Livsmedelsverket
National Food Agency

European Union Reference Laboratory
for Foodborne Viruses

EURL – FOODBORNE VIRUSES

FINAL REPORT

PROFICIENCY TESTING SCHEME EFV 01, 2018

Detection of norovirus and hepatitis A virus in raspberries

Final Report – Version 1 (13/02/2019)

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INTRODUCTION

Proficiency test (PT) EFV 01 was organised and funded by the European Union Reference Laboratory (EURL) for Foodborne Viruses in December 2018 to support the official controls on foodborne viruses in line with Regulation (EU) 2017/625. Twenty-five laboratories were invited to take part in the PT and twenty participated. The PT scheme was designed for qualitative detection of hepatitis A virus (HAV) and norovirus genogroup I (GI) and genogroup II (GII) in two samples of frozen raspberries. The participating laboratories were requested to examine the samples using their routine methods, however the EURL recommended to analyse the samples according to ISO/TS 15216-2. A Standard Operating Procedure (SOP) for qualitative detection of norovirus and hepatitis A virus in soft fruit was therefore provided, as well as external control (EC) RNA and process control virus upon request.

LEGISLATION

Since 2018, the Swedish National Food Agency has been appointed EURL for Foodborne Viruses according to Regulation (EU) 2017/625 and under Article 94 the EURL is responsible to organise PTs for the National Reference Laboratories (NRLs) for Foodborne Viruses. Participation in EURL PTs is mandatory for NRLs appointed in line with Regulation (EU) 2017/625.

PT EFV 01 of 2018 was dedicated to the identification of HAV and norovirus GI and GII in soft fruits. Partly because of the temporary legislation to control norovirus in imported frozen raspberries from Serbia (EU 669/2009, EU 2298/2017) and partly to implement the EURLs SOP based on ISO/TS 15216-2 to contribute to method harmonisation (EU 2017/625).

SAMPLES

The dispatched material was comprised of artificially contaminated frozen raspberries inoculated with characterised norovirus GI and GII from human faecal material and HAV from cell culture supernatant. Table 1 shows detailed information of the viruses used for preparation of the samples.

Table 1: Description of the viruses used for the PT EFV 01, 2018

Viruses	Origin	Strain ID/genotype
Hepatitis A virus	Cell culture supernatant	HM 175/18f
Norovirus genogroup I	Faecal material	GI.3 (capsid sequence)
Norovirus genogroup II	Faecal material	GII.4 Sydney_2012 (capsid sequence)

The matrices were inoculated with approximately 10^5 virus genome copies per 25 gram raspberries given the intended results as shown in Table 2.

Table 2: Intended results of PT EFV 01, 2018

Sample	Norovirus GI	Norovirus GII	HAV
EFV1801A	+	-	-
EFV1801B	-	+	+

DISTRIBUTION OF THE PROFIENCY TEST ITEMS

Samples were prepared and dispatched on dry ice by courier to the 20 participating laboratories on December 10th 2018, in accordance with IATA packing instructions 650 for UN3373. All participating laboratories received two samples and those who had requested also received EC RNA and/or process

control virus (mengovirus). Instruction sheet, results form and EURLs SOP (version 2018-11-05) were sent by email to the contact person(s) at each laboratory.

Upon arrival, each participant had until 21th of December 2018 to perform analyses using their routine methods and report their results.

CONFIDENTIALITY

The procedures used for the organisation of PTs assure that the identity of the participating laboratories and the information provided by them is treated as confidential. The participating laboratories are assigned a unique laboratory code used all through this report. However, NRLs appointed in line with Regulation (EU) 2017/625 will be disclosed to DG SANTE for (long-term) performance assessment.

QUALITY CONTROL

The base material used to produce the two test items was frozen raspberries purchased from retail. Analysed according to EURLs SOP based on ISO/TS 15216-2, the purchased raspberry base material batch tested negative for HAV, norovirus GI and GII.

STABILITY STUDY

In order to investigate possible virus instability in inoculated raspberry samples stored in the freezer, a stability study was made. Samples were inoculated with the target viruses and kept at -20 °C and one sample of each EFV1801A and EFV1801B were analysed at day 2, 4, 7 and 28 according to EURLs SOP based on ISO/TS 15216-2 and quantification according to ISO 15216-1. The results indicated that the samples were relatively stable for up to 7 days but after 28 days, the virus genome copies per 25 gram raspberries had reduced one log (Appendix A). In this PT the participants had a time period of approximately 11 days to perform the analyses and report their results.

HOMOGENEITY STUDY

The homogeneity of the samples was tested by inoculating 10 samples each of EFV1801A and EFV1801B followed by a storage period of approximately 24 hours at -20 °C. The samples were then analysed according to EURLs SOP based on ISO/TS 15216-2 and quantified according to ISO 15216-1. The results for the virus genome copies per 25 gram raspberries were between $7.0 \times 10^1 – 3.0 \times 10^2$, 1.6×10^2- 7.9×10^3 and 9.9×10^1- 1.4×10^3 for norovirus GI, norovirus GII and HAV respectively (Appendix B). The PT EFV 01 of 2018 was designed for qualitative detection of norovirus and HAV with relatively high contamination levels and the variation was considered acceptable for this PT.

ASSESSING THE PERFORMANCE

Performance was assessed according to three criteria:

- Relative specificity (SP)
  Percentage relative specificity = SP (%) = [TN/(TN+FP)]*100
- Relative sensitivity (SE)
  Percentage relative sensitivity = SE (%) = [TP/(TP+FN)]*100
- Relative accuracy (AC)
  Percentage relative accuracy = AC (%) = [(TP+TN)/N]*100

Where:
TN: True negatives
FP: False positives
TP: True positives
FN: False negatives
N: Total number of tests

RESULTS

REFERENCE RESULTS

Reference analyses were performed by the EURL on samples prepared on the same day as the dispatched samples (10th of December 2018), stored on dry ice for 24 hours followed by an additional 48 hours for EFV1801A and 72 hours for EFV1801B at -20 °C to mimic realistic shipping conditions. Five samples of each EFV1801A and EFV1801B were analysed and quantified according to EURLs SOP based on ISO/TS 15216-2 and ISO 15216-1 respectively. Reference results for each sample are shown in Table 3, with box and whisker plots included in Appendix C.

Table 3: Reference results for PT EFV 01, 2018

Sample	Norovirus genogroup I	Norovirus genogroup II	Hepatitis A virus
EFV1801A	Virus genome detected in 25 g	Virus genome not detected in 25 g	Virus genome not detected in 25 g
EFV1801B	Virus genome not detected in 25 g *	Virus genome detected in 25 g	Virus genome detected in 25 g

*One out of five samples was positive in one out of five RT-PCR replicates

PARTICIPANTS' RESULTS

Twenty laboratories received samples, whereof two laboratories did not return results for any analyses and another two laboratories did not test for HAV. Ten laboratories performed the analyses under accreditation whereof three laboratories had invalid results caused by inhibition and low extraction efficiency. There were one false negative result and one false positive result.

Table 4: Overview of participants´ results

Target viruses	N	Sample EFV1801A				Sample EFV1801B			
		SF	FP	FN	NV	SF	FP	FN	NV
Norovirus GI	18	17	0	1	0	17	0	0	1
Norovirus GII	18	15	1	0	2	18	0	0	0
Hepatitis A virus	16	13	0	0	3	16	0	0	0

N: Number of laboratories that reported results for the analysis, SF: Number of laboratories with satisfactory result
FP: False positive, FN: False negative, NV: Not valid

The participants´ results were assessed as presence–absence data in concordance with intended results as percentage relative accuracy, specificity and sensitivity as previously described and are shown in Table 5. Detailed information about the participating laboratories quantification cycle (Cq) values and methodology can be found in Annex D and E. Quantitative results reported beside Cq values are displayed in Annex E.

Table 5: Participants´ results for PT EFV 01, 2018

Lab. ID	Norovirus genogroup I			Norovirus genogroup II			Hepatitis A virus		
No.	AC (%)	SP (%)	SE (%)	AC (%)	SP (%)	SE (%)	AC (%)	SP (%)	SE (%)
101	100	100	100	100	100	100	100	100	100
102[a]	100	100	100	100	100	100	100[b]	100[b]	100[b]
103[a]	100	100	100	100	100	100	100	100	100
104[a]	100	100	100	100	100	100	100	100	100
105[a]	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]
106	100	100	100	100	100	100	NT	NT	NT
107[a]	100	100	100	100	100	100	100	100	100
108[a]	100	100	100	100	100	100	100	100	100
109[a]	100	100	100	100	100	100	100	100	100
110	100	100	100	50	0	100	100	100	100
111	100	100	100	100	100	100	100	100	100
112[a]	100	100	100	100[c]	100[c]	100[c]	100[c]	100[c]	100[c]
113[a]	100	100	100	100	100	100	100	100	100
114[a]	100	100	100	100	100	100	100	100	100
115	100	100	100	100	100	100	100	100	100
116	NR	NR	NR	NR	NR	NR	NR	NR	NR
117	100	100	100	100	100	100	100	100	100
118	100	100	100	100	100	100	100	100	100
119	50	100	0	100	100	100	NT	NT	NT
120	NR	NR	NR	NR	NR	NR	NR	NR	NR

AC: Relative accuracy, SP: Relative specificity, SE: Relative sensitivity, NT: Not tested, NR: No results returned
a: Accredited for detection of norovirus and HAV in soft fruit, b: Invalid negative results, unacceptable inhibition
c: Invalid negative results, unacceptable extraction efficiency

DISCUSSION

A majority of the participating laboratories obtained intended results (Table 4) and analysed the samples according to ISO 15216 (appendix D). The laboratories overall accuracies for all reported results were 97 % for norovirus GI and GII and 100 % for HAV (Table 5). Besides the two laboratories that did not return any results at all, there were two laboratories that for unknown reason did not test for HAV. However, the EURL recommends that all designated NRLs for Foodborne Viruses have the ability to analyse for HAV, even though Regulation (EU) 2017/2298 only stipulates the analyses of norovirus and not HAV in imported frozen raspberries from Serbia. The EURL willingly offers technical support to any NRL upon request.

According to ISO/TS 15216-2 one laboratory obtained unacceptable inhibition, in both samples for all targets. The ΔCq (Cq value (sample RNA + EC RNA) − Cq value (water + EC RNA)) for RNA extracts diluted 10^{-1} were all greater than 4. That laboratory used a modified version of ISO/TS 15216-2 with RNA extraction with EZ1® Virus Mini Kit v2.0 (Qiagen) on Qiagen Biorobot EZ1. Potential inhibitors were reduced with OneStep™ PCR Inhibitor Removal Kit (Zymo Research) before running duplex real-time RT-PCR for norovirus and singleplex real-time RT-PCR for HAV with QuantiTect® Probe RT-PCR kit (Qiagen). The laboratory suspects that some complications with the Qiagen Biorobot EZ1 caused the invalid results, which seems probable since the inhibition was high even with the use of an inhibitor removal kit and after diluting the samples.

Another laboratory that performed the analysis according to ISO/TS 15216-2 got unacceptable inhibition for HAV in both samples. The ΔCq values for the EC RNA extracts for sample EFV1801A and EFV1801B diluted 10^{-1} were greater than 3. Raspberries are fragile and contain various substances, which may inhibit RNA detection. In addition to gentle handling of the raspberries, the use of an inhibitor removal kit may potentially reduce inhibiting substances.

One laboratory reported unacceptable extraction efficiency for sample EFV1801A. The laboratory performed the analysis according to ISO/TS 15216-2. No explanation for the low extraction efficiency could be identified. Nonetheless, norovirus GI was correctly detected in the sample.

If a valid result is not obtained, results should normally be expressed as “no result”. However, a positive result, in an otherwise not valid analysis, should be reported as ”virus genome detected in 25 g” with details about the analysis included in the report. Therefore, the positive results are accepted but the negative results are not valid and in reality should be re-tested. Since the laboratories did not get the opportunity to retest the samples, all of them were scored 100 % with a remark on their accuracy, specificity and sensitivity (Table 5).

One laboratory got a false negative result for norovirus GI in sample EFV1801A. That laboratory used Oasig™ OneStep qRT-PCR Mastermix (Primerdesign Ltd) with cycling conditions according to the manufacturer's protocol with primers and probes from genesig™ Advanced Detection Kit, Norovirus Genogroups 1 and 2 (Primerdesign Ltd). The design of primers and probe is not known in this case, and the negative result may be caused by primer or probe mismatches. It is recommended to use primers and probes that are described in the informative Annex C of ISO/TS 15216-2.

There was also one false positive result for norovirus GII in sample EFV1801A. The sample was weakly positive for norovirus GII only in one undiluted RT-PCR replicate. The laboratory extracted the virus with an alternative robot system with NucliSens® (BioMérieux) reagents and used QuantiTect® Probe RT-PCR kit (Qiagen) as RT-PCR reagent. The positive result could be due to contamination either during preparation of the sample by the EURL or at the analysing laboratory. Another explanation could be that the specific raspberry sample analysed was naturally contaminated with the virus.

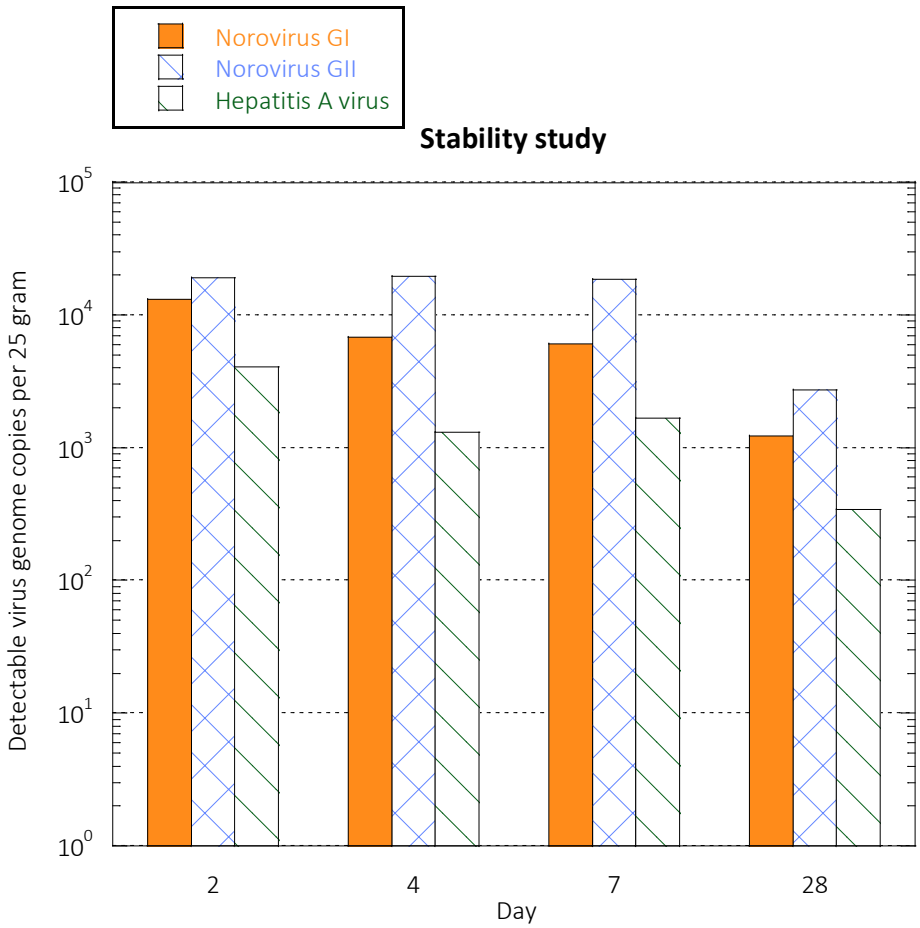
The EURL experienced a similar result when analysing the reference samples for the PT, one out of five samples of EFV1801B was weakly positive for norovirus GI in one out of five RT-PCR replicates. As mentioned previously, this may be due to contamination but it could also be a true positive – even if the raspberries were pre-tested for the target viruses this does not guarantee absence of the target viruses in the entire batch. One of the major challenges when analysing food matrices is to detect low levels of viral contamination heterogeneously distributed in a batch, while at the same time analysing a limited amount of individual samples and small sample sizes.

CONCLUSION

In 2018, the PT EFV 01 organised by EURL of Foodborne Viruses was dedicated for qualitative detection of HAV and norovirus GI and GII in frozen raspberries. Twenty laboratories participated in the PT and the majority of reporting laboratories followed the standard method ISO/TS 15216-2 with some modifications. In a few cases, the quantitative method ISO 15216-1 was used and quantitative results were reported beside Cq values. The performance of the participating laboratories was satisfactory with overall accuracies of 97 % for norovirus GI and GII and 100 % for HAV for all reported results.

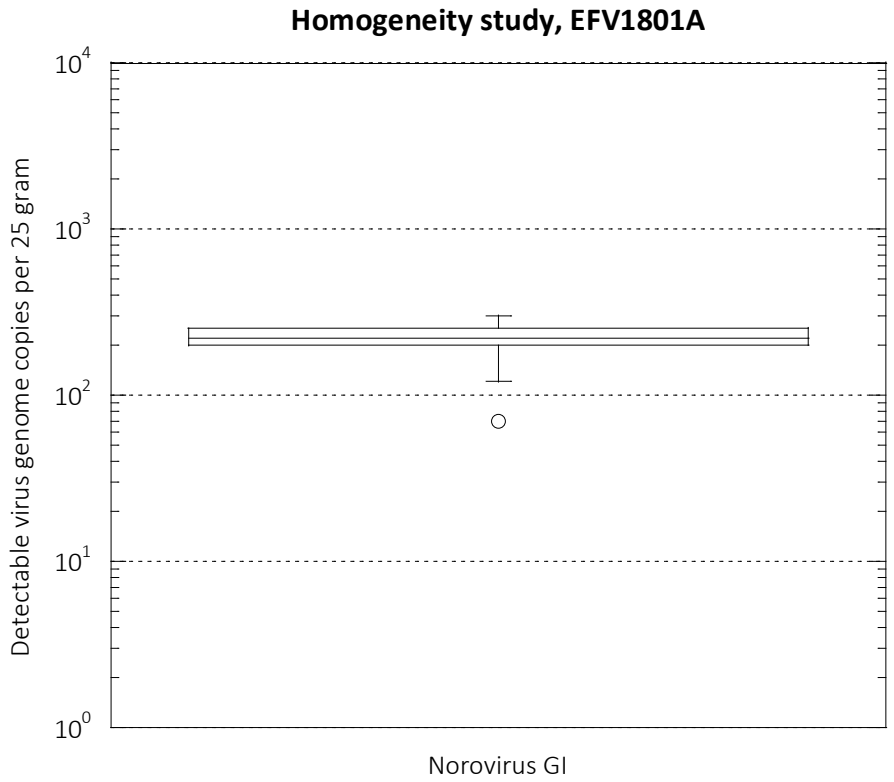
APPENDIX A

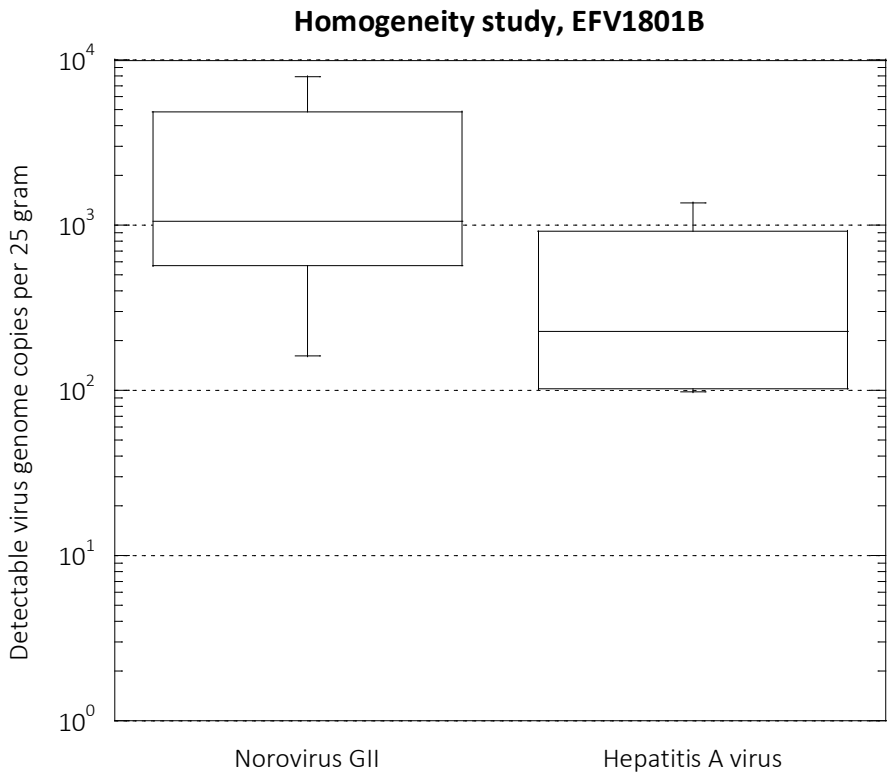
Stability study of frozen raspberries inoculated with the target viruses used for PT EFV 01, 2018.



APPENDIX B

Homogeneity study displayed as box and whisker plots of detectable genome copies per 25 gram. *The box includes 50 % of the results (25 % of the results above the median and 25 % of the results below the median). The remaining 50 % are illustrated by lines and circles outside the box. A circle is for technical reasons shown in the plot when a value deviates to certain degree[*] from the other values. This does not by itself indicate that the value is an outlier.*

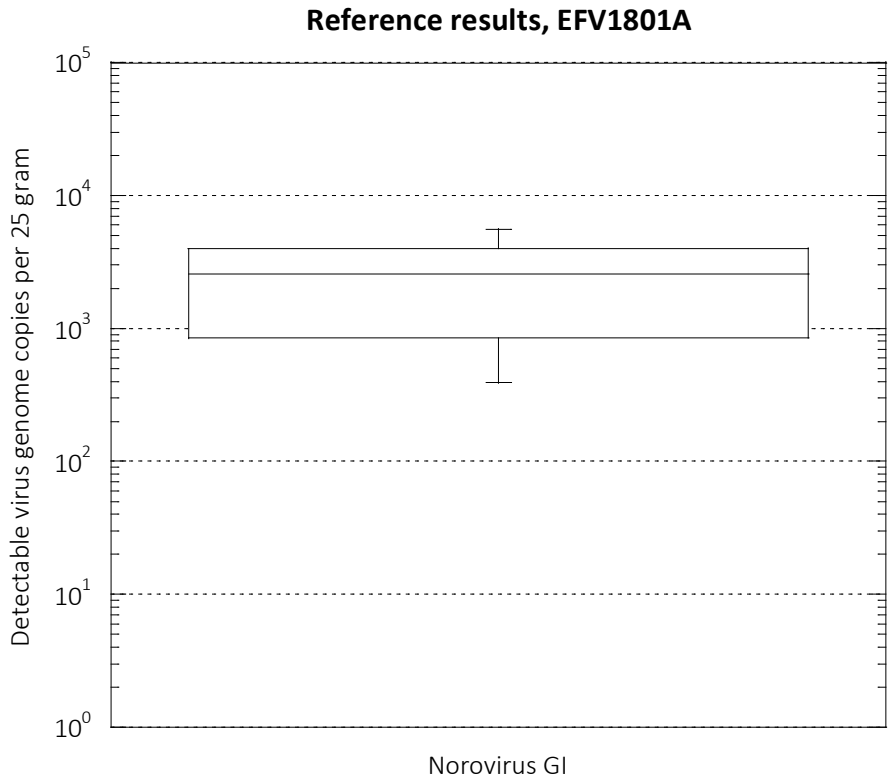


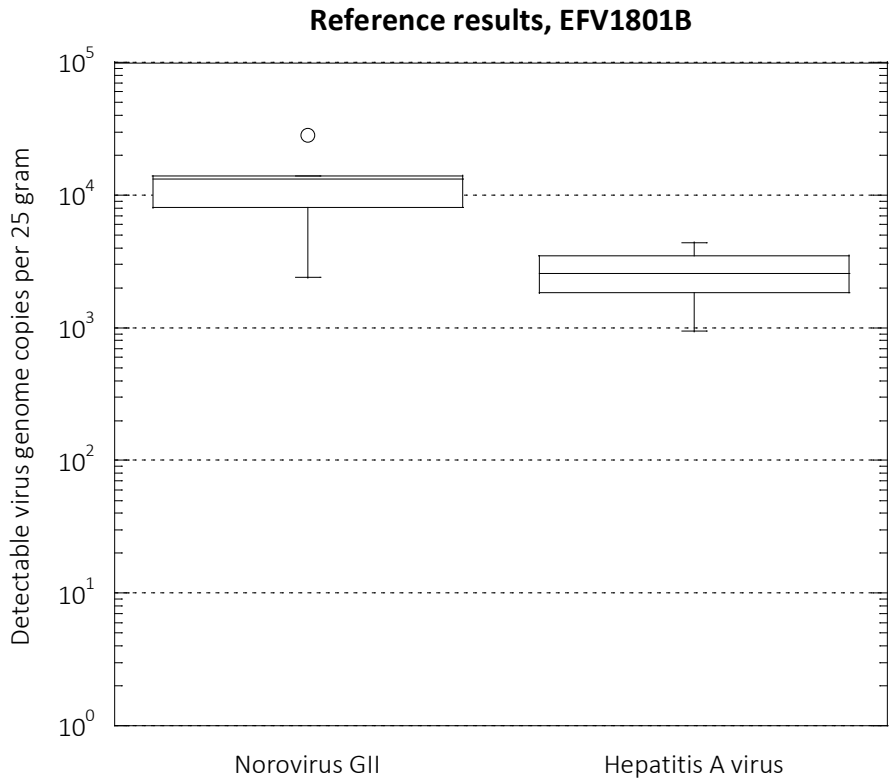


[*] < [lowest value in the box −1.5 × (highest value in the box− lowest value in the box)] or > [highest value in the., box + 1.5 × (highest value in the box − lowest value in the box)]

APPENDIX C

Reference results displayed as box and whisker plots of detectable genome copies per 25 gram. *The box includes 50 % of the results (25 % of the results above the median and 25 % of the results below the median). The remaining 50 % are illustrated by lines and circles outside the box. A circle is for technical reasons shown in the plot when a value deviates to certain degree[†] from the other values. This does not by itself indicate that the value is an outlier.*





[†] < [lowest value in the box −1.5 × (highest value in the box− lowest value in the box)] or
> [highest value in the., box + 1.5 × (highest value in the box − lowest value in the box)]

APPENDIX D

Results and methods used for PT EFV 01, 2018. *For key to method codes see next page.*

Lab. ID No.	Sample EFV01A			Sample EFV01B			Sample preparation	RNA extraction	RT-PCR reagents	Primers & probes
	GI (Cq)	GII (Cq)	HAV (Cq)	GI (Cq)	GII (Cq)	HAV (Cq)				
101	29.97				30.19	32.38	A	D	I	O
102[a]	39.27[b]		NV		34.82	36.53	A	D	I	O
103[a]	33.84				33.04	34.66	A	D	I	O
104[a]	30.77[c]				30.07[c]	30.39[c]	A	D	I	O
105[a]	38.0	NV	NV	NV	35.6	38.4	B	F	J	O
106	36.5		NT		35.5	NT	A	D	I	O
107[a]	28.15				25.07	27.03	B	G	K	P
108[a]	29.10				26.51	30.71	A	D	L	Q
109[a]	30.00				28.10	31.93	A	D	I	O
110	36.79[b]	37.30			30.32	33.81	A	E	J	O
111	31.08				30.80	34.23	A	D	M	O
112[a]	32.27	NV	NV		28.84	33.63	A	D	I	O
113[a]	31.52[b]				29.57[b]	33.36[b]	B	D	L	Q
114[a]	29.62				29.78	31.11	A	D	I	O
115	32.68				29.11	31.01	A	D	I	O
116	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
117	30.47				29.66	29.78	C	H	I	O
118	34.42				32.70	35.99	A	D	I	O
119			NT		32.78	NT	A	D	N	R
120	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

NT: Not tested, NR: No results returned, NV: Not valid

a: Accredited for detection of norovirus and HAV in soft fruit, b: RNA extract diluted 10^{-1}, c: Mean Cq calculated by the EURL

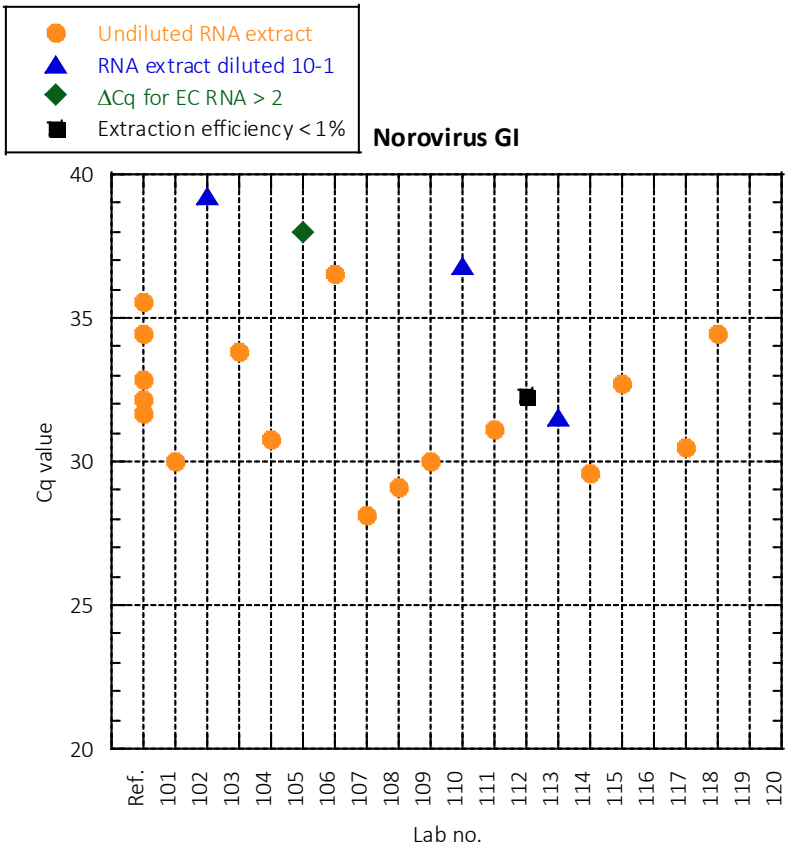
Cells highlighted in red indicate false positive and negative results. Cells highlighted in grey indicate modifications from ISO-15216 including informative material. This do not necessarily mean noncompliance with the ISO standard.

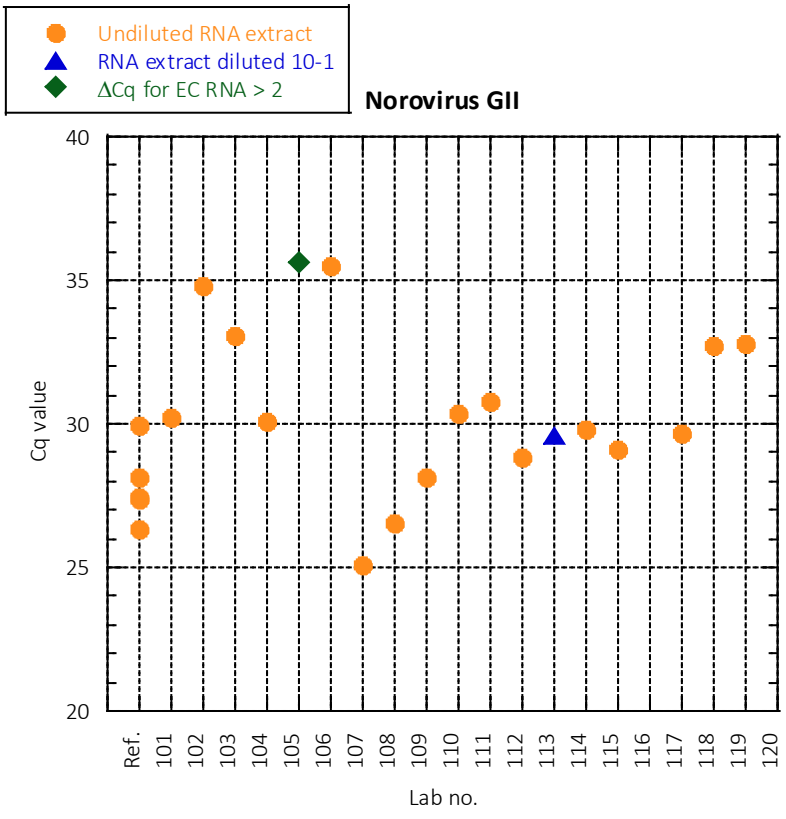
Key to method codes

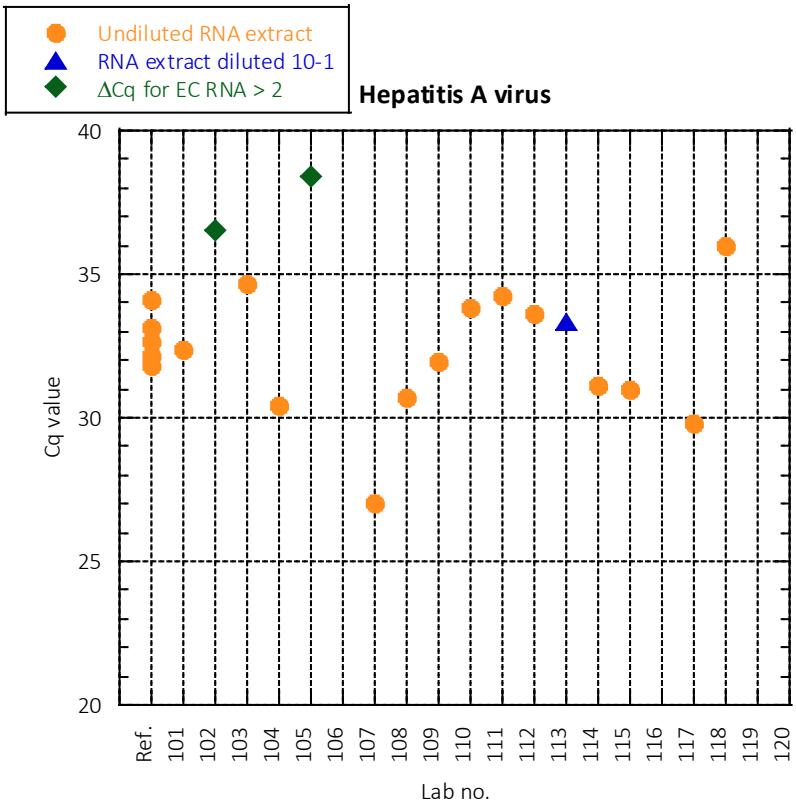
Sample preparation method	
A	ISO/TS 15216-2
B	Modified ISO/TS 15216-2
C	Modified ISO 15216-1
RNA extraction reagents	
D	NucliSens® (BioMérieux)
E	NucliSens® (BioMérieux), alternative robot system
F	EZ1® Virus Mini Kit v2.0 (Qiagen)
G	QIAamp® Viral RNA Mini Kit (Qiagen)
H	Ambion® Plant RNA Isolation Aid (Life technologies)
RT-PCR reagents	
I	RNA UltraSense™ One-Step Quantitative RT-PCR System
J	QuantiTect® Probe RT-PCR kit (Qiagen)
K	GoTaq® Probe 1-Step RT-qPCR System (Promega)
L	CeeramTools® real time RT-PCR kits (Ceeram)
M	TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems)
N	Oasig™ OneStep qRT-PCR Mastermix (Primerdesign Ltd)
Primers and probes	
O	ISO-15216 *(The probe, NVGG1p or TM9, for norovirus GI was not asked to be specified)*
P	ISO 15216, with different fluorophores & quenchers
Q	CeeramTools®
R	genesig™ Advanced Detection Kit, Norovirus Genogroups 1 and 2 (Primerdesign Ltd)

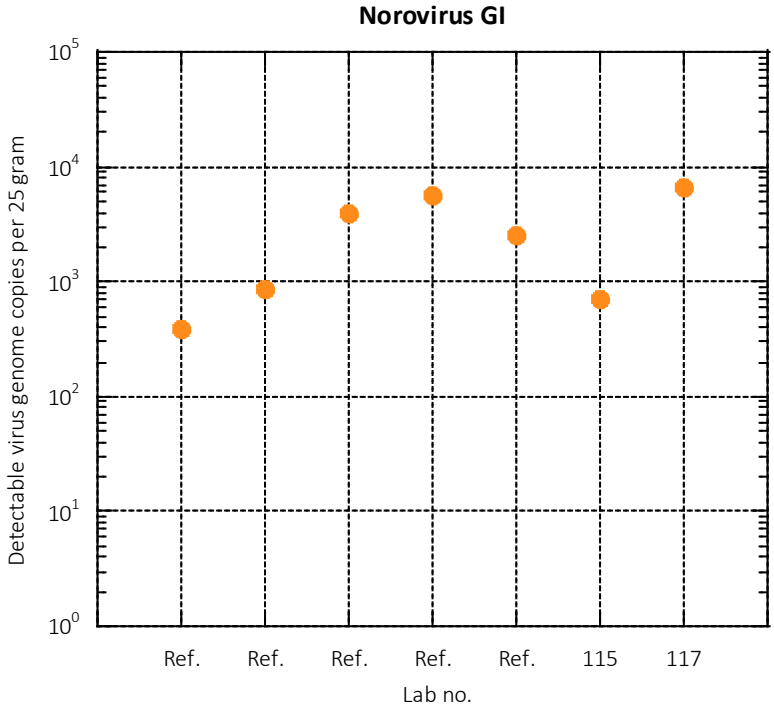
APPENDIX E

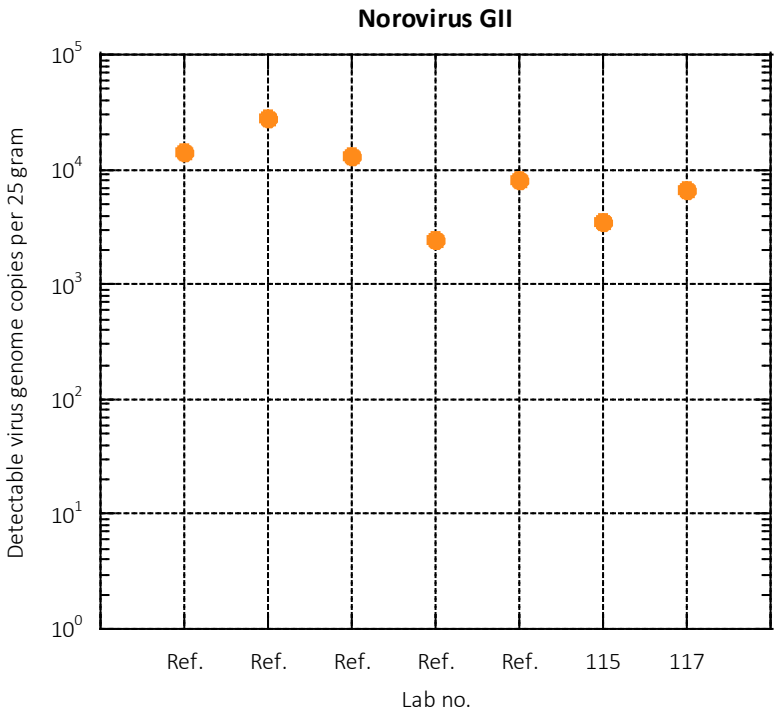
The participating laboratories Cq values and quantitative results compared with reference results.

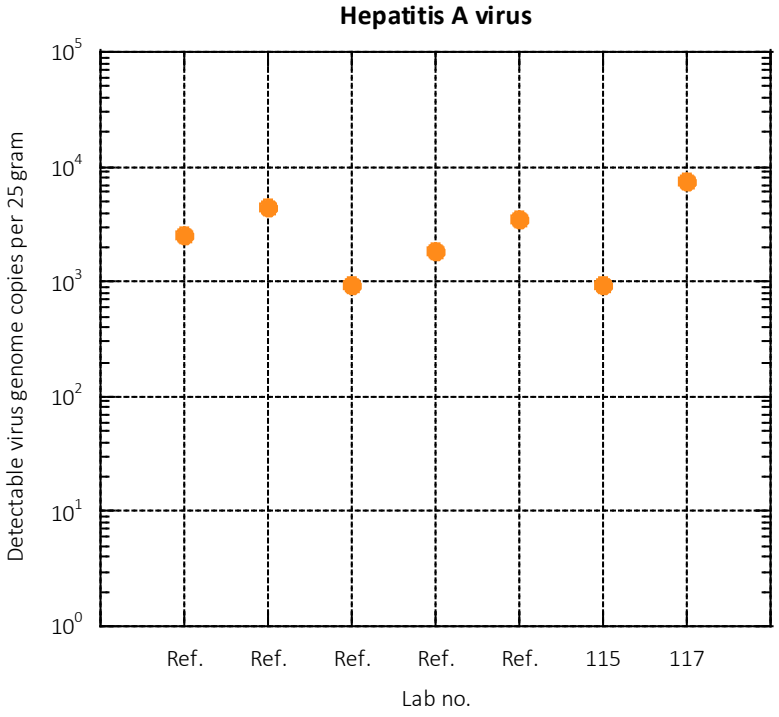





Undiluted RNA extract
RNA extract diluted 10-1
ΔCq for EC RNA > 2
Hepatitis A virus
Cq value
40
35
30
25
20
Ref.
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
Lab no.


Norovirus GI
Detectable virus genome copies per 25 gram
10^5
10^4
10^3
10^2
10^1
10^0
Ref.
Ref.
Ref.
Ref.
Ref.
115
117
Lab no.


Norovirus GII
Detectable virus genome copies per 25 gram
10^5
10^4
10^3
10^2
10^1
10^0
Ref.
Ref.
Ref.
Ref.
Ref.
115
117
Lab no.


Hepatitis A virus
Detectable virus genome copies per 25 gram
10^5
10^4
10^3
10^2
10^1
10^0
Ref.
Ref.
Ref.
Ref.
Ref.
115
117
Lab no.