Food and Agriculture Organization of the United Nations

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Centre for Environment Fisheries & Aquaculture Science

World Class Science for the Marine and Freshwater Environment

FAO Reference Centre for Bivalve Mollusc Sanitation

Norovirus (Genogroup I and II) and Hepatitis A virus Proficiency Testing (PT 79)

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Issue Date: 05.09.19 (Final V1)

Cefas Document Control

Submitted to:	PT 79 participants
Date submitted:	05.09.19
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Quality control by:	Dr. James Lowther
Approved by and date:	Dr. James Lowther 04.09.19
Version:	Final V1

Version Control History			
Version	Author	Date	Comment
Draft V1	L. Stockley	05.08.19	Released for internal review
Draft V2	L. Stockley	08.08.19	Version updated following JL comments
Draft V3	L. Stockley	28.08.19	No further changes required, submitted to participants for comments
Final V1	L. Stockley	05.09.19	Updated following participants comments

Contents **Page number**

Samples	2
Results	3
Performance Scoring	3
Discussion	5
References	6
Appendices	7

Samples

Materials dispatched consisted of whole Pacific oysters (*Crassostrea 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 in bioaccumulation are given in Table 1. All samples were held at <-15°C until required for quality control testing, dispatch and/or reference analysis.

Preparation of negative digestive gland blend

A batch of approximately 3000 Pacific oysters (*C. gigas*) collected from a UK commercial harvesting area were tested to demonstrate the absence of norovirus (NoV) genogroups I and II (GI and GII) and hepatitis A virus (HAV). Following testing, the remaining shellfish were shucked, and the digestive glands removed. The digestive glands were pooled together before being blended to form a homogenous mixture.

Preparation of highly contaminated digestive gland blends

Approximately 1000 Pacific oysters (*C. gigas*) collected from a UK commercial harvesting area were tested to demonstrate the absence of NoV GI, NoV GII and HAV before being placed in trays and immersed in 500 litres of re-circulating natural seawater at 16±1 °C. The shellfish were left to acclimatise for approximately 24 hours before 50 ml of shellfish food containing high levels of NoV GI from human faeces was added to the tank. After approximately 16 hours to allow bioaccumulation to occur, the shellfish were removed from the tank, shucked and the digestive glands removed. The digestive glands were pooled together before being blended to form a homogenous mixture. Separately this procedure was repeated with NoV GII from human faeces and HAV cell culture supernatant to produce three highly contaminated blends, each contaminated with a single target virus.

Shellfish Sample 1

Approximately 400 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area during the summer of 2018. In May 2019, subsamples of 10 oysters were randomly selected and placed in sample bags and stored at <-15°C.

Shellfish sample 2

Blended negative glands (see above) were mixed with the highly contaminated blends containing NoV GI and GII (as described above) 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.

Shellfish sample 3

Blended negative glands (see above) were mixed with the highly contaminated blends containing NoV GI and HAV (as described above) 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.

Shellfish sample 4

Blended negative glands (see above) were mixed with the highly contaminated blends containing NoV GII and HAV (as described above) 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 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.7 (based on capsid sequence)
Norovirus genogroup II	Faecal material	

Sample distribution

Samples were dispatched on dry ice in accordance with IATA packing instructions 650 for UN3373 'Diagnostic Specimens' on 17th June 2019 to 24 participating laboratories. Participants were requested 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.

Results

Reference results

Reference analyses were performed by the FAO Reference Centre 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 I.

Table 2: Reference results for PT 79 proficiency testing material

	Norovirus		
	GI	GII	
Shellfish sample 1 (Whole animal)	-	-	-
Shellfish sample 2 (Digestive gland)	+ ($3.25 \times 10^2 - 7.24 \times 10^2$)	+ ($1.10 \times 10^3 - 2.14 \times 10^3$)	-
Shellfish sample 3 (Digestive gland)	+ ($6.55 \times 10^2 - 9.57 \times 10^2$)	-	+ ($1.02 \times 10^3 - 2.23 \times 10^3$)
Shellfish sample 4 (Digestive gland)	-	+ ($1.98 \times 10^3 - 2.85 \times 10^3$)	+ ($9.98 \times 10^2 - 2.09 \times 10^3$)

Quantities in copies/g.

Note: Ranges based on a 95% confidence limit determined as 2 geometric standard deviations above and below the geometric mean.

Participants' results

Participant's results are tabulated in Appendices II, III and IV and quantitative results are shown in graphical form alongside the reference values in Appendix V.

Performance scoring

Presence/absence

For all laboratories, performance scoring was undertaken on 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. For each laboratory an overall score (out of 8) is provided for each target virus, taking into account the results of all 4 samples (Table 4).

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/TS 22117 Microbiology of food and animal feeding stuffs – 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 assessment. Where laboratories submitted quantitative results determined using both their own quantification standards, and those provided by the FAO Reference centre, only the results using their own standards were considered for performance scoring; however, where laboratories submitted quantitative results using the FAO Reference centre standards only, these were considered.

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 (σ_{MAD}; Table 3). For each individual result, its absolute deviation from the participants' median was compared with the calculated σ_{MAD} to determine its acceptability and score as follows:-

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

Where laboratories reported quantities for some samples as below or above a specific limit, these samples were not considered for quantitative performance scoring. The differences between individual participants' results and the participants' median, expressed in terms of σ_{MAD} are shown in Appendix IV, and the graphs in Appendix V 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

Quantity	Shellfish sample 2		Shellfish sample 3		Shellfish sample 4	
	GI	GII	GI	HAV	GII	HAV
MEDIAN	2.476	3.024	2.468	3.034	3.256	3.025
MAD	0.308	0.342	0.264	0.402	0.320	0.335
σMAD	0.456	0.508	0.391	0.596	0.474	0.497

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

Lab ID No.	Presence / Absence			Quantification		
	NoV		HAV	NoV		HAV
	GI	GII		GI	GII	
2	8 out of 8	8 out of 8	8 out of 8	NQ	NQ	NQ
3	8 out of 8	8 out of 8	8 out of 8	7 out of 8	8 out of 8	NQ
10	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
20	8 out of 8	8 out of 8	8 out of 8	NQ	NQ	NQ
21	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
24	8 out of 8	8 out of 8	8 out of 8	6 out of 8	8 out of 8	8 out of 8
25	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
35	8 out of 8	8 out of 8	6 out of 8	NQ	NQ	NQ
53	NE	NE	8 out of 8	NE	NE	NQ
57	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 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
113	4 out of 8	8 out of 8	4 out of 8	4 out of 8	4 out of 8	4 out of 8
157	8 out of 8	8 out of 8	6 out of 8	NQ	NQ	NQ
158	8 out of 8	8 out of 8	8 out of 8	NQ	NQ	NQ
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
193	8 out of 8	8 out of 8	8 out of 8	7 out of 8	8 out of 8	8 out of 8
203	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
214	8 out of 8	6 out of 8	4 out of 8	NQ	NQ	NQ
225	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
237	8 out of 8	8 out of 8	8 out of 8	5 out of 8	8 out of 8	8 out of 8
239	8 out of 8	8 out of 8	4 out of 8	4 out of 4*	6 out of 6*	4 out of 8
242	6 out of 8	8 out of 8	6 out of 8	NQ	NQ	NQ
263	8 out of 8	8 out of 8	8 out of 8	NQ	NQ	NQ
257	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8

Key: NE = Target virus not examined; NQ = Target virus not quantified; * = Results for one or more samples reported were positive but not quantified, these were excluded from quantification scoring.

Discussion

Twenty-four laboratories received samples, with all returning results. Laboratory 53 only examined the samples for HAV.

Methods used by participants to analyse the test samples are shown in Appendix VI, while brief details of the types of materials used as quantification standards are included as Appendix VII.

Presence/absence determination

Eighteen laboratories (75%) obtained the intended presence/absence result (as determined by the FAO Reference Centre) for all samples and all target viruses tested. Overall sensitivity, specificity and accuracy levels were 91%, 100% and 95% respectively.

For NoV, 20 out of 23 labs (87%) obtained the intended presence/absence result (as determined by the FAO Reference Centre) for all samples and both genogroups. A total of 4 false negative results (3 for GI, 1 for GII) were reported by 3 different laboratories, resulting in an overall sensitivity of 96%. No false positive results were reported (overall specificity 100%). Overall accuracy for norovirus results was 98%.

For HAV, 18 out of 24 labs (75%) obtained the intended presence/absence result (as determined by the FAO Reference Centre) for all samples. A total of 9 false negative results were reported by 6 different laboratories, resulting in an overall sensitivity of 81%. No false positive results were reported (overall specificity 100%). Overall accuracy for HAV results was 91%.

Quantification

All 24 laboratories reported Ct values, and in addition 15 laboratories (63%) reported quantitative data for at least one sample/virus combination. One of these laboratories (239) reported results for most positive sample/virus combinations as not quantifiable however. Results reported in this way were excluded from the quantification scoring. Of the 15 laboratories reporting quantitative data, 9 (60%) reported all results in the satisfactory range, scoring full marks for quantification for the three target viruses. Three laboratories (20%) reported at least one unsatisfactory result (including false negatives). Laboratory 113 reported unsatisfactory results for all positive sample/virus combinations. The FAO Reference Centre recommends any laboratory with unsatisfactory results for either presence/absence or quantification refers to the trouble shooting guide in Appendix VIII.

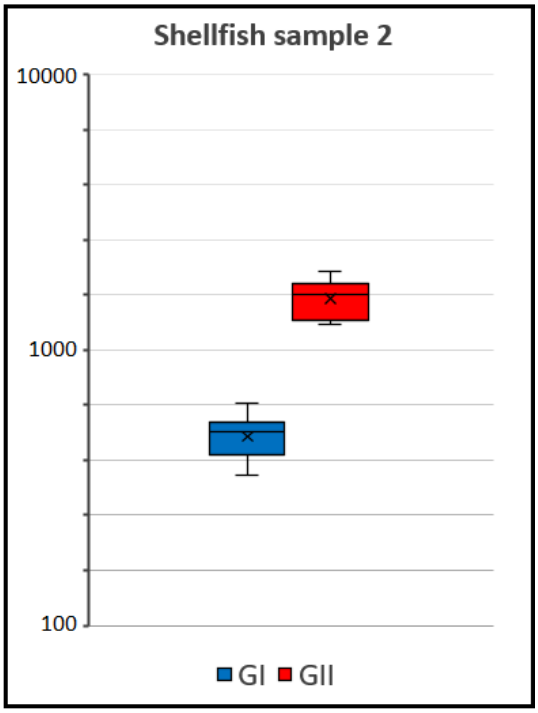
References

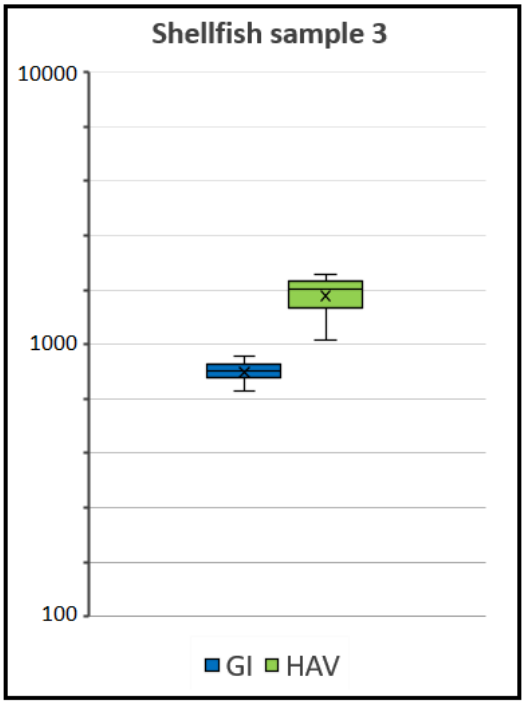
Codd AA, Richardson IR, Andrews N. 1998. Lenticules for the control of quantitative methods in food microbiology. J Appl Microbiol. 85(5):913–7.

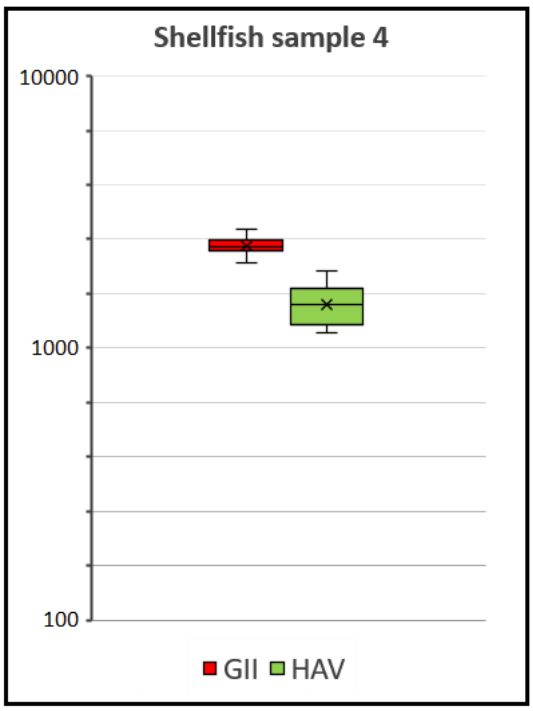
Anon 2017. ISO 15216-1:2017 Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR -- Part 1: Method for quantification.

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

Appendix I: FAO Reference Centre results displayed as box and whisker plots (log scale) of detectable genome copies per gram.







Appendix II: Participants' 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	
No.	-	Ct	-	Ct	-	Ct	+	Ct	+	Ct	-	Ct	+	Ct	-	Ct	+	Ct	-	Ct	+	Ct	+	Ct
2	-		-		-		+	35.977	+	35.015	-		+	35.974	-		+	33.22	-		+	35.605	+	32.73
3	-		-		-		+	39.95 / 39.59	+	35.39 / 36.10	-		+	39.52 / 38.98	-		+	34.91 / 35.51	-		+	35.55 / 34.96	+	34.99 / 34.73
10	-		-		-		+	36.18	+	35.1	-		+	36.48	-		+	36.04	-		+	33.3	+	35.27
20	-		-		-		+	33.37	+	31.9	-		+	33.08	-		+	33.34	-		+	32.08	+	37.43
21	-		-		-		+	32.1	+	33.2	-		+	33.2	-		+	35.3	-		+	33.2	+	35.3
24	-		-		-		+	35.4	+	31.6	-		+	35.7	-		+	32.7	-		+	30.9	+	32.5
25	-		-		-		+	36.13	+	34.11	-		+	36.44	-		+	36.81	-		+	33.25	+	36.27
35	-		-		-		+	36.77	+	38.16	-		+	39.52	-		+	39	-		+	39.21	-	
53	NE		NE		-		NE		NE		-		NE		NE		+	34.81	NE		NE		+	33.86
57	-		-		-		+	38.71	+	35,77	-		+	36.77	-		+	35.49	-		+	33.84	+	35.09
96	-		-		-		+	37.32 / 36.54	+	34.18 / 33.60	-		+	36.26 / 36.55	-		+	32.72 / 32.91	-		+	33.72 / 34.15	+	33.63 / 33.28
113	-		-		-		-		+	41.38	-		-		-		-		-		+	40.41	-	
157	-		-		-		+	28.5	+	30	-		+	33.2	-		+	34.3	-		+	32.2	-	
158	-		-		-		+	34.39	+	32.33	-		+	32.89	-		+	34.89	-		+	31.52	+	34.78
168	-		-		-		+	34.94	+	33.48	-		+	35.4	-		+	34.37	-		+	33.27	+	34.82
193	-		-		-		+	37.36	+	33.29	-		+	39.17	-		+	35.48	-		+	33.28	+	35.98
203	-		-		-		+	35.8 / 35.37	+	33.04 / 33.44	-		+	34.32 / 35.93	-		+	34.65 / 34.91	-		+	32.62 / 32.69	+	34.72 / 35.49
214	-		-		-		+	39.2	-		-		+	39	-		-		-		+	39.15	-	
225	-		-		-		+	34.62	+	35.41	-		+	34.89	-		+	34.89	-		+	35.96	+	34.13
237	-		-		-		+	40.26	+	31.61	-		+	35.13	-		+	34.21	-		+	31.76	+	35.12
239	-		-		-		+	36.09	+	33.36	-		+	36.2	-		-		-		+	36.7	-	
242	-		-		-		+	42.68	+	38	-		-		-		+	42.59	-		+	34.1	-	
257	-		-		-		+	34.88	+	35.7	-		+	34.92	-		+	34.9	-		+	35.67	+	34.16
263	-		-		-		+	35.395	+	34.247	-		+	35.291	-		+	33.007	-		+	34.549	+	32.89

Key: NE= sample/target virus combination not examined (shaded grey). Yellow shading denotes false negative results.

Appendix III: **Participants reported quantities for each target (copies/g)**

Lab ID No.	Shellfish sample 2				Shellfish sample 3				Shellfish sample 4			
	GI		GII		GI		HAV		GII		HAV	
	A	B	A	B	A	B	A	B	A	B	A	B
3	1.53E+03	1.02E+03	6.32E+03	8.11E+03	2.06E+03	2.20E+03			8.58E+03	8.24E+03		
10	3.31E+02		7.37E+02		2.70E+02			7.18E+02	2.57E+03			1.18E+03
21		6.07E+02		1.59E+03		2.64E+02		1.74E+03		1.56E+03		1.62E+03
24		2.60E+03	2.70E+03	7.80E+03		2.30E+03	1.20E+03	9.10E+03	4.40E+03	1.10E+04	1.40E+03	9.90E+03
25		5.80E+02		2.20E+03		4.80E+02		3.30E+02		3.80E+03		4.60E+02
57	1.70E+02	4.80E+01	4.30E+02	4.70E+02	5.40E+02	1.70E+02	2.90E+03	4.80E+02	1.50E+03	1.50E+03	3.80E+03	6.20E+02
96	1.78E+02	1.48E+02	2.56E+03	2.71E+03	2.46E+02	2.04E+02	1.43E+03	2.12E+03	2.08E+03	2.21E+03	9.49E+02	1.43E+03
113			1.94E+01						3.71E+01			
168	1.20E+03	1.49E+03	1.96E+03	3.45E+03	8.73E+02	1.08E+03		2.75E+03	2.40E+03	4.22E+03		2.15E+03
193		1.33E+02	9.38E+03	1.61E+03		3.80E+01		9.76E+02	6.19E+03	1.63E+03		7.31E+02
203		1.03E+02		1.06E+03		1.09E+02		4.31E+02		1.49E+03		3.68E+02
225	2.99E+02	1.59E+03	4.80E+02	1.01E+03	2.81E+02	1.20E+03	1.20E+02	2.78E+02	2.92E+02	3.00E+02	2.10E+02	2.32E+02
237	1.02E+00	1.02E+00	9.55E+02	6.61E+02	4.27E+03	4.79E+02	1.55E+04	1.55E+03	8.71E+02	6.03E+02	9.11E+03	8.13E+02
239	<LC	<LC	1.18E+02	<LC	<LC	<LC			<LC	<LC		
257	2.87E+02	1.52E+03	4.93E+02	1.03E+03	2.94E+02	1.25E+03	1.25E+02	2.90E+02	3.00E+02	3.10E+02	2.14E+02	2.36E+02

Key: A = Results obtained with lab's own quantification standards; B = Results obtained with FAO Reference Centre quantification standards; results reported as <x or >x are included for information and are presented as reported by the relevant participant.

Appendix IV: **Differences between participants' results and the participants' median, expressed in terms of σ_{MAD}**

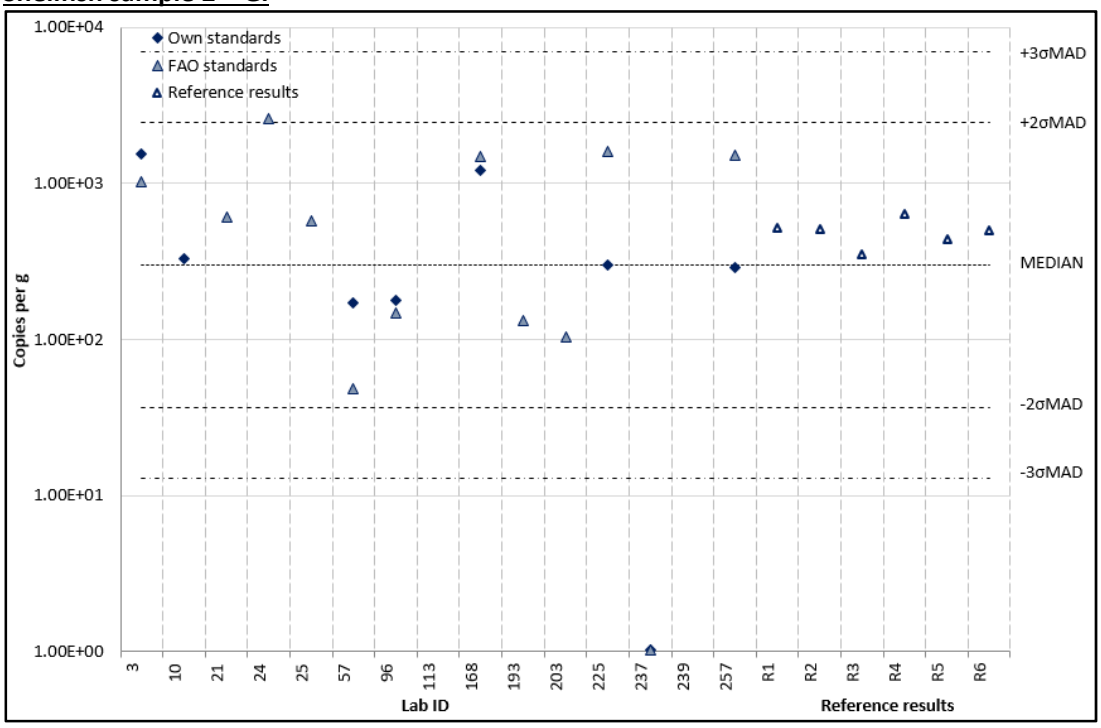
Lab ID No.	Shellfish sample 2		Shellfish sample 3		Shellfish sample 4	
	GI	GII	GI	HAV	GII	HAV
3	1.56	1.53	2.16	NQ	1.43	NQ
10	0.10	-0.31	-0.09	-0.30	0.32	0.10
21	0.67	0.35	-0.12	0.35	-0.13	0.37
24	2.06	0.80	2.28	0.08	0.82	0.24
25	0.63	0.63	0.54	-0.87	0.68	-0.73
57	-0.54	-0.77	0.67	0.72	-0.17	1.12
96	-0.49	0.76	-0.20	0.20	0.13	-0.10
113	Negative	-3.42	Negative	Negative	-3.56	Negative
168	1.32	0.53	1.21	0.68	0.26	0.62
193	-0.77	1.87	-2.27	-0.08	1.13	-0.32
203	-1.02	0.00	-1.10	-0.67	-0.18	-0.92
225	0.00	-0.67	-0.05	-1.60	-1.67	-1.41
237	-5.41	-0.09	2.97	1.94	-0.67	1.88
239	NQ	-1.87	NQ	Negative	NQ	Negative
257	-0.04	-0.65	0.00	-1.57	-1.64	-1.40

Key: Red shading = Unsatisfactory results (false negative, or magnitude of difference between result and participants' median >3 σ_{MAD}); Orange shading = Questionable results (magnitude of difference between result and participants' median >2 σ_{MAD} and <3 σ_{MAD}); Grey shading (NQ) = quantitative results not reported, excluded from scoring.

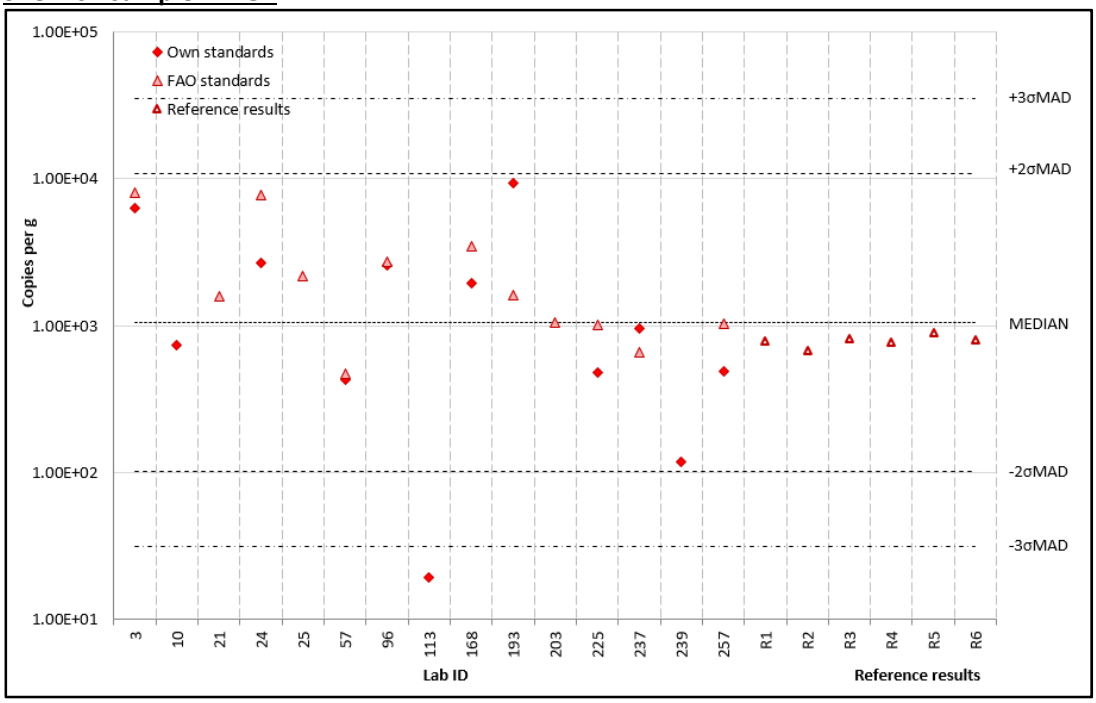
Appendix V: Participants' and reference quantities for each sample.

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.

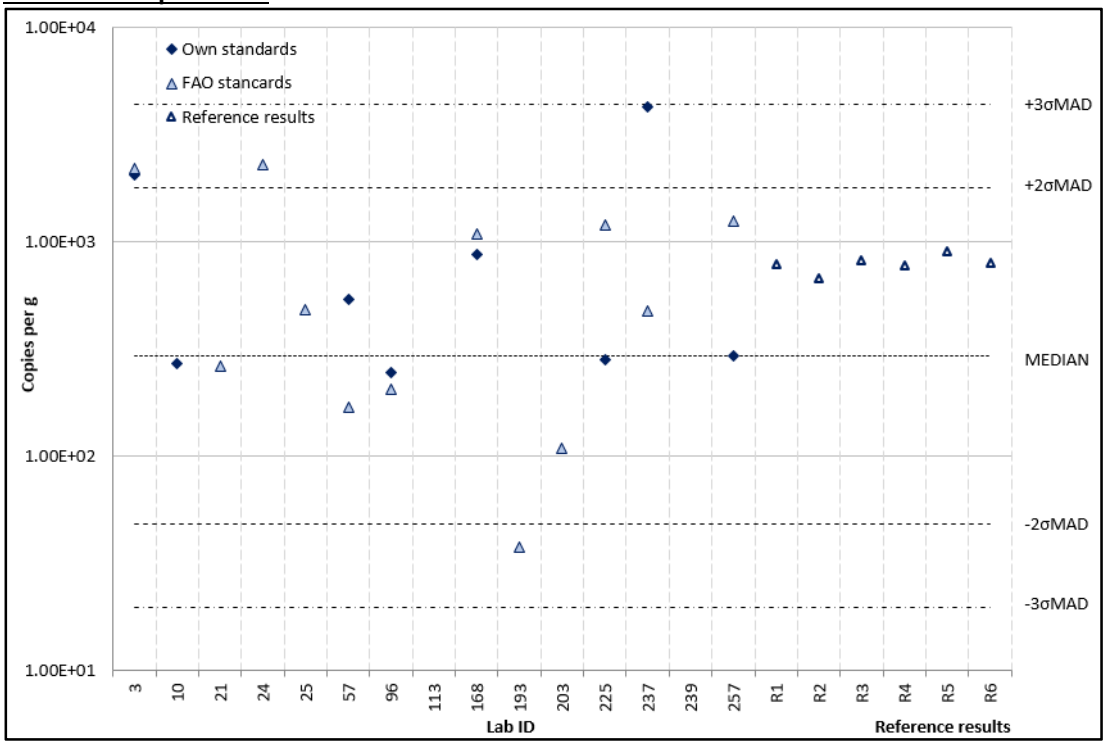
Shellfish sample 2 – GI



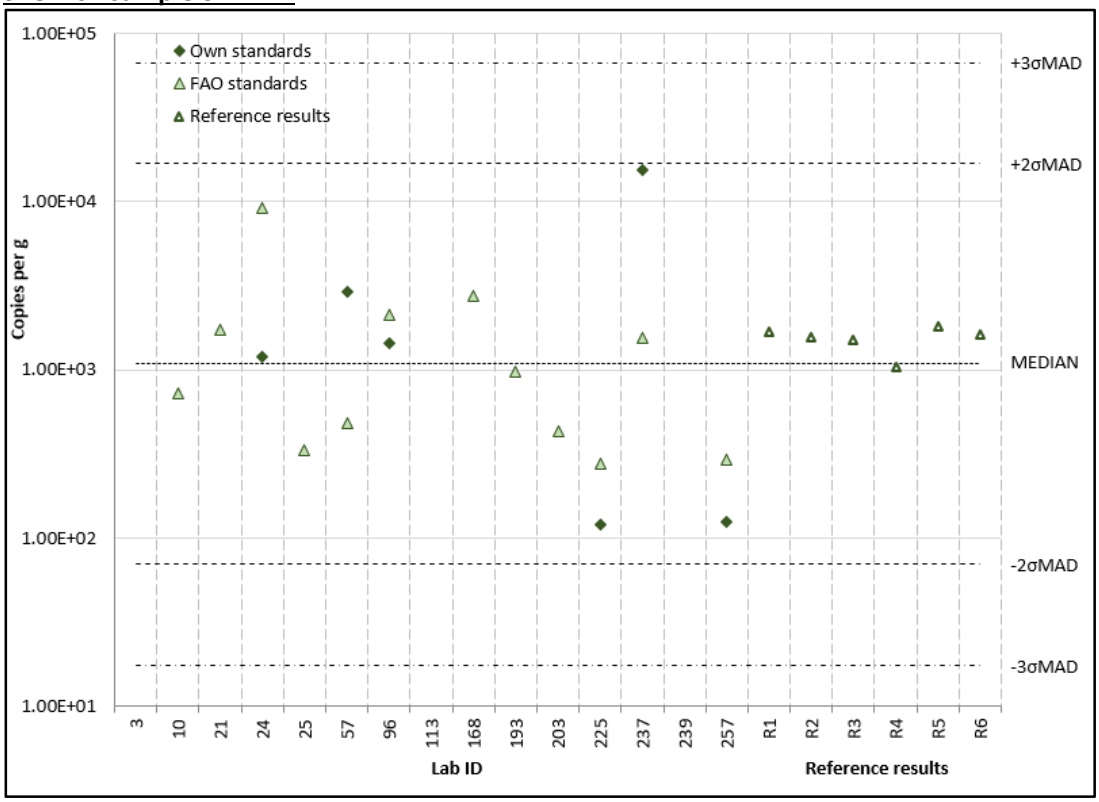
Shellfish sample 2 – GII



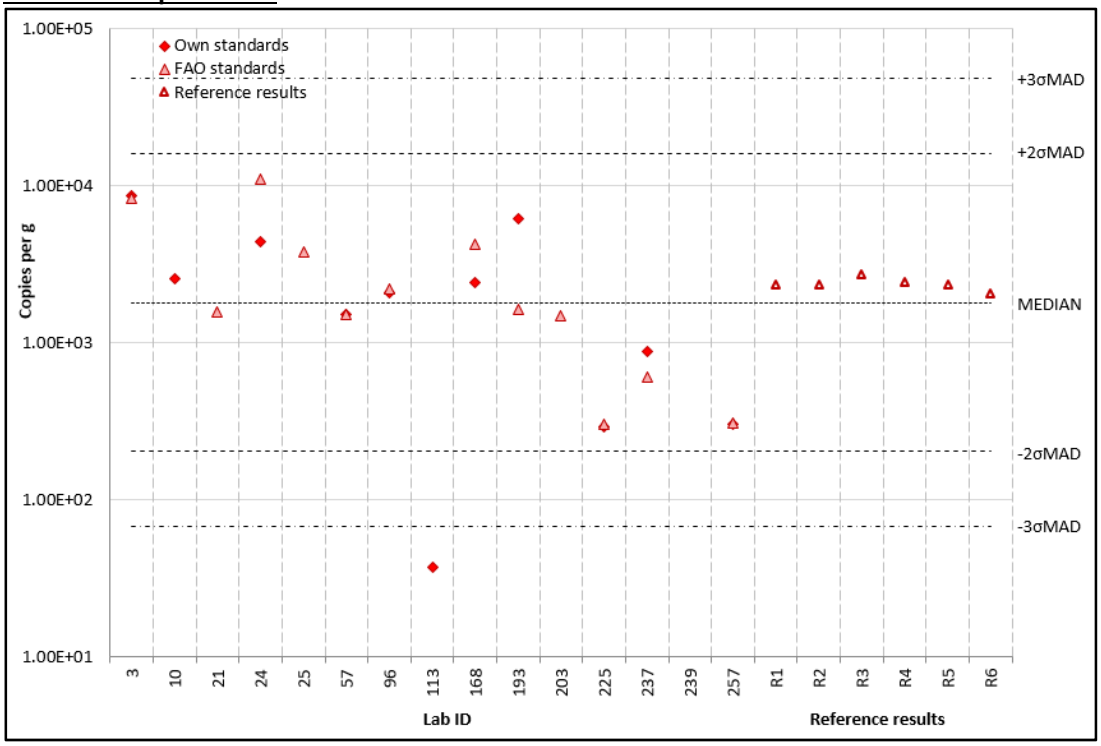
Shellfish sample 3 – GI



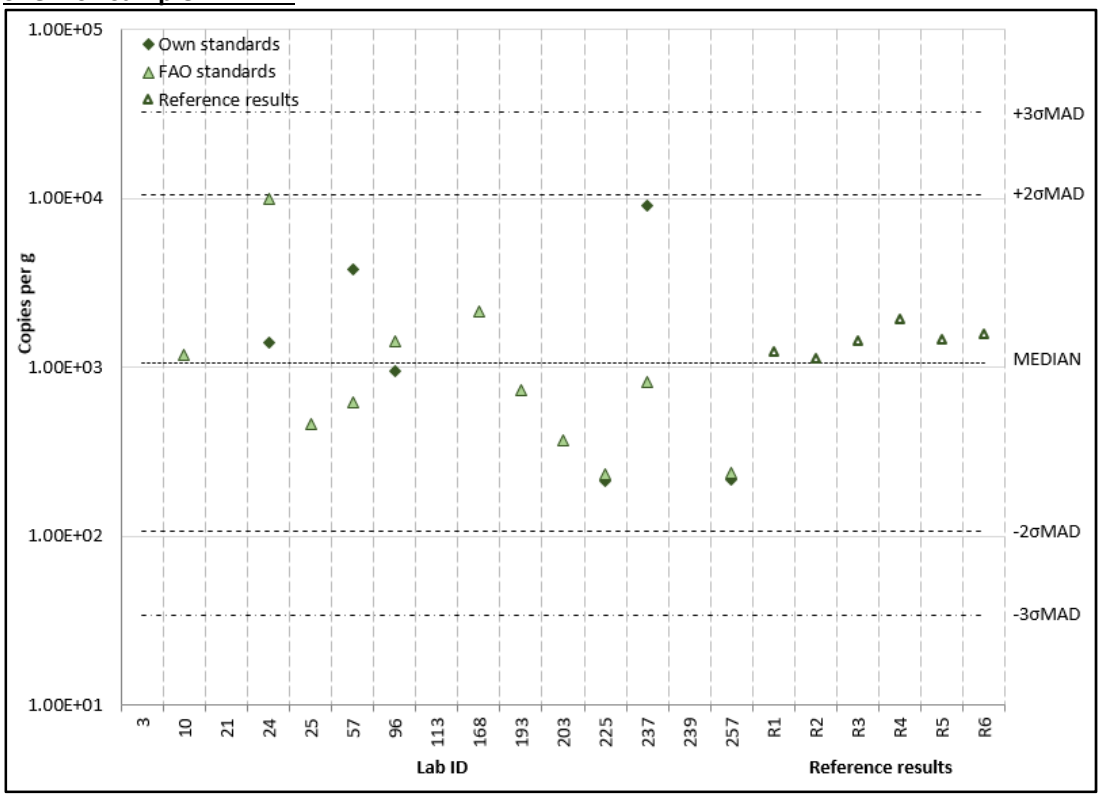
Shellfish sample 3 – HAV



Shellfish sample 4 – GII



Shellfish sample 4 – HAV



Appendix VI: Results and methods used. (For key to method codes see page 15)

LAB ID	Shellfish Sample 1			Shellfish Sample 2			Shellfish Sample 3			Shellfish Sample 4			Virus extraction	RNA extraction	RT-PCR method	RT-PCR reagents	Primers		
	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV	GI	+	HAV					GI	GII	HAV
2	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	L	AA-1	AA	EE
3	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-1	AA	AA
10	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-1	AA	AA
20	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	N	BB	BB	BB
21	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-2	AA	AA
24	-	-	-	+	+	-	+	-	+	-	+	+	A	D	K	O	AA-2	DD	AA
25	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-2	AA	AA
35	-	-	-	+	+	-	+	-	+	-	+	-	A	C	J	P	AA-1	AA	AA
53	NE	NE	-	NE	NE	-	NE	NE	+	NE	NE	+	A	C	J	N	-	-	BB
57	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-2	AA	AA
96	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-2	AA	AA
113	-	-	-	-	+	-	-	-	-	-	+	-	A	C	J	Q	AA-2	AA	AA
157	-	-	-	+	+	-	+	-	+	-	+	-	B	E	J	R	CC	CC	CC
158	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	N	BB	BB	BB
168	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-1	AA	AA
193	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	N	BB	BB	BB
203	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	S	AA-1	AA	AA
214	-	-	-	+	-	-	+	-	-	-	+	-	A	F	J	N	BB	BB	BB
225	-	-	-	+	+	-	+	-	+	-	+	+	A	D	J	T	AA-2	AA	AA
237	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	M	AA-2	AA	AA
239	-	-	-	+	+	-	+	-	-	-	+	-	A	G	J	N	BB	BB	BB
242	-	-	-	+	+	-	-	-	+	-	+	-	A	H	J	N	BB	BB	BB
257	-	-	-	+	+	-	+	-	+	-	+	+	A	I	J	T	AA-2	AA	AA
263	-	-	-	+	+	-	+	-	+	-	+	+	A	C	J	U	AA-1	AA	FF

Key: NE = target virus not examined, yellow shading = Ddenotes false negative results, grey shading = denotes method elements as described in the main body and informative annexes of ISO 15216-1

Key to method codes

Virus extraction methods	
A	Proteinase K digestion
B	Proteinase K digestion with chloroform:butanol clean-up
RNA extraction methods	
C	NucliSens Magnetic extraction reagents (BioMerieux)
D	High Pure Viral RNA Kit (Roche)
E	QIAmp viral RNA mini kit (Qiagen)
F	foodproof® Sample Preparation Kit IV (BIOTECON Diagnostics GmbH)
G	MagMAX kit (Applied Biosystems)
H	High Pure Viral RNA Kit (Roche) & OneStep PCR Inhibitor Removal Kit (Zymo Research)
I	IQ2000 kit (GeneReach Biotechnology)
RT-PCR methods	
J	Real-time one-step
K	Real-time two-step
RT-PCR reagents	
L	Norovirus GI and HAV: TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems). Norovirus GII: RNA Ultrasense (Invitrogen)
M	RNA Ultrasense (Invitrogen)
N	Ceeram Tools
O	Superscript III (RT) & Platinum® qPCR SuperMix-UDG (Invitrogen)
P	Quantitect RT-PCR kits (Qiagen)
Q	One Step RT-PCR kit (Qiagen)
R	foodproof® Detection Kits (BIOTECON Diagnostics GmbH)
S	Platinum quantitative RT-PCR Thermoscript One-step system (Invitrogen)
T	Probe 1-step master mix (Promega)
U	TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems) & RNA Ultrasense (Invitrogen)
Primers/probes	
AA	ISO 15216-1; 1) with TM9 probe for noV GI; 2) with NVGG1p probe for noV GI
BB	Ceeram Tools (sequences as AA-2)
CC	foodproof® Detection Kits (BIOTECON Diagnostics GmbH)
DD	Kageyama *et al*, (2003)
EE	OPFLP-07
FF	Guevremont *et al*., 2006; Houde *et al*., 2007

Appendix VII: Details of laboratory's own quantification standards (preparation and quantification)

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
24	Linearised plasmid DNA
57	Non-linearised plasmid DNA - quantified using A260 spectrophotometry
96	Linearised ISO 15216-1 plasmid DNA, quantified using A260 spectrophotometry and fluorimetry
113	Ceeram tools quantification standards (quantified by supplier)
168	Linearised plasmid DNA
193	*No information provided*
225	RNA quantified using A260 spectrophotometry
237	Commercially produced linear dsDNA (quantified by supplier)
239	*No information provided*
257	RNA quantified using A260 spectrophotometry

Appendix VIII: Guidance for troubleshooting problematic results in virus PT

Introduction

Evidence from Proficiency Testing (PT) suggests that different methods can give different results in the detection and quantification of viruses in bivalve shellfish samples. It is therefore as a first principle recommended that laboratories experiencing problems in PT adopt a method based on ISO 15216-1:2017. Comparison of the ISO with an in-house method can help to isolate the root causes of problems. ISO 15216-1:2017 provides flexibility in the choices of reagents for RNA extraction and RT-PCR, however specific reagents and protocols are provided in the informative annexes; these methods were tested by 13 labs during the validation of ISO 15216-1. The specific methods in the informative annexes are also included in the former EURL generic protocol (available at https://eurlcefas.org/public-documents/methods.aspx). Alternative in-house methods should be tested against the ISO method (including the informative annexes) on a range of naturally contaminated positive and negative samples to ensure they are comparable before adoption for routine testing.

Problematic results in PT can be broadly classified into 3 types:

- False positives (positive results in samples intended as negative)
- False negatives (negative results in samples intended as positive)
- Results outside the acceptable quantification limits

Further details for troubleshooting results of these types are given below.

False positives (Positive results in samples intended as negative)

False positive results are most often caused by contamination, either between samples (cross-contamination) or through contamination of samples with the products of PCR amplification.

Prevention of contamination

It is imperative that laboratories separate areas used for sample preparation and PCR/post-PCR activities (including preparation of positive control materials) in accordance with ISO 22174. Failure to understand the important of strict separation, particularly in laboratories unfamiliar with the use of PCR for diagnostic detection in low titre samples, is the root cause of many contamination events. In addition to strict separation, the use of separate workspaces for testing unknown food samples and potentially highly contaminated clinical or environmental samples (e.g. wastewater) is desirable to avoid cross-contamination. Where laboratory separation is not currently implemented, it is advisable that the laboratory prioritises this, otherwise repeat problems with false positive results can be anticipated.

To identify contamination rapidly it is vital that laboratories follow the guidance in the EURL generic protocol and ISO 15216 on the use of negative controls.

Removal of contamination

The severity of contamination can be approximated by assessing the equivalent concentrations in the affected samples. Where these are low, close to the limit of detection of the assay, and where there is a mixture of positive and negative PCR replicates from a sample or set of samples, the contamination issue can normally be rectified taking simple steps as described below. Where positive results equivalent to high concentrations are present across all samples intended as negative this is indicative of a severe contamination problem that will require more radical attention. The physical source of contamination must be identified and either thoroughly cleaned with a product that can degrade nucleic acid or removed and replaced. Swabbing and testing using PCR of

multiple different areas within the laboratory can aid identification of the contamination source. It may also be useful to determine whether the contamination is caused by RNA (e.g. from highly contaminated clinical/environmental samples) or DNA (from positive controls/PCR products); this can be achieved through testing in parallel using mastermixes that can amplify DNA and RNA, or DNA only. Where contamination is severe it is likely that even after removal of the source of contamination residual contaminating nucleic acid will be widespread in the laboratory environment. The laboratory must be thoroughly cleaned and potentially contaminated reagents replaced. Following these steps, absence of contamination should be confirmed by testing multiple aliquots of negative material (e.g. water) and checking for complete absence of amplification.

Where levels of contamination are low it is likely that thorough cleaning of the laboratory followed by replacement of reagents will be sufficient to remove contamination. This should be confirmed by testing multiple aliquots of negative material as above however.

False negatives (Negative results in samples intended as positive)
False negative results can arise due to a variety of different issues with sample processing, virus extraction, RNA extraction or PCR.

If quantification standards are provided by the FAO Reference Centre, poor sensitivity (negative or high Cq values [e.g. over 25] with the undiluted controls) may indicate an issue with the PCR, for example poor quality reagents. Note however, that the quantification controls used by the FAO Reference Centre are designed to react with the primers in the informative annexes of ISO 15216, and the controls may not work with all primer/probe combinations. False positive results for one target virus but not for the others may also be indicative of a problem with the PCR primers and probes for the affected target virus.

Where results with unextracted positive material are as intended but all/most extracted samples intended as positive (including non-matrix samples e.g. Lenticules if provided) produce negative results, this may indicate a problem with RNA extraction.

Where results are as intended with non-matrix samples (e.g. Lenticules if provided), but false negative results are obtained with matrix samples, then this indicates a problem with the matrix-specific parts of the procedure e.g. sample processing or virus extraction. It should be noted that PCR is particularly sensitive to ineffectively extracted shellfish matrix which is known to cause inhibition.

In all cases affected laboratories should ensure that the method specifications provided in the normative part of ISO 15216 should be followed, and to ensure best practise, the specific methods and reagents for samples processing, virus extraction, RNA extraction and PCR provided in the informative annexes of the ISO and also the EURL generic protocol should be used where possible. Proper storage of reagents is also important and appropriate recommendations should be followed.

Where results are indicative of a serious failure of the detection method, it may be possible for the FAO Reference Centre to provide training.

Results outside the acceptable quantification limits
Where results reported are above the upper acceptable quantification limit (determined through analysis of all participants' results) this is likely to be caused by either an incorrect concentration ascribed to the laboratories quantification standard, or by mistakes in the quantity calculations. Experience suggests it is unlikely that results of this type are caused by "super-efficient" extraction of the samples. Where results are below the lower acceptable limit, in addition to an incorrect concentration ascribed to the laboratories quantification standard,

or mistakes in the quantity calculations as described above, problems with incorrect or inefficient sample processing, virus or RNA extraction, or PCR can contribute as with false negative results (see above).

Comparison of the laboratory's Cq values for the affected sample with the reference values in the PT, and also those of other laboratories, can help with the identification of the problem; where these are similar it is most likely that problems with the quantification of the standard or with the quantification calculations are major factors in the unacceptable results. However, this is only an indication since different PCR reagents and platforms can produce widely different Cq values from equivalent starting material.

For many PT distributions the FAO Reference Centre will provide ready-to-use quantification standards. If possible, the results for the laboratory's own standards should be compared with these. If the results are significantly different this is indicative of a problem with the laboratory's own standards. Laboratories should ensure that they are using quantification standards consistent with the instructions in ISO 15216-1 i.e. linear dsDNA quantified diluted to working concentrations using an appropriate buffer (e.g. TE). A method for generation of such a standard is provided in the EURL generic protocol at https://eurlcefas.org/public-documents/methods.aspx. Quantification of the standards should use spectrophotometry, fluorimetry or digital PCR; ideally, independent confirmation of the concentration by more than one method is valuable. Care should also be taken to ensure that dilution of the standards to working concentration is carried out correctly and should be double checked during troubleshooting.

Errors in quantity calculations can also result in results outside the acceptable range. Laboratories should check their calculations carefully against the formulae given in ISO 15216-1 and the EURL generic protocol, again double-checking during troubleshooting. In addition a ready-to-use quantification spreadsheet for use with the ISO method is provided on the former EURL website at https://eurlcefas.org/public-documents/methods.aspx.

Where problems with quantification standards or quantity calculations can be ruled out, and where Cq values are higher, and quantities lower, for the affected samples, the root cause of the problem may be inefficient extraction of virus RNA from the samples. The rectification approach should be as for false negative results (see above).

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