

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

Noroviruses and hepatitis A virus

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Samples

Material dispatched consisted of laboratory constructed LENTICULES™ (Vials 1 and 2). Table 1 shows details of the stock viruses used in the preparation of LENTICULE™ 2.

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.7 (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™ 2 included known levels of GI and 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 22nd June 2015 to 33 participating laboratories. Laboratory 78 received multiple sets of samples. 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 24th July 2015.

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 53 proficiency testing material

Sample	Norovirus		HAV
	GI	GII	
LENTICULE™ 1	-	-	-
LENTICULE™ 2	+ (2.03 x 10 ² – 3.76 x 10 ²)	+ (2.53 x 10 ² – 6.59 x 10 ²)	+ (9.45 x 10 ² – 2.70 x 10 ³)

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 assessed as percentage relative accuracy, specificity and sensitivity for each determinant (Table 3) according to the calculations described in Appendix II.

Note: Participants' results were expressed as percentage concordance with intended results generated by the EURL. In this assessment presence/absence data was used and no consideration of quantitative measurements or Ct values were made.

Table 3: Participants' results for all LENTICUE™ samples

Lab ID No.	GI			GII			HAV			Performance score	
	AC	SP	SE	AC	SP	SE	AC	SP	SE	NoV	HAV
3*	100	100	100	100	100	100	100	100	100	A	A
7*	100	100	100	100	100	100	100	100	100	A	A
10*	100	100	100	100	100	100	100	100	100	A	A
13*	100	100	100	100	100	100	100	100	100	A	A
17*	100	100	100	100	100	100	100	100	100	A	A
19*	100	100	100	100	100	100	100	100	100	A	A
20	100	100	100	100	100	100	100	100	100	A	A
21*	100	100	100	100	100	100	100	100	100	A	A
22*	100	100	100	100	100	100	100	100	100	A	A
25*	100	100	100	100	100	100	100	100	100	A	A
27*	100	100	100	100	100	100	100	100	100	A	A
39*	100	100	100	100	100	100	100	100	100	A	A
41*	100	100	100	100	100	100	100	100	100	A	A
43*	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	-
47*	100	100	100	100	100	100	100	100	100	A	A
48	100	100	100	100	100	100	100	100	100	A	A
49	100	100	100	50	100	0	100	100	100	B	A
72	100	100	100	100	100	100	NE	NE	NE	A	-
78 ^a	100	100	100	100	100	100	100	100	100	A	A
78 ^b	100	100	100	100	100	100	100	100	100	A	A
94	100	100	100	100	100	100	100	100	100	A	A
95	100	100	100	50	100	0	50	100	0	B	B
98	100	100	100	100	100	100	100	100	100	A	A
113	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	-
122	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	-
132	100	100	100	100	100	100	50	0	100	A	A
147*	100	100	100	100	100	100	100	100	100	A	A
168	100	100	100	100	100	100	100	100	100	A	A
198	100 ^a	100 ^a	100 ^a	n/a	n/a	n/a	100	100	100	A	A
200	100	100	100	100	100	100	50	0	100	A	B
203	100	100	100	100	100	100	100	100	100	A	A
214	100	100	100	100	100	100	100	100	100	A	A
228	100 ^a	100 ^a	100 ^a	n/a	n/a	n/a	50	100	0	A	B
250	100 ^a	100 ^a	100 ^a	n/a	n/a	n/a	100	100	100	A	A

* = Designated NRL, NE= Not examined, NR = Results not returned, a = non-genogroup discriminating method used for norovirus, n/a = not applicable, AC =Relative accuracy, SP = Relative specificity, SE = Relative sensitivity, Performance scoring; **A** = satisfactory (100% accuracy), **B** = questionable (one incorrect result), **C** = unsatisfactory (two or more incorrect results). Norovirus (NoV) and HAV scored separately.

Note Lab 78 received multiple sets of samples and returned two sets of results.

Note 26/30 laboratories that returned results scored 100% overall accuracy for all determinands tested.

Conclusion and discussion

General comments

Thirty-three laboratories (15 NRLs and 18 other laboratories) received samples. Laboratories 43, 113 and 122 did not return results. Laboratory 72 did not examine for HAV. Laboratory 78 received multiple sample sets and returned two sets of results. For purposes of calculation of overall laboratory performance, results from a single set only (78 a) are considered. Laboratories 198, 228 and 250 used a non-genogroup discriminating method for norovirus. Results for these laboratories are not considered for purposes of calculation of overall laboratory performance for GI and GII. Results reported to the EURL are shown in Appendices III and IV.

Discussion

26/30 (86%) of the laboratories that returned results obtained the intended results (as determined by EURL reference designations) for all the sample/determinand combinations which they tested. The overall accuracies across all laboratories were 100%, 96% and 95% for GI, GII and HAV respectively. The false positive reporting rates for GI, GII and HAV were 0%, 0% and 3% respectively. The false negative reporting rates for GI, GII and HAV were 0%, 7% and 7% respectively.

Seventeen laboratories (57%) returned data expressed as both C_t values and quantities for at least one sample/determinand combination (Appendix III). 12 laboratories (40%) returned data expressed as C_t values only. One laboratory (3%) returned results as quantities only. Quantitative results from individual labs alongside reference results are shown in Appendix IV. Reference results were not corrected using extraction efficiency data.

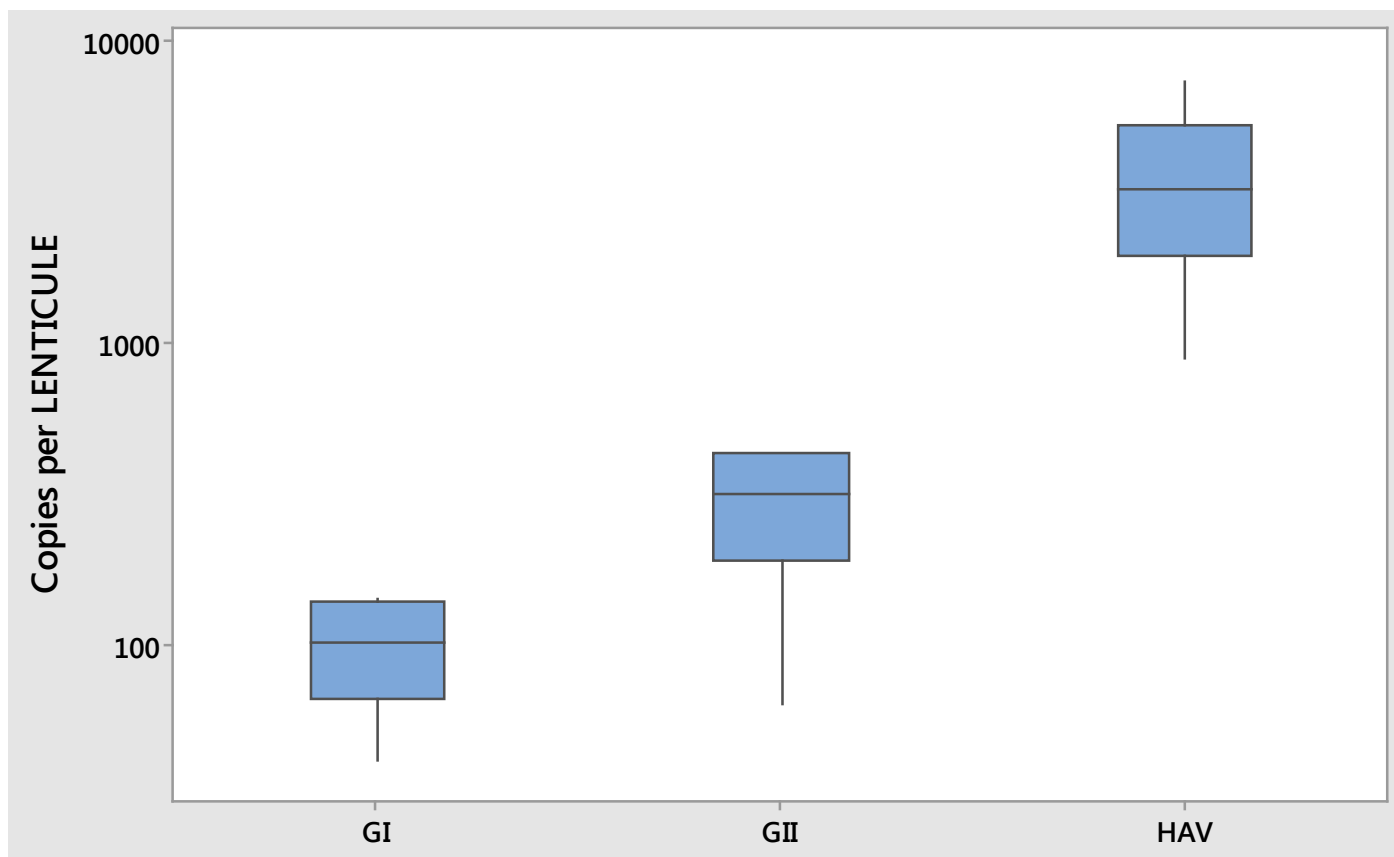
Methods used by participants to analyse the test samples are shown in Appendix V.

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.

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

LENTICULE 2



Appendix II:

Percentage relative sensitivity:	Relative sensitivity (SE) =	$\frac{TP}{(TP+FN)}$	
Percentage relative specificity:	Relative specificity (SP) =	$\frac{TN}{(TN+FP)}$	x 100%
Percentage relative accuracy:	Relative accuracy (AC) =	$\frac{TP+TN}{N}$	x 100%

Where TP = true positives

FN = false negatives

FP = false positives

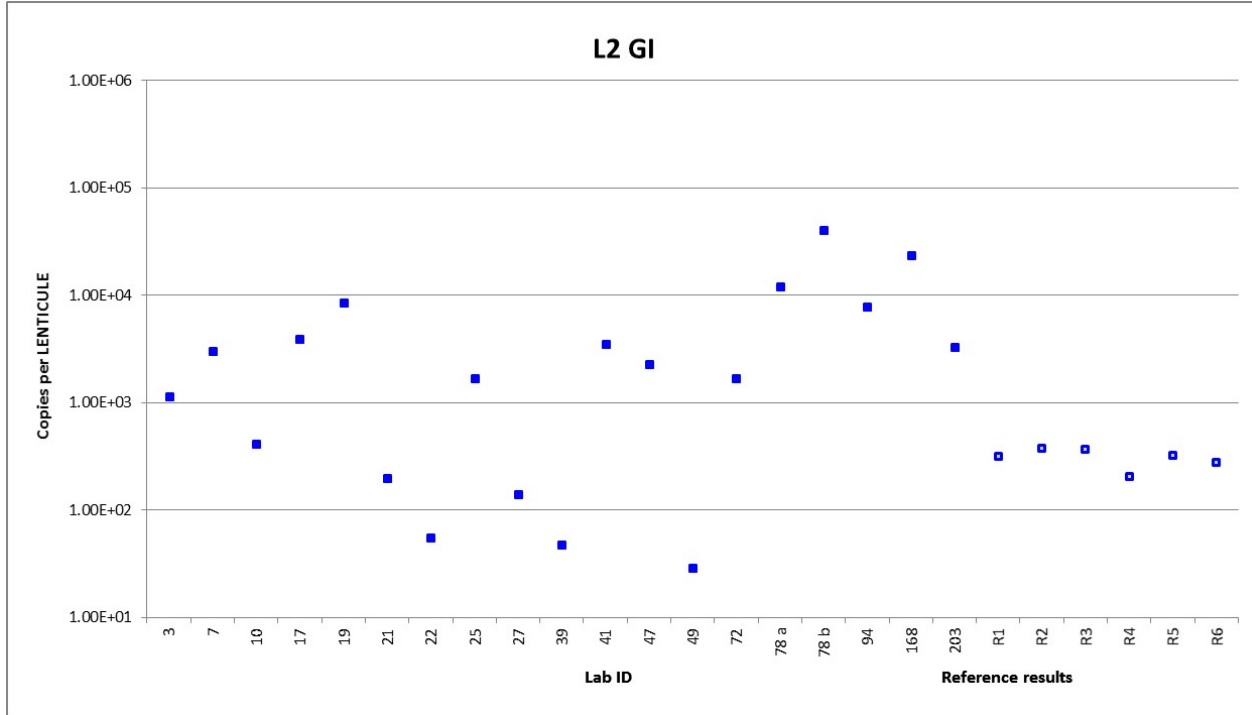
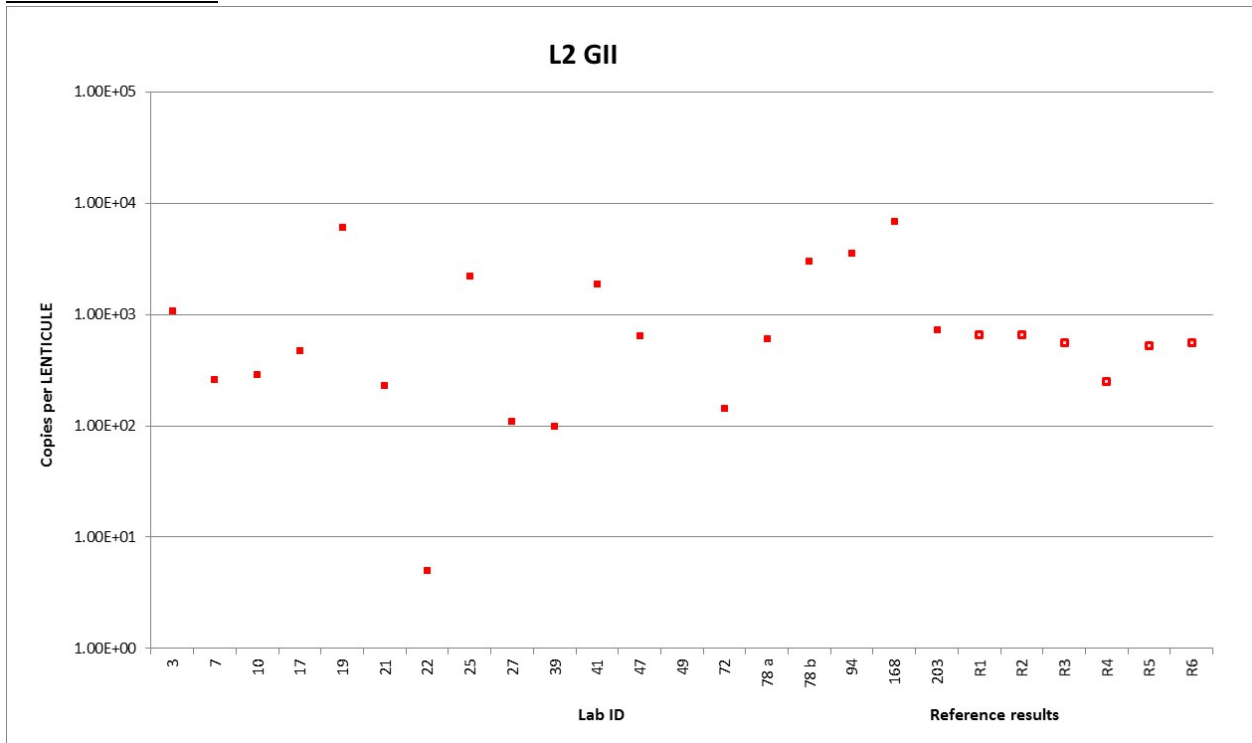
TN = true negatives

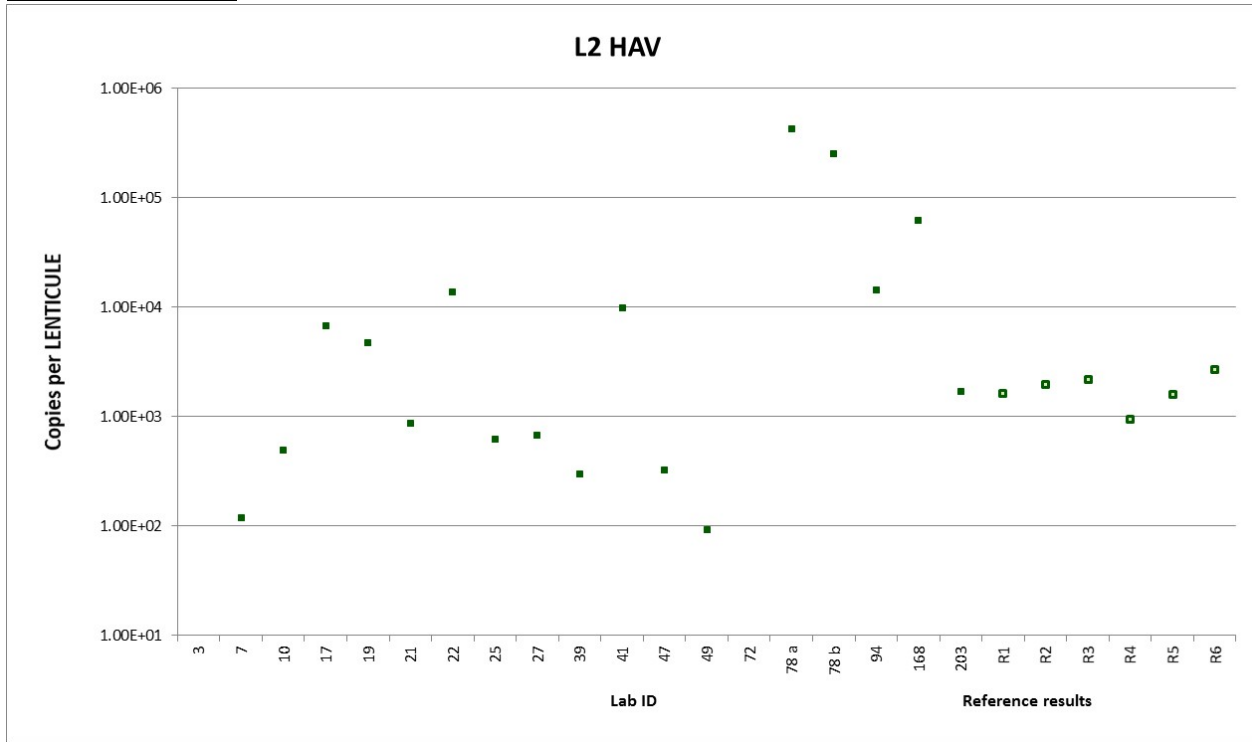
N = total number of tests

Note: Participants' results were expressed as percentage concordance with intended results generated by the EURL. In this assessment presence/absence data was used and no consideration of quantitative measurements (C_t values) was made.

Lab ID No.	GI			GII			HAV					
	-	-	-	+	CT	copies/ LENTICULE	+	CT	copies/ LENTICULE	+	CT	copies/ LENTICULE
3*	-	-	-	+	36.79	1.15E+03	+	35.85	1.09E+03	+	34.86	-
7*	-	-	-	+	35.95	3.00E+03	+	37.81	2.60E+02	+	38.75	1.20E+02
10*	-	-	-	+	34.74	4.13E+02	+	35.59	2.90E+02	+	34.5	4.96E+02
13*	-	-	-	+	37.61	-	+	37.53	-	+	38.92	-
17*	-	-	-	+	33.49	3.96E+03	+	37.24	4.78E+02	+	31.41	6.79E+03
19*	-	-	-	+	33.13	8.53E+03	+	37.09	6.14E+03	+	33.02	4.70E+03
20	-	-	-	+	32.16	-	+	36.53	-	+	33.09	-
21*	-	-	-	+	35.1	2.00E+02	+	33.6	2.30E+02	+	34.7	8.65E+02
22*	-	-	-	+	36.3	5.50E+01	+	38.57	5.00E+00	+	31.71	1.38E+04
25*	-	-	-	+	34.02	1.68E+03	+	32.86	2.21E+03	+	34.8	6.21E+02
27*	-	-	-	+	39.15	1.40E+02	+	39.48	1.10E+02	+	37.63	6.80E+02
39*	-	-	-	+	37.8	4.80E+01	+	36.71	1.00E+02	+	35.05	3.00E+02
41*	-	-	-	+	35.6	3.50E+03	+	36.51	1.90E+03	+	35.46	9.80E+03
43*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
47*	-	-	-	+	35	2.27E+03	+	35.8	6.42E+02	+	35.7	3.29E+02
48	-	-	-	+	35.57	-	+	35.69	-	+	31.52	-
49	-	-	-	+	41.03	2.90E+01	-	-	-	+	36.95	9.20E+01
72	-	-	-	+	34.01	1.69E+03	+	35.9	1.45E+02	NE	-	-
78 a	-	-	-	+	-	1.22E+04	+	-	6.05E+02	+	-	4.25E+05
78 b	-	-	-	+	-	4.07E+04	+	-	3.00E+03	+	-	2.56E+05
94	-	-	-	+	33.46	7.84E+03	+	34.32	3.56E+03	+	32.72	1.45E+04
95	-	-	-	+	39.35	-	-	-	-	-	-	-
98	-	-	-	+	35.2	-	+	36.26	-	+	35.61	-
113	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
122	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
132	-	-	NE	+	35.6	-	+	35.3	-	+	34.3	-
147*	-	-	-	+	34.32	-	+	34.63	-	+	32.33	-
168	-	-	-	+	32.58	2.36E+04	+	33.36	6.92E+03	+	35.09	6.28E+04
198	- ^a	n/a	-	+ ^a	36 ^a	-	n/a	n/a	n/a	+	34	-
200	-	-	+	+	36.25	-	+	36.04	-	+	34.34	-
203	-	-	-	+	40.4	3.30E+03	+	37.91	7.30E+02	+	35.19	1.70E+03
214	-	-	-	+	35	-	+	35	-	+	35	-
228	- ^a	n/a	-	+ ^a	36.06 ^a	-	n/a	n/a	n/a	-	-	-
250	- ^a	n/a	-	+ ^a	-	-	n/a	n/a	n/a	+	33.97	-

* = Designated NRL, NE= determinand not examined, NR= results not returned, a = non-genogroup discriminating method used for norovirus, n/a = not applicable, Yellow shading denotes false positives, Red shading denotes false negatives.

Appendix IV: Participants' and reference quantities for each sample.
LENTICULE 2 – GI

LENTICULE 2 – GII


LENTICULE 2 – HAV


Appendix V: Results and methods used for test samples. (For key to method codes see page 11)

LAB ID	LENTICULE 1			LENTICULE 2			RNA extraction	RT-PCR method	RT-PCR reagents	Primers		
	GI	GII	HAV	GI	GII	HAV				GI	GII	HAV
3*	-	-	-	+	+	+	A	J	M	AA-1	AA	AA
7*	-	-	-	+	+	+	A	J	M	AA-2 ^a	AA ^a	AA ^a
10*	-	-	-	+	+	+	A	J	M	AA-1	AA	AA
13*	-	-	-	+	+	+	A	J	M	AA-2	AA	AA
17*	-	-	-	+	+	+	A	J	M	AA-2	AA	AA
19*	-	-	-	+	+	+	A	J	M	AA-2	AA	AA
20	-	-	-	+	+	+	A	J	N	BB	BB	BB
21*	-	-	-	+	+	+	A	J	M	AA-2	AA	AA
22*	-	-	-	+	+	+	B	J	M	AA-2	AA	AA
25*	-	-	-	+	+	+	A	j	M	AA-2	AA	AA
27*	-	-	-	+	+	+	A	J	O	AA-1	AA	AA
39*	-	-	-	+	+	+	A	J	M	CC	CC	AA
41*	-	-	-	+	+	+	A	J	M	AA-2	AA	AA
47*	-	-	-	+	+	+	A	J	P	AA-2 ^b	AA ^b	AA
48	-	-	-	+	+	+	B	J	M	AA-1	AA	AA
49	-	-	-	+	-	+	C	J	O	AA-1	AA	KK
72	-	-	NE	+	+	NE	A	J	Q	AA-1	AA	-
78 a	-	-	-	+	+	+	B	K ^c	R	DD	DD	LL
78 b	-	-	-	+	+	+	B	K ^c	R	DD	DD	LL
94	-	-	-	+	+	+	A	J	N	BB	BB	BB
95	-	-	-	+	-	-	B	L ^d	S	CC	CC	MM
98	-	-	-	+	+	+	D	J	T	EE	EE	GG
132	-	-	-	+	+	+	A	J	U	AA-1	AA	AA
147	-	-	-	+	+	+	B	J	V	FF	FF	BB
168	-	-	-	+	+	+	A	J	M	AA-2	AA	AA
198	- ^e	n/a	-	+ ^e	n/a	+	nr	nr	W	GG ^e	n/a	GG
200	-	-	+	+	+	+	A	J	M	AA-2	AA	AA
203	-	-	-	+	+	+	E	J	X	AA-1	AA	AA
214	-	-	-	+	+	+	F	J	N	BB	BB	AA
228	- ^e	n/a	-	+ ^e	n/a	-	G	J	Y	HH ^e	n/a	AA
250	- ^e	n/a	-	+ ^e	n/a	+	H	K ^f	Z	JJ ^e	n/a	AA

* = Designated NRL, Red = false negative results; 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 PCR with sequencing confirmation used as detection method, followed by droplet digital RT-PCR for quantification; d = conventional two-step used for HAV only; e = non-genogroup discriminating method used for NoV; f = real-time one-step used for HAV only

Key to method codes

RNA extraction methods

A	NucliSens Magnetic extraction reagents (BioMerieux)
B	QIAamp/Rneasy kits (Qiagen)
C	Dynabeads® MyOne™ SILANE (LifeTech) /Rneasy kit (QIAGEN)
D	Column extraction (r-Biopharm)
E	NucleoSpin® RNA Virus (Macherey-Nagel)
F	foodproof® Virus Sample Preparation Kit (Bioteccon)
G	High Pure Viral RNA Kit (Roche)
H	iPrep Virus Kit (Invitrogen)

RT-PCR methods

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

RT-PCR reagents

M	RNA Ultrasense (Invitrogen)
N	ceeram Tools
O	TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems)
P	Quantitect/Quantifast RT-PCR kits (Qiagen)
Q	RNA to Ct One step kit (Applied Biosystems)
R	One-Step RT-PCR kit (Qiagen)/One-Step RT-ddPCR kit for Probes (Bio-Rad)
S	High Capacity cDNA RT Kit & Taqman Universal Mastermix (Applied Biosystems)
T	Surefood kit (r-Biopharm)/foodproof® Detection Kit (Bioteccon)
U	One step qRT-PCR System (Invitrogen)
V	NoV; Ridagene Norovirus detection kit (r-Biopharm)/CeeramTools for genogroup determination. HAV; CeeramTools
W	Surefood kit (Congen)
X	SuperScript® III Platinum One-Step qRT-PCR System (Invitrogen)
Y	NoV; virellaNoro real time RT-PCR Kit (Gerbion). HAV; Quantitect Probe RT-PCR Kit (Qiagen)
Z	NoV; SuperScript® III Platinum One-Step qRT-PCR System (Invitrogen). HAV; RNA Ultrasense (Invitrogen)

Primers/probes

AA	Primre/probe sets from 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 <i>et al.</i> , (2003)
DD	Kojima <i>et al.</i> , (2003)/Kageyama <i>et al.</i> , (2003)/Vinje <i>et al.</i> , (2011)
EE	Surefood kit (r-Biopharm)/foodproof® Detection Kit (Bioteccon)
FF	Ridagene Norovirus detection kit (r-Biopharm)/CeeramTools for genogroup determination
GG	Surefood kit (Congen/r-Biopharm)
HH	virellaNoro real time RT-PCR Kit (Gerbion)
JJ	Vennema <i>et al.</i> , (2002)
KK	Guevremont <i>et al.</i> , (2006), Houde <i>et al.</i> , (2007)
LL	Guevremont <i>et al.</i> , (2006)
MM	FDA/BAM Chapter 26 “Detection and Quantification of Hepatitis A virus in Shellfish by the Polymerase Chain Reaction”

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