FAO Reference Centre for Bivalve Mollusc Sanitation

Norovirus (Genogroup I and II) and Hepatitis A virus Proficiency Testing (PT 99)

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Cefas

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Draft V1	L. Stockley	25/09/24	Sent to JL for review
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Draft V3	L. Stockley	08/11/24	Minor amendments prior to executive approval
Draft V4	L. Stockley	13/11/24	Updated to include missing results
Final V1	L. Stockley	27/11/24	Distributed to participants

Contents

1. Preparation of sample material

1.1. Sample and virus strain origin

Materials dispatched consisted of whole Pacific oysters (*Magallana gigas*), blended digestive glands from the same species and dsDNA control solutions for quantification (1 x 10^5 copies/µl) for each target virus. The origin of the viruses used for preparing the samples are given in Table 1. All samples were held at <-15°C until required for quality control testing, dispatch and/or reference analysis.

Table 1 - Origin and strain/genotype of viruses used for shellfish contamination

Description	Source	Strain ID / genotype
Hepatitis A virus	Cell culture supernatant	HM175/43c
Norovirus genogroup I	Faecal material	GI.3 (based on capsid sequence)
Norovirus genogroup II	Faecal material	GII.4 (based on capsid sequence)

1.2. Preparation of digestive gland base material

A single batch of approximately 3000 Pacific oysters (*M. gigas*) was collected from a UK commercial harvesting area in August 2023. A proportion of the shellfish was shucked, and the digestive glands removed, pooled together and blended to form a homogenous mixture. The mixture was tested for norovirus (NoV) genogroups I and II (GI and GII) and hepatitis A virus (HAV) prior to being used to prepare different samples.

1.3. Sample preparation

1.3.1. Sample 1

Pacific oysters (*M. gigas*) collected from a UK commercial harvesting area in August 2023 were initially tested to demonstrate the absence of all 3 target viruses. The shellfish were then placed in a large sterile container and thoroughly mixed before subsamples of 10 oysters were randomly selected and placed in sample bags and stored at <-15°C.

1.3.2. Sample 2

Blended digestive gland base material (see above) was mixed with NoV GI, NoV GII and HAV to obtain the desired target levels before being split into 2 g aliquots. The samples were held

at <-15 °C until required for quality control testing, dispatch and/or reference analysis (Table 2).

1.3.3. Sample 3

Blended negative glands (see above) were split into 2 g aliquots. The samples were held at <-15 °C until required for quality control testing, dispatch and/or reference analysis.

1.3.4. Sample 4

Blended digestive gland base material (see above) was mixed with NoV GI, NoV GII and HAV to obtain the desired target levels before being split into 2 g aliquots. The samples were held at <-15 °C until required for quality control testing, dispatch and/or reference analysis (Table 2).

2. Sample distribution

Samples were dispatched on dry ice in accordance with IATA packing instructions for UN3373 'Diagnostic Specimens' on 8th July 2024 or after to 14 participating laboratories.

On arrival, participants were given a month to analyse the test samples using their routine method. Those laboratories using quantitative real-time RT-PCR were requested to calculate the quantity of target virus in each sample using both their own standard material and using the dsDNA control solutions provided with this PT distribution.

3. Results

3.1. Reference results

Reference analyses were performed by the FAO Reference Centre (FAORC) for Bivalve Mollusc Sanitation on samples stored at <-15˚C. Six randomly selected samples from each sample type were extracted in duplicate and qRT-PCR (TaqMan™) was carried out using duplicate PCR reactions for each RNA extract and each target. Reference results for each sample are shown in Table 2, with box and whisker plots included in Appendix 1.

Table 2 - Reference results for PT 99 Proficiency testing material

Sample	NoV GI	NoV GII	HAV
Sample 1 (Whole animal)	-	-	-
Sample 2 (Digestive gland)	+ $2.45 \times 10^3 – 7.75 \times 10^3$	+ $5.85 \times 10^2 – 1.69 \times 10^3$	+ $3.16 \times 10^3 – 1.11 \times 10^4$
Sample 3 (Digestive gland)	-	-	-
Sample 4 (Digestive gland)	+ $7.71 \times 10^2 – 1.35 \times 10^3$	+ $1.03 \times 10^3 – 1.96 \times 10^3$	+ $2.05 \times 10^3 – 3.79 \times 10^3$

KEY: - = Negative, + = Positive, Quantities in copies/g, ranges based on a 95% confidence limit determined as 2 geometric standard deviations above and below the geometric mean.

3.2. Participants' results

Participants' results are tabulated in Appendices 2, 3 and 4 and quantitative results are shown in graphical form alongside the reference values in Appendix 5.

3.3. Performance scoring

3.3.1. Presence / absence

Performance scoring was undertaken on each participant's presence/absence results. A single score for each sample and each target virus (NoV GI, NoV GII and HAV) was assigned as follows: Correct = 2 points, Incorrect = 0 points. Any results reported as invalid due to quality control issues (e.g. unacceptable extraction efficiencies) were not subject to performance scoring for presence/absence. For each laboratory an overall score is provided for each target virus, taking into account the results of all samples (Table 4).

3.3.2. Quantification

For those laboratories submitting quantitative results, an additional performance scoring for quantification was undertaken following the median absolute deviation from the median (MAD) approach described in ISO 22117 Microbiology of the food chain – Specific requirements and guidance for proficiency testing by interlaboratory comparison (ISO, 2019). The MAD approach is recommended for assessment of PT data where less than 50 participants return quantitative results and/or for new proficiency assessments. Where laboratories submitted quantitative

results determined using both their own quantification standards, and those provided by the FAORC, only the results using their own standards were considered for performance scoring; however, where laboratories submitted quantitative results using the FAORC standards only, these were considered. Where laboratories reported positive results at levels below their limit of quantification (LOQ), or positive; not quantifiable results due to quality control issues (e.g. unacceptable extraction efficiencies), these results were not subject to performance scoring for quantification; where laboratories reported positive results at levels below LOQ determined using their own quantification standards, but quantifiable positive results using the FAORC standards, results using the FAORC standards were used for performance scoring. Laboratories were given a score for each virus for which they reported at least one quantitative result.

For each sample/target virus combination where the intended result was positive, a statistically robust acceptability range was determined by calculation of the median absolute deviation (MAD) of each participant's result from the median of all participants' results. This figure was then multiplied by a constant (1.4826) to obtain a robust estimate of the standard deviation (σMAD; Table 3). For each individual result, its absolute deviation from the participants' median was compared with the calculated σMAD to determine its acceptability and score as follows:-

- Difference between result and participants' median <2 σMAD = satisfactory (2 points)
- Difference between result and participants' median >2 σMAD and <3 σMAD = questionable (1 point)
- Difference between result and participants' median >3 σMAD = unsatisfactory (0 points)
- Result reported as negative = unsatisfactory (0 points)

The differences between individual participants' results and the participants' median, expressed in terms of σMAD are shown in Appendix 4, and the graphs in Appendix 5 include lines showing the boundaries of the satisfactory and questionable ranges for each sample/target matrix combination.

For each sample/target virus combination where the intended result was negative, its acceptability and score was determined as follows:-

- Result reported as negative = satisfactory (2 points)
- Result reported as positive = unsatisfactory (0 points)

Table 3 - Dataset characteristics for quantitative results

Characteristic	Shellfish sample 2			Shellfish sample 4		
	GI	GII	HAV	GI	GII	HAV
MEDIAN	3.792	2.948	3.984	3.204	2.761	3.210
MAD	0.359	0.312	0.404	0.378	0.177	0.474
σMAD	0.533	0.462	0.599	0.560	0.263	0.703

Values in $\log_{10}$ copies/g

For each laboratory an overall score (usually out of 8) is provided for each target virus, taking into account the results of all 4 samples (Table 4).

Table 4 - Performance scoring

	Presence / absence			Quantification		
Lab ID	**NoV GI**	**NoV GII**	**HAV**	**NoV GI**	**NoV GII**	**HAV**
3	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	NE
10	8 out of 8	8 out of 8	8 out of 8	6 out of 6 [a]	8 out of 8	8 out of 8
20	8 out of 8	8 out of 8	8 out of 8	NE	NE	NE
24	8 out of 8	4 out of 8	6 out of 8	NE	NE	NE
47	8 out of 8	4 out of 8	4 out of 8	8 out of 8	4 out of 8	4 out of 8
96	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
143	8 out of 8	6 out of 8	8 out of 8	8 out of 8	6 out of 8	8 out of 8
168	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
190	8 out of 8	8 out of 8	8 out of 8	6 out of 6 [a]	4 out of 4 [a]	6 out of 6 [a]
203	8 out of 8	6 out of 8	8 out of 8	6 out of 6 [a]	6 out of 8	8 out of 8
237	8 out of 8	8 out of 8	8 out of 8	8 out of 8	4 out of 4 [a]	8 out of 8
242	6 out of 8	4 out of 8	4 out of 8	NE	NE	NE
423	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	5 out of 6 [a]
462	8 out of 8	8 out of 8	8 out of 8	8 out of 8	7 out of 8	7 out of 8

Key: NE = Target virus not examined or not quantified; [a] = score is out of less than 8 as some samples not scored due to positive results at <LOQ or positive; not quantifiable results. Labs that scored less than full marks for any criteria are highlighted in yellow.

4. Discussion

Fourteen laboratories received samples. Methods used by participants to analyse the test samples are shown in Appendix 6, while brief details of the types of materials used as

quantification standards are included as Appendix 7.

4.1. Material despatch

Eleven laboratories received material within 4 days of dispatch. A number of participants experienced delays at customs; the causes included incorrect paperwork supplied or communication issues between the courier and the receiving laboratory. The FAORC recommends those laboratories who experience problems to review the documentation requested by customs and/or brokers prior to dispatch in future PT schemes. Please ensure the documents are available and that the contact details provided are correct and suitable for the week of dispatch.

4.2. Presence / absence determination

For NoV, 9 out of 14 labs that tested for NoV (64%) obtained the intended presence/absence result (as determined by the FAORC) for all samples and both genogroups. A total of 6 false negative results (1 for GI, 5 for GII) were reported by laboratories 24, 203 and 242, resulting in an overall sensitivity for NoV of 89%. Three false positive results (for GII) were reported by laboratories 47 and 143, resulting in an overall specificity for NoV of 95%. The overall accuracy for norovirus results was 92%.

For HAV, 11 out of 14 labs that tested for HAV (79%) obtained the intended presence/absence result (as determined by the FAORC) for all samples. A total of 5 false negative results for HAV were reported by laboratories 24, 47 and 242, resulting in an overall sensitivity of 82%. No false positives were reported. Overall specificity and accuracy levels for HAV were 100% and 91% respectively.

4.3. Quantification

A total of 11 laboratories (79%) reported quantitative data for at least one sample/virus combination and were subject to performance scoring; 6 laboratories (55%) received maximum scores for all target viruses for which they were assessed. Laboratory 423 reported one quantitative result for HAV in the questionable range and laboratory 462 reported one quantitative result each for GII and HAV in the questionable range. Laboratories 47, 143 and 203 additionally received one or more zero scores for a false positive or false negative result, however across the entire PT no reported quantities for positive sample/target virus combinations were considered to be in the unsatisfactory range. Where a small number of laboratories report quantitative results, this will tend to result in relatively wide ranges for

satisfactory and questionable results in some sample/virus combinations which may partly explain the low number of unsatisfactory and questionable results.

The FAORC recommends any laboratory with unsatisfactory results for either presence/absence or quantification refers to the trouble shooting guide available on the FAORC website Troubleshooting guidance for virus PT (cefas.co.uk)

5. References

Codd AA, Richardson IR, Andrews N. 1998. Lenticules for the control of quantitative methods in food microbiology. J Appl Microbiol. 85(5):913–7.

Anon. 2017. ISO 15216-1:2017 Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR -- Part 1: Method for quantification.

Anon. 2019. ISO 22117:2019 Microbiology of the food chain – Specific requirements and guidance for proficiency testing by interlaboratory comparison.

6. Appendices

6.1. Appendix 1 – FAORC Results

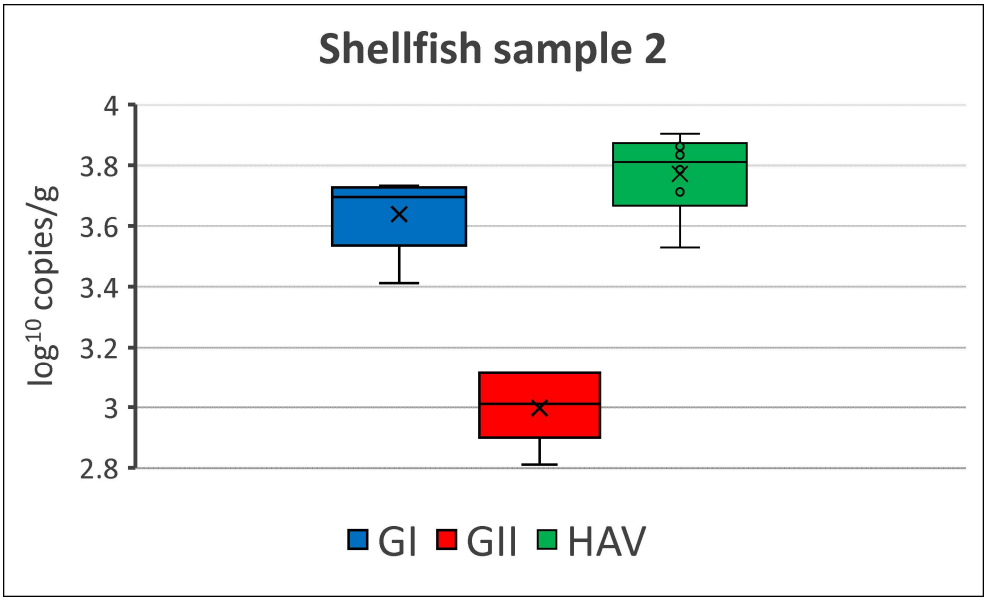

Figure 1. Whisker plots showing log10 copies per gram of norovirus genogroup I, norovirus genogroup II, and hepatitis A virus for shellfish sample 2.

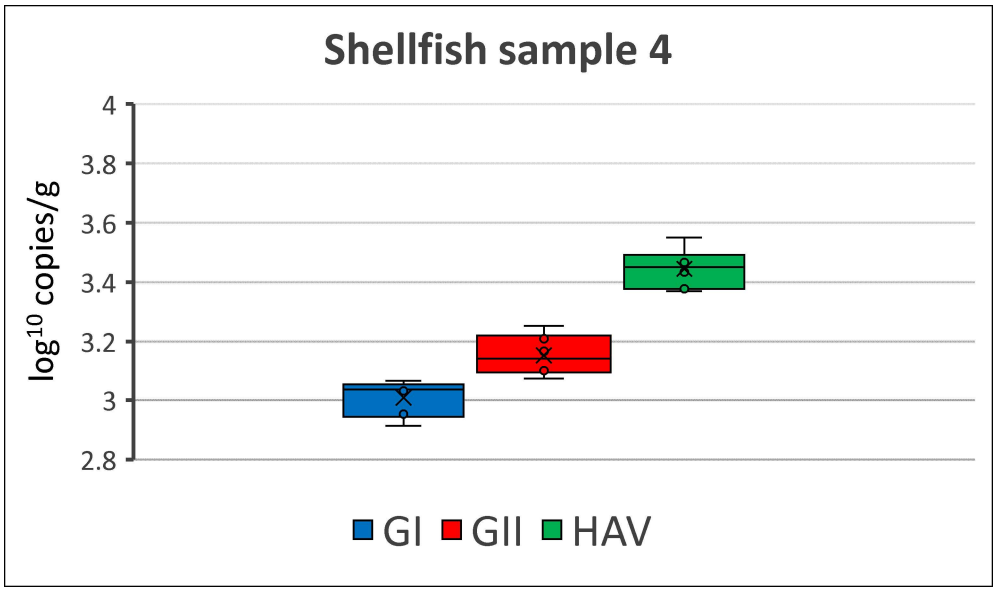

Figure 2. Whisker plots showing log10 copies per gram of norovirus genogroup I, norovirus genogroup II, and hepatitis A virus for shellfish sample 4.

6.2. Appendix 2 - Participants' presence/absence results and C_t values

Lab ID	Shellfish sample 1						Shellfish sample 2						Shellfish sample 3						Shellfish sample 4					
	GI		GII		HAV		GI		GII		HAV		GI		GII		HAV		GI		GII		HAV	
	-	Ct	-	Ct	-	Ct	+	Ct	+	Ct	+	Ct	-	Ct	-	Ct	-	Ct	+	Ct	+	Ct	+	Ct
3	-		-		-		+	37.22 / 37.11	+	38.80 / 40.96	+	36.4	-		-		-		+	36.73 / 36.63	+	40.89 / 37.86	+	37.94
10	-		-		-		+	35.15	+	36.6	+	34.49	-		-		-		+	39.8	+	37.4	+	36.98
20	-		-		-		+	32.53	+	37.25	+	33.33	-		-		-		+	33.99	+	35.95	+	34.45
24	-		-		-		+	NP	-		-		-		-		-		+	NP	-		+	NP
47	-		+	34.7	-		+	33.62	+	33.4	-		-		+	35.25	-		+	36.22	+	35.19	-	
96	-		-		-		+	30.97	+	34.34	+	29.94	-		-		-		+	33.4	+	35.02	+	32.43
143	-		+	38.46	-		+	33.2	+	37.2	+	33.58	-		-		-		+	34.61	+	35.56	+	34.61
168	-		-		-		+	33.49	+	35.47	+	31.05	-		-		-		+	35.48	+	35.41	+	33.39
190	-		-		-		+	33.87	+	40.05	+	33.85	-		-		-		+	35.53	+	38.42	+	35.4
203	-		-		-		+	32.89 / 32.1	+	43.14 / 33.88	+	32.01 / 31.97	-		-		-		+	36.03 / 35.91	-		+	35.64 / 35.56
237	-		-		-		+	33.23	+	34.06	+	31.38	-		-		-		+	35.13	+	34.19	+	33.02
242	-		-		-		+	38.9	-		-		-		-		-		-		-		-	
423	-		-		-		+	31.46	+	35.71	+	36.62	-		-		-		+	33.82	+	35.63	+	38.31
462	-		-		-		+	36.66	+	37.13	+	34.82	-		-		-		+	37.46	+	37.35	+	36.90

Key: - = negative; **+** = positive; **NP** = Not provided; **Yellow shading** denotes false negative results; **Red shading** denotes false positive results.

6.3. Appendix 3 – Participants' reported quantities for positive results

Reported quantities for each target given in genome copies per gram for positive sample/target virus combinations

Lab ID	Shellfish sample 2						Shellfish sample 4					
	GI		GII		HAV		GI		GII		HAV	
	A	B	A	B	A	B	A	B	A	B	A	B
3	$7.37x10^3$	$8.83x10^3$	$1.45x10^2$	<LOQ	NE	NE	$1.02x10^3$	$1.24x10^3$	<LOQ	$4.05x10^2$	NE	NE
10	$2.30x10^3$	$1.31x10^3$	$1.03x10^3$	$1.06x10^3$	$6.72x10^3$	$3.84x10^3$	<LOQ	<LOQ	$6.04x10^2$	$6.35x10^2$	$1.27x10^3$	$6.76x10^2$
47	$1.07x10^4$	$7.41x10^3$	$2.41x10^3$	$2.43x10^3$	ND	ND	$1.60x10^3$	$1.22x10^3$	$6.90x10^3$	$7.37x10^2$	ND	ND
96	$4.90x10^4$	$1.57x10^4$	$8.87x10^2$	$1.61x10^3$	$2.73x10^4$	$2.52x10^4$	$9.06x10^3$	$3.33x10^3$	$5.52x10^2$	$1.00x10^3$	$5.03x10^3$	$4.91x10^3$
143	$3.53x10^4$	$2.39x10^4$	$3.78x10^3$	$1.43x10^3$	$2.05x10^4$	$7.08x10^3$	$6.66x10^3$	$4.35x10^3$	$1.60x10^3$	$6.03x10^2$	$2.07x10^3$	$6.60x10^2$
168	$4.61x10^3$	NE	$8.05x10^2$	NE	$6.81x10^4$	NE	$1.31x10^3$	NE	$9.16x10^2$	NE	$1.44x10^4$	NE
190	NQ	NQ	NQ	NQ	NQ	NQ	<LOQ	$6.70x10^2$	<LOQ	<LOQ	<LOQ	$8.00x10^2$
203	NE	$2.41x10^3$	NE	$5.10x10^2$	NE	$4.25x10^3$	NE	<LOQ	NE	ND	NE	$3.84x10^2$
237	$1.26x10^4$	$9.03x10^2$	<LOQ	<LOQ	$1.38x10^4$	$3.69x10^3$	$4.17x10^3$	$2.83x10^2$	<LOQ	<LOQ	$4.63x10^3$	$1.41x10^3$
423	$5.20x10^3$	$1.50x10^3$	<LOQ	$2.80x10^2$	$3.20x10^2$	$7.20x10^2$	$1.65x10^3$	$4.40x10^2$	$2.20x10^2$	$3.90x10^2$	<LOQ	<LOQ
462	$1.56x10^3$	$9.05x10^2$	$1.82x10^3$	$4.08x10^2$	$5.81x10^2$	$1.19x10^4$	$3.54x10^2$	$3.90x10^2$	$1.59x10^2$	$3.78x10^2$	$1.87x10^2$	$3.18x10^3$

Key: A = results obtained with lab's own quantification standards; **B** = results obtained with FAO Reference Centre quantification standards; **NE** = sample/target virus/quantification standard combination not tested (shaded grey); **ND** = target virus not detected (shaded yellow); **<LOQ** = positive results at below the laboratory LOQ reported (shaded blue); **NQ** = positive; not quantifiable results reported (shaded blue)

6.4. Appendix 4 - Comparison of results and median

Differences between participants' results and the participants' median, expressed in terms of σMAD, for positive sample/target virus combinations.

Lab ID	Shellfish sample 2			Shellfish sample 4		
	GI	GII	HAV	GI	GII	HAV
3	0.14	-1.70	NE	-0.35	-0.59	NE
10	-0.81	0.14	-0.26	NS	0.07	-0.15
47	0.45	0.94	ND	0.00	0.29	ND
96	1.69	0.00	0.76	1.34	-0.07	0.70
143	1.42	1.36	0.55	1.11	1.69	0.15
168	-0.24	-0.09	1.42	-0.16	0.76	1.35
190	NS	NS	NS	-0.67	NS	-0.44
203	-0.77	-0.52	-0.59	NS	ND	-0.89
237	0.58	NS	0.26	0.74	NS	0.65
423	-0.14	-1.08	-2.47	0.02	-1.59	NS
462	-1.12	0.67	-2.04	-1.17	-2.13	-1.34

Key: NE = Quantitative results for sample/target virus combination not reported (grey shading); **NS** = sample/target virus quantification not scored due to positive, <LOQ or positive, or not quantifiable results (shaded blue); **ND** = target virus not detected; **orange shading** = questionable results (magnitude of difference between result and participants' median >2 σMAD and <3 σMAD); **red shading** = unsatisfactory results (magnitude of difference between result and participants' median >3 σMAD) or false negative results.

6.5. Appendix 5 - Participants' and reference quantities by sample

Note: Where quantities were reported using both the laboratory's own quantification standards and those provided by the FAO Reference Centre, only those using the lab's own standards are considered for performance scoring.

Shellfish sample 2 – GI

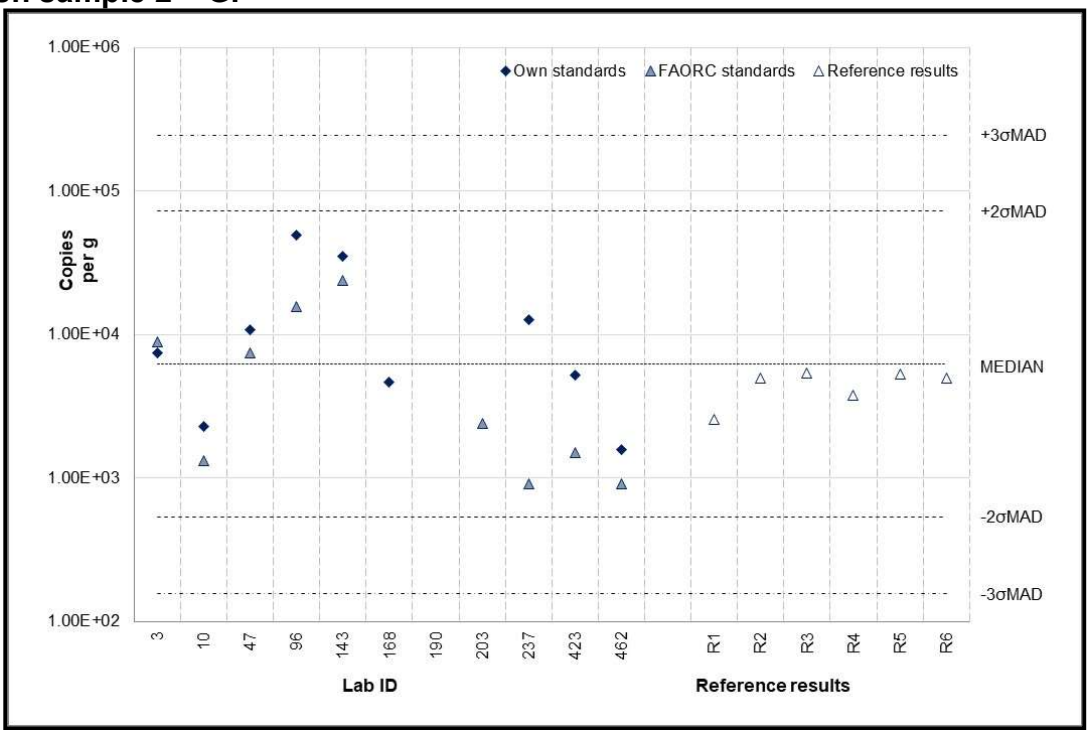

Figure 3. Scatter graph showing $\log^{10}$ copies per gram of norovirus genogroup I for shellfish sample 2.

Shellfish sample 2 – GII

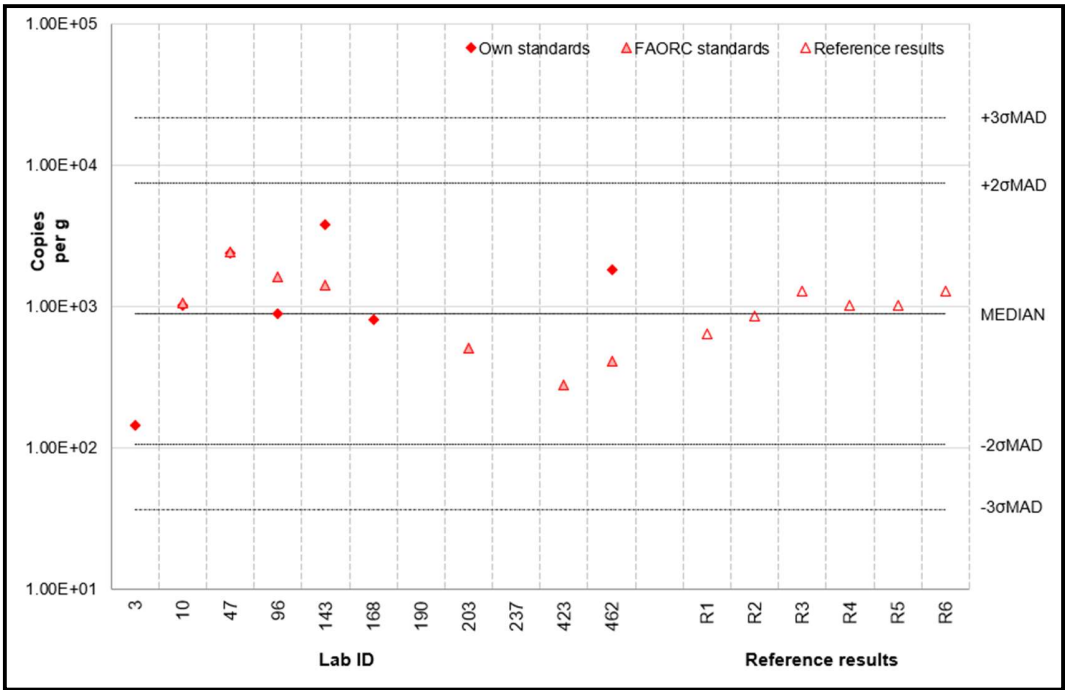

Figure 4. Scatter graph showing log[10] copies per gram of norovirus genogroup II for shellfish sample 2.

Shellfish sample 2 – HAV

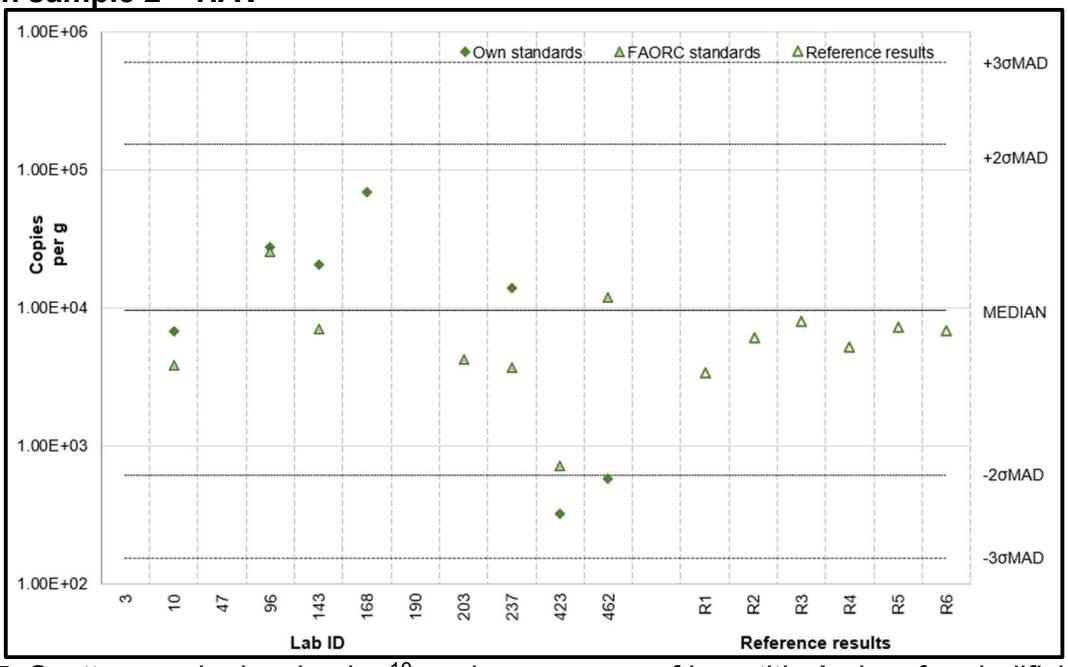

Figure 5. Scatter graph showing log[10] copies per gram of hepatitis A virus for shellfish sample 2.

Shellfish sample 4 – GI

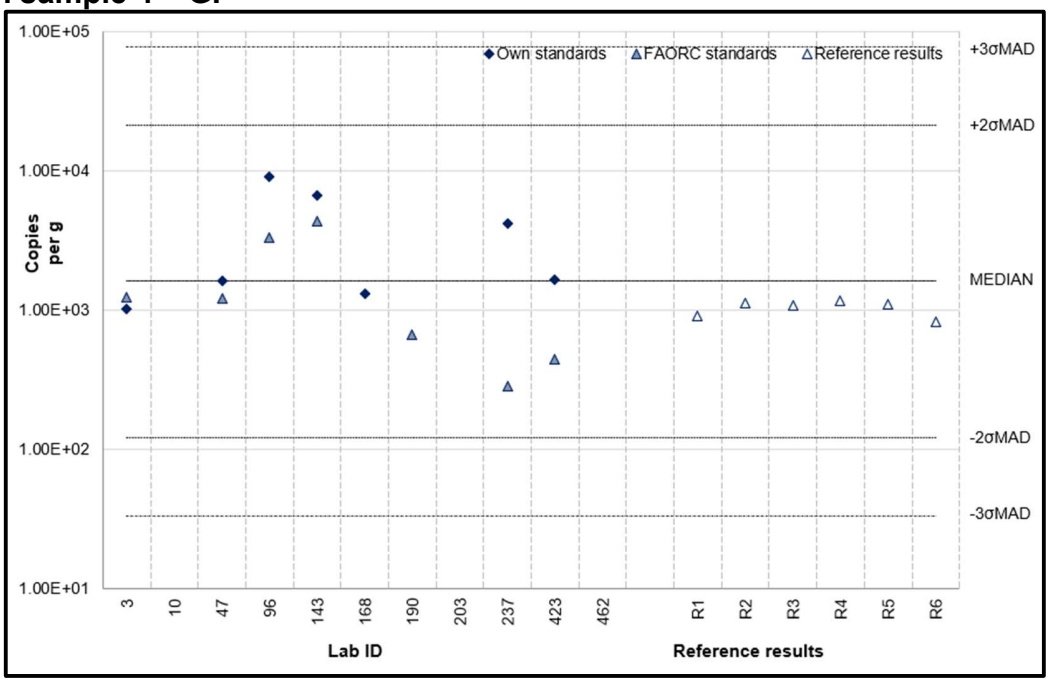

Figure 6. Scatter graph showing $\log^{10}$ copies per gram of norovirus genogroup I for shellfish sample 4.

Shellfish sample 4 – GII

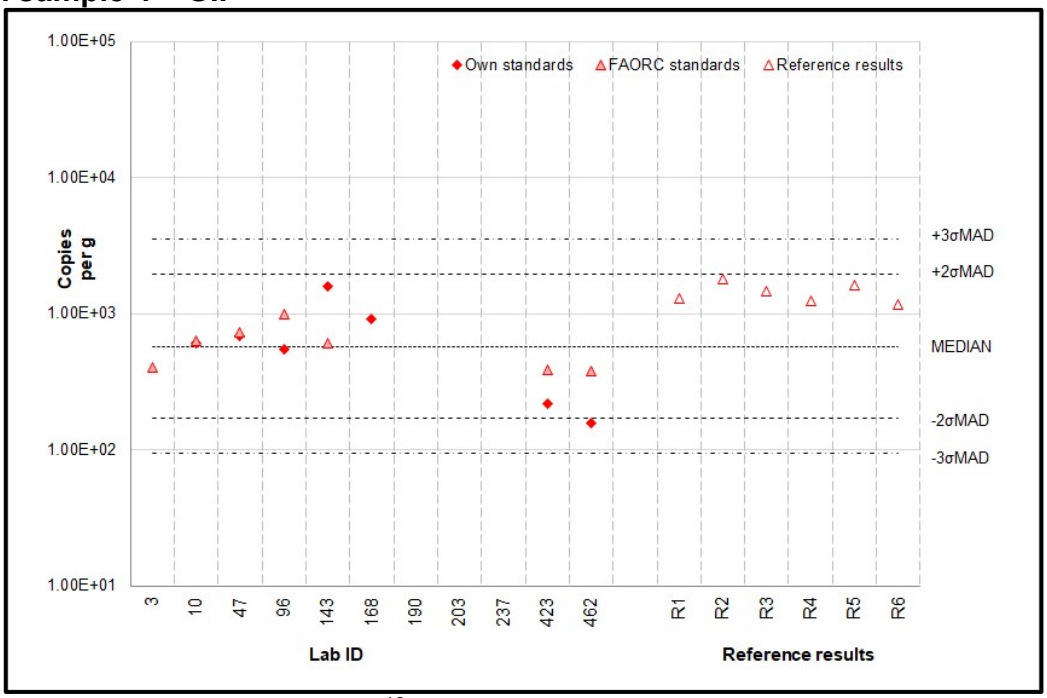

Figure 7. Scatter graph showing $\log^{10}$ copies per gram of norovirus genogroup II for shellfish sample 4.

Shellfish sample 4 – HAV

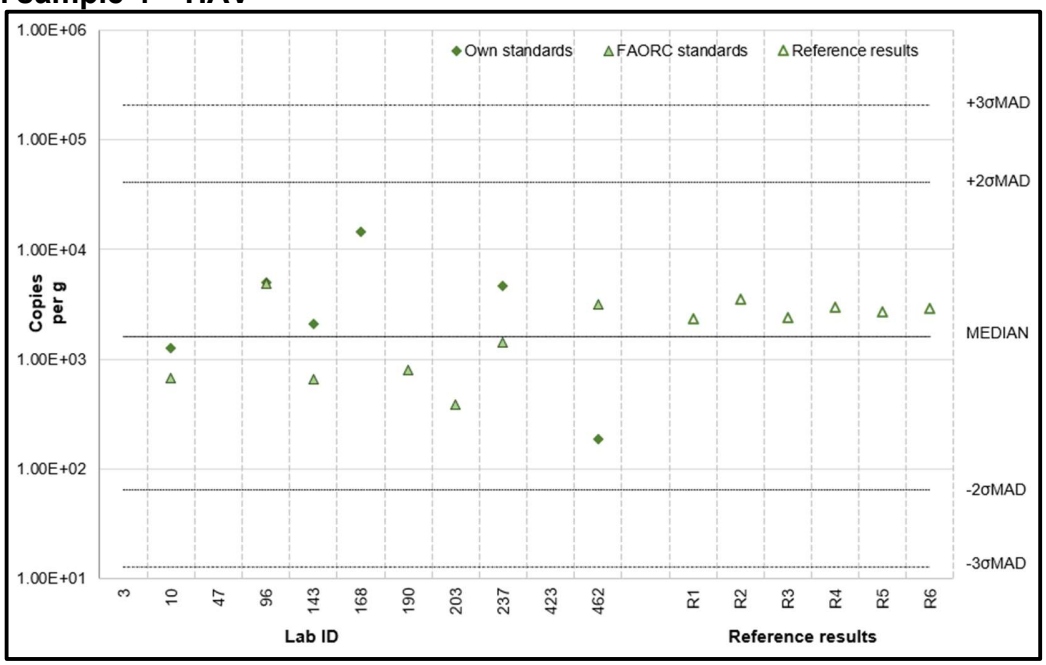

Figure 8. Scatter graph showing $\log^{10}$ copies per gram of hepatitis A virus for shellfish sample 4

6.6. Appendix 6 - Methods used by participants

LAB ID	Virus extraction	RNA extraction	RT-PCR method	RT-PCR reagents	Primers		
					GI	GII	HAV
3	A	B	D	F	N-1	N	N
10	A	B	D	F	N-1	N	N
20	A	B	D	G	O	O	O
24	A	C	E	H	P	Q	N
47	A	B	D	F	N-1	N	N
96	A	B	D	J	N-2	N	N
143	A	B	D	G	O	O	O
168	A	B	D	F	N-1	N	N
190	A	B	D	G	O	O	O
203	A	B	D	K	N-1	N	N
237	A	B	D	F	N-2	N	N
242	A	C	D	G	O	O	O
423	A	B	D	L	N-2	N	N
462	A	B	D	F	N-1	N	N

Method elements as described in the informative annexes of ISO 15216-1 are shaded grey.

Key to method codes

Virus extraction methods

A Proteinase K digestion

RNA extraction methods

B NucliSens Magnetic extraction reagents (BioMerieux)

C Roche High Pure Viral Nucleic Acid Kit

RT-PCR methods

D Real-time (quantitative) PCR - one-step

E Real-time (quantitative) PCR - two-step

RT-PCR reagents

F RNA Ultrasense (Invitrogen)

G Ceeram Tools

H RT: Invitrogen Superscript III ; PCR: Invitrogen Platinum® qPCR SuperMix-UDG

J Fast Virus 1-step MasterMix (ThermoFisher Scientific)

K Platinum quantitative RT-PCR Thermoscript One-step system (Invitrogen)

L GoTaq Probe qPCR and RT-qPCR Systems (One-Step RT-qPCR) (Promega)

Primers/probes

N ISO 15216-1; 1) with TM9 probe for NoV GI; 2) with NVGG1p probe for NoV GI

O Ceeram Tools (sequences as N-2)

P Wolf et al, 2010

Q Kageyama et al, 2003

6.7. Appendix 7 - Laboratory quantification standards

Details of each laboratory's own quantification standards

LAB ID	
3	Linearised ISO 15216-1 plasmid DNA, quantified using fluorimetry
10	PCR product amplified from ISO 15216-1 plasmid, quantified using A260 spectrophotometry
47	Linearised ISO 15216-1 plasmid DNA, quantified using A260 spectrophotometry
96	Linearised ISO 15216-1 plasmid DNA, quantified using A260 spectrophotometry and fluorimetry
143	Standards provided in Ceeram Tools kit
168	Linearised plasmid DNA, quantified using A260 spectrophotometry
190	Standards provided in Ceeram Tools kit
237	Commercially produced linear dsDNA (quantified by supplier)
423	Commercially produced linear dsDNA (quantified by supplier)
462	PCR product amplified from ISO 15216-1 plasmid, quantified using A260 spectrophotometry

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