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Swedish Food Agency

European Union Reference Laboratory
for Foodborne Viruses

EURL – FOODBORNE VIRUSES

FINAL REPORT

PROFICIENCY TESTING SCHEME EFV 04, 2020

Detection of norovirus and hepatitis A virus in lettuce

Final Report – Version 1 (19/04/2021)

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INTRODUCTION

The Swedish Food Agency has been appointed European Union Reference Laboratory (EURL) for Foodborne Viruses according to Regulation (EU) 2017/625, since 2018. Under Article 94, the EURL is responsible for organizing Proficiency Tests (PTs) for the National Reference Laboratories (NRLs) for Foodborne Viruses. Participation in EURL PTs is mandatory for relevant NRLs in each Member State appointed in line with Regulation (EU) 2017/625.

This report describes the performance of NRLs for detection of viral contamination of lettuce samples PT scheme EFV04, organised by the EURL for Foodborne Viruses.

Distribution was made 6th of October 2020 to 21 laboratories that signed up to take part in the PT and was designed for the detection of hepatitis A virus (HAV) and norovirus genogroup I (GI) and genogroup II (GII) in three samples of fresh lettuce.

The participating laboratories were requested to examine the samples using their routine method, however the EURL recommended to analyse the samples according to ISO 15216-2. A Standard Operating Procedure (SOP) for qualitative detection of norovirus and hepatitis A virus in soft fruit, based on ISO 15216-2, was therefore provided. External control (EC) RNA and process control virus were distributed together with PT samples, upon request.

In order to ensure confidentiality, all participants are assigned a unique laboratory identification number. Only staff within the PT team and the laboratory itself have access to this ID. However, results from NRLs appointed in line with Regulation (EU) 2017/625 will be disclosed to DG SANTE for performance assessment.

SAMPLES

Materials dispatched consisted of artificially contaminated refrigerated lettuce samples inoculated with characterised norovirus GI and GII from human faecal material and HAV from cell culture supernatant. Detailed information of the viruses used for preparation of the samples is demonstrated in Table 1.

Table 1: Description of the viruses used for the PT EFV 04

Viruses	Origin	Strain ID/genotype
Hepatitis A virus	Cell culture supernatant	ATCC® VR-1402™ (HM 175/18f)
Norovirus genogroup I	Faecal material	GI.3 (capsid sequence)
Norovirus genogroup II	Faecal material	GII.6 (capsid sequence)

Sample A was inoculated with Hepatitis A virus, norovirus GI and GII in approximately 10^5 virus genome copies per 25 gram lettuce and sample B with approximately 10^5 Hepatitis A virus genome copies per 25 gram lettuce. Sample C however, was not inoculated with any of the target viruses. Concentration values are shown in Table 2.

Table 2: Spiking of PT EFV 04 samples

Sample	Norovirus GI	Norovirus GII	HAV
20EFV04 A	≈10^5*	≈10^5*	≈10^5*
20EFV04 B	–	–	≈10^5*
20EFV04 C	–	–	–

* *Detectable virus genome copies spiked to each sample*

PREPARATION OF SAMPLES

Approximately 2.5 kg lettuce of the same batch was purchased from a retail in Sweden. A homogenous mixture was prepared by chopping the lettuce leaves in into pieces of mixing the material together to make it more homogenous. The material was then divided into 25 grams, transferred to plastic bags, spiked with the target viruses, sealed and stored in 4° C for approximately one hour before dispatching.

DISTRIBUTION OF THE PROFIENCY TEST ITEMS

Samples were dispatched in refrigerated condition by courier in accordance with IATA packing instructions 650 for UN3373, on October 6th. All 21 laboratories received three refrigerated lettuce samples and the ones that so requested also received EC RNA and/or process control virus (mengovirus). Instruction sheet and results form were sent by email to the contact person(s) at each laboratory. The deadline for submitting the results was October 13th.

QUALITY CONTROL

Lettuce used to produce the test samples was tested negative for HAV, norovirus GI and norovirus GII. Spiked material were also examined for homogeneity and stability. Inhibition and extraction efficiency were acceptable for all the samples used for homogeneity and stability test.

STABILITY OF VIRUS LEVELS IN LETTUCE SAMPLES

In order to investigate the stability of spiked viruses in samples stored in refrigerator, a study was conducted before and after dispatch. The preliminary test showed that the virus levels have a tendency to decrease after 4 days and therefore the participants were asked to start the virus extraction within 24 hours upon the samples' arrival. The procedure and results of the stability test done after dispatch are presented in the reference samples section together with the homogeneity test.

REFERENCE RESULTS- HOMOGENEITY AND STABILITY OF VIRUS LEVELS IN LETTUCE SAMPLES

In order to mimic realistic shipping conditions, storage conditions at the participating laboratories as well testing the homogeneity, ten samples each of 20EFV04A, 20EFV04B and 20EFV04C were randomly picked on the dispatch date (October 6th 2020). Two samples of each were tested immediately after the inoculation (day -1), and the rest of samples were stored in refrigerator and tested at day 0, 1, 2 and 5. Samples we analysed according to EURL SOP based on ISO 15216-2 and ISO 15216-1 for qualitative and quantitative detection of target viruses respectively. The results are shown in Table 3 and 4, with box and whisker plots included in graph 1. Results of day 2 are also included in Appendix A, as it is most likely the day analyses started at the participating laboratories. Inhibition and extraction efficiency were calculated for all the reference samples. PT samples are considered to be homogenous enough for the trial 04 purposes.

Table 3: Qualitative results for reference samples for PT EFV 04

Sample	Norovirus GI	Norovirus GII	HAV
20EFV04 A	Detected	detected	Detected
20EFV04 B	not detected	not detected	Detected
20EFV04 C	not detected	not detected	not detected

Table 4: Quantitative results for ten reference samples for PT EFV 04

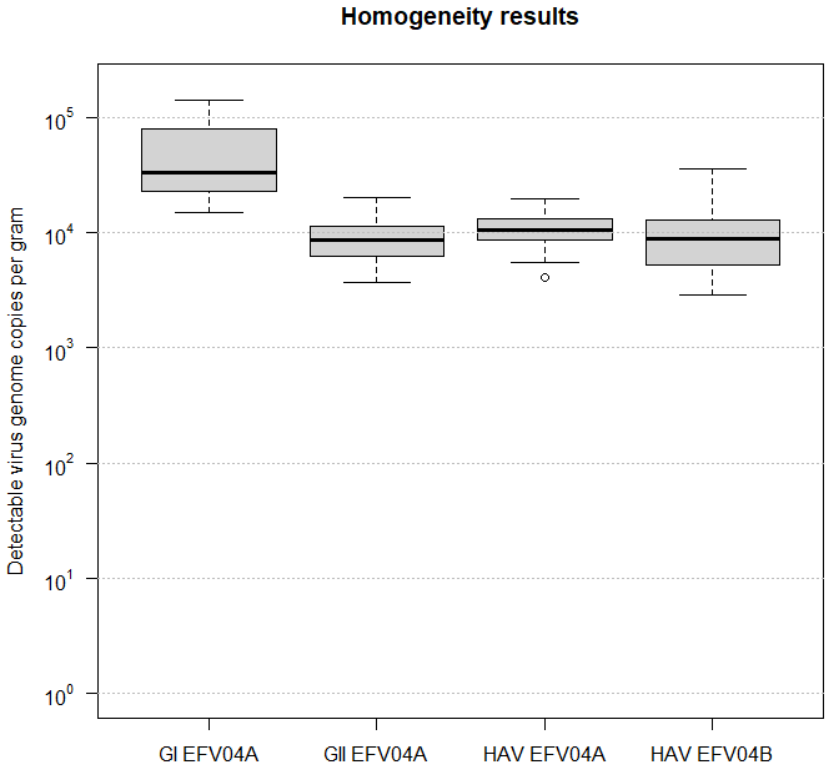
Ranges based on a 95 % confidence limit determined as two geometric standard deviations above and below the geometric mean.

Sample	Norovirus GI	Norovirus GII	HAV
20EFV04 A	$1.1 \times 10^4 – 1.2 \times 10^5$ c/g*	$3 \times 10^3 – 2.2 \times 10^4$ c/g	$4.9 \times 10^3 – 2.2 \times 10^4$ c/g
20EFV04 B	not detected	not detected	$2.6 \times 10^3 – 3.2 \times 10^4$ c/g
20EFV04 C	not detected	not detected	not detected

**detectable virus genome copies per gram sample*

Graph 1: Box and whisker plots for homogeneity test of samples 20EFV04 A and B

The box includes 50 % of the results from 10 samples for samples A and B which were spiked with target viruses. 25 % of the results set above the median, 25 % of the results set below the median and the remaining 50 % are illustrated by lines outside the box. A circle in the plot indicates a value that deviates from the other values but is not defined as an outlier.[1]



[1] R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

RESULTS AND DISCUSSION

Samples were sent to 21 laboratories and 19 laboratories returned their results (16 NRLs and two in the process of becoming NRL). Information provided by laboratories showed that samples temperature upon arrival was between 3.8- 5.4 ^{0}C. The majority received the samples the day after dispatch (October 7th), two laboratories on October 8th and one laboratory on October 15th. All the laboratories analysed the samples within 24 hours after arrival as they were instructed.

In total, no false negative or false positive results were reported by the laboratories. However, some of the true negative results were not valid due to unacceptable inhibition and/or extraction efficiency. Since re-testing was not possible, such non-valid results were accounted as correct in the scoring of participants. Overview of results are demonstrated in Table 5.

It is observed that laboratory 114 reported Cq values for not detected viruses (Cq=45) which is assumed to be the number of cycles that PCR plate was subjected to in accordance to ISO 15216-2 and not the actual Cq for a not detected virus. In similar case, lab 116 also reported Cq values for not detected viruses and it was presumed to be Cq value from water+EC RNA. The results were corrected later on by the laboratory. Moreover their Cq results are much lower than other participant's results.

Detailed information about the participating laboratories results can be found in Appendix A.

Table 5: Overview of participants´ results for samples 20EFV04 A, B and C

Target viruses	N	Sample 20EFV04A				Sample 20EFV04B				Sample 20EFV04C			
		T	FP	FN	NV	T	FP	FN	NV	T	FP	FN	NV
Norovirus GI	19	19	-	0	0	19	0	-	4	19	0	-	4
Norovirus GII	19	19	-	0	0	19	0	-	4	19	0	-	4
Hepatitis A virus	19	19	-	0	0	19	-	0	0	19	0	-	4

N: Number of laboratories that reported results for the analysis, T: true results, FP: False positive, FN: False negative, NV: Not valid negative results, -: not possible outcome

PERFORMANCE ASSESMENT

All the results were firstly assessed as presence–absence data in concordance with intended results as followed:

- 2 points: correct result for each target virus, regardless valid or non-valid results for negative samples.
- 0 points: Incorrect results for each target virus

The maximum score for each laboratory (for each target virus), taking into account the results of all three samples is therefore six (Table 6).

Table 6: Calculated data used for scoring assessment

Presence/absence			
Lab ID	GI	GII	HAV
101*	6 out of 6	6 out of 6	6 out of 6
103	6 out of 6	6 out of 6	6 out of 6
104*	6 out of 6	6 out of 6	6 out of 6
105*	6 out of 6	6 out of 6	6 out of 6
107*	6 out of 6	6 out of 6	6 out of 6
108*	6 out of 6	6 out of 6	6 out of 6
109*	6 out of 6	6 out of 6	6 out of 6
110*	6 out of 6	6 out of 6	6 out of 6
111*	6 out of 6	6 out of 6	6 out of 6
112*	6 out of 6	6 out of 6	6 out of 6
113*	6 out of 6	6 out of 6	6 out of 6
114*	6 out of 6	6 out of 6	6 out of 6
115*	6 out of 6	6 out of 6	6 out of 6
116*	6 out of 6	6 out of 6	6 out of 6
119*	6 out of 6[e]	6 out of 6[e]	6 out of 6[e]
122*	6 out of 6[i]	6 out of 6[i]	6 out of 6[i]
129*	6 out of 6[ei]	6 out of 6[ei]	6 out of 6[ei]
130	6 out of 6	6 out of 6	6 out of 6
131*	6 out of 6[ei]	6 out of 6[ei]	6 out of 6[ei]

** Designated EU/EFTA member state NRL*

e: unacceptable efficiency, i: unacceptable inhibition

INHIBITION and EFFICIENCY RESULTS

The results were also evaluated based on inhibition and extraction efficiency outcomes. In total, 15 out of 19 laboratories (79 %) reported acceptable results for both inhibition and efficiency values. It means that some negative results were not valid due to unacceptable inhibition and/or efficiency results. Furthermore, it was observed that some laboratories had some problems regarding the calculating and reporting of inhibition and extraction efficiency results. However, since it was not possible to provide the laboratories with a retest option, this evaluation is not a part of performance assessment. However, it can provide a guidance for valid reporting in official control according to ISO 15216-2. It should be noted that lettuce contain much less inhibitors comparing to for instance raspberries and therefore only few unacceptable inhibition results were reported in this PT trial.

The majority of the laboratories reported acceptable inhibition (<2 or ≤75 %) and extraction efficiency results (≥1%). Laboratory 131 did not report any inhibition and extraction efficiency results. NRL 122 did not report inhibition results for all not detected viruses and laboratory 129 experienced troubles with their own process control virus and therefore could not report extraction efficiency results. One laboratory (101) reported ISO 15216-2 as their used method, but reported inhibition in percentage and did not provide any quantitative results. Participant 119 could not report extraction efficiency results due to some practical complications and reported inhibition for 10^{-1} sample RNA.

According to ISO 15216-2, for matrices where RT-PCR inhibition is normally within the acceptable parameters (surfaces, bottled water, BMS), it is therefore permitted for laboratories to omit 10^{-1}

sample RNA from the initial analysis of target virus and process control virus. In this case, where RT-PCR inhibition is outside the acceptable parameters for undiluted sample RNA, real-time RT-PCR analysis for any affected target viruses and for the process control virus shall be repeated using 10^{-1} sample RNA. 10^{-1} sample RNA shall not be omitted from the initial analysis for soft fruits and leaf, stem and bulb vegetables (matrices where RT-PCR inhibition is frequently outside the acceptable parameters). Despite the fact that EURL is committed to follow ISO, it preserves the right to reflect NRLs opinion as well as itself. If undiluted sample RNA could not produce acceptable inhibition results, then acceptable inhibition results from 10^{-1} sample RNA could be taken to account. Moreover, the inhibition results submitted by PT participants could provide a clear picture of inhibition's range for matrices produced by EURL and used in the PT.

According to ISO 15216-1 and 2, negative results are not valid in absence of inhibition or/and extraction efficiency values as well as in case of unacceptable inhibition or/and extraction efficiency results and shall be reported as invalid. Positive results on the other hand could be considered valid despite unacceptable inhibition and extraction efficiency results and details shall be included in the test report. All the results reported for norovirus GI, GII and hepatitis A virus in sample A and hepatitis A virus in sample B are valid regardless the inhibition and extraction efficiency values, since the respective samples were positive for the respective target viruses. Results are presented in Appendix B.

Based on ISO/TS 15216-2, external control (EC) RNA should serve as a control for RT-PCR inhibition. Inhibition is calculated as Cq value (sample RNA + EC RNA) − Cq value (water + EC RNA). When the ΔCq in undiluted sample is ≥2, the calculation shall be repeated for diluted (1:10) samples. If ΔCq in diluted samples still is ≥2, negative results are not valid.

Some laboratories performed quantitative analyses and calculated inhibition by using both ΔCq and m (slope of the dsDNA standard curve), $(1 - 10^{(\Delta Cq/m)}) \times 100$ %. According to ISO 15216-1, when the inhibition in undiluted samples is >75 %, calculation shall be repeated for diluted samples and if it still is >75 %, negative results are not valid.

According to ISO 15216, process control virus (for instance mengovirus) must be added to the samples prior to virus extraction. A process control virus standard curve is produced in order to estimate extraction efficiency. Extraction efficiency is calculated as $10^{(\Delta Cq/m)} \times 100$ %, where ΔCq is the Cq value for process control virus in sample RNA − Cq value for undiluted process control virus RNA (the first point in the process control virus RNA standard curve) and m is the slope of the process control virus RNA standard curve. If the extraction efficiency is <1 %, negative results are not valid. If 10^{-1} sample RNA results are used, multiply by 10 to correct for the dilution factor.

METHODS USED BY THE PARTICIPANTS

Eleven laboratories were accredited according to ISO/IEC 17025 or detection of norovirus GI and norovirus GII and 10 laboratories for detection of HA. The majority followed ISO 15216-2 with exception of two laboratories which used their own internal method. Detailed information on the methodologies used is shown in Appendix C.

CONCLUSION

PT EFV04 organized by EURL for Foodborne Viruses in 2020, aimed at assessing the NRLs abilities to qualitatively detect HAV, norovirus GI and norovirus GII in refrigerated lettuce samples. Nineteen laboratories participated in the PT and all the reporting laboratories obtained satisfactory results. The proportion of valid results reported has been improved compared to previous qualitative PT distributions.

APPENDIX A

Participants' results

with EURL standards with own standards false results

Lab. ID No.	20EFA04 A			20EFA04 B			20EFA04 C		
	GI (Cq)	GII (Cq)	HAV (Cq)	GI (Cq)	GII (Cq)	HAV (Cq)	GI (Cq)	GII (Cq)	HAV (Cq)
101*	26.08	30.10	29.08			28.90			
103	28.08	27.59	29.98			31.12			
104*	27.76	30.15	28.87			28.18			
105*	27.83	29.31	31.33			32.78			
107*	27.00	27.84	29.56			29.07			
108*	30	31.51	27.75			30.31			
109*	30.5	27.90	33.86			35.14			
110*	27.17	26.48	28.14			28.12			
110*	27.17	26.48	28.14			28.12			
111*	26.29	27.78	28.25			30.04			
112*	26.57	29.21	30.91			30.55			
113*	29.41	29.08	31.75			32.59			
113*	28.04	30.83	32.40			32.20			
114*	23.16	24.73	27.39	45.00**	45.00**	27.79	45.00**	45.00**	45.00**
114*	23.16	24.13	27.45	45.00**	45.00**	27.85	45.00**	45.00**	45.00**
115*	30.77	32.05	32.34			30.09			
116*	31	34.39	33.56			33.97			
119*	29.03	29.75	30.84			31.07			

** Designated EU/EFTA member state NRL, **Reported as not detected; the Cq value indicated is the maximum cycles recommended in ISO 15216.*

Lab. ID No.	20EFA04 A			20EFA04 B			20EFA04 C		
	GI (Cq)	GII (Cq)	HAV (Cq)	GI (Cq)	GII (Cq)	HAV (Cq)	GI (Cq)	GII (Cq)	HAV (Cq)
122*	34.40	34.98	34.98			35.85			
129*	25.8	27.6	29.9			29.9			
130	28.95	32.12	29.95			32.12			
131*	31.45	30.24	30.84			31.76			
EURL	27.58	26.29	31.56			31.74			

** Designated EU/EFTA member state NRL, ** Reference results from day 1*

APPENDIX B

Inhibition and extraction efficiency results for sample 20EFV04A

	Inhibition			Efficiency	Results		
Lab. ID	GI^t	GII^t	HAV^t		GI^t	GII^t	HAV^t
101*	A	A	A	A	V	V	V
103	A	A	A	A	V	V	V
104*	A	A	A	A	V	V	V
105*	A	A	A	A	V	V	V
107*	A	A	A	A	V	V	V
108*	A	A	A	A	V	V	V
109*	A	A	A	A	V	V	V
110*	A	A	A	A	V	V	V
111*	A	A	A	A	V	V	V
112*	A	A	A	A	V	V	V
113*	A	A	A	A	V	V	V
114*	A	A	A	A	V	V	V
115*	A	A	A	A	V	V	V
116*	A	A	A	A	V	V	V
119*	A(1:10)	A(1:10)	A(1:10)	U	V	V	V
122*	A	A	A	A	V	V	V
129*	A	A	A	U	V	V	V
130	A	A	A	A	V	V	V
131*	NR	NR	NR	NR	V	V	V

** Designated EU/EFTA member state NRL*

A: Acceptable, f: false results, NR: not reported, NV: not valid, t: target virus, U: Unacceptable, V: valid results

Inhibition and extraction efficiency results for sample 20EFV04B

	Inhibition			Efficiency	Results		
Lab. ID	GI	GII	HAV^{t}		GI	GII	HAV^{t}
101*	A	A	A	A	V	V	V
103	A	A	A	A	V	V	V
104*	A	A	A	A	V	V	V
105*	A	A	A	A	V	V	V
107*	A	A	A	A	V	V	V
108*	A	A	A	A	V	V	V
109*	A	A	A	A	V	V	V
110*	A	A	A	A	V	V	V
111*	A	A	A	A	V	V	V
112*	A	A	A	A	V	V	V
113*	A	A	A	A	V	V	V
114*	A	A (1:10)	A	A	V	V	V
115*	A	A	A	A	V	V	V
116*	A	A	A	A	V	V	V
119*	A(1:10)	A (1:10)	A(1:10)	U	NV	NV	V
122*	NR	NR	A	A	NV	NV	V
129*	NR	NR	A	U	NV	NV	V
130	A	A	A	A	V	V	V
131*	NR	NR	NR	NR	NV	NV	V

** Designated EU/EFTA member state NRL*

A: Acceptable, f: false results, NR: not reported, NV: not valid, t: target virus, U: Unacceptable V: valid results

Inhibition and extraction efficiency results for sample 20EFV04C

	Inhibition			Efficiency	Results		
Lab. ID	GI	GII	HAV		GI	GII	HAV
101*	A	A	A	A	V	V	V
103	A	A	A	A	V	V	V
104*	A	A	A	A	V	V	V
105*	A	A	A	A	V	V	V
107*	A	A	A	A	V	V	V
108*	A	A	A	A	V	V	V
109*	A	A	A	A	V	V	V
110*	A	A	A	A	V	V	V
111*	A	A	A	A	V	V	V
112*	A	A	A	A	V	V	V
113*	A	A	A	A	V	V	V
114*	A	A (1:10)	A (1:10)	A	V	V	V
115*	A	A	A	A	V	V	V
116*	A	A	A	A	V	V	V
119*	A (1:10)	A (1:10)	A (1:10)	U	NV	NV	NV
122*	NR	NR	NR	A	NV	NV	NV
129*	NR	NR	NR	U	NV	NV	NV
130	A	A	A	A	V	V	V
131*	NR	NR	NR	NR	NV	NV	NV

** Designated EU/EFTA member state NRL*

A: Acceptable, f: false results, NR: not reported, NV: not valid, t: target virus, U: Unacceptable V: valid results

APPENDIX C

General information on methods

Lab. ID No.	1	2	3	4	5	6	7
101*	A	D	H	J	R		W
103	A	D	H	J	R		X
104*	A	D	H	J	R	UV	W
105*	A	D	H	J	R (TM9)	UV	Wi
107*	A	E	H	P	S	UV	Za
108*	A	D	H	L	T	UV	X
109*	A	D	H	J	R		Yy
110*	A	F	H	M	R	UV	W
111*	A	D	H	N	R		Y
112*	A	D	H	J	R	UV	Yr
113*	B	D	H	L	T	UV	W
114*	A	D	H	J	R	UV	Z
115*	B	D	H	J	R (TM9)		Zb
116*	A	D	I	J	R		W
119*	A	D	H	J	R	UV	Z
122*	A	D	H	O	R		X
129*	A	D	H	L	T		W
130	A	D	H	J	R	UV	W
131*	A	D	H	M	T	UV	Y

** Designated EU/EFTA member state NRL*

Key to method codes

1. Virus isolation and concentration method	
A	ISO 15216-2
B	Modified ISO 15216-2
2. RNA extraction methods/reagents	
D	NucliSens® (BioMérieux)
E	NucliSens® (BioMérieux), TANBead Maelstrom™ 8 Autostage
F	NucliSens® (BioMérieux), alternative robot system QuikPick Tool
3. RNA extraction (PCR method)	
H	One step
I	Two step
4. RT-PCR reagents	
J	RNA UltraSense™ One-Step Quantitative RT-PCR System
L	CeeramTools® real time RT-PCR kits (Ceeram)
M	QuantiTect® Probe RT-PCR kit (Qiagen)
N	Applied Biosystems™ TaqMan® Fast virus 1-Step Master Mix
O	SensiFAST™ Probe Hi-ROX One-Step Kit
P	GoTaq® Probe 1-Step RT-qPCR System
5. Primers and probes	
R	ISO 15216 *(The probe, NVGG1p or TM9, for norovirus GI was not asked to be specified)*
S	ISO 15216, with some modifications
T	CeeramTools®

6. Accreditation	
U	Norovirus
V	HAV
7. PCR system	
W	CFX96™ Real-Time PCR Detection System (Biorad)
X	AriaMx Real-time PCR System
Y	Applied Biosystems™ 7500 Fast Real-Time PCR System
Z	Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System
Wi	LightCycler® 96 System (Roche)
Yy	Applied Biosystems™ 7900HT Fast Real-Time PCR System
Yr	Applied Biosystems™ 7500 Real-Time PCR System
Za	Rotor-Gene Q (Qiagen)
Zb	Stratagene MX3005P® QPCR System