

European Union Reference Laboratory (EURL) Proficiency Testing Scheme

**Noroviruses and hepatitis A virus
EURL PT reference number: PT53**

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Samples

Materials dispatched consisted of laboratory constructed LENTICULES™ (Vials 1 and 2). The reference results for each sample are shown in Table 2.

Sample preparation

LENTICULES™ 1 and 2

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. The mix prepared for LENTICULE™ 2 included known levels of GII norovirus and HAV. 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™ samples

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	GI.4 (based on capsid sequence)

Sample distribution

Samples were dispatched in accordance with IATA packing instructions 650 for UN3373 'Diagnostic Specimens' on 23rd June 2014 to 28 participating laboratories. Samples were dispatched to an additional 3 participating laboratories between 25th June 2014 and 30th July 2014. 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 required to submit their results by 8th August 2014. The final laboratory to receive samples (197) was allowed an additional 3 weeks to complete the analysis.

Results

Reference results

Reference analyses were performed by the EURL 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 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 GI	GII	HAV
LENTICULE™ 1	+ (5.09 x 10 ² – 4.77 x 10 ³)	-	-
LENTICULE™ 2	-	+ (1.05 x 10 ³ – 3.13 x 10 ³)	+ (3.72 x 10 ² – 5.68 x 10 ²)

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 (Ct values) were made.

Table 3: Participants' results for all test 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	50	0	100	50	0	100	B	B
9*	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	-
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
20	100	100	100	100	100	100	100	100	100	A	A
21*	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
34	100	100	100	100	100	100	NE	NE	NE	A	-
35*	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
47*	100	100	100	100	100	100	100	100	100	A	A
49	100	100	100	100	100	100	100	100	100	A	A
72	100	100	100	50	100	0	NE	NE	NE	B	-
90*	100	100	100	100	100	100	50	0	100	A	B
94	100	100	100	100	100	100	100	100	100	A	A
95	100	100	100	100	100	100	100	100	100	A	A
98	100	100	100	100	100	100	100	100	100	A	A
106	50	0	100	100	100	100	100	100	100	B	A
113	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	-
132	100	100	100	100	100	100	100	100	100	A	A
143	100	100	100	100	100	100	NE	NE	NE	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
197	100	100	100	100	100	100	50	100	0	A	B
203	100	100	100	100	100	100	100	100	100	A	A
207	100	100	100	100	100	100	100	100	100	A	A
222	100	100	100	100	100	100	NE	NE	NE	A	-

* - Designated NRL, NE= Not examined, NR = Results not returned at date of report. 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 21/29 laboratories that returned results tested for all determinands and scored 100% overall accuracy. 3/29 laboratories scored 100% overall accuracy but did not test for HAV.

Conclusion and discussion

General comments

Thirty-one laboratories (14 NRLs and 17 other laboratories) received samples. Laboratories 34, 72, 143 and 222 did not examine for HAV. Laboratories 9 and 113 did not return results. Results reported to the EURL are shown in Appendices III and IV.

Discussion

24/29 (83%) of the laboratories which 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 98%, 97% and 94% for GI, GII and HAV respectively. The false positive reporting rates for GI, GII and HAV were 3%, 3% and 8% respectively. The false negative reporting rates for GI, GII and HAV were 0%, 3% and 4% respectively.

Twenty nine laboratories (100%) returned data expressed as C_t values for at least one sample/determinand combination (Appendix IV). Fifteen laboratories (52%) returned quantitative data for at least one sample/determinand combination. Quantitative results from individual labs alongside reference results are shown in Appendix V. Reference results were not corrected using extraction efficiency data.

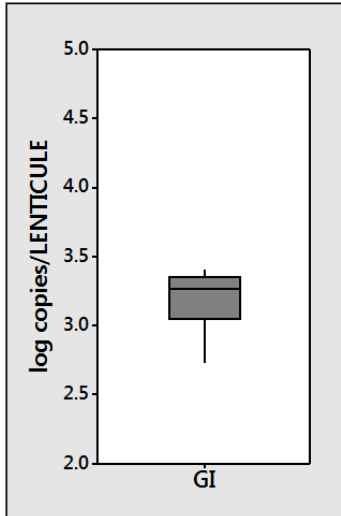
Methods used by participants to analyse the test samples are shown in Appendix VI.

References

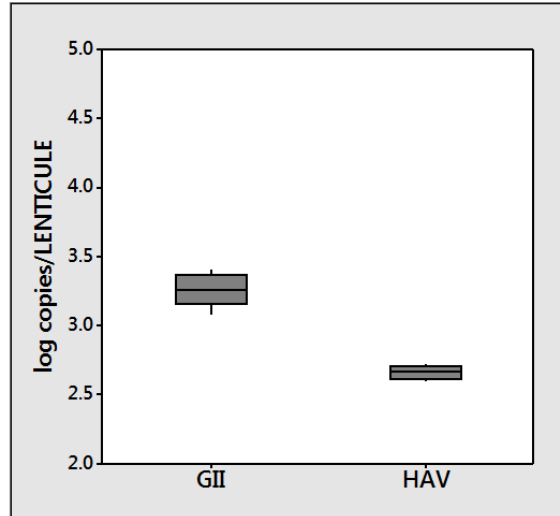
Codd AA, Richardson IR, Andrews N. 1998. Lenticules for the control of quantitative methods in food microbiology. *J Appl Microbiol.* **85(5)**:913–7.

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

LENTICULE 1



LENTICULE 2



Appendix II:

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

Where TP = true positives
FN = false negatives
FP = false positives
TN = true negatives
N = total number of tests

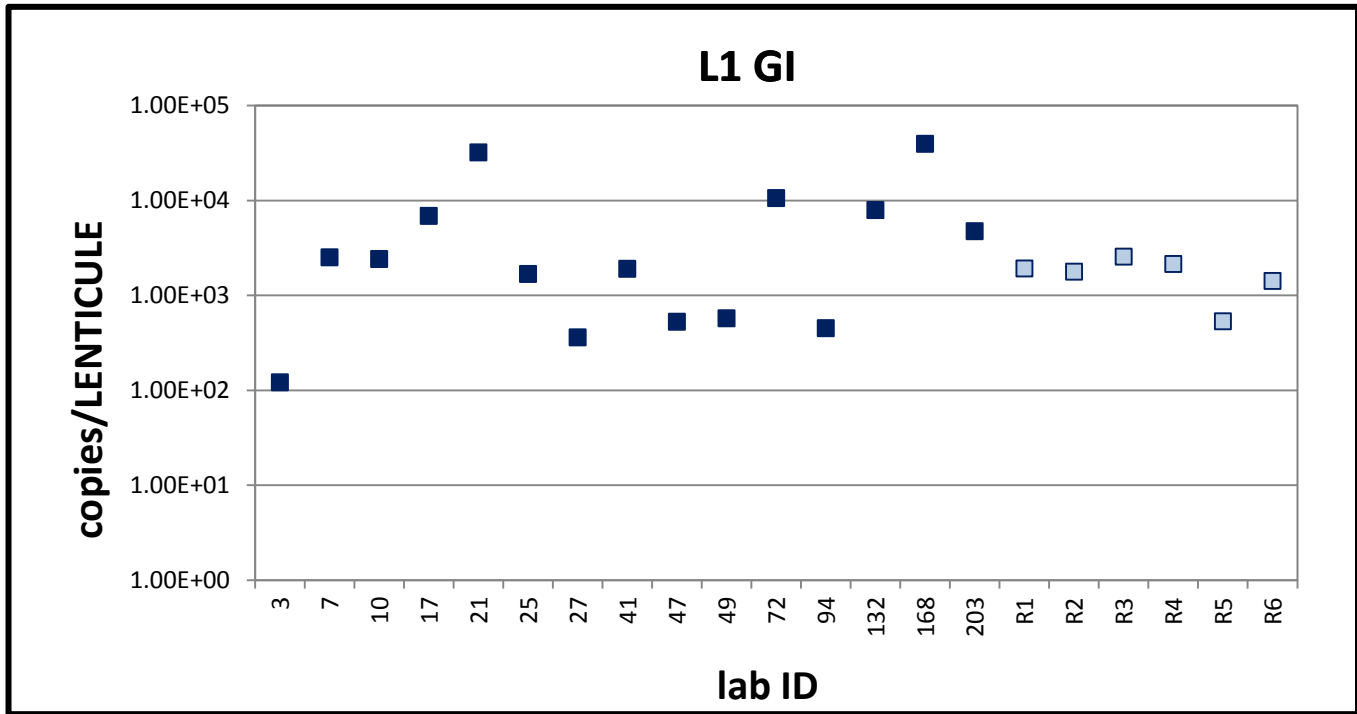
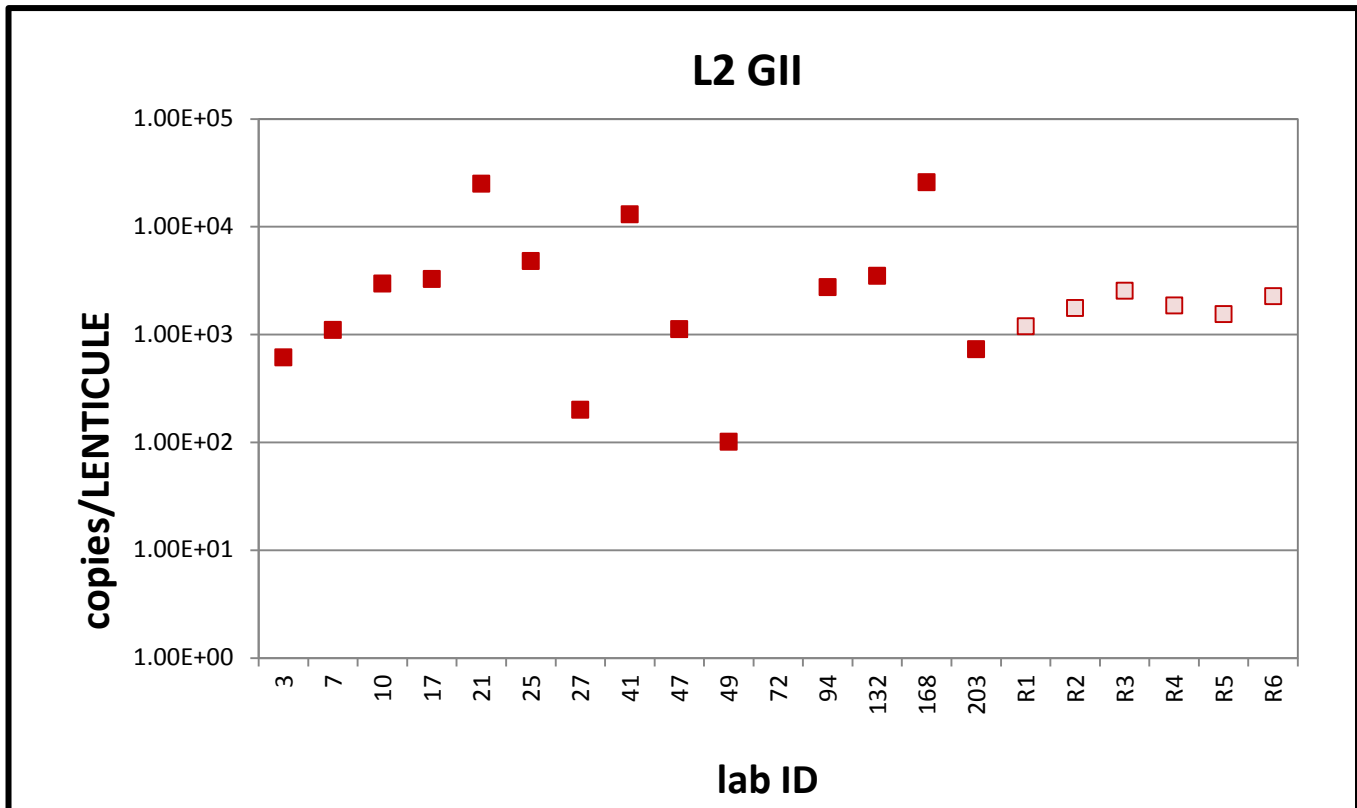
Note: Participants' results were expressed as percentage concordance with intended results generated by the EURL. In this assessment presence/absence data was used and no consideration of quantitative measurements (C_t values) was made.

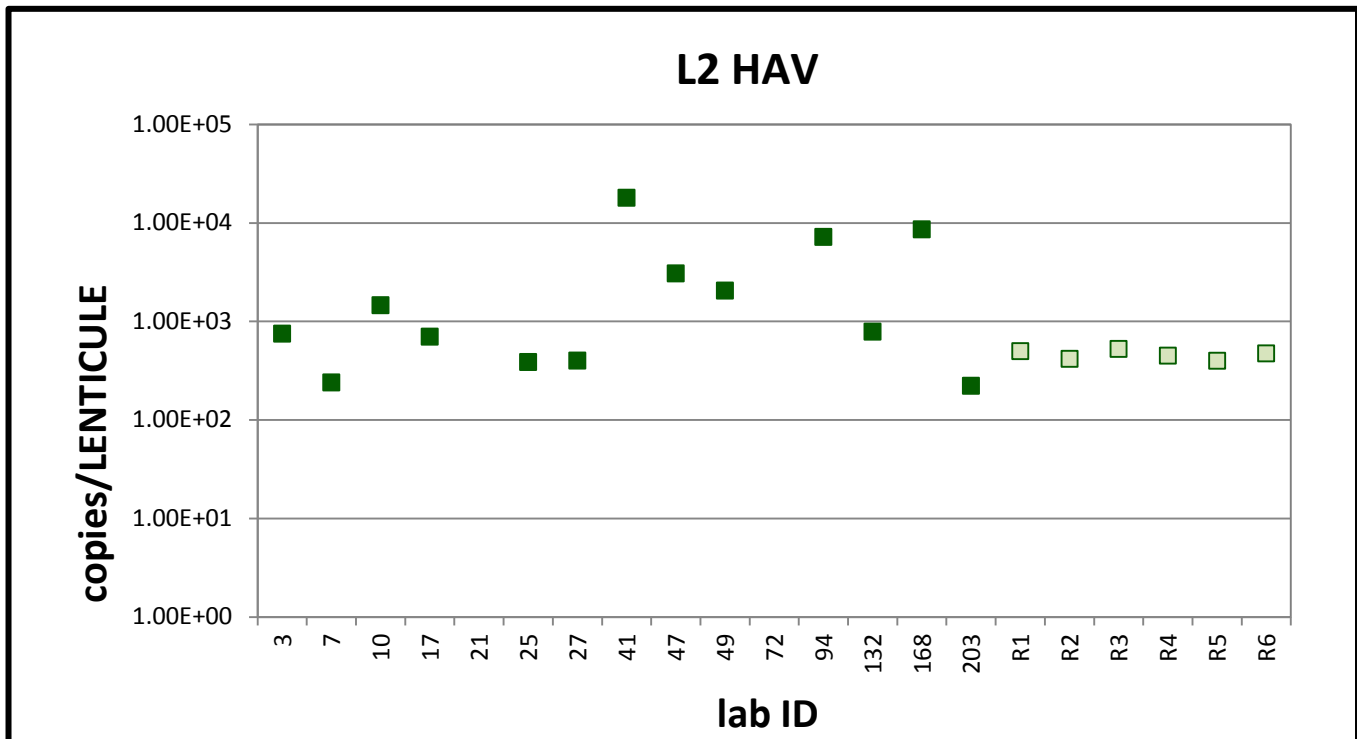
Appendix III: Participants' results

Lab ID No.	LENTICULE 1							LENTICULE 2							
	GI +	CT	copies/ LENTICULE	GII -	CT	HAV -	CT	GI +	CT	GII +	CT	copies/ LENTICULE	HAV +	CT	copies/ LENTICULE
3*	+	39.24	1.21E+02	-	-	-	-	-	+	35.60	6.12E+02	+	36.77	7.51E+02	
7*	+	32.53	2.51E+03	+	42.74	+	37.97	-	+	35.69	1.10E+03	+	36.05	2.40E+02	
9*	NR			NR		NR		NR		NR		NR			
10*	+	35.50	2.41E+03	-	-	-	-	-	+	34.00	2.96E+03	+	36.50	1.46E+03	
13*	+	33.91		-	-	-	-	-	+	33.20		+	36.81		
17*	+	31.45	6.86E+03	-	-	-	-	-	+	31.12	3.27E+03	+	35.73	7.00E+02	
20	+	32.99		-	-	-	-	-	+	32.60		+	33.02		
21*	+	34.80	3.20E+04	-	-	-	-	-	+	34.30	2.50E+04	+	37.00		
25*	+	32.36	1.68E+03	-	-	-	-	-	+	30.71	4.79E+03	+	35.32	3.87E+02	
27*	+	38.20	3.60E+02	-	-	-	-	-	+	34.70	2.00E+02	+	38.00	4.00E+02	
34	+	35.38		-		NE		-	+	33.17		NE			
35*	+	32.54		-		-		-	+	34.11		+	33.18		
39*	+	34.94		-		-		-	+	33.77		+	36.18		
41*	+	35.78	1.90E+03	-	-	-	-	-	+	33.87	1.30E+04	+	36.15	1.80E+04	
47*	+	34.90	5.26E+02	-	-	-	-	-	+	34.30	1.12E+03	+	35.70	3.07E+03	
49	+	36.99	5.72E+02	-	-	-	-	-	+	38.11	1.01E+02	+	34.05	2.06E+03	
72	+	35.97	1.06E+04	-		NE		- ^a		- ^a		NE			
90*	+	38.60		-		+	36.61	-		+	36.23		+	36.54	
94	+	38.01 / 39.49 ^b	4.50E+02	-		-		-		+	33.81 / 36.84 ^b	2.75E+03	+	34.94 / 35.43 ^b	7.21E+03
95	+	39.52		-		-		-		+	39.06		+		
98	+	38.96		-		-		-		+	36.47		+	35.13	
106	+	32.29		-		-		+	37.70	+	32.03		+	37.30	
113	NR			NR		NR		NR		NR		NR			
132	+	34.24	7.90E+03	-	-	-	-	-	+	34.27	3.50E+03	+	35.95	7.87E+02	
143	+	34.86		-		NE		-		+	34.32		NE		
147	+	39.20		-		-		-		+	38.20		+	38.72	
168	+	32.78	3.94E+04	-	-	-	-	-		+	35.76 ^c	2.58E+04	+	35.99	8.60E+03
197	+	37.40 ^d		-		-		-		+	29.90 ^e		-		
203	+	37.07	4.73E+03	-	-	-	-	-		+	38.04	7.29E+02	+	38.47	2.22E+02
207	+	32.00		-		-		-		+	33.00		+	33.00	
222	+	34.58		-		NE		-		+	34.91		NE		

NE= not examined, NR= results not returned at time of report, Yellow denotes false positives, Red denotes false negatives.

a; some damage to LENTICULE 2 noted by participating laboratory, b; results for undiluted and 1/10 diluted RNA shown, c; Ct value for 1/10 diluted RNA shown, d; positive result with primer set U (see Appendix V) only e; positive result with primer set CC (see Appendix V) only .

Appendix IV: Participants' and reference quantities for each sample.
LENTICULE 1 - 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			Virus extraction	RNA extraction	RT-PCR method	RT-PCR reagents	Primers		
	GI	GII	HAV	GI	GII	HAV					GI	GII	HAV
3*	+	-	-	-	+	+	-	B	J	L	V	U	U
7*	+	+	+	-	+	+	-	B	J	L	U ^a	U ^a	U ^a
10*	+	-	-	-	+	+	-	B	J	L	V	U	U
13*	+	-	-	-	+	+	-	B	J	L	U	U	U
17*	+	-	-	-	+	+	-	B	J	L	U	U	U
20	+	-	-	-	+	+	-	B	J	M	W	W	W
21*	+	-	-	-	+	+	-	B	J	L	U	U	U
25*	+	-	-	-	+	+	-	B	J	L	U	U	U
27*	+	-	-	-	+	+	A	B	J	N	V	U	U
34	+	-	NE	-	+	NE	-	C	J	O	U	U	-
35*	+	-	-	-	+	+	-	B	J	O	V	U	U
39*	+	-	-	-	+	+	-	B	J	L	X	X	U
41*	+	-	-	-	+	+	-	B	J	L	U	U	U
47*	+	-	-	-	+	+	-	B	J	O	U ^b	U ^b	U
49	+	-	-	-	+	+	-	D	J	N	V	U	Z
72	+	-	NE	-	-	NE	-	B	J	P	V	U	-
90*	+	-	+	-	+	+	-	E	J	O	U	U	U
94	+	-	-	-	+	+	-	B	J	M	W	W	W
95	+	-	-	-	+	+	-	E	K ^c	Q	X	X	AA
98	+	-	-	-	+	+	A	B	J	M	W	W	W
106	+	-	-	+	+	+	-	B	J	L	U	U	U
132	+	-	-	-	+	+	-	B	J	R	U	U	U
143	+	-	NE	-	+	NE	-	B	J	M	W	W	-
147	+	-	-	-	+	+	-	E	J	M	W	W	W
168	+	-	-	-	+	+	-	B	J	L	U	U	U
197	+ ^f	-	-	-	+ ^g	-	-	B	J	O	U/BB ^e	U/CC ^e	U/DD ^e
203	+	-	-	-	+	+	A	F	J	S	V	U	U
207	+	-	-	-	+	+	A	G	J	L	U	U	U
222	+	-	NE	-	+	NE	-	H	J	T	Y ^d	Y ^d	-

Red = false negative results; yellow = false positive results; dark grey = CEN TAG4 (or very similar) method; light grey = CEN TAG4 method with modifications

- 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 GI, GII & MS2 (process control assay) as multiplex
- e two primer/probe sets run in parallel
- f positive with primer set U only
- g positive with primer set CC only

Key to method codes

Virus extraction methods

A	Pro K (CEN or minor modifications)
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RNA extraction methods

B	NucliSens Magnetic extraction reagents (BioMerieux)
C	Thermo Scientific MagJET Viral DNA and RNA Kit
D	Trizol/Chloroform/Isopropyl alcohol/Dynabeads Oligo (dT)25
E	QIAamp/Rneasy kits (Qiagen)
F	High Pure Viral RNA Kit (Roche)
G	5X MagMax Pathogen RNA / DNA KIT (Lifescience), automatic extractor MegMax Express 96
H	foodproof® Virus Sample Preparation Kit (Biotecon)

RT-PCR methods

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

RT-PCR reagents

L	RNA Ultrasense (Invitrogen)
M	ceeram Tools
N	TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems)
O	Quantitect/Quantifast RT-PCR kits (Qiagen)
P	RNA to Ct One step kit (Applied Biosystems)
Q	High Capacity cDNA RT Kit & Taqman Universal Mastermix (Applied Biosystems)
R	One step qRT-PCR System (Invitrogen)
S	SuperScript® III PlatinumOne-Step qRT-PCR System (Invitrogen)
T	foodproof® Norovirus Detection Kit (Biotecon)

Primers/probes

U	CEN bench protocols
V	Primers; CEN, Probe; Hohne and Schreier, (2006)
W	Ceeram Tools (sequences as CEN)
X	Kageyama et al, (2003)
Y	foodproof® Norovirus Detection Kit (Biotecon)
Z	Guevremont et al, (2006), Houde et al, (2007)
AA	FDA/BAM Chapter 26 "Detection and Quantification of Hepatitis A virus in Shellfish by the Polymerase Chain Reaction"
BB	Dreier et al, (2010)
CC	Hohne and Schreier, (2006)
DD	Hu and Arsov, (2009)