

# **European Union Reference Laboratory (EURL) Proficiency Testing Scheme**

**Noroviruses and hepatitis A virus**

**EURL PT reference number: PT 65**

**Final report version 1**

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<b>Document prepared by:</b>	<b>Louise Stockley</b>	<b>Location</b>	



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### Samples

Material dispatched consisted of two laboratory constructed LENTICULES™. Table 1 shows details of the stock viruses used in the preparation of the LENTICULES™.

**Table 1: Origin and strain/genotype of viruses used for preparation of LENTICULE™ sample**

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

### Sample preparation

Two batches of laboratory constructed LENTICULES™ were prepared following the method of Codd *et al* (1998) with minor modifications. The mix prepared for LENTICULE™ 1 included known levels of GI norovirus from human faeces, while the mix for LENTICULE™ 2 included known levels of GII norovirus from human faeces and HAV cell culture supernatant.

### Sample distribution

Samples were dispatched in accordance with IATA packing instructions 650 for UN3373 'Diagnostic Specimens' on 4<sup>th</sup> July 2016 to 43 participating laboratories. All participants were requested to examine the samples using their routine method. Those laboratories using quantitative real-time PCR were requested to calculate the quantity of target virus in each sample using their standard quantification methods. Laboratories were requested to submit their results by 12<sup>th</sup> August 2016.

### Results

#### Reference results

Reference analyses were performed by the EURL on samples stored at <-15°C. Six randomly selected LENTICULES™ from each sample were extracted in duplicate and qRT-PCR (TaqMan™) was carried out using triplicate 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 65 proficiency testing material**

Sample	Norovirus GI	GII	HAV
LENTICULE™ 1	+ (1.60 x 10 <sup>4</sup> – 2.86 x 10 <sup>4</sup> )	-	-
LENTICULE™ 2	-	+ (1.72 x 10 <sup>3</sup> – 2.81 x 10 <sup>3</sup> )	+ (3.01 x 10 <sup>4</sup> – 4.24 x 10 <sup>4</sup> )

Results expressed as copies/LENTICULE. Ranges based on a 95% confidence limit determined as 2 geometric standard deviations above and below the geometric mean.

#### Participants' results

Performance assessment was undertaken on participant's presence / absence data for each parameter. Scores were assigned as follows: A = satisfactory (100% accuracy), B = questionable (one incorrect result), C = unsatisfactory (two or more incorrect results). For those participants reporting quantitative data, an additional assessment of the quantities ascribed to positive samples based on the approach described in ISO/TS 22117 (Anon 2010) was adopted according to the calculations described in Appendix II. Allocated scores for NoV (GI and GII) and HAV are shown in table 3.

**Table 3: Participants' results for all LENTICUE™ samples**

ID No.	Presence / Absence		Quantification		ID No.	Presence / Absence		Quantification	
	NoV (GI and GII)	HAV	NoV (GI and GII)	HAV		NoV (GI and GII)	HAV	NoV (GI and GII)	HAV
2	A	A	A	A	96	A	A	A	A
3 *	A	A	A	-	98	A	A	-	-
7 *	A	A	B	A	102 *	A	A	-	-
9 *	C	NE	B	-	110	A	A	A	C
10 *	A	A	A	A	147 *	A	A	-	-
17 *	A	A	A	A	161	A	NE	-	-
19 *	A	A	A	A	163	A	A	B	A
20	A	A	-	-	168	A	A	C	B
22 *	NR	NR	-	-	169	A	A	-	-
25 *	A	A	A	B	177	A	A	-	-
27 *	A	A	A	A	183	A	A	-	-
35 *	A	A	-	-	188	A	A	-	-
39 *	A	A	C	A	193	NR	NR	-	-
41 *	A	A	A	A	203 *	A	A	A	A
42 *	A	NE	-	-	205	A	A	-	-
47 *	B	A	B	A	218	A	A	-	-
48	A	A	-	-	227	A	A	A	A
49	A	A	C	A	229	A	A	-	-
72	B	NE	C	-	242	A	A	-	-
90 *	A	A	C	B	245	NR	NR	-	-
94	NR	NR	-	-	250	NE	A	-	-
95	A	A	-	-					

\* = Designated NRL, NE= Not examined, NR = Results not returned. Norovirus (NoV) and HAV scored separately.

Presence / absence performance scoring; **A** = satisfactory (100% accuracy), **B** = questionable (one incorrect result), **C** = unsatisfactory (two or more incorrect results).

Quantification performance scoring; **A** = satisfactory (all results fall within  $\pm 2 \delta\text{MAD}$  of participants' median), **B** = questionable (no more than one result falls outside  $\pm 2 \delta\text{MAD}$  of participants' median, and no result falls outside  $\pm 2.58 \delta\text{MAD}$  of participants' median), **C** = unsatisfactory (two or more results fall outside  $\pm 2 \delta\text{MAD}$  of participants' median, or one or more result falls outside  $\pm 2.58 \delta\text{MAD}$  of participants' median).

## **Conclusion and discussion**

### **General comments**

Forty-three laboratories (18 NRLs and 25 other laboratories) received samples. Laboratories 22, 94, 193 and 245 did not return results. Laboratory 250 did not examine for norovirus and laboratories 9, 42, 72 and 161 did not examine for HAV. Results reported to the EURL are shown in Appendix III.

### **Discussion**

36/39 (93%) of laboratories reported correct presence/absence results (as determined by the EURL reference samples) for all determinands that they tested. For NoV (GI and GII) 35 laboratories achieved a satisfactory performance score for presence/absence determination, with 2 (47 and 72) and 1 (9) laboratories obtaining questionable and unsatisfactory performance scores respectively. In all cases questionable and unsatisfactory scores related to false positive results (no false negative results were reported for NoV). For HAV 35 laboratories achieved a satisfactory performance score.

Twenty-one laboratories (54%) returned data expressed as both  $C_t$  values and quantities for at least one sample/determinand combination (Appendix IV; all 21 laboratories reported quantities for NoV and 18 did so for HAV). Twelve laboratories (31%) returned data expressed as  $C_t$  values but no quantities. Of the 21 laboratories providing quantitative data, 12 laboratories obtained a satisfactory performance score for quantification of positive samples for NoV with 4 and 5 laboratories obtaining questionable and unsatisfactory performance scores respectively. For HAV 14 laboratories achieved a satisfactory performance score for quantification with 3 and 1 obtaining questionable and unsatisfactory performance scores respectively. Quantitative results and scoring are shown in Appendix V.

Methods used by participants to analyse the test samples are shown in Appendix VI.

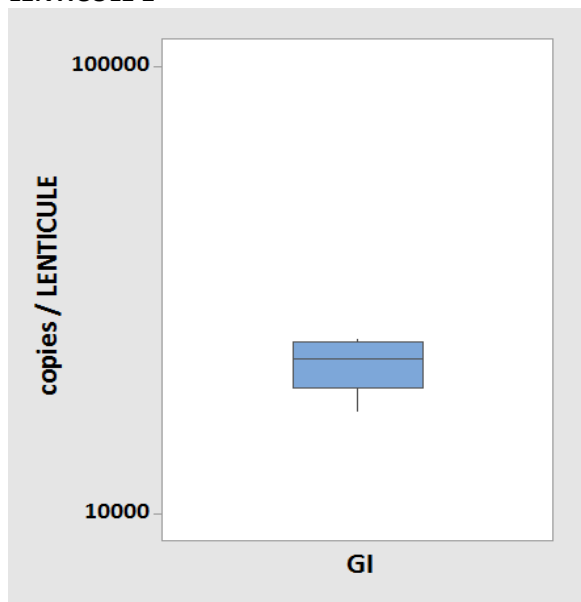
### **References**

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

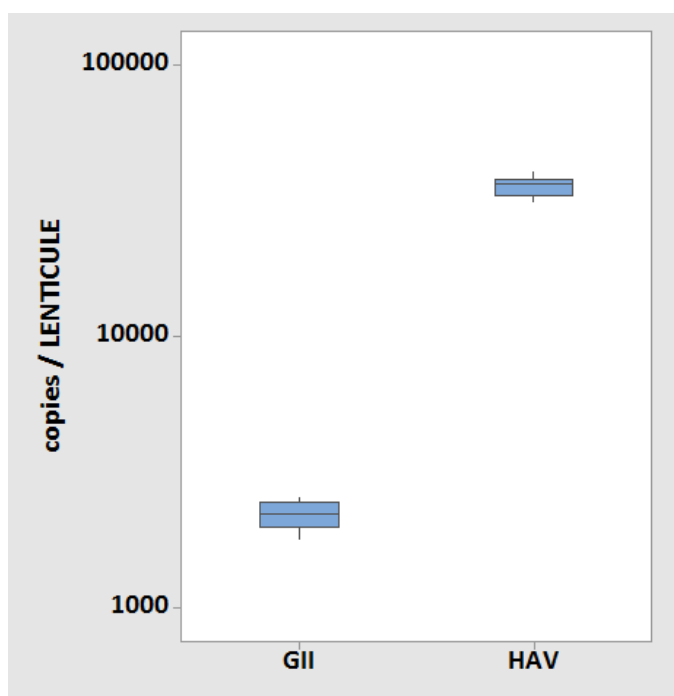
Anon 2010 ISO/TS 22117 Microbiology of food and animal feeding stuffs – Specific requirements and guidance for proficiency testing by interlaboratory comparison.

Appendix I : EURL reference results displayed as box and whisker plots of detectable genome copies per LENTICULE™

**LENTICULE 1**



**LENTICULE 2**



## Appendix II : Competency assessment of quantification data

Competency assessment was undertaken following the approach described in ISO/TS 22117 Microbiology of food and animal feeding stuffs – specific requirements and guidance for proficiency testing by interlaboratory comparison (ISO 2010). The MAD approach was used to assess proficiency testing data where less than 50 participants return quantitative results and/or for new proficiency assessment.

### 1. Where the intended result is positive

Assessment was performed by evaluation of the median absolute deviation from the median (MAD) value for each laboratories individual samples. The MAD approach produces a statistically robust acceptability range by calculation of the median difference from the median for every participant's result multiplied by a constant (1.4826) to obtain a robust estimate of the standard deviation (MAD value or  $\delta_{MAD}$ ).

It is proposed that the individual results from laboratories will be interpreted as follows:-

- Difference between result and participants' median  $<2 \delta_{MAD}$  = satisfactory
- Difference between result and participants' median  $>2 \delta_{MAD}$  and  $<2.58 \delta_{MAD}$  = questionable
- Difference between result and participants' median  $>2.58 \delta_{MAD}$  = unsatisfactory
- Result reported as negative = unsatisfactory

### 2. Where the intended result is negative

Assessment of results from samples with sample results intended as negative will be as follows:-

- Result reported as negative = satisfactory
- Result reported as positive = unsatisfactory

**Appendix III : Participants' results**

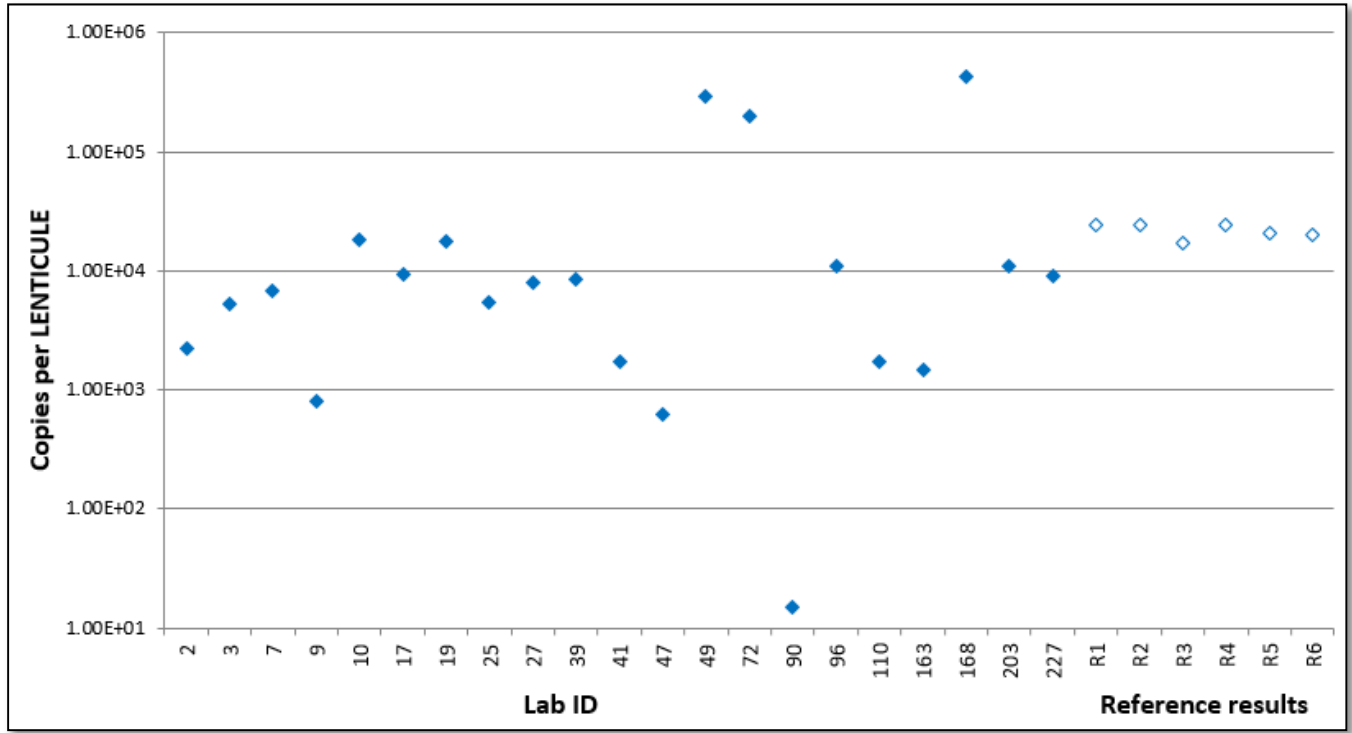
ID No.	L1				L2				L3			
	GI	CT	Copies / LENTICULE	GII	HAV	GI	GII	CT	Copies / LENTICULE	HAV	CT	Copies / LENTICULE
	+			-	-	-	+			+		
2	+	39.94	2.20E+03	-	-	-	+	34.53	2.05E+03	+	26.67	6.80E+05
3 *	+	32.97, 32.37	5.20E+03	-	-	-	+	34.90, 34.78	1.19E+03	+	29.32, 29.28	NQ
7 *	+	32.3	6.70E+03	-	-	-	+	37.5	1.40E+02	+	29.1	4.70E+04
9 *	+	29.44, 29.86	7.99E+02	+	-	+	+	33.27, 33.24	1.60E+02	-		
10 *	+	29.4	1.80E+04	-	-	-	+	31.3	1.65E+03	+	27.67	5.25E+04
17 *	+	30.96, 30.22	9.30E+03	-	-	-	+	32.3, 32.81	2.00E+03	+	28.79, 29.02	5.50E+04
19 *	+	30.38	1.79E+04	-	-	-	+	36.02	1.16E+03	+	27.47	9.98E+04
20	+	30.46	NQ	-	-	-	+	34.63	NQ	+	25.8	NQ
22 *	NR			NR	NR	NR	NR			NR		
25 *	+	31.39	5.50E+03	-	-	-	+	32.39	1.00E+03	+	28.42	4.70E+03
27 *	+	34.23	8.00E+03	-	-	-	+	36.46	7.80E+02	+	30.01	6.30E+04
35 *	+			-	-	-	+			+		
39 *	+	33.03	8.40E+03	-	-	-	+	39.35	9.40E+01	+	29.14	1.00E+05
41 *	+	33.16	1.70E+03	-	-	-	+	36.13	1.60E+03	+	28.96	1.70E+05
42 *	+			-	NE	-	+			NE		
47 *	+	35.9	6.20E+02	+	-	-	+	34.3	1.10E+03	+	29.4	9.90E+03
48	+	31.96, 31.34	NQ	-	-	-	+	34.57, 34.31	NQ	+	30.77, 30.83	NQ
49	+	26.32	2.92E+05	-	-	-	+	31.74	7.77E+03	+	24.32	5.59E+05
72	+	33.83	1.97E+05	-	-	+	+	36.98	1.13E+03			
90 *	+	38.38	1.50E+01	-	-	-	+	37.28	6.50E+01	+	31.33	4.88E+03
94	NR			NR	NR	NR	NR			NR		
95	+	39.75	NQ	-	-	-	+	37.69	NQ	+		
96	+	31.22, 31.26	1.08E+04	-	-	-	+	33.07, 32.46	3.14E+03	+	26.49, 27.71	1.23E+05
98	+	27.82	NQ	-	-	-	+	32.57	NQ	+	31.24	NQ
102 *	+	36.3	NQ	-	-	-	+	32.2	NQ	+	27.9	NQ
110	+	32.36	1.70E+03	-	-	-	+	31.92	3.30E+02	+	30.29	2.80E+06
147 *	+	34.09	NQ	-	-	-	+	33.06	NQ	+	26.37	NQ
161	+	34.33	NQ	-	NE	-	+	33.44	NQ	NE		
163	+	33.01	1.45E+03	-	-	-	+	33.43	1.93E+02	+	28.41	3.01E+04
168	+	28.54	4.26E+05	-	-	-	+	32.38	9.88E+03	+	29.9	1.14E+06
169	+			-	-	-	+			+		
177	+	32.82	NQ	-	-	-	+	36.44	NQ	+	32.75	NQ
183	+	32.4749	NQ	-	-	-	+	32.4772	NQ	+	28.9615	NQ
188	+	30	NQ	-	-	-	+	32	NQ	+	26	NQ
193	NR			NR	NR	NR	NR			NR		
203 *	+	29.19	1.10E+04	-	-	-	+	33.22	1.40E+03	+	26.01	1.40E+05
205	+	30.74	NQ	-	-	-	+	35.19	NQ	+	27.28	NQ
218	+			-	-	-	+			+		
227	+	29.91	9.00E+03	-	-	-	+	31.74	7.74E+02	+	29.16	1.95E+04
229	+			-	-	-	+			+		
242	+	31.82	NQ	-	-	-	+	34.24	NQ	+	33.66	NQ
245	NR			NR	NR	NR	NR			NR		
250	NE			NE	-	NE	NE			+		

\* = Designated NRL, NE= determinand not examined, NR= results not returned, NQ = Not quantified, Yellow shading denotes false positives.

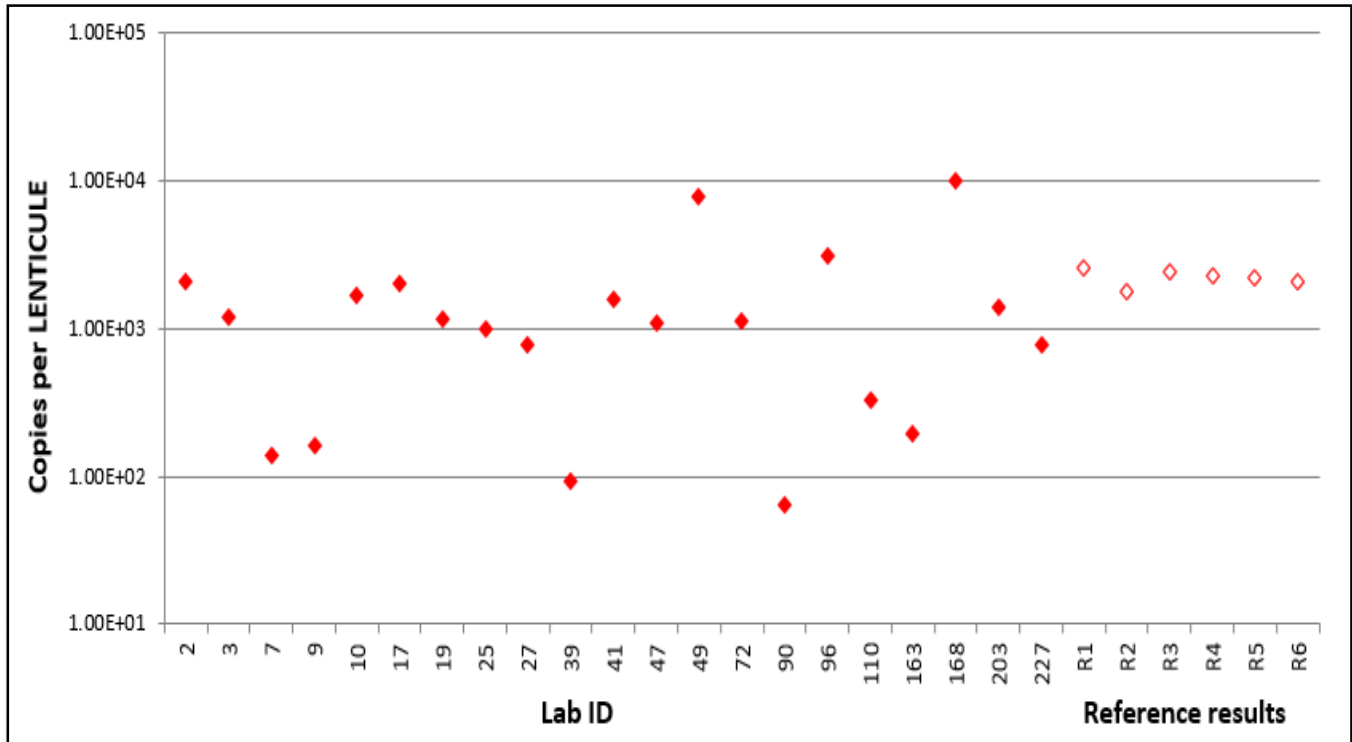


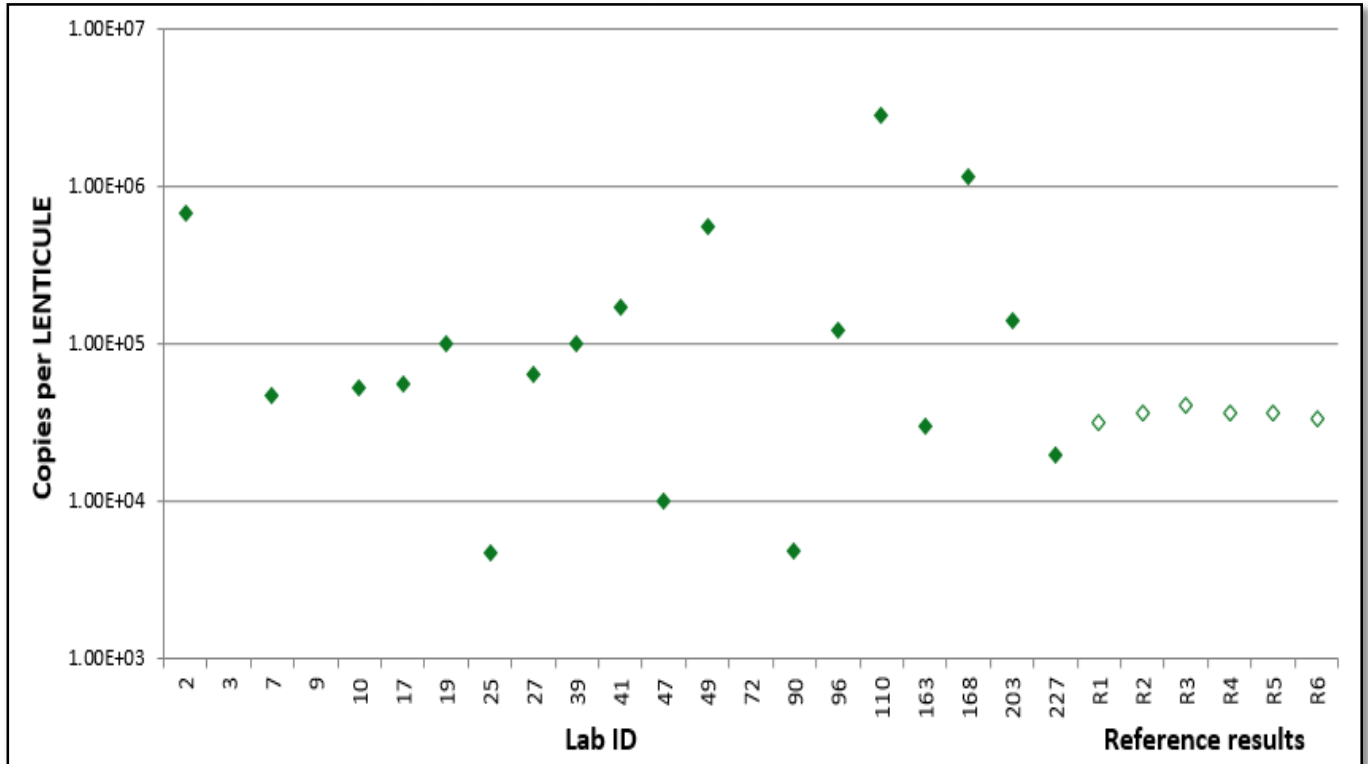
Appendix IV : Participants' and reference quantities for each sample.

**LENTICULE 1 – GI**



**LENTICULE 2 – GII**

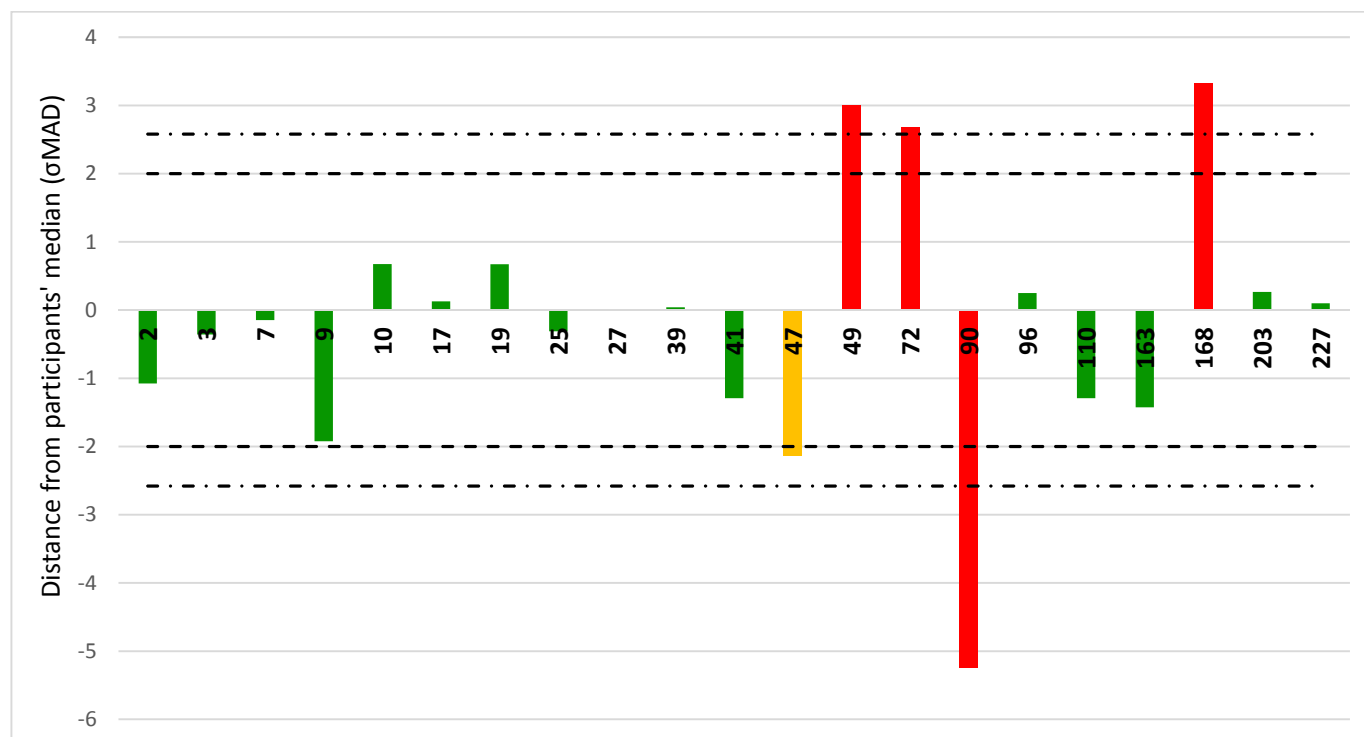


**LENTICULE 2 – HAV**


Appendix V : Participants quantification data and allocated performance scores

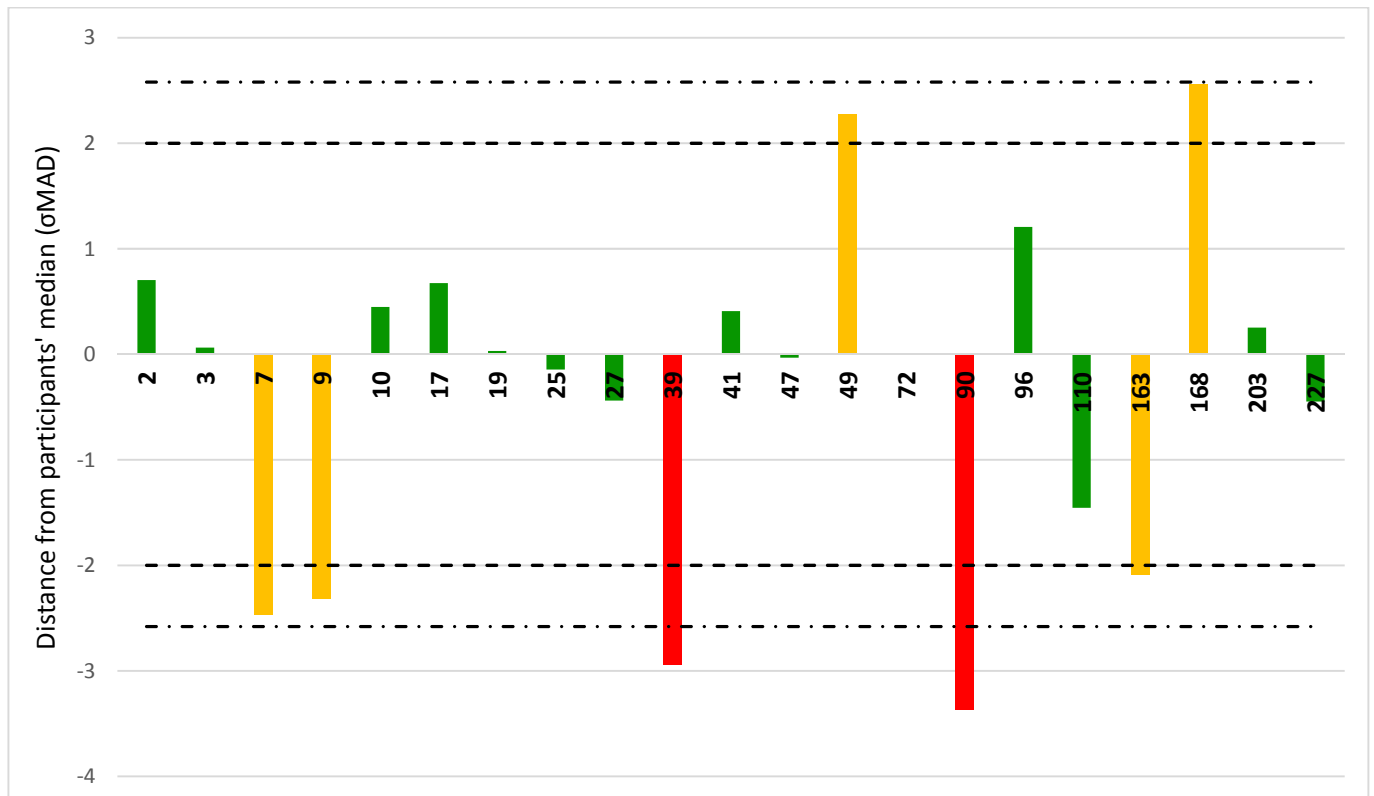
LENTICULE 1 - GI quantification data

ID No.	Copies / LENTICULE	Within $\pm 2 \sigma$ MAD of the participants' median	Within $\pm 2.58 \sigma$ MAD of the participants' median	Outside $\pm 2.58 \sigma$ MAD of the participants' median
2	2.20E+03	YES	-	-
3	5.20E+03	YES	-	-
7	6.70E+03	YES	-	-
9	7.99E+02	YES	-	-
10	1.80E+04	YES	-	-
17	9.30E+03	YES	-	-
19	1.79E+04	YES	-	-
25	5.50E+03	YES	-	-
27	8.00E+03	YES	-	-
39	8.40E+03	YES	-	-
41	1.70E+03	YES	-	-
47	6.20E+02	NO	YES	-
49	2.92E+05	NO	-	YES
72	1.97E+05	NO	-	YES
90	1.50E+01	NO	-	YES
96	1.08E+04	YES	-	-
110	1.70E+03	YES	-	-
163	1.45E+03	YES	-	-
168	4.26E+05	NO	-	YES
203	1.10E+04	YES	-	-
227	9.00E+03	YES	-	-



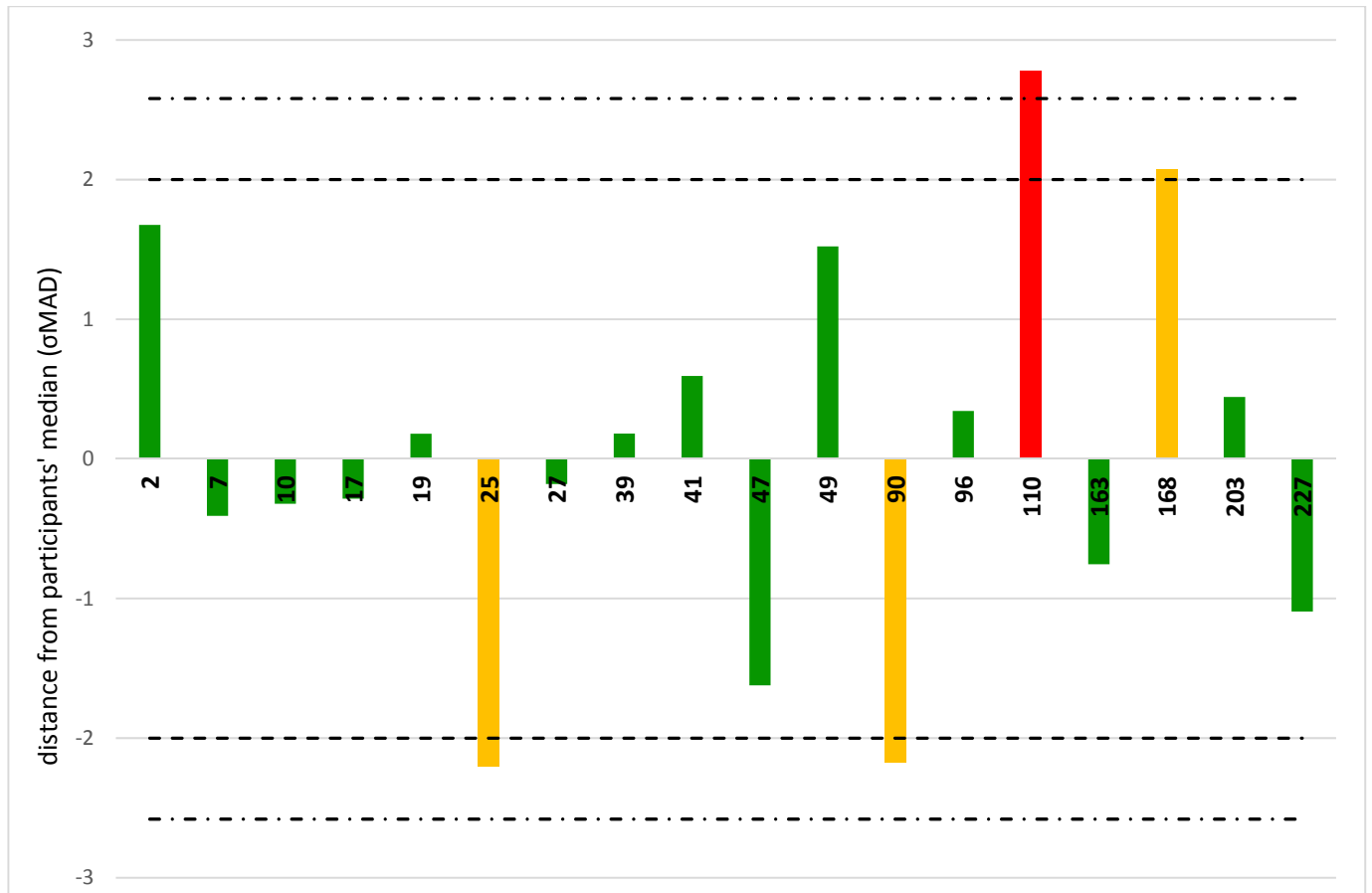
**LENTICULE 2 - GII quantification data**

ID No.	Copies / LENTICULE	Within $\pm 2 \sigma$ MAD of the participants' median	Within $\pm 2.58 \sigma$ MAD of the participants' median	Outside $\pm 2.58 \sigma$ MAD of the participants' median
2	2.05E+03	YES	-	-
3	1.19E+03	YES	-	-
7	1.40E+02	NO	YES	-
9	1.60E+02	NO	YES	-
10	1.65E+03	YES	-	-
17	2.00E+03	YES	-	-
19	1.16E+03	YES	-	-
25	1.00E+03	YES	-	-
27	7.80E+02	YES	-	-
39	9.40E+01	NO	-	YES
41	1.60E+03	YES	-	-
47	1.10E+03	YES	-	-
49	7.77E+03	NO	YES	-
72	1.13E+03	YES	-	-
90	6.50E+01	NO	-	YES
96	3.14E+03	YES	-	-
110	3.30E+02	YES	-	-
163	1.93E+02	NO	YES	-
168	9.88E+03	NO	YES	-
203	1.40E+03	YES	-	-
227	7.74E+02	YES	-	-



**LENTICULE 2 - HAV quantification data**

ID No.	Copies / LENTICULE	Within $\pm 2 \sigma$ MAD of the participants' median	Within $\pm 2.58 \sigma$ MAD of the participants' median	Outside $\pm 2.58 \sigma$ MAD of the participants' median
2	6.80E+05	YES	-	-
7	4.70E+04	YES	-	-
10	5.25E+04	YES	-	-
17	5.50E+04	YES	-	-
19	9.98E+04	YES	-	-
25	4.70E+03	NO	YES	-
27	6.30E+04	YES	-	-
39	1.00E+05	YES	-	-
41	1.70E+05	YES	-	-
47	9.90E+03	YES	-	-
49	5.59E+05	YES	-	-
90	4.88E+03	NO	YES	-
96	1.23E+05	YES	-	-
110	2.80E+06	NO	-	YES
163	3.01E+04	YES	-	-
168	1.14E+06	NO	YES	-
203	1.40E+05	YES	-	-
227	1.95E+04	YES	-	-



**Appendix VI: Results and methods used for test samples. (For key to method codes see page 13)**

LAB ID	LENTICULE 1			LENTICULE 2			RNA extraction	RT-PCR method	RT-PCR reagents	Primers		
	GI	GII	HAV	GI	GII	HAV				GI	GII	HAV
2	+	-	-	-	+	+	A	K	N	CC	CC	GG
3*	+	-	-	-	+	+	A	K	O	AA-1	AA	AA
7*	+	-	-	-	+	+	A	K	O	AA-2 <sup>a</sup>	AA <sup>a</sup>	AA <sup>a</sup>
9*	+	+	NE	+	+	NE	A	K	O	AA-1	AA	-
10*	+	-	-	-	+	+	A	K	O	AA-1	AA	AA
17*	+	-	-	-	+	+	A	K	O	AA-1	AA	AA
19*	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
20	+	-	-	-	+	+	A	K	P	BB	BB	BB
25*	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
27*	+	-	-	-	+	+	A	K	Q	AA-1	AA	AA
35*	+	-	-	-	+	+	A	K	R	AA-2	AA	AA
39*	+	-	-	-	+	+	A	K	O	CC	CC	AA
41*	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
42*	+	-	NE	-	+	NE	B	K	S	AA-1	AA	-
47*	+	+	-	-	+	+	A	K	R	AA-2 <sup>b</sup>	AA <sup>b</sup>	AA
48	+	-	-	-	+	+	A	K	O	AA-1	AA	AA
49	+	-	-	-	+	+	A	K	T	AA-1	AA	HH
72	+	-	NE	+	+	NE	A	K	O	AA-1	AA	-
90*	+	-	-	-	+	+	A	K	R	AA-2	AA	AA
95	+	-	-	-	+	+	C	L <sup>c</sup>	U	CC	CC	JJ
96	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
98	+	-	-	-	+	+	D	K	V	DD	DD	DD
102*	+	-	-	-	+	+	C	K	W	AA-2	AA	BB
110	+	-	-	-	+	+	A	K	P	BB	BB	BB
147*	+	-	-	-	+	+	A	K	X	EE	EE	BB
161	+	-	NE	-	+	NE	E	K	R	AA-2	AA	-
163	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
168	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
169	+	-	-	-	+	+	A	K	P	BB	BB	BB
177	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
183	+	-	-	-	+	+	C	K	P	BB	BB	BB
188	+	-	-	-	+	+	F	K	V	DD	DD	DD
203	+	-	-	-	+	+	G	K	Y	AA-1	AA	AA
205	+	-	-	-	+	+	A	K	O	AA-2	AA	AA
218	+	-	-	-	+	+	H	K	O	AA-2	AA	AA
227	+	-	-	-	+	+	A	K	P	BB	BB	BB
229	+	-	-	-	+	+	C	M <sup>d</sup>	Z	FF	FF	KK
242	+	-	-	-	+	+	I	K	V	DD	DD	DD
250	NE	NE	-	NE	NE	+	J	K	O	-	-	AA

\* = Designated NRL, Yellow = false positive results; Grey = method as in annexes of ISO/DIS 15216-1 draft standard (or very similar); a = IAC assay run as multiplex with target assays; b = norovirus analysis carried out as multiplex; c = conventional two-step used for HAV only; d = real-time one-step used for HAV only.

**Key to method codes**
**RNA extraction methods**

A	NucliSens Magnetic extraction reagents (BioMerieux)
B	PureLink Viral RNA/DNA mini Kits (Invitrogen)
C	QIAamp/Rneasy kits (Qiagen)
D	Column extraction
E	MagJET Viral DNA and RNA Kit (Thermo Scientific)
F	SureFast PREP DNA/RNA Virus (CONGEN)
G	NucleoSpin® RNA Virus (Macherey-Nagel)
H	Viral Carrier RNA Kit (SYNGEN)
I	High Pure Viral RNA Kit (Roche) & OneStep PCR Inhibitor Removal Kit (Zymo Research)
J	iPrep Virus Kit (Invitrogen)

**RT-PCR methods**

K	Real-time one-step
L	Real-time two-step
M	Conventional one-step

**RT-PCR reagents**

N	NoV: Brilliant II QRT-PCR Core Reagent Kit (Agilent), HAV: TAQMAN Fast 1-Step RT-QPCR Kit (Thermofisher)
O	RNA Ultrasense (Invitrogen)
P	ceeram Tools
Q	TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems)
R	Quantitect/Quantifast RT-PCR kits (Qiagen)
S	AgPath-ID One-Step RT-PCR Reagents (Applied biosystems)
T	GI & HAV; TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems). GII; RNA Ultrasense (Invitrogen)
U	High Capacity cDNA RT Kit & Taqman Universal Mastermix (Applied Biosystems)
V	SureFast reagents (CONGEN)
W	NoV; AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems). HAV; Ceeram Tools
X	NoV; SureFast reagents (CONGEN) & Ridagene Norovirus detection kit (R-biopharm). HAV; CeeramTools
Y	Platinum quantitative RT-PCR Thermoscript One-step system (Invitrogen)
Z	NoV; PrimeScript One Step RT-PCR Kit and Taq Hot Start kit (TaKaRa). HAV; One Step PrimeScript RT-PCR Kit - Perfect Real Time (TaKaRa)

**Primers/probes**

AA	ISO/DIS 15216-1; 1) with TM9 probe for noV GI; 2) with NVGG1p probe for noV GI
BB	Ceeram Tools (sequences as AA-2)
CC	Kageyama et al, (2003)
DD	SureFast reagents (CONGEN)
EE	SureFast reagents (CONGEN) & Ridagene Norovirus detection kit (R-biopharm)
FF	Kojima et al, (2003)
GG	Health Canada method OPFLP-07
HH	Guevremont et al, (2006), Houde et al, (2007)
JJ	FDA/BAM Chapter 26 "Detection and Quantification of Hepatitis A virus in Shellfish by the Polymerase Chain Reaction"
KK	diPasquale et al, 2010

## About us

Cefas is a multi-disciplinary scientific research and consultancy centre providing a comprehensive range of services in fisheries management, environmental monitoring and assessment, and aquaculture to a large number of clients worldwide.

We have more than 500 staff based in 2 laboratories, our own ocean-going research vessel, and over 100 years of fisheries experience.

We have a long and successful track record in delivering high-quality services to clients in a confidential and impartial manner.  
([www.cefas.defra.gov.uk](http://www.cefas.defra.gov.uk))

Cefas Technology Limited (CTL) is a wholly owned subsidiary of Cefas specialising in the application of Cefas technology to specific customer needs in a cost-effective and focussed manner.

CTL systems and services are developed by teams that are experienced in fisheries, environmental management and aquaculture, and in working closely with clients to ensure that their needs are fully met.  
([www.cefastechnology.co.uk](http://www.cefastechnology.co.uk))

### Head office

Centre for Environment,  
Fisheries & Aquaculture Science  
Pakefield Road, Lowestoft,  
Suffolk NR33 0HT UK

Tel +44 (0) 1502 56 2244  
Fax +44 (0) 1502 51 3865  
Web [www.cefas.defra.gov.uk](http://www.cefas.defra.gov.uk)

## Customer focus

With our unique facilities and our breadth of expertise in environmental and fisheries management, we can rapidly put together a multi-disciplinary team of experienced specialists, fully supported by our comprehensive in-house resources.

Our existing customers are drawn from a broad spectrum with wide ranging interests. Clients include:

- international and UK government departments
- the European Commission
- the World Bank
- Food and Agriculture Organisation of the United Nations (FAO)
- oil, water, chemical, pharmaceutical, agro-chemical, aggregate and marine industries
- non-governmental and environmental organisations
- regulators and enforcement agencies
- local authorities and other public bodies

We also work successfully in partnership with other organisations, operate in international consortia and have several joint ventures commercialising our intellectual property

Centre for Environment,  
Fisheries & Aquaculture Science  
Weymouth Laboratory,  
Barrack Road, The Nothe, Weymouth,  
Dorset DT4 8UB

Tel +44 (0) 1305 206600  
Fax +44 (0) 1305 206601

