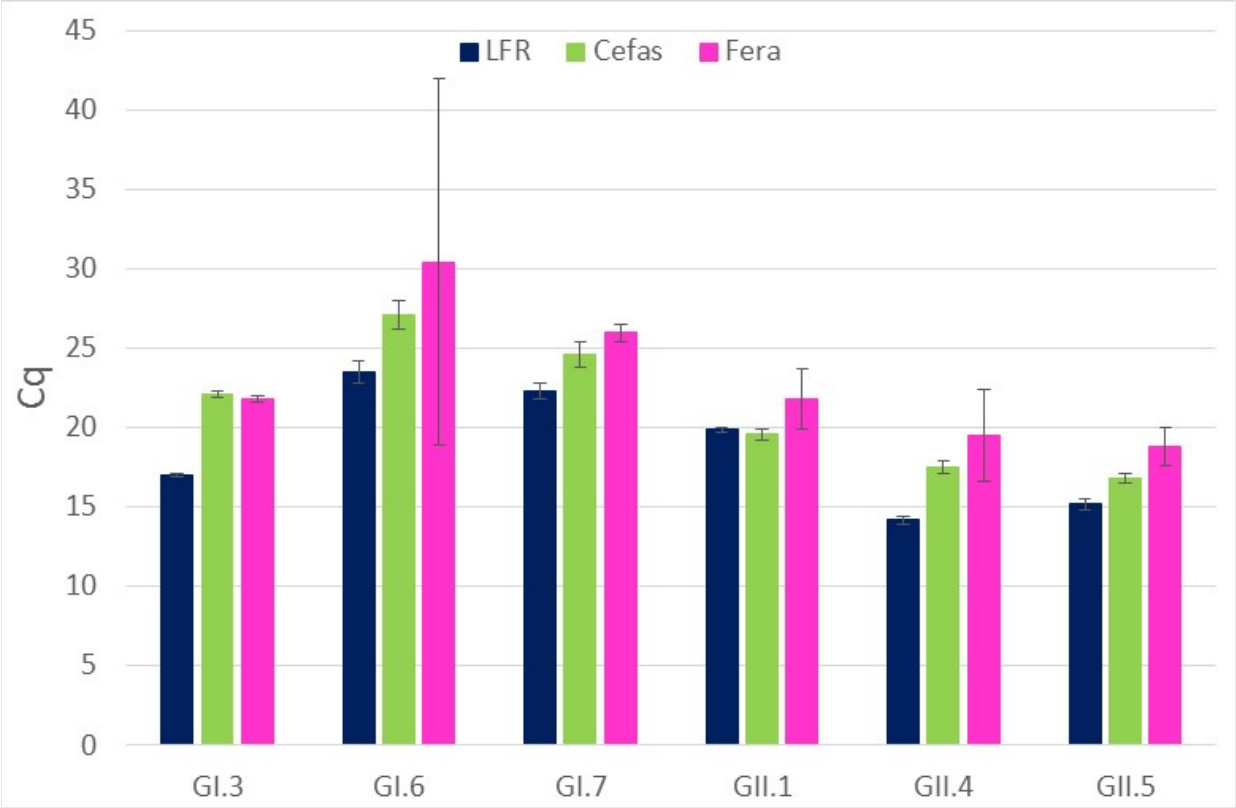
Stool sample	Norovirus genotype
NVREFFS0001	GI.3
NVREFFS0002	GI.6
NVREFFS0003	GI.7
NVREFFS0004	GII.1
NVREFFS0005	GII.4
NVREFFS0006	GII.5

Results

Inter-laboratory comparison of the ISO/TS 15216 RT-qPCR method

Independent blind RT-qPCR of hNoV samples using the ISO real-time PCR protocol showed that Cq determinations obtained from the three test laboratories were very similar despite differences in sampling, reagents, equipment, personnel, and extraction methods (Figure 1).

Figure 1. Cq values obtained for the different hNoV samples obtained in the different laboratories at the commencement of this study.



Inter-laboratory comparison of the CIA method

Each participant evaluated the CIA assay in a blind study according to the supplied SOP using independent stool samples and dilution in PBS to 0.1% (v/v), the ISO RT-qPCR protocol (with DNA standards supplied by Cefas), independent sources of all reagents and different RNA extraction protocols (Cefas, Biomerieux, LFR and FERA,

Qiagen). Following confirmation by partners during the course of this study control B was not performed in all experiments, since no evidence could be found for the occurrence of “naked” RNase sensitive RNA in any of the samples tested (in agreement with published data).

Results for heat treatment at 80^0C for 2 minutes are shown in Figures 2 & 3. Figure 2 shows the ΔCq values obtained in the different laboratories comparing results obtained following heat treatment at 80^0C for 2 minutes, followed by either addition of RNase and incubation at 37^0C for 15 minutes or RNase buffer alone i.e. comparing results C and D above. The results were very similar between laboratories with 4/6 isolates showing significant RNA exposure (ΔCq values of 5-6 or greater), however two hNoV samples (GI.3 and GII.1) showed minimal exposure.

Overall the comparison of ΔCq values obtained between the different laboratories shown in Figure 2 & Figure 3 was very similar showing that the heating of samples without adding RNase did not affect the ΔCq value obtained. However the GI.3 sample which showed a minimal differences in ΔCq in comparison with the heated control without added RNase (as shown in Figure 2.) shows a significant (>6) ΔCq increase when compared with the unheated control. However, and in contrast, the GII.1 sample showed minimal ΔCq differences upon heat treatment independent of the choice of control.

Figure 2. ΔCq values obtained from a blind inter-laboratory comparison of the CIA using the six different 0.1% (v/v) hNoV samples comparing values obtained following heat treatment at 80^{0}C for 2 minutes with control values obtained after heating to 80^{0}C for 2 minutes without added RNase. * denotes a ΔCq > 8.

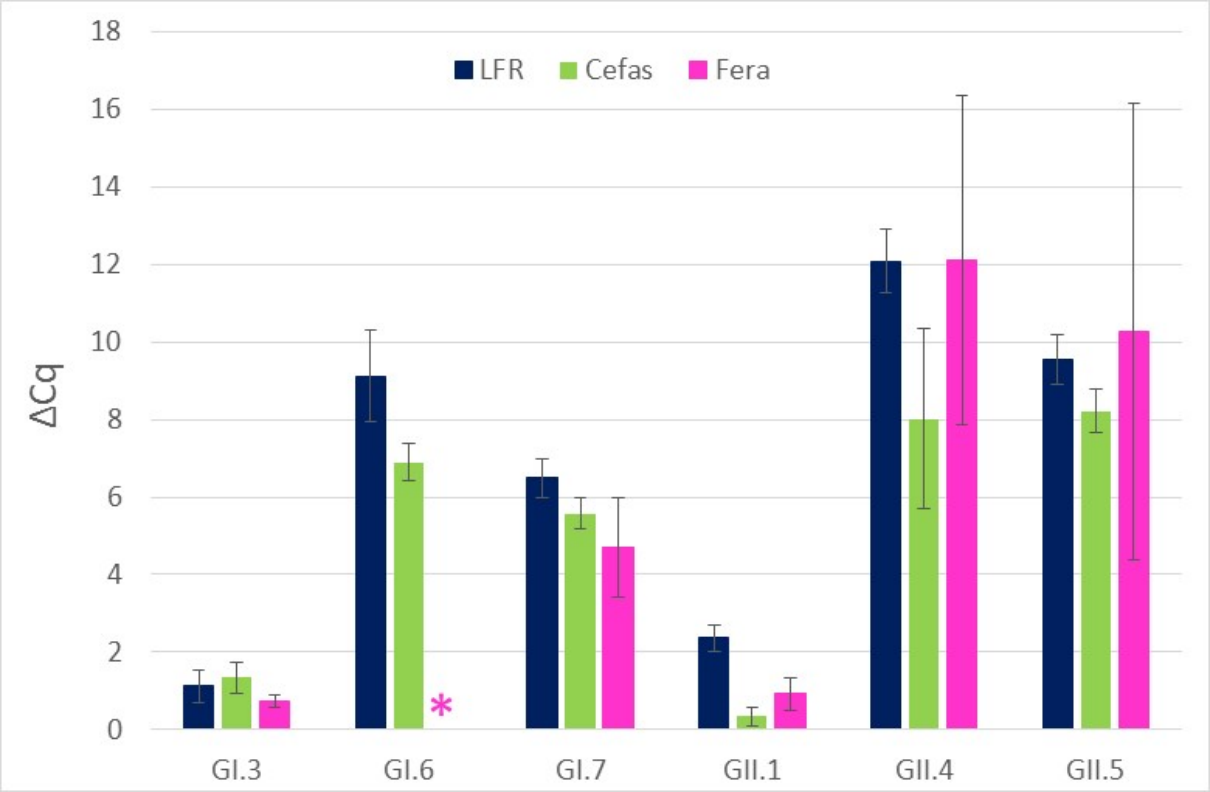
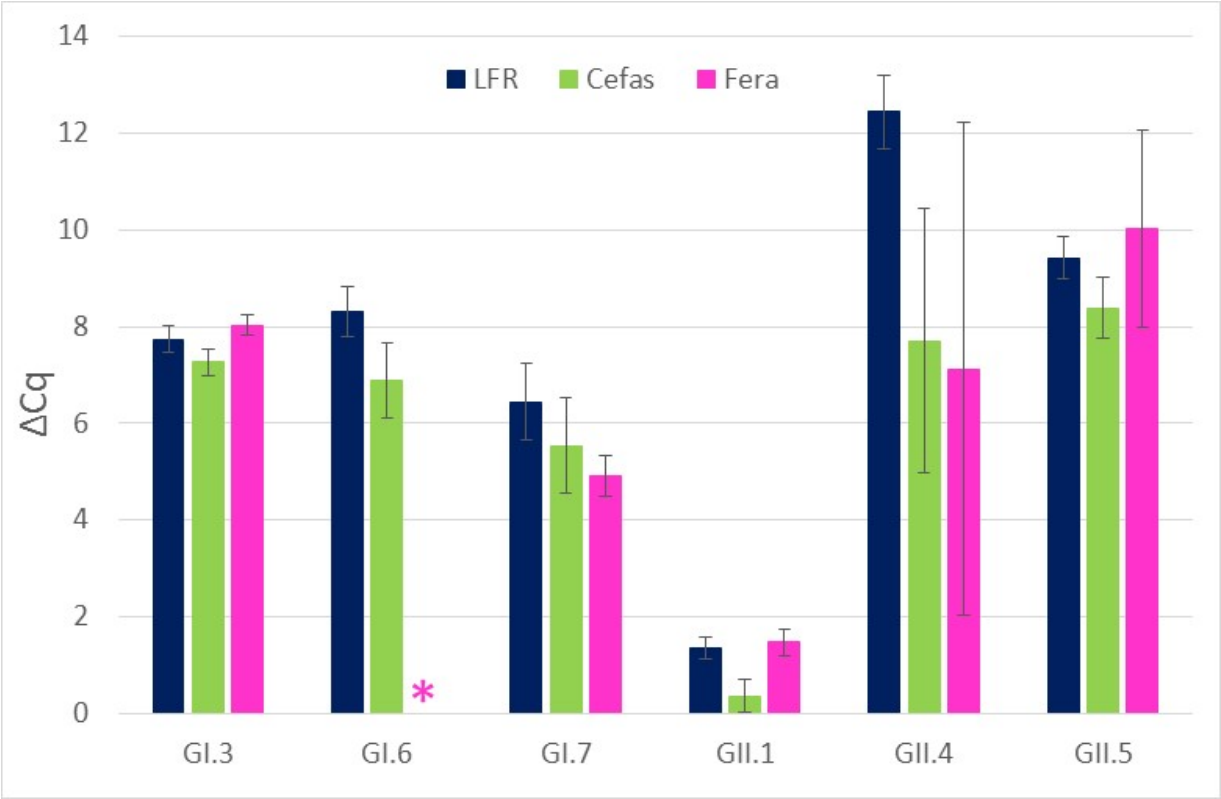


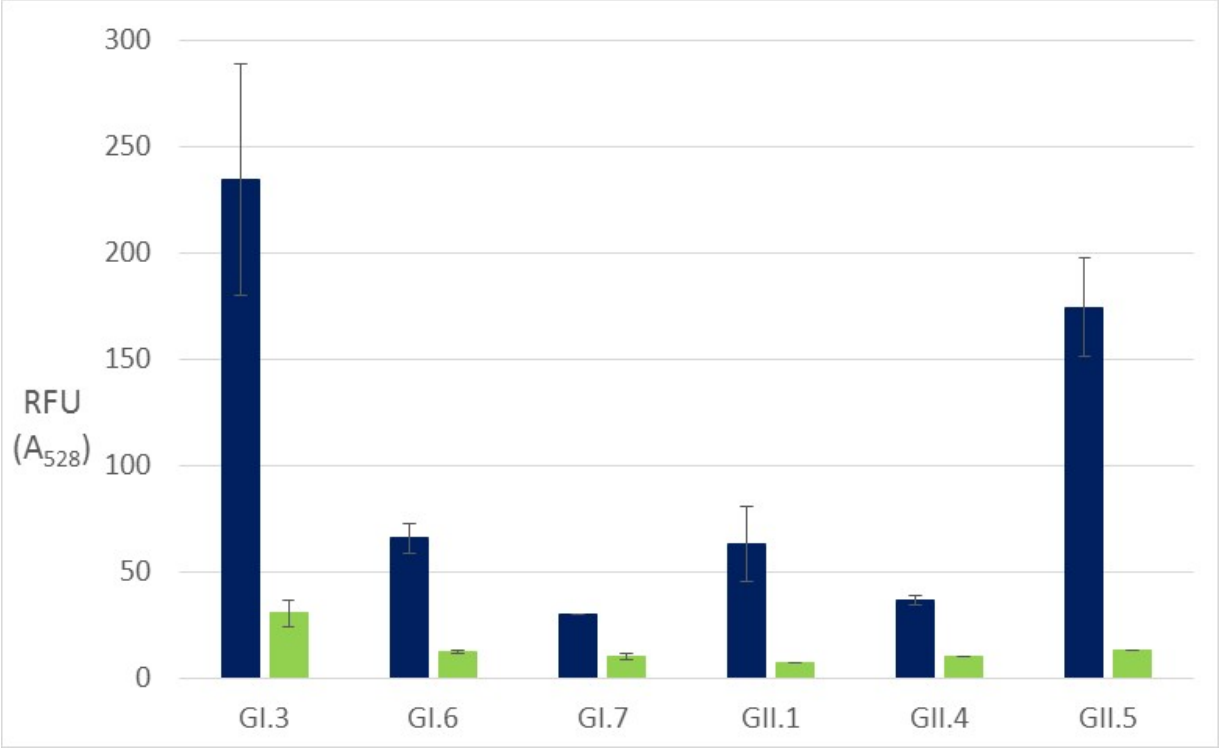
Figure 3. ΔCq values obtained from a blind inter-laboratory comparison of the CIA using the six different hNoV 0.1% (v/v) stool samples and comparing results obtained following heat treatment at 80^{0}C for 2 minutes with added RNase compared with unheated (4^{0}C) controls without added RNase. * denotes a ΔCq > 8.



RNase activity in hNoV samples

The differences in ΔCq values obtained for the GI.3 strain following heat treatment in comparison to an unheated control was considered attributable to endogenous RNase activity present in the sample. Therefore RNase activity was measured in the diluted extracts using a commercially available kit (Figure 4).

Figure 4. Endogenous RNase activity present in the six diluted (0.1% v/v) hNoV stool samples before [■] and after heating [■] to 80^{0}C for 2 minutes.

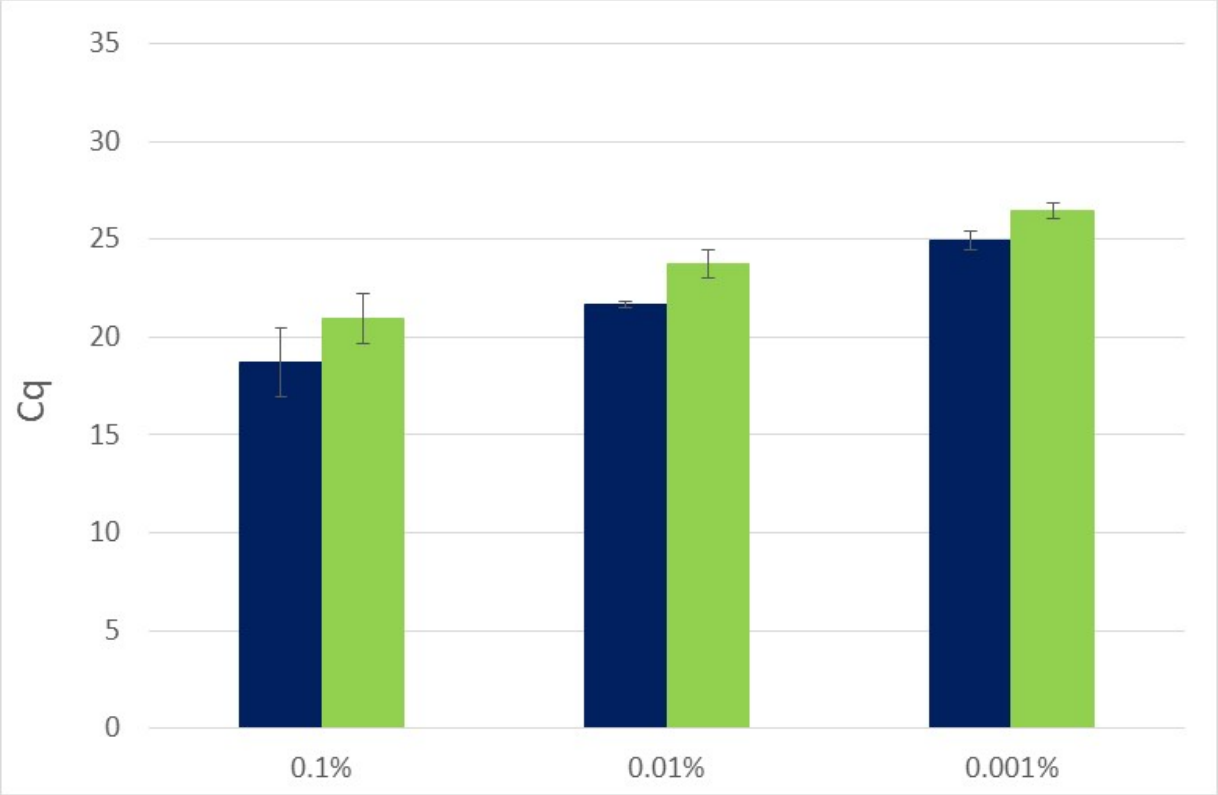


These data also show that data comparison using the CIA only requires a comparison between a heated sample + RNase and an unheated control sample i.e. results from D (typically heating to 80^{0}C for 2 minutes followed by RNase digestion) vs A (unheated, +4^{0}C control) this eliminates the need for controls B and C and consequently minimises the impact of sample splitting when testing very low Cq value samples typically found in foods and the environment.

Thermal Stability of hNoV GII.1

The apparent failure of the GII.1 sample to expose RNA following heat treatment in comparison to the other hNoV samples suggested that this was owing to either a matrix effect, or an intrinsic property of the virus capsid within this sample resulting in an increased thermal stability of the virus capsid or a potentially false positive result. To investigate any dilutable matrix effect the sample was further diluted and the ΔCq values measured. Figure 5 shows the effect of further dilution of the sample.

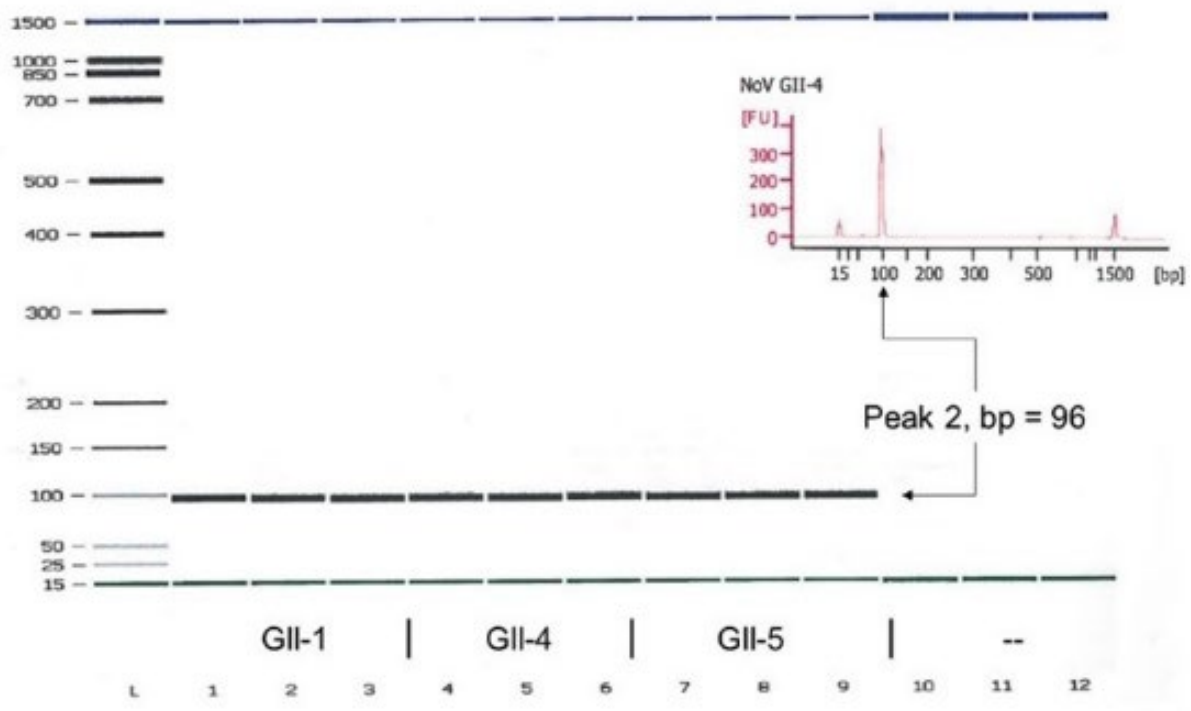
Figure 5. Cq values obtained using the CIA and 0.1 – 0.001 % (v/v) dilutions of GII.1 stool sample before [■] and after heating [■] to 80^{0}C for 2 minutes.



The data shows that the ΔCq values obtained using different dilutions of stool samples for the heat resistant GII.1 sample did not change significantly showing that there was not a dilutable matrix effect resulting in increased capsid stability. Additionally spiking the GII.1 with GI.6 hNoV in GI RT-qPCR did not confer a protective effect on the GI.6 sample (data not shown) again showing that no diffusible protective effect was present.

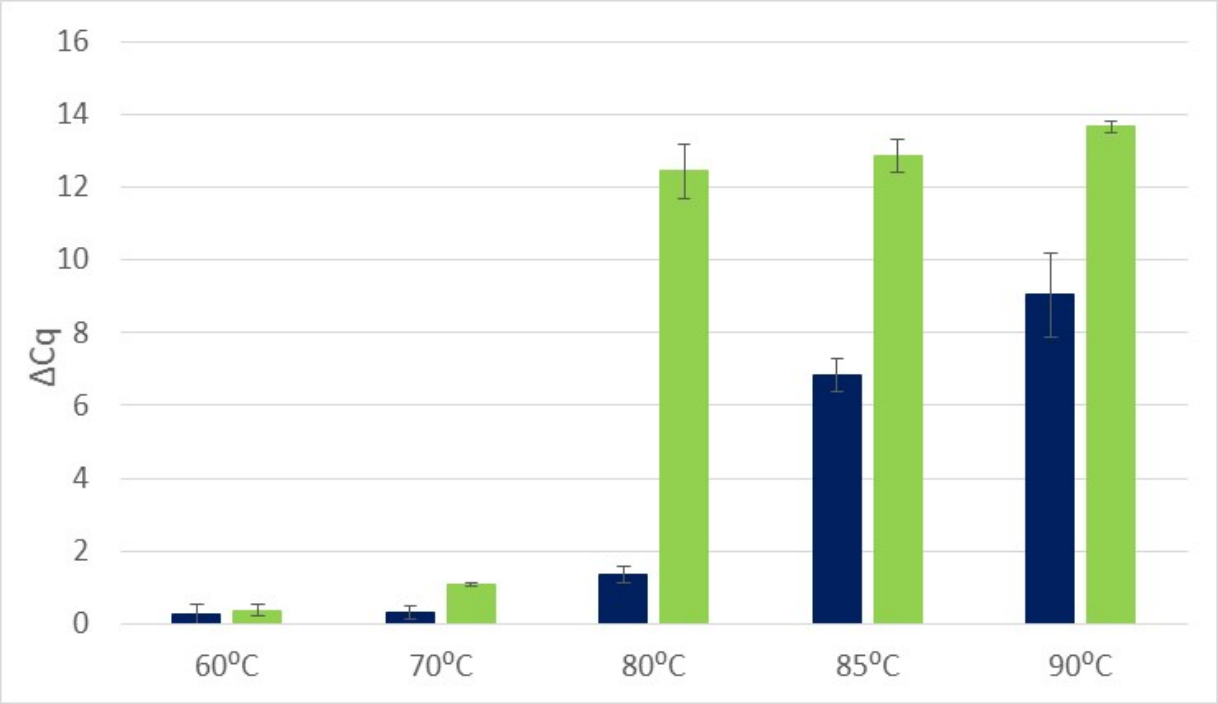
Evidence that the GII.1 RT-qPCR product was of the expected size and not owing to a false positive was provided by Lab-on-a- Chip (LOC) capillary electrophoresis (Figure 6).

Figure 6. LOC capillary electrophoresis of RT-qPCR products derived from GII.1, GII.4 and GII.5 samples.



Finally the thermal stability of the GII.1 sample was compared with that of the GII.4 sample (Figure 7).

Figure 7. ΔCq values obtained using the CIA following heating at different temperature for 2 minutes for the GII.1 sample [■] in comparison to the GII.4 sample [■].

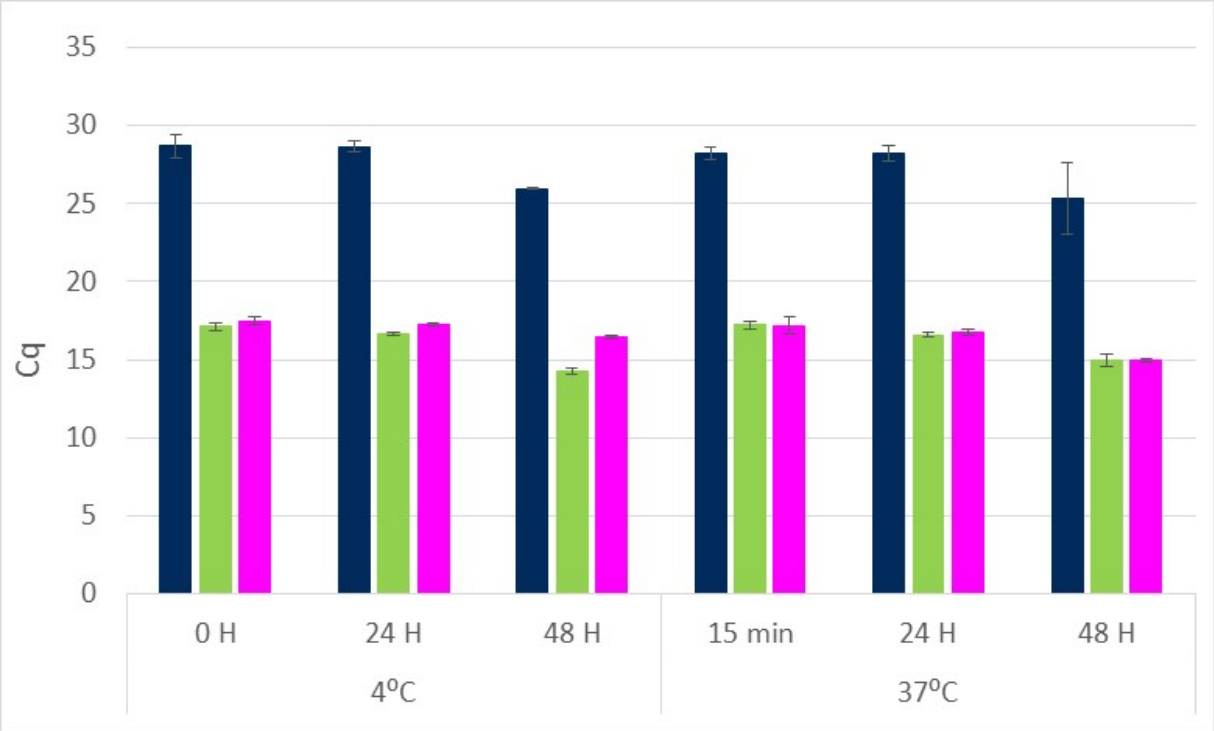


Capsid Stability

Thermal stability of GII.4 hNoV at 37^{0}C

Although heat treatment results in the formation of RNase resistant RT-qPCR signals due to RNP there are currently no data to show that RNP occurs naturally. We therefore conducted an accelerated test by incubating 0.1% hNoV in PBS at 37^{0}C for 48hr in comparison to normal storage at 4^{0}C. The results are shown in Figure 8.

Figure 8. Accelerated stability testing of GII.4 hNoV at 37^{0}C, with RNase treatment [■] and without RNase treatment [■] after heating at 80^{0}C for 2 minutes and at 4^{0}C [■].



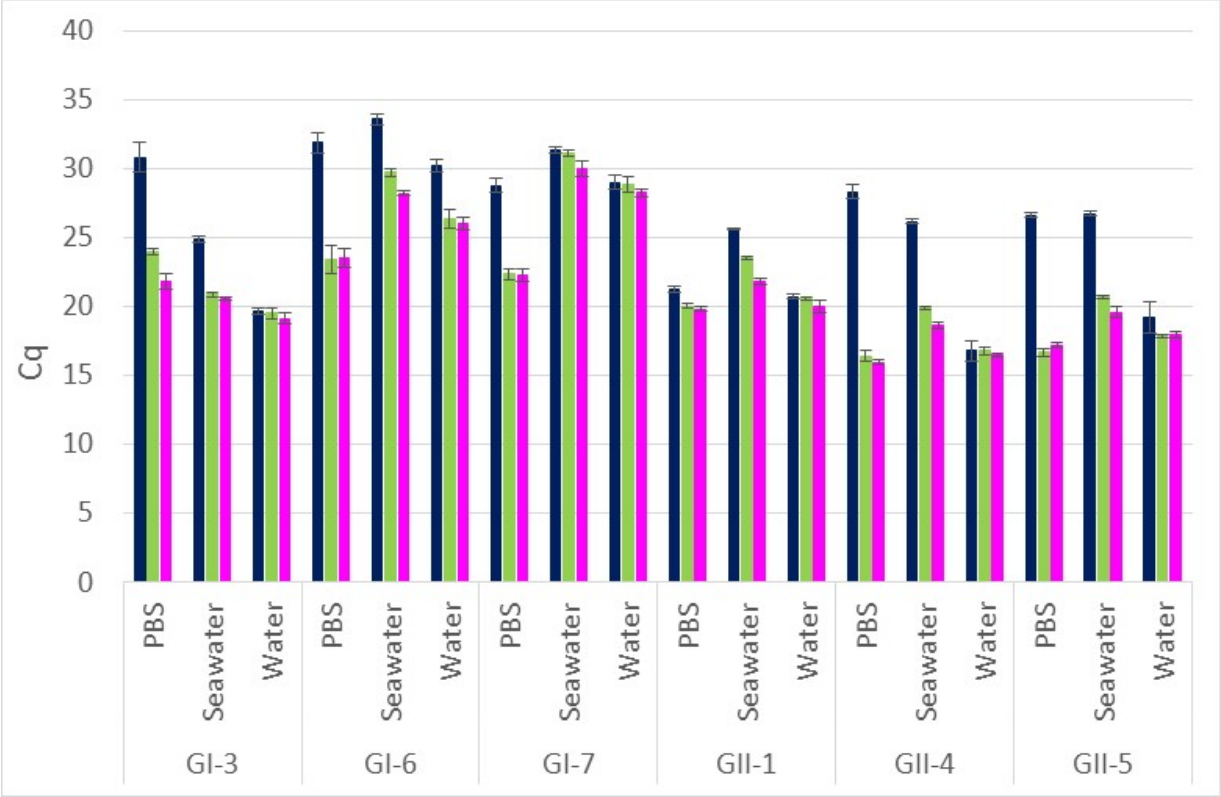
The results (Figure 8) show that the GII.4 hNoV sample was stable at 37^{0}C for 48h in comparison to the controls kept at 4^{0}C.

Capsid stability in PBS, water, and seawater

Capsid stability was determined in PBS, water, and finally in seawater using the CIA and heat treatment at 80^{0}C for 2 minutes to assess the impact of simple environmental conditions on capsid stability. As anticipated RNase I activity was unaffected by PBS, water, or seawater (data not shown), For GI.3, GI.4 and GII.5 hNoV samples the capsid protected RNA was exposed when heated in PBS or seawater but was stabilised in water. The GI.6 sample showed exposure of capsid protected RNA in PBS, seawater and to a lesser extent in water. Results for GII.1 did not expose capsid protected RNA at 80ºC in PBS (as already shown at this temperature above) in water or seawater. The GI.7 sample showed exposure of capsid protected RNA in PBS but not in water or seawater; however this difference was considered attributable to a sample storage effect since repeat analysis in PBS showed an increased starting Cq value with concomitant loss of the ability

of the capsid to expose capsid protected RNA. A similar overall effect is seen for the seawater test samples where the capsid exposure was often decreased compared with PBS but was accompanied by an increased starting Cq suggesting that intact particles had become degraded with concomitant loss of capsid integrity resulting in residual RNP.

Figure 9. Comparison of hNoV capsid stability in the CIA assay following heat treatment at 80^{0}C for 2 minutes for 0.1% (v/v) hNoV stool samples diluted in PBS (pH 7.2), artificial seawater (pH 8.0) and water (pH 5.2), heated with RNase treatment [■] heated without RNase treatment [■] and at 4^{0}C [■].

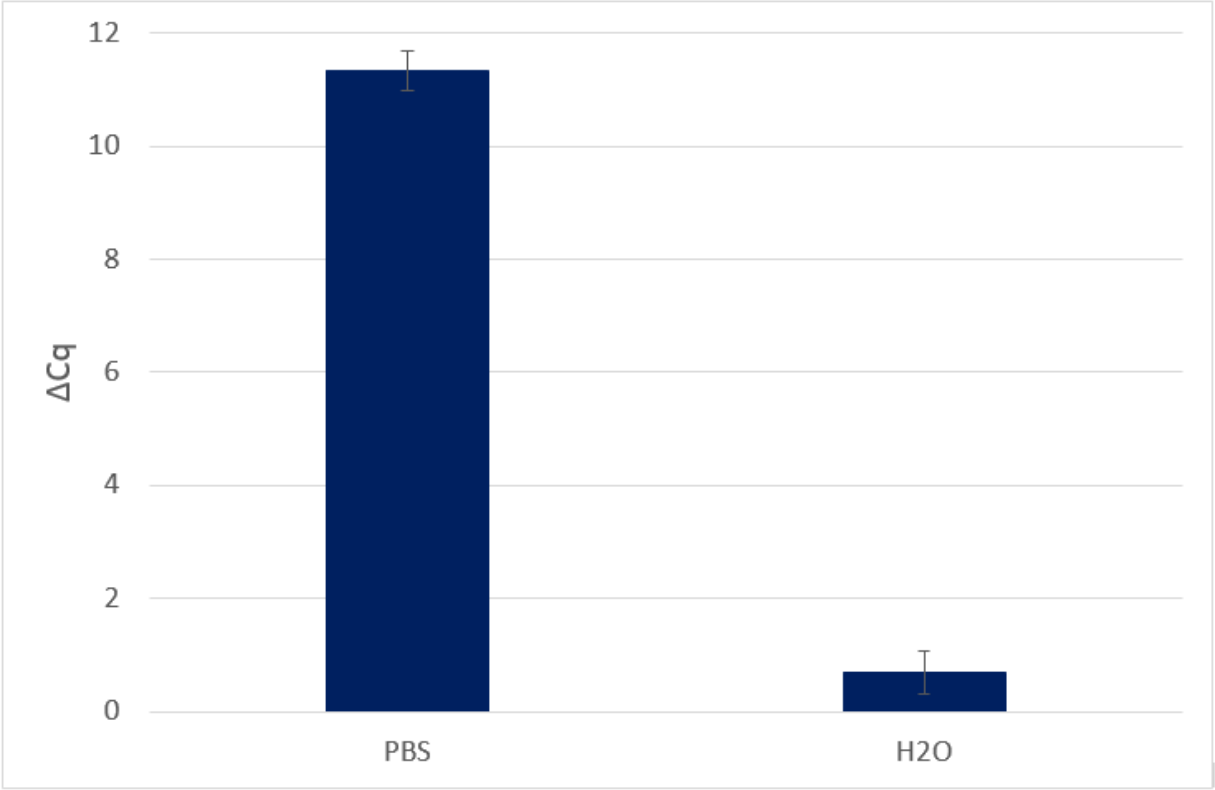


Freeze-thaw stability of GII.4 hNoV

Freeze-thaw stability of the GII.4 hNoV capsid was investigated in water and PBS by repeatedly (3X) freezing 0.01% hNoV samples (100 µl) at -80^{0}C for 1 hour followed by defrosting at 4^{0}C for 10 minutes and then testing for capsid integrity using the CIA. The results are shown in Figure 10. The results for PBS show that GII.4 hNoV continues to expose capsid protected RNA following three freeze thaw cycles indicating that the capsid remained intact. However freezing in water appeared to result in a loss of the ability of the capsid to expose RNA suggesting that the capsid had either been

damaged resulting in RNP or stabilised by the water. This was further investigated by freeze thawing 10% hNoV stool in water followed by dilution in PBS. The results showed that the GII.4 capsid regained the ability to expose RNase sensitive RNA and therefore that water stabilised the capsid (data not shown) consistent with the observed increased capsid stability when hNoVs were analysed using the CIA following dilution in water (above).

Figure 10. ΔCq values obtained using the CIA following three freeze thaw cycles at -80ºC in PBS or water.



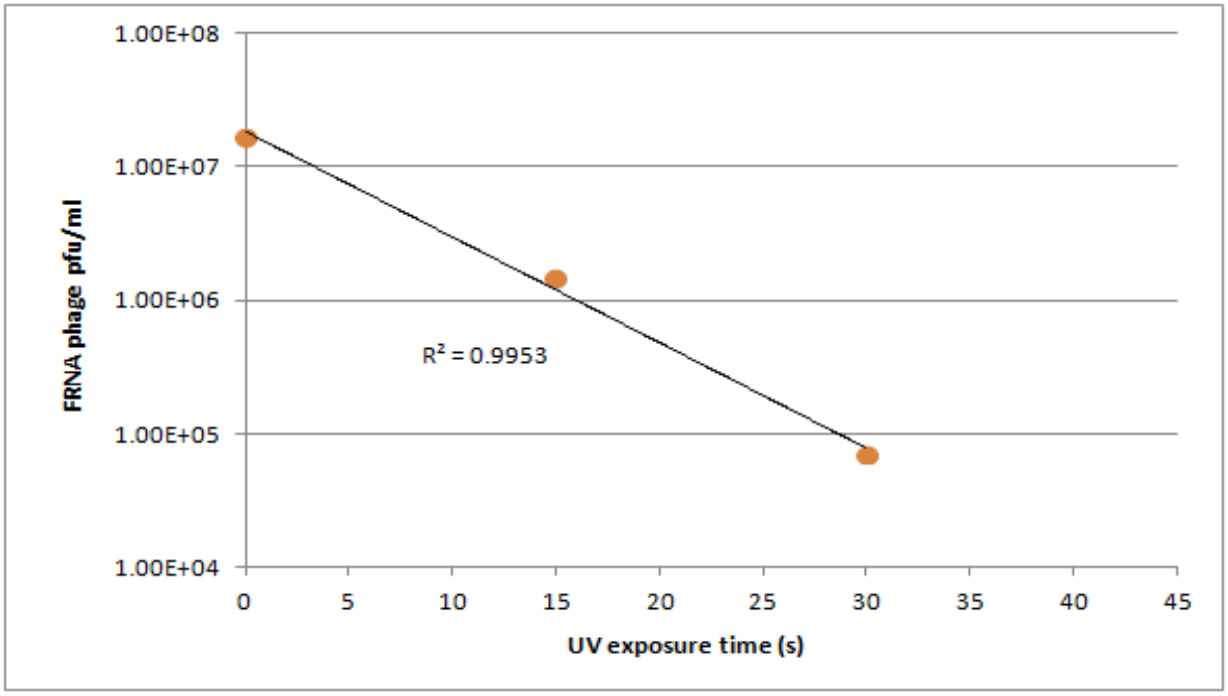
Application of the CIA to Shellfish

A comparison of the effect of UV light treatment on MS2 infectivity, MS2 and hNoV capsid integrity.

UV treatment is commonly used as a final step in the treatment of sewage effluent before discharge, and UV disinfected virus may therefore contribute to RT-qPCR positive results in bivalve shellfish samples. UV treatment resulting in a 2 log reduction

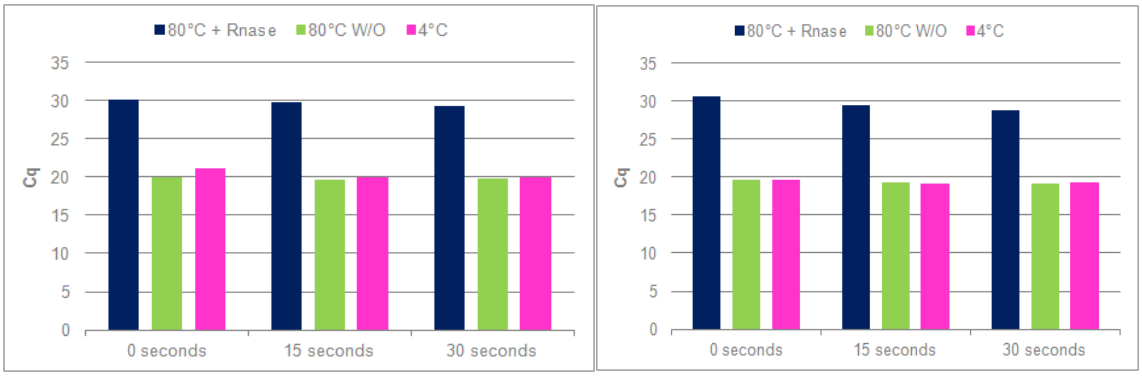
in infectivity for FRNA bacteriophage is considered effective. Experiments were therefore performed to investigate if such UV treatments had any effect on RT-qPCR measurements or CIA results for both FRNA bacteriophage and hNoV. Following calibration experiments to determine appropriate UV exposure times a virus mix was prepared using MS2 strain FRNA bacteriophage and GII.5 hNoV. Portions of this mix were then exposed to germicidal UV light at 254 nm for different time-periods; 0 seconds, 15 seconds (calibrated to achieve a ~1 log reduction in viable FRNA bacteriophage) and 30 seconds (calibrated to achieve a ~2 log reduction). Measurements of viable FRNA bacteriophage in the different portions (using the plaque assay) are shown in Figure 11.

Figure 11. Reductions in infectivity of MS2 FRNA bacteriophage in a norovirus/MS2 mix following a time-course of germicidal UV exposure.



The CIA was then applied to each UV-treated portion of the virus mix. After the CIA separate RT-qPCR analyses for hNoV GII (Figure 12A) and MS2 strain FRNA bacteriophage (Figure 12B) were performed on each RNA extract.

Figure 12. CIA data obtained using A) hNoV GII and B) MS2 RT-qPCR assays from a norovirus/MS2 mix following a time-course of germicidal UV exposure. For each data set results are shown following incubation; heated with RNase treatment [■] heated without RNase treatment [■] and at 4^{0}C [■].



12 A **12 B**

Effect of the Proteinase K step on extraction efficiency of hNoV from shellfish using the ISO/TS 15216 method

The ISO/TS 15216 extraction method for the purification of RNA from bivalve mollusc and shellfish (section 8.2.6) includes a proteinase K digestion step whereby a proteinase K solution is added to the digestive tissues of the shellfish sample then incubated at 37^{0}C for 60 minutes followed by a secondary incubation at 60 ^{0}C for 15 minutes. Potentially the use of Proteinase K in this step might expose RNA from initially viable virus particles and therefore distort the results of the CIA if this is subsequently applied to the extract. Experiments were therefore performed at Cefas to determine if this step of the extraction protocol could be modified to accommodate the CIA. Results showed that replacement of proteinase K solution with water only had a minor effect on virus recoveries (data not shown). In the experiments below, virus extraction protocols where proteinase K solution was replaced with either water, 1X PBS or 10X PBS were therefore used.

Application of the CIA to hNoV in shellfish extracts

In order to investigate the application of the CIA to shellfish extracts Cefas conducted a series of experiments designed to investigate capsid exposure in virus extracts from

shellfish naturally contaminated with hNoV GI and GII as well as extracts spiked with hNoV and/or MS2 bacteriophage (all results shown in Table 3). Through the course of these experiments minor modifications to the virus extraction and CIA protocols were trialled, including the use of 1x or 10x PBS as sample diluent in preference to water, in an attempt to neutralise the extract. In addition in later experiments a centrifugation step (10,000g for 1 minute) was added to the CIA protocol after heating, to clear extracts of the precipitate that had been observed in earlier experiments following the heat treatment of shellfish extract, and which had negatively impacted extraction efficiencies. To compensate for the difficulties with RNA extraction following heating possibly distorting results (artificially increasing ΔCqs), mengo virus was added to all samples as a specific RNA extraction control after virus extraction and CIA. Raw Cq values obtained for the target viruses were then normalised using the mengo virus Cq values prior to determination of ΔCqs (NOTE: in ISO/TS 15216 mengo virus is not used as a control for the efficiency of RNA extraction, but for the entire extraction process, and mengo virus Cq values are not used to normalise results for target viruses). For each set of experiments using spiked MS2 or hNoV a reference ΔCq (average normalised Cq following 80^0C for 2 minutes + RNase I, minus average normalised Cq following 4^0C w/o RNase I) was determined using the spike virus in buffer only (no shellfish extract).

Where hNoV was spiked into SF extracts (experiments 1 and 5) ΔCqs were significantly reduced (range -1.5 to 1.8) compared with the reference ΔCqs (determined with the same virus stock in buffer only, range 7.0 to 9.3). These results indicate a protective effect of the shellfish matrix for the samples tested (SF1, SF2 and SF8) on the hNoV capsid. In addition for the other five naturally contaminated shellfish samples tested in experiments 2-4, ΔCqs for GI and GII hNoV were also small (maximum 0.5) indicating that the same protective effect may have occurred, although reference ΔCqs cannot be determined for naturally contaminated samples and other virus-related mechanisms leading to low ΔCqs in these cases cannot therefore be ruled out. In addition the low levels of virus present in these samples, coupled with reductions in sensitivity due to the modifications applied to the ISO/TS 15216 method, means that accurate determination of ΔCqs were not always possible.

Interestingly for MS2 bacteriophage, although the protective effect of the shellfish extract on the capsid was observed to some extent in samples SF3 and SF4 in experiment 3 (ΔCqs 1.6 to 3.3 cf. reference ΔCq of 8.4), for samples SF7 and SF8 tested in experiments 4 and 5 no protective effect on MS2 was observed, even though these SF extracts exerted a protective effect on hNoV.

The theoretical possibility that the observed protective effect of the shellfish extracts resulted from inhibition of RNase by substances in the matrix was excluded, both by the observed ΔCqs for MS2 in experiment 5, and by direct measurement of RNase activity on the extracts used in the same experiment (Figure 11).

Taking into account the results of previous experiments on the stabilising effects of low pH on virus capsids, it is possible that the protective effect observed was a function of the acidic pH of the shellfish digestive tissues. In experiments 4 and 5 treatment of the tissues with buffers (1x PBS, 10x PBS) resulted in no observable effect compared with the use of water only. However direct pH measurements on the extracts in experiment 5 (pH 6.2) indicated that even the use of 10 x PBS as diluent was not sufficient to fully neutralise the extract. Further neutralising modifications of the method for preparing shellfish samples for the CIA may therefore eliminate the protective effect of the matrix, and allow further investigation of the application of the CIA to shellfish.

An additional complication with the application of the CIA protocol to SF extracts was observed with the formation of precipitates following heating at 80°C, leading to difficulties with RNA extraction. In the majority of cases heated extracts showed RNA extraction efficiencies that were markedly reduced compared to non-heated extracts, leading to a significant reduction in sensitivity and corresponding problems with determinations of reliable ΔCqs. In experiment 5 a centrifugation step was trialled to remove the precipitate. Although this step was successful in reducing the problems with RNA extraction as measured by mengo virus Cq values, centrifugation also resulted in a reduction in recovery of hNoVs from the samples cf. heat-treatment only (non-normalised Cq for GII hNoV for 80^0C + centrifugation + RNase = 22.1, for 80^0C + RNase =19.3), presumably due to co-sedimentation of the spiked hNoV particles with the precipitate. Due to this additional reduction in recovery of hNoV leading to lower

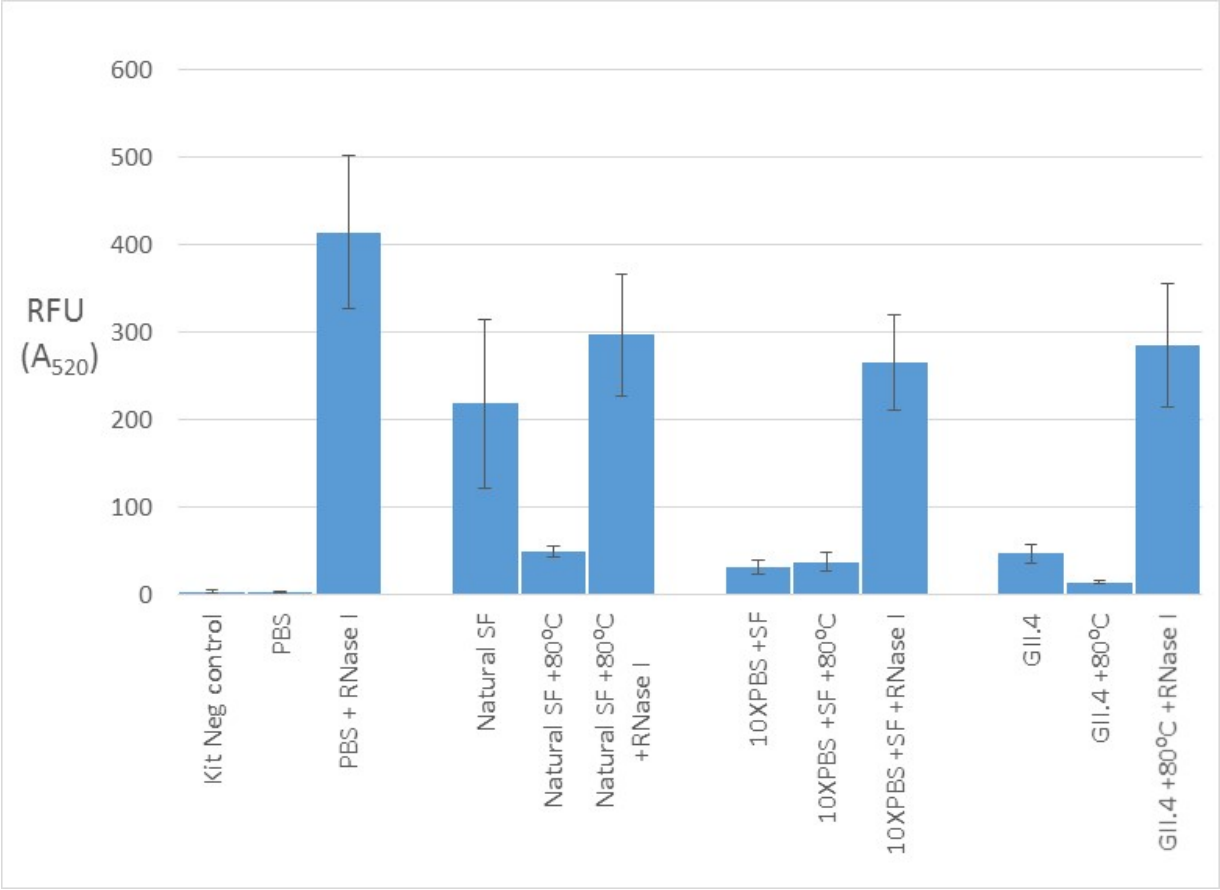
overall sensitivity, removal of precipitates by the particular centrifugation step as trialled here is not a suitable modification to the extraction/CIA protocol.

If the temperature applied for the heat treatment step is increased as may be necessary to expose RNA from certain hNoV strains it is to be anticipated that the problem with reduced sensitivity due to the formation of precipitates upon heating will be further exacerbated, complicating application of the CIA to shellfish samples.

Table 3. Application of the CIA to shellfish extracts. NC = ΔCq not calculated (one or other result negative), NQ = ΔCq not quantifiable (one or other result less than the limit of quantification), x = no reference ΔCq available (naturally contaminated samples).

Experiment number	Sample number	diluent	reduction in RNA extraction efficiency due to heating	centrifugation after heat treatment?	ΔCq (normalised for extraction efficiency)						source of norovirus
					MS2		GI		GII		
					sample	**reference (no SF extract)**	sample	**reference (no SF extract)**	sample	**reference (no SF extract)**	
Exp 1	SF1	h2o	7.7x	x	-	**-**	-	**-**	0.4	**9.3**	NVREFFS0006 (GII.5)
"	SF2	h2o	3.6x	x	-	**-**	-	**-**	-0.1	**9.3**	
Exp 2	SF3	h2o	4.6x	x	-	**-**	NC	**x**	-3.2 (NQ)	**x**	naturally contaminated
"	SF4	h2o	8.8x	x	-	**-**	0.3	**x**	0.5	**x**	
"	SF5	h2o	14.5x	x	-	**-**	0.2 (NQ)	**x**	NC	**x**	
"	SF6	h2o	1.3x	x	-	**-**	-1.1 (NQ)	**x**	-0.2 (NQ)	**x**	
Exp 3	SF3	h2o	76.4x	x	3.3	**8.4**	-0.7 (NQ)	**x**	NC	**x**	naturally contaminated
"	SF4	h2o	2.6x	x	1.6	**8.4**	-0.2	**x**	-0.3	**x**	
Exp 4	SF7	1 x PBS	1.3x	x	8.1	**7.3**	-0.3	**x**	0.2	**x**	naturally contaminated
"	"	h2o	23.0x	x	6.3	**7.3**	0.4	**x**	0.5 (NQ)	**x**	
Exp 5	SF8	10 x PBS	6.2x	x	7.0	**7.0**	-	**-**	-1.2	**7.0**	NVREFFS0005 (GII.4)
"	"	10 x PBS	none	YES	9.5	**7.0**	-	**-**	1.8	**7.0**	
"	"	h2o	9.0x	x	7.8	**7.0**	-	**-**	-1.5	**7.0**	

Figure 13. Endogenous RNase activity and added RNase I activity in PBS, shellfish extract, shellfish extract in 10X PBS, and in GII.4 hNoV (data supplied by LFR).



Application of the CIA to berries and leafy greens

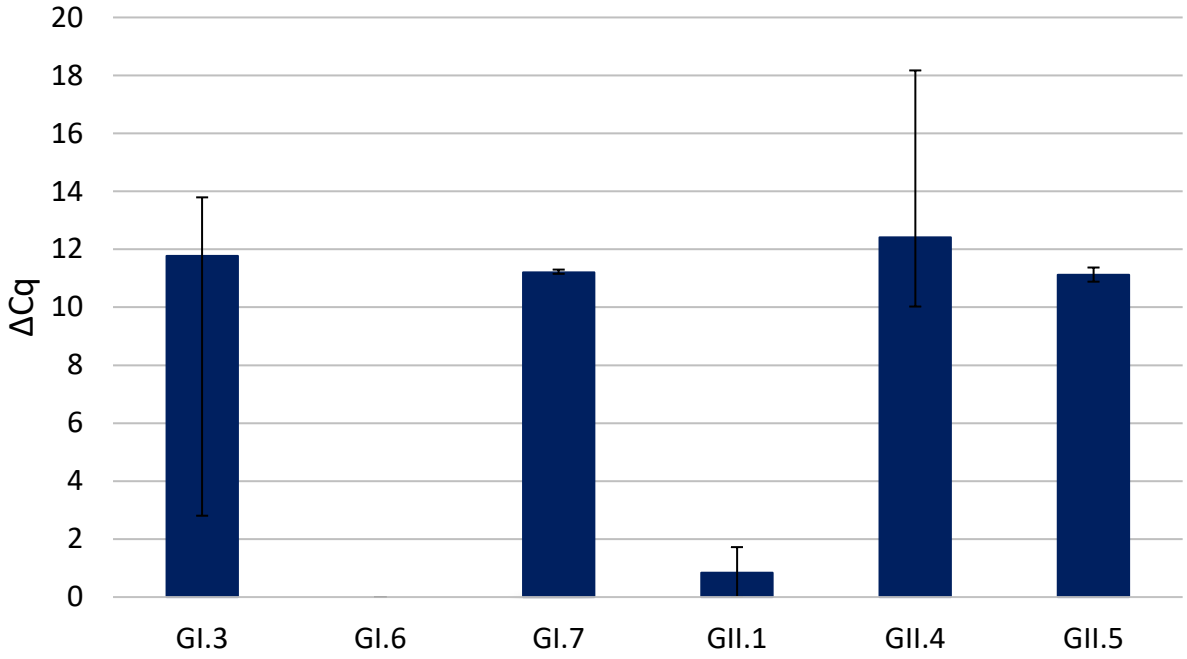
Re-analysis of faecal extracts containing hNoV strains

This test was performed to ascertain whether any loss of capsid integrity occurred in the hNoV strains through storage in faecal extracts at 4^0C prior to the analysis of raspberries

The faecal extracts used in the inter-laboratory comparison of the CIA method were re-analysed 3 months after the analysis reported above. This was required to assess any potential storage effects that might influence the analysis of raspberries. The faecal samples had been stored at 4^0C and diluted in PBS to 0.1% (v/v). Figure 14, shows the results obtained. The main difference from the first set of results is that the mean ΔCq obtained from the GI.3 is greater than the first time the CI assay was performed on this

strain. This may signify that the virus capsids have become more fragile over the period of storage and therefore more susceptible to disruption by the 80ºC treatment. The GI.6 sample appeared to have completely degraded

Figure 14. ΔCqs obtained from the CIA on 6 hNoV strains 3 months after the interlaboratory comparison.



Application of the CIA to raspberries

This test was performed to determine whether the CI assay could be applied to Norovirus extracted from raspberries by the CEN method.

Two samples of 25-30 g fresh raspberries were each spiked with 10 μl of a faecal extract (10 % neat extract in PBS) containing Norovirus GII.4. Each sample was extracted following the CEN method. After chloroform: isobutanol extraction, the extracts were divided into replicate 4 aliquots of 100 μl. 2 aliquots of each extract were kept at 4^0C prior to nucleic acid extraction. 2 aliquots of each extract were heated to 90^0C for 2 minutes treated, then RNase was added and the extracts incubated at 37^0C for 15 minutes; nucleic acid extraction was then performed. RT-qPCR was then performed in duplicate on all nucleic acid extracts. The results are shown in Table 4.

Table 4. Cq values obtained from RT-qPCR amplification of hNoV GII.4 sequences extracted from spiked-raspberry extracts subjected to the capsid integrity assay.

Sample	Treatment	
	4ºC +RNase buffer	90ºC+ RNase
1	25.8	35.9
	25.7	>40*
	25.7	>40
	25.9	>40
2	26.5	>40
	26.9	37.6
	26.7	>40
	26.8	>40

***No Cq values above 40 were recorded by the thermocycler.**

Cq values obtained from RTPCR analysis of each unheated sample were similar. After 90ºC / RNase treatment, ΔCqs from ~10 to >14 were obtained. Following the capsid integrity hypothesis this indicates that the majority of the hNoV particles extracted from the raspberries were intact, and that few RNP complexes were present. The results also demonstrate that the CIA can be applied successfully to raspberry samples, and consequently it will be applied to extracts from retained berries from hNoV-positive samples during the survey in WP4.

Application of the CIA after freezing of fresh raspberries spiked with hNoV strains

This test was performed to test whether freezing of raspberries at -20ºC resulted in any loss of capsid integrity of contaminating hNoV strains.

Six samples of 25-30 g fresh raspberries were spiked with 10 μl of a faecal suspension containing hNoV GI.3, and 6 similar samples were spiked with 10 μl of a faecal suspension containing GII.4. 3 samples of each spiking type were immediately treated

following the CEN method, and 3 samples were placed in a freezer at 20ºC and stored overnight prior to treatment. For each sample, after chloroform ; isobutanol extraction the extracts were divided into 2 replicate aliquots of 100 µl. 1 aliquot was kept at 4ºC prior to nucleic acid extraction.1 aliquot of each extract was heat treated to 80ºC for 2 minutes, then RNase was added and the extracts incubated at 37ºC for 15 minutes; nucleic acid extraction was then performed. RT-qPCR was then performed in duplication all nucleic acid extracts. The results are shown in Table 5.

Table 5. Cq values obtained from RT-qPCR amplification of hNoV sequences following the CIA using fresh or frozen raspberries.

		Before freezing		After freezing	
Virus	**Sample**	**4ºC + RNase buffer**	**80ºC + RNase 1**	**4ºC + RNase buffer**	**80ºC + RNase 1**
GI.3	1	30.0	>40*	29.8	>40
		30.2	>40	29.8	>40
	2	30.6	>40	29.8	>40
		31.3	>40	29.9	>40
	3	30.6	>40	29.1	>40
		30.5	>40	29.3	>40
GII.4	1	28.1	>40	26.8	37.0
		28.7	>40	26.7	37.9
	2	28.7	>40	27.7	35.6
		28.9	>40	27.7	>40
	3	27.8	37.9	27.4	36.7
		27.8	>40	27.4	35.6

***No Cq values above 40 were recorded by the thermocycler.**

Before freezing, a measurable ΔCq was only observed from one replicate of hNoV GII.4-spiked berries. Heating of the fresh raspberry extracts to 80ºC then RNase treatment resulted in undetectable hNoV in all other replicates. After freezing, similar

results were obtained for all hNoV GI.3-spiked berries. ΔCqs could however be obtained from most replicates of hNoV GII.4-spiked berries; around 8-10 ΔCq was observed. This could have been the result of improved recovery of virus from the frozen fruit, as seen by the lower Cq values compared to fresh berry samples. It could not be concluded from these result that freezing of raspberries had any measurable effect on hNoV capsid integrity. Then successful application of the CIA in this experiment does provide a demonstration of its applicability to the analysis of raspberries within the project.

Discussion

Capsid Integrity Assay

The results of a blind trial have shown that the CIA method is readily transferrable between laboratories. Results have shown that the assay only requires comparison between an unheated control and a heated sample, RNase digested. For the first time a comprehensive range of hNoV genogroups and genotypes have been compared simultaneously. The GII.7 sample used in this study appeared to possess a more heat resistant capsid. The reasons for this are not known although the sample matrix did not confer a dilutable protective effect and the RT-qPCR resulted in a product of the expected size when analysed directly by LOC. It may be that this effect is owing to virus bound stool specific matrix components e.g. fats, protein, antibody, gastric mucin etc. The CIA cannot distinguish differences associated with matrix effects or the particular strain. These data suggest that the temperature of the CIA requires increasing to 85-90°C in PBS in order to observe capsid RNA exposure in all samples.

The capsid stability of GII.4 hNoVs was retained following three freeze thaw cycles in PBS suggesting that freezing or samples may be useful for the archiving of samples during the attribution survey. Surprisingly no significant capsid exposure occurred upon freezing in water. This result was found owing to an increased capsid stability in water (pH5.2) that could be regained by diluting the sample in PBS (pH7.2). This was considered most likely a pH effect and was supported by the results of stability studies in seawater (pH 8.0), in comparison to water and PBS.

All of these CIA data showed the persistence of RNase resistant RT-qPCR (RNP) signals following heat treatment. Potentially RT-qPCR signals resulting from RNP might compromise the analysis of survey data since they do not represent infectious particles; however the natural occurrence of RNPs is unknown. Attempts to model this by accelerated storage at 37ºC for 48h failed to show any evidence for capsid degradation and RNP formation. However the stability trials in PBS, water and seawater (which were performed in that order chronologically) suggested that the ageing of stool samples was accompanied by higher Cq values and reduced capsid integrity (i.e. more RNP).

Application of the CIA to Shellfish

The results of the application of the CIA for MS2 and hNoV from a UV treated virus mix were similar and showed that the capsid integrity of neither MS2 nor hNoV was affected by UV treatment (sufficient to produce a 2 Log reduction in MS2 infectivity). Therefore if UV treatment similarly affects hNoV infectivity, the CIA cannot be used to distinguish UV inactivated and viable hNoV in shellfish. It is possible that the CIA may be able to identify the presence of other types of non-viable hNoV particles in these samples e.g. RNPs.

Although the potentially problematic inclusion of digestion with proteinase K (as in the ISO 15216 method) was found to be not essential for virus extraction, the application of CIA to shellfish extract has not proven successful for hNoV (although the available data does show greater RNA exposure for spiked MS2). Two significant problems have been identified. The first of these is associated with the formation of a precipitate upon heating at 80°C that subsequently reduces extraction efficiency. This is a common problem with measuring l virus inactivation in food matrices that is usually reduced by centrifugation. However the addition of the centrifugation step used in these experiments was not helpful and led to further reductions in recovery. The second problem appears to be associated with protection of the hNoV capsid by the shellfish matrix, likely due to the acidic nature of shellfish digestive tissues (although a more specific protection mechanism associated with shellfish extract cannot be excluded). Attempts to remove this protective effect b attempting by neutralising shellfish extract were made by using 10X PBS instead of water as diluent, however the pH of extract produced using this buffer was still <7.0.

Before the application of the CIA to naturally contaminated and bioaccumulated shellfish can be properly assessed, the CIA protocol requires further modification. In particular, further studies are required to:

- Neutralise the pH of the shellfish extract and investigate whether this reduces the protective effect of the matrix
- Minimise of the effect of precipitates on hNoV recovery

Application of the CIA to berries and leafy greens

Data obtained by Fera showed that the CIA could be applied to fresh and frozen raspberries when spiked with human GII.4 hNoV.

Part 2 Application of a combined capsid and genomic integrity assay to CEN methods

Introduction

This interim report provides preliminary data on the development of a novel assay using VPg magnetic immuno-capture PCR as novel method for measuring capsid and genomic integrity. This assay was the subject of a feasibility study for the FSA strategic call submitted to the FSA by LFR in 2012.

A limitation of the CIA as a molecular assay for infectivity is its inability to detect genomic degradation, this is important since single genomic lesions inactivate virus particles. A simple approach to the problem of identifying genomic degradation might be to design full length RT-PCR reactions to amplify whole genomes, however PCR efficiency decreases with fragment length and although such reactions have been previously described they are currently insensitive (Kostela *et al* . 2008) and not compatible with the ISO 15216 methods. An alternative approach might investigate the use of novel RT approaches capable of efficient full length cDNA synthesis, followed by small fragment RT-qPCR reactions targeted at the 5' end of the genome. This would ensure that the resulting amplicon is derived from full length cDNA and include a more efficient detection step compared with full length amplification. Although this approach is feasible, and has been previously demonstrated (Wolf *et al*., 2009) it has the disadvantage in that it would require new consensus RT-qPCR assays targeted at the 5' end of the genome and control templates for copy number determination. Such assays would then require extensive validation in conjunction with the already developed ISO reactions. Alternatively, the potential to use 5' RNA hybridisation capture followed by ISO RT-qPCR could allow detection of genomes of a minimum size of 5000b since the ISO RT-qPCR target is located 5,000 bases downstream of the 5' end. However this approach (and 5'RT-qPCR assays) is limited by sequence variation within hNoVs at the 5' end of the virus. We have therefore investigated an alternative hNoV RNA capture system capable of isolating full length NoV RNA from intact particles using a novel approach that is potentially applicable to all NoVs. This approach simultaneously

detects an intact capsid with the largest size intact genome detectable by the ISO methods following the heat treatment of intact particles.

Noroviruses like certain other RNA viruses possess a covalently attached protein at the 5' end of the virus known as VPg. This 15 KDa protein is considered to be involved in transcription initiation and binds transcription factor eIF3 (Daughenbaugh *et al*., 2003). Purification of full length RNA by conventional methods necessarily co-purifies this protein (albeit probably denatured) owing to its covalent bonding to the 5' end of the viral RNA. Intriguingly this protein exhibits a very high level of sequence conservation within human GI and GII NoVs (68% overall aa identity) and the hNoV surrogate mouse norovirus MNV-1 (54% overall aa identity) but less homology with the alternative surrogate feline calicivirus (18% overall aa identity).

A sequence alignment comparing VPg from MNV-1 and human GI Norwalk norovirus is shown below (Daughenbaugh *et al*., 2006) and shows a very high level of sequence conservation particularly at the N and C termini.

Figure 15. MNV-1 VPg and NV VPg share significant amino acid sequence identity. Sequences encoding MNV-1 VPg and NV VPg were aligned with ClustalW. Asterisks indicate identical residues. Semicolons are conservative substitutions. Amino acid numbers correspond to the position in the ORF1 polyprotein. Reproduced from Daughenbaugh *et al.,* 2006.

```
MNV-1  GK-KGKNKKGRGRPG---VFRTRGLTDEEYDEFKKRRESRGGKYSIDDYLADRER-   921
NV     GKNKGKTKKGRGRKNNYNAFSRRGLSDEEYEEYKKIREEKNGNYSIQEYLEDRQRY  1018
       ** *** ******       *  ***:****:*:** ** : *:***::** **:*

MNV-1  EEELLERDE---------EEAIFGDGFGLKATRRSRKAERAKLGLVSGGDIRARKPIDWN   972
NV     EEELAEVQAGGDGGIGETEMEIRHRVFYKSKSKKHQQEQRRQLGLVTGSDIRKRKPIDWT  1078
       **** * :           *  *    *    ::: :: :* :****:* *** ******

MNV-1 VVGPSWADDDRQVDYGEKINFE  994
NV    PPKNEWADDDREVDYNEKINFE 1100
           ****** *** ******
```

In this study we have therefore raised polyclonal antibodies against conserved VPg peptide sequences and developed magnetic immuno-capture reagents for VPg-linked NoV RNA in conjunction with detection using the existing ISO hNoV PCR reactions. The

use of magnetic RNA binding reagents should minimise the degree of modification to the ISO/TS 15216 method required as the ISO method already recommends (non-specific) magnetic RNA binding reagents for RNA extraction. This approach therefore potentially allows detection of near full length viral RNA following heat treatment of intact virus particles whilst still allowing detection using the already developed and well-characterised ISO RT-qPCR assays. Since VPg linked RNA is only located within intact virus capsid such signals can theoretically only be obtained following heat treatment of intact capsid particles.

One potential difficulty of this approach is that proteinase K is used in the ISO virus extraction method for shellfish (but not other foodstuffs), and therefore it might be considered that proteinase K could cleave VPg from the genome. This could possibly prevent the use of this approach however data from CIA studies reported in Part 1 shows that proteinase K treatment is not required absolutely for successful virus extraction from shellfish suggesting that modification to the ISO method is feasible. Another possible limitation of the VPg approach may occur for UV inactivated virus particles in that although UV damage to RNA resulting in strand cleavage will be detectable, inactivation owing solely to base dimerization may not.

Using the VPg immuno-capture PCR approach in conjunction with temperature treatment (to expose VPg linked RNA) it should be possible to detect NoV RNA genomes that are a minimum of 5000 nucleotides in length and that are derived from virus particles that have intact capsids. Detection of such material will provide strong evidence of viability. Additionally, evidence that this approach can be applied using antibodies to selected VPg peptides is already present in the literature (Margis *et al*., 1993; Weitz *et al*., 1986).

Methods

Database searching for conserved peptides was based upon the sequence alignment shown above and optimised for GII and GII.4 hNoV using the BLAST facility available at pubmed.

Polyclonal antibodies to each of three identified conserved peptides were raised in rabbits and protein A purified (Pierce). Since the relative affinity of the antibodies for different peptides and hNoVs was not known equal volume mixtures of antisera were pooled and used for preliminary analysis. Binding of pooled sera used protein A magnetic beads in accordance with manufacturers' instructions (Life sciences). PCR following immuno-capture used 5ul magnetic bead solution directly in RT-qPCR and the CEN protocol.

hNoV positive stool samples were kindly supplied by D. Allen Public Health England (PHE) and are listed in Table 6. Unless otherwise stated all experiments were performed at least in duplicate with triplicate RT-qPCR.

Table 6. hNoV stool samples provided by PHE.

Stool sample	Norovirus genotype
NVREFFS0001	GI.3
NVREFFS0002	GI.6
NVREFFS0003	GI.7
NVREFFS0004	GII.1
NVREFFS0005	GII.4
NVREFFS0006	GII.5

Results

Identification of conserved sequences

Sequence alignment and BLAST searches identified three conserved hNoV peptide regions as:

WADDDREVDYNEKINFE 17aa (NV 1083-1100 above)

LGLVTGSDIRKRKPIDW 17 aa (NV 1058-1075 as above)

GLSDEEYDEYKRIREERNG 19 aa (NV 985-1004 as above)

Searches showed that these peptides were 80 - 100% conserved between human GI and GII noroviruses and in the selected GII samples used in this study. Although the peptides were also highly conserved amongst GI sequences in general, no homologies were identified for the selected GI samples used in this study owing to an absence of available sequence for non-structural proteins within the NCBI database.

Development of VPg immuno-capture

Trial experiments have resulted in the development of a starting protocol for the preparation of beads and their use in VPg immuno-capture using dilute (1/100) pooled antisera. Beads were washed and tested to ensure the absence of RNase activity. Control beads without antibody were prepared and treated in the same manner.

Testing of VPg immuno-capture PCR

Testing of VPg immuno-capture PCR used 0.1% hNoV dilute stool samples and tested VPg binding following heat treatment at 80^0C for 2 minutes either in the presence or absence of VPg antibody beads. Similar experiments were also performed with whole RNA preparations extracted from the GII.4 sample. Results of preliminary duplicate experiments to test VPg immuno-capture PCR are shown in Table I for the GII isolates used in this study.

Table 7. Results of VPg magnetic immuno-capture PCR using dilute 0.1% hNoV stool samples and purified GII.4 RNA.

	GII.4				GII.1		GII.5	
	Experiment 1		Experiment 2		Experiment 2		Experiment 2	
	Mean	σ	Mean	σ	Mean	σ	Mean	σ
80ºC + VPg Ab_Beads	22.36	0.34	25.25	0.97	>38		42.72	0.47
80ºC + Beads control	28.67	0.49	28.71	0.99	>38		30.48	1.22
∆Cq	6.32		3.46				5.75	
RNA + VPg Ab_Beads	18.21	1.10						
RNA + Beads control	20.26	0.33						
∆Cq	2.14							
RNA + Ab_Beads + RNase I	>38							

Both GII.4 and GII.5 dilute stool samples showed significantly greater binding in the presence of VPg antibody beads than to beads without antibody in all experiments following heat treatment at 80^0C for 2 minutes. Background ΔCq values independent of VPg antibody resulting from non-specific binding was also observed. The GII.1 sample did not show either specific or non-specific binding in the duplicate experiments. Data for purified RNA showed binding to the beads irrespective of the presence of antibody. Bound RNA was completely digested by RNase I showing that the residual Cq values obtained for GII.4 and GII.5 resulted from non-specific virus binding

Discussion

Preliminary results to date have demonstrated the feasibility of applying VPg immuno-capture to detection of near full length RNA from intact virus particles. Significant differences have been obtained for two GII hNoV isolates demonstrating proof of principle. This method and approach is novel and should be patented.

Available data shows that the VPg immuno-capture method does not bind purified RNA presumably owing to conformational differences between heated and guanidinium denatured epitopes (resulting from the RNA extraction procedure). Additionally some background binding is evident in the absence of heat treatment, whether this is owing to non-specific binding or RNP within the sample is unknown currently. Intriguingly the GII.I isolate that showed increased temperature resistance in the CIA (Part 1) has failed to bind VPg antibodies in immuno-capture PCR (as might be expected) but also failed to exhibit any non-specific binding suggesting that the virus surface properties of this sample may be different to others perhaps owing to bound stool specific matrix components e.g. fats, protein, antibody, gastric mucin etc. further experiments are required to confirm this data.

The method is potentially quick and simple only requiring heat-treatment followed by VPg immuno-capture PCR and direct RT-qPCR using the capture beads. However at present the technique has not been fully tested or optimised and further work is required to:

- Obtain more repeat data and extend studies to GII and GI samples
- Consider the inclusion of RNase inhibitors
- Investigate optimal antibody selection for capture
- Reduce or eliminate non-specific binding to increase sensitivity and specificity
- Trial and compare the assay using shellfish or berry extract and the ISO methods

In summary these studies have demonstrated proof of principle of a unique assay that simultaneously measures capsid and genomic integrity for the first time.

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Appendix 1

Protocol for determining the exposure of capsid RNA using RT-QPCR and RNase ONE

1. All experiments are performed at a minimum of containment level 2 conditions to minimise the risk of aerosols and contamination. A risk assessment should be performed ensuring safe handling and decontamination procedures.

2. Resuspend a loopful (100 µL) of stool sample in 900 µL ice cold Dulbecco's PBS pH 7.2 (without magnesium or calcium, Sigma cat no. D8537) to obtain a stock 10% PBS stool suspension. Ensure the sample is adequately dispersed by vortexing and micro centrifuge for 30 seconds. As far as possible use "fresh" stool. Stock 10% PBS stool suspension should be kept at 4°C and used within one month.

3. Construct a working stock of dilute stool suspension by adding 10 µL of the stock 10% PBS stool suspension from step 2 to 990 µL cold PBS in a screw cap microfuge tube and mix by vortexing briefly.

4. Prepare RNase ONE stock by mixing 90 µl of the 10 x reaction buffer supplied with 10 µl (100 units) of RNase ONE.

5. Label each of 8 thin walled PCR microfuge tubes 1-8. On ice add 100 µL of working stock stool suspension from step 3 in preparation for the different sample treatments below.

Sample No	RNase treatment	Heat treatment
1	No	No
2	No	No
3	Yes	No
4	Yes	No
5	No	Yes
6	No	Yes
7	Yes	Yes
8	Yes	Yes

6. Keep samples 1-4 on ice.

7. Submit samples 5-8 to heat treatment at 80°C for 2min in a thermal cycler using the max/standard ramp rates with cooling to 4°C at the end of the heating period. Keep samples 5-8 in the block at 4C until required.

8. Add 11 µL RNase ONE stock to samples 3,4,7, and 8, mix by gently pipetting, and incubate at 37°C for 15 min in a water bath, and then cool in ice-water to 4°C.

9. Add 11 µL ice cold RNase reaction buffer to samples 1,2,5 & 6 and keep in ice-water

10. Add 29 µL ice cold PBS to each sample to increase the total volume to 140 µL and extract RNA immediately.

RNA extraction

Any method of RNA extraction may be utilised that allows consistent results between replicate extractions.

Qiagen Method

Label 8 X 1.5 ml microfuge tubes 1-8 (for samples 1-8 above)

1. Extract viral RNA by QIAamp viral RNA mini kit (Cat no 52906) according to the manufacturer's instructions.

2. QIAamp - Carrier RNA preparation. Prepare the Carrier RNA according to the QIAamp instruction on page 15, by adding 310 µL Buffer AVE, elution buffer, to 310 µg of lyophilized carrier RNA. Place the carrier RNA at 4°C in a fridge or in ice-cold water awaiting further use. Remaining carrier RNA is to be stored at -80°C.

3. QIAamp - Buffer AVL preparation. Prepare the Buffer AVL (Virus lysis buffer) according to the QIAamp instruction on page 16, table 1, in conjunction with the amount of sample numbers.

4. QIAamp – Lysis buffer addition. Add 560 µL of prepared Buffer AVL (Virus lysis buffer) (Step 3) to each of the 8 X pre-labelled 1.5 mL Eppendorf centrifuge tube. Leave at ROOM TEMPERATURE

5. Sample addition. Add 140 µL of each of the test samples. Mix thoroughly by gentle inversion. Incubate the samples at room temperature for 10 minutes.

6. Add 560 µL of 100% Ethanol to each sample and mix thoroughly by gentle inversion

7. QIAamp - Mini column clean up

a. Add 630 µL of the sample-ethanol (Step 6) to the Mini column and centrifuge for 1 minute at 8000 RPM. Take care to remove all the droplets from the lid.
b. Discard the filtrate, add the remaining 630 µL (Step 7a) and centrifuge, again, for 1 minute at 8000 RPM
c. Add 500 µL of Buffer AW1 (wash buffer 1) to the Mini column and centrifuge for 1 minute at 8000 RPM.
d. Discard the filtrate and add 500 µL of Buffer AW2 (wash buffer 2) to the Mini column and centrifuge for 3 minute at 13000 RPM.
e. Discard the filtrate and centrifuge again, for 1 minute at 13000 RPM

8. Remove the bottom tube and insert the Mini column into a new, pre-labelled, 2 mL collection tube (T5449-70E, Sigma-Aldrich). Snip off the Mini column lid as it will not fit into the micro centrifuge.

9. Carefully add 60 µL of Buffer AVE, elution buffer, to the centre of the Mini column and centrifuge for 1 minute at 8000 RPM.

10. Discarded the Mini column and store the tube with the purified RNA at 4°C in a fridge or in ice-cold water (Freeze at -80°C, when not needed).

RT-qPCR

1. RT-qPCR utilises the ISO/TS 15216 methods.

2. Perform RT-qPCR reactions for controls and test samples in triplicate and simultaneously.

3. Record Ct values and calculated copy number. The % reduction is calculated in comparison to the mean value obtained for the control samples (4°C without RNase) and can be calculated by reference to a standard curve or by –Δ Ct.

Typically heat treatment followed by RNase digestion results in a difference of 5-6 Ct values compared with unheated controls or heated samples that have been kept at 4°C