

# EURL Guidance document for verification of ISO 15216-1:2017

## Version history

Version	Date	Changes
Version 1	231024	

## Table of contents

1	Introduction and scope .....	3
2	Validation of ISO 15216-1:2017 .....	3
3	Aim of the user laboratory verification .....	4
4	Terms and definitions.....	4
5	Notes .....	4
6	Recommended procedure for user laboratory verification .....	5
6.1	Overview.....	5
6.2	Number of contamination levels and subsamples .....	7
6.3	Analysis of non-spiked foodstuff .....	7
6.3.1	Laboratory procedure.....	7
6.4	Preparation and quantification of viruses for artificial contamination.....	8
6.4.1	Laboratory procedure.....	9
6.5	Initial calibration extractions.....	9
6.5.1	Laboratory procedure.....	9
6.6	Preparation of target viruses for the dilution series .....	11
6.6.1	Laboratory procedure.....	12
6.7	Determination of LOD95, LOQ and $s_r$ .....	12
6.7.1	Laboratory procedure.....	12
6.7.2	Data collection and quality control .....	13
6.7.3	Determination of LOD95, LOQ and $s_r$ .....	15
7	Support for calculation of anticipated values and LOQ .....	18
8	Acceptance criteria.....	18
8.1	A special note on LOQ in oysters.....	18
	Appendix I.....	20
	References.....	22

## 1 Introduction and scope

As part of the quality assurance process, a laboratory wishing to implement a new method for accreditation must determine whether the method is fit for its intended purpose. Therefore, laboratories must *validate* or *verify* the performance of the method. If the method has already been validated elsewhere, verification shows that the user laboratory can correctly apply the validated method. This document provides guidance for laboratories wishing to verify the method: Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification (ISO 15216-1:2017) (1) as amended by ISO 15216-1:2017/AMD 1:2021 (2).

## 2 Validation of ISO 15216-1:2017

The methods described in ISO 15216-1:2017 (1) were validated in a study that included both single-laboratory and interlaboratory elements. The validation was organised by the former European Union Reference Laboratory (EURL) for Monitoring Bacteriological and Viral Contamination of Bivalve Molluscan Shellfish at the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth, United Kingdom. The validation included quantitative detection of three targets: HAV, norovirus GI and norovirus GII on seven different foodstuffs: surfaces (the outer surfaces of bell peppers), raspberries, lettuce, green onions, bottled water, pacific oysters and mussels (3). The following performance parameters were determined:

**Repeatability standard deviation ( $s_r$ ):** the standard deviation for test results obtained under repeatability conditions.

Repeatability conditions apply when independent test results are generated using the same measurement method for identical samples, in the same laboratory, with the same operator, with the same equipment and under a short period of time (4).

**Repeatability limit ( $r$ ):** a value to which the absolute difference between two independent  $\log_{10}$  transformed test results provided under repeatability conditions will be less or equal to, in 95% of the cases. For instance, if a first test result of 4.0  $\log_{10}$  detectable viral genome copies/g of a food item is observed in a laboratory, and the repeatability limit is 0.6, a second test result of that food item is expected to be within 3.4 and 4.6  $\log_{10}$  detectable viral genome copies/g in 95% of the cases. Repeatability limit is calculated directly from the repeatability standard deviation by multiplying by 2.8

**Reproducibility standard deviation ( $S_R$ ):** the standard deviation for test results provided under reproducibility conditions.

Reproducibility conditions apply when independent test results are generated using the same measurement method for identical samples, in different laboratories, with different operators and with the use of different equipment (4).

**Reproducibility limit ( $R$ ):** a value to which the absolute difference between two independent  $\log_{10}$  transformed test results provided under reproducibility conditions will be less or equal to, in 95% of the cases. As above, reproducibility limit is calculated directly from the reproducibility standard deviation by multiplying by 2.8.

In addition, the following performance parameters were also determined for raspberries, lettuce, bottled water and pacific oysters:

**95% limit of detection (LOD95):** the concentration of target (in copies/cm<sup>2</sup>, copies/g or copies/ml) in a test sample for which the probability of detection is 95%.

**Limit of quantification (LOQ):** the lowest concentration of target that is possible to be determined with a certain accuracy and precision.

The values for  $s_r$ ,  $r$ ,  $S_R$  and  $R$  are given in Annex J of ISO 15216-1:2017 (1) and the values for LOD95 and LOQ for certain matrices are given in a paper by Lowther *et al.* (3), describing the validation procedure of ISO 15216-1:2017. The values for the performance parameters relevant to this verification guide ( $s_r$ , LOD95 and LOQ) are summarised in Appendix I to this document.

### 3 Aim of the user laboratory verification

The aim of the user laboratory verification is to determine whether the implemented method is suitable for the intended application. For this purpose, the results of the verification by the user laboratory are compared with the results of the inter-laboratory validation of ISO 15216-1:2017 (1, 3). The user laboratory verification should be performed for all relevant targets and foodstuffs and include the determination of LOD95, LOQ and  $s_r$ .

### 4 Terms and definitions

This document uses, as far as possible, the same terms and definitions as described in section 3 of ISO 15216-2:2017 (1).

### 5 Notes

- The method implemented in the user laboratory shall be consistent with the method described in ISO 15216-1:2017 (1). If the user laboratory has made significant changes to the method, verification cannot be performed but validation is required, which usually covers more performance parameters than those described in this document.
- The procedure for user laboratory verification described in this document is intended as a recommendation and may be modified by the user laboratory as necessary, e.g. if a different experimental set-up would facilitate the practical aspects of the laboratory work.
- The results from the user laboratory verification may not be applicable to foodstuffs other than those included in the validation of ISO 15216-1:2017 (1), i.e., surfaces, raspberries, lettuce, green onions, bottled water, pacific oysters and mussels.
- There is an ISO method for the verification of methods for the analysis of microorganisms in food: Microbiology of the food chain - Method validation - Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory (ISO 16140-3:2021). However, ISO 16140-3:2021 mainly refers to bacteria and fungi and therefore many parts are not relevant to methods for the analysis of foodborne viruses. Instead, the procedure for user laboratory verification described in this document have been specifically developed to allow comparison of performance parameters with the validation of ISO 15216-1:2017 (1, 3).
- Since the actual level of viral contamination in a food sample is usually unknown and certified reference materials for viruses in food are not available, it is not possible to determine systematic measurement errors (trueness) by the verification procedure described in this document. It is therefore important for the user laboratory to monitor the level of agreement

with expected values through the participation in proficiency testing programmes organised by the EURL or others to be able to compare its performance with other laboratories.

## 6 Recommended procedure for user laboratory verification

### 6.1 Overview

This document describes a procedure in which a twofold dilution series of HAV, norovirus GI and norovirus GII is added to the foodstuff(s) to be verified. Multiple subsamples are tested at each level of the dilution series under repeatability conditions, i.e. using the same measurement method, in the same laboratory, with the same operator, with the same equipment and under a short period of time. The results are used to determine the LOD95 using a detection probability function (5). The LOQ is determined as the lowest level above the LOD95 where the  $s_r$  (repeatability standard deviation) of the  $\log_{10}$  transformed results is less than 0.5 (3). The completion of the verification procedures described in this document is estimated to take approximately three working weeks. To fulfil the repeatability conditions, make sure to complete the procedure within days. Figure 1 shows an overview of the procedure.

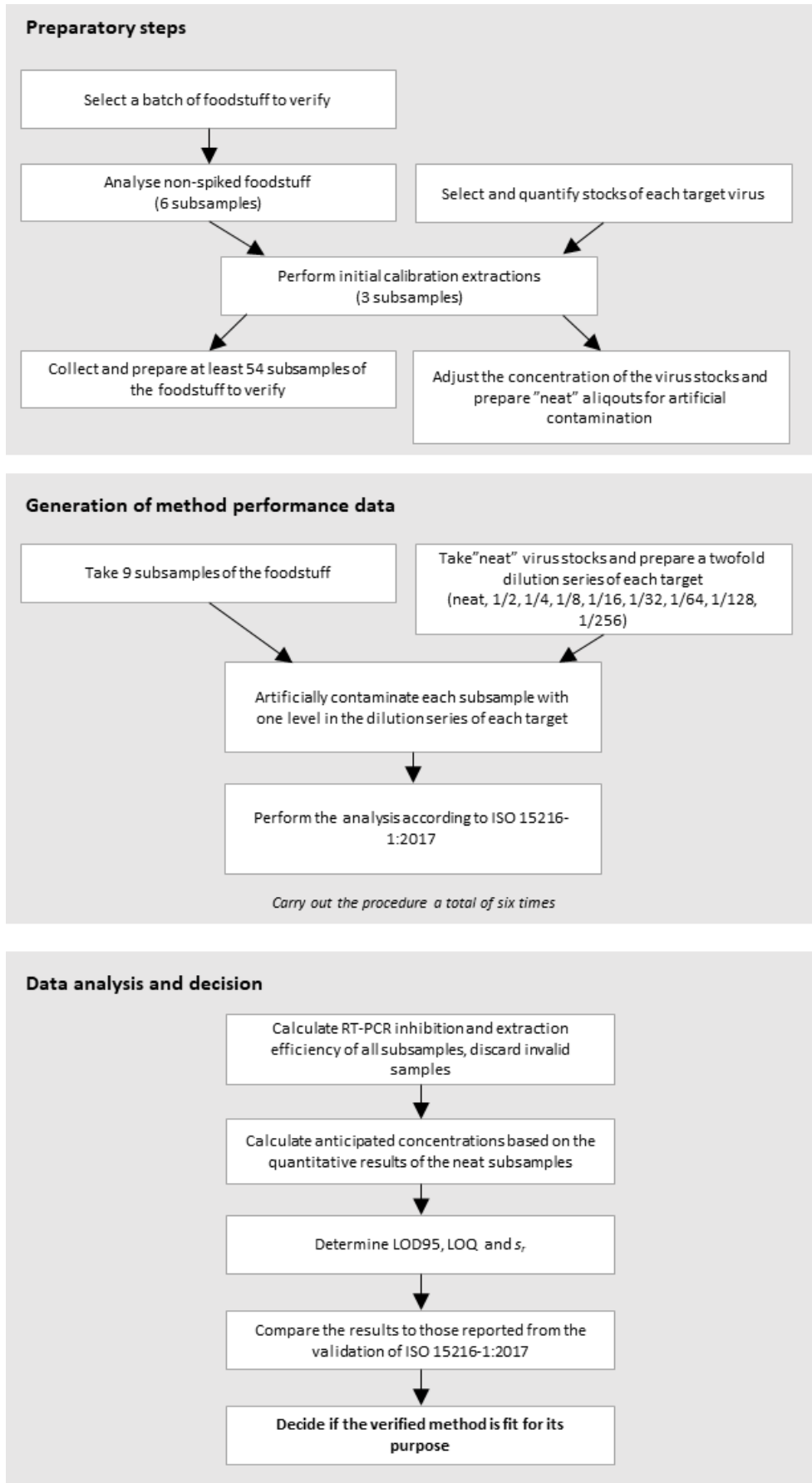


Figure 1. Schematic procedure for user laboratory verification.

## 6.2 Number of contamination levels and subsamples

At least nine contamination levels with at least six replicate subsamples at each level should be used. This allows for a small number of missing data points due to unacceptable RT-PCR inhibition or extraction efficiency results. The use of fewer replicates or the presence of a large number of missing data points will result in less robust estimates of the values of LOD95 and LOQ.

## 6.3 Analysis of non-spiked foodstuff

The first step of the verification procedure is to select a representative batch of the foodstuff of interest to verify the method on. It is important to confirm that the batch of the foodstuff intended for verification is not naturally contaminated with any of the target viruses of interest and that it gives acceptable RT-PCR inhibition and extraction efficiency results.

### 6.3.1 Laboratory procedure

Select six subsamples from the selected batch of foodstuff (e.g. 6 × 25 g of raspberries) and analyse them for all target viruses of interest according to the protocol in ISO 15216-1:2017 (1). Do not forget to add a process control virus to each sample to monitor the extraction efficiency. Figure 2 shows a proposed plate layout for the analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Subsample 1	Subsample 1	Subsample 1 + HAV EC RNA	Subsample 2	Subsample 2	Subsample 2 + HAV EC RNA	Subsample 3	Subsample 3	Subsample 3 + HAV EC RNA	Subsample 4	Subsample 4	Subsample 4 + HAV EC RNA	HAV assay (22 wells)
B	Subsample 5	Subsample 5	Subsample 5 + HAV EC RNA	Subsample 5	Subsample 5	Subsample 6 + HAV EC RNA	H <sub>2</sub> O + HAV EC RNA	-ve extraction control	H <sub>2</sub> O	H <sub>2</sub> O			
C	Subsample 1	Subsample 1	Subsample 1 + GI EC RNA	Subsample 2	Subsample 2	Subsample 2 + GI EC RNA	Subsample 3	Subsample 3	Subsample 3 + GI EC RNA	Subsample 4	Subsample 4	Subsample 4 + GI EC RNA	GI assay (22 wells)
D	Subsample 5	Subsample 5	Subsample 5 + GI EC RNA	Subsample 6	Subsample 6	Subsample 6 + GI EC RNA	H <sub>2</sub> O + GI EC RNA	-ve extraction control	H <sub>2</sub> O	H <sub>2</sub> O			
E	Subsample 1	Subsample 1	Subsample 1 + GII EC RNA	Subsample 2	Subsample 2	Subsample 2 + GII EC RNA	Subsample 3	Subsample 3	Subsample 3 + GII EC RNA	Subsample 4	Subsample 4	Subsample 4 + GII EC RNA	GI assay (22 wells)
F	Subsample 5	Subsample 5	Subsample 5 + GII EC RNA	Subsample 6	Subsample 6	Subsample 6 + GII EC RNA	H <sub>2</sub> O + GII EC RNA	-ve extraction control	H <sub>2</sub> O	H <sub>2</sub> O			
G	Subsample 1	Subsample 2	Subsample 3	Subsample 4	Subsample 5	Subsample 6	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H <sub>2</sub> O	Process control virus assay (12 wells)
H													

Figure 2. Proposed plate layout for control of non-spiked foodstuff.

Ensure that both wells of all subsamples are negative for all targets and that all undiluted subsamples give acceptable RT-PCR inhibition and extraction efficiency results. If this is not the case, consider selecting another batch of the foodstuff and repeat the procedure. Table 1 provides a template for summarising the results. Note that according to ISO 15216-1:2017 (10<sup>-1</sup>) diluted RNA samples are required for the analysis of fruits and vegetables, as these matrices often cause RT-PCR inhibition. However, for verification purposes, we recommend selecting a batch that provides acceptable inhibition for undiluted samples. By testing several (i.e. 6) subsamples of this batch in this first experiment and monitoring inhibition during all subsequent experiments to ensure that it is always ≤75%, the user laboratory can make the exception to remove (10<sup>-1</sup>) dilutions during the verification process as they take up a lot of space on the RT-PCR plate.

Table 1. Reporting template for analysis of non-spiked foodstuff.

Subsample	HAV		Norovirus GI		Norovirus GII		Process control virus
	Real-time RT-PCR result (pos/neg)	Inhibition (%)	Real-time RT-PCR result (pos/neg)	Inhibition (%)	Real-time RT-PCR result (pos/neg)	Inhibition (%)	Extraction efficiency (%)
1							
2							
3							
4							
5							
6							
Mean							

Once a suitable batch of the foodstuff of interest is identified, collect sufficient material for subsequent experiments to determine LOD<sub>95</sub>, LOQ and  $s_r$ . Table 2 shows the quantities of foodstuff needed for all subsequent analyses.

Table 2. Amounts of the foodstuffs required to perform the verification study.

Category of foodstuff	Amount required per sample	Number of samples required for verification*	Minimum amount of foodstuff required for verification (including 20% extra)
Bivalve molluscan shellfish	2 g of digestive glands	57	137 g
Bottled water	Up to 2000 ml (i.e. one water bottle)	57	Up to 70 bottles
Leaf, stem and bulb vegetables	25 g	57	1710 g
Soft fruit	25 g	57	1710 g
Surface	5 × 5 cm (25 cm <sup>2</sup> )	57	342 cm × 342 (116,964 cm <sup>2</sup> )

\*This includes estimation of the matrix effect (n = 3) and determination of LOD<sub>95</sub>, LOQ and  $s_r$  (n = 54)

#### 6.4 Preparation and quantification of viruses for artificial contamination

Viruses to be used for artificial contamination can be obtained from faecal suspensions (HAV, norovirus GI and norovirus GII), cell culture supernatants (HAV) or, alternatively, from commercially available Lenticule® discs (6). If the virus concentrations are not known in advance, the virus stocks must be quantified as described below.



### 6.4.1 Laboratory procedure

Prepare the stock of target virus(es) using a procedure appropriate for the sample type<sup>1</sup>. Extract the RNA and perform real-time RT-PCR analysis according to the protocol in ISO 15216-1:2017 (1). Include both undiluted and 10<sup>-1</sup> and 10<sup>-2</sup> diluted samples in case the undiluted samples show signs of RT-PCR inhibition that could lead to an underestimation of the virus concentration. If this is the case, use the data from the 10<sup>-1</sup> or 10<sup>-2</sup> diluted samples and adjust by the dilution factor to quantify the sample. Figure 3 shows a proposed plate layout for the analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	HAV virus stock	HAV virus stock	HAV virus stock + HAV EC RNA	HAV virus stock (-1)	HAV virus stock (-1)	HAV virus stock (-1) + HAV EC RNA	HAV virus stock (-2)	HAV virus stock (-2)	HAV virus stock (-2) + HAV EC RNA	H <sub>2</sub> O + HAV EC RNA	-ve extraction control	-ve extraction control	HAV assay (24 wells)
B	HAV dsDNA (undiluted)	HAV dsDNA (undiluted)	HAV dsDNA (-1)	HAV dsDNA (-1)	HAV dsDNA (-2)	HAV dsDNA (-2)	HAV dsDNA (-3)	HAV dsDNA (-3)	HAV dsDNA (-4)	HAV dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
C	GI virus stock	GI virus stock	GI virus stock + GI EC RNA	GI virus stock (-1)	GI virus stock (-1)	GI virus stock (-1) + GI EC RNA	GI virus stock (-2)	GI virus stock (-2)	GI virus stock (-2) + GI EC RNA	H <sub>2</sub> O + GI EC RNA	-ve extraction control	-ve extraction control	GI assay (24 wells)
D	GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
E	GII virus stock	GII virus stock	GII virus stock + GII EC RNA	GII virus stock (-1)	GII virus stock (-1)	GII virus stock (-1) + GII EC RNA	GII virus stock (-2)	GII virus stock (-2)	GII virus stock (-2) + GII EC RNA	H <sub>2</sub> O + GII EC RNA	-ve extraction control	-ve extraction control	GII assay (24 wells)
F	GII dsDNA (undiluted)	GII dsDNA (undiluted)	GII dsDNA (-1)	GII dsDNA (-1)	GII dsDNA (-2)	GII dsDNA (-2)	GII dsDNA (-3)	GII dsDNA (-3)	GII dsDNA (-4)	GII dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
G													
H													

Figure 3. Proposed plate layout for quantification of viral stocks of HAV, norovirus GI and norovirus GII.

Calculate and note the viral copy number per µl of stock sample. The desired concentration is about 1000 copies/µl. If possible, consider another stock solution if the concentration falls below about 1000 copies/µl. If using Lenticule® discs, a higher concentration can be obtained by pooling several discs together, effectively by resuspending in a smaller volume than recommended. Table 3 provides a template for summarising the results.

Table 3. Reporting template for quantification of viral stocks.

Target virus	Stock concentration (viral copies/µl)
HAV	
Norovirus GI	
Norovirus GII	

### 6.5 Initial calibration extractions

A considerable matrix effect can be expected from foodstuffs. Therefore, an estimation of this matrix effect is required to find a suitable target virus concentration for subsequent artificial contamination.

#### 6.5.1 Laboratory procedure

Dilute each stock of target virus of interest to a final concentration of approximately 1000 copies/µl.

<sup>1</sup>If required, the EURL can provide specific protocols for sample preparation.

Prepare three subsamples from the selected batch of the foodstuff of interest (e.g. 3 × 25 g of raspberries or 3 × 2 g of homogenised digestive glands from oysters). If frozen, let the material thaw for at least 20 min before proceeding.

Artificially contaminate each subsample by carefully pipetting 10 µl of each target virus solution (approximately 10,000 copies) as indicated below. Do not forget to add a process control virus to each subsample.

For **soft fruit, surface and leaf, stem and bulb vegetables**, pipet each target virus solution across the surface of the subsample, and let the sample air dry for 20 min in ambient temperature before proceeding with the analysis.

For **bivalve molluscan shellfish**, pipet 10 µl of each target virus solution across the surface of 3 × 2 g of homogenised digestive tissue subsample (i.e. each 2 g) and vortex the sample to mix. Proceed to Proteinase K extraction according to the protocol in ISO 15216-1:2017 (1). Note that bioaccumulated bivalve molluscan shellfish were used for validation of ISO 15216-1:2017 (1). However, due to the limited availability of bioaccumulation facilities, we describe here a simpler approach. The approach is similar to that used during a baseline study organised by EFSA for noroviruses in oysters to determine the LOD95 and LOQ for each participating laboratory (7, 8). Although bioaccumulation can be used, the EURL recommends the use of spiking to obtain a harmonised procedure between EU Member States.

For **bottled water**, pipet each target virus solution directly to the subsample and mix by inversion or vortexing.

Perform the subsequent analysis according to the protocol in ISO 15216-1:2017 (1). Figure 4 shows a proposed plate layout for the analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Subsample 1	Subsample 1	Subsample 1 + HAV EC RNA	Subsample 2	Subsample 2	Subsample 2 + HAV EC RNA	Subsample 3	Subsample 3	Subsample 3 + HAV EC RNA	H <sub>2</sub> O + HAV EC RNA	-ve extraction control	-ve extraction control	HAV assay (24 wells)
B	HAV dsDNA (undiluted)	HAV dsDNA (undiluted)	HAV dsDNA (-1)	HAV dsDNA (-1)	HAV dsDNA (-2)	HAV dsDNA (-2)	HAV dsDNA (-3)	HAV dsDNA (-3)	HAV dsDNA (-4)	HAV dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
C	Subsample 1	Subsample 1	Subsample 1 + GI EC RNA	Subsample 2	Subsample 2	Subsample 2 + GI EC RNA	Subsample 3	Subsample 3	Subsample 3 + GI EC RNA	H <sub>2</sub> O + GI EC RNA	-ve extraction control	-ve extraction control	GI assay (24 wells)
D	GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
E	Subsample 1	Subsample 1	Subsample 1 + GII EC RNA	Subsample 2	Subsample 2	Subsample 2 + GII EC RNA	Subsample 3	Subsample 3	Subsample 3 + GII EC RNA	H <sub>2</sub> O + GII EC RNA	-ve extraction control	-ve extraction control	GII assay (24 wells)
F	GII dsDNA (undiluted)	GII dsDNA (undiluted)	GII dsDNA (-1)	GII dsDNA (-1)	GII dsDNA (-2)	GII dsDNA (-2)	GII dsDNA (-3)	GII dsDNA (-3)	GII dsDNA (-4)	GII dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
G	Subsample 1	Subsample 2	Subsample 3	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H <sub>2</sub> O				Process control virus assay (9 wells)
H													

Figure 4. Proposed plate layout for estimation of the matrix effect on analysis of HAV, norovirus GI and norovirus GII in foodstuff.

Calculate the obtained value of each sample, expressed as copies per g, ml or cm<sup>2</sup>, depending on foodstuff, as described in ISO 15216-1:2017 (1).

Calculate the RT-PCR inhibition and extraction efficiency as described in ISO 15216-1:2017 (1). Table 4 provides a template for summarising the results.

Table 4. Reporting template for initial calibration extractions.

Subsample	HAV		Norovirus GI		Norovirus GII		Process control virus
	Obtained concentration (copies per g, ml or cm <sup>2</sup> )	Inhibition (%)	Obtained concentration (copies per g, ml or cm <sup>2</sup> )	Inhibition (%)	Obtained concentration (copies per g, ml or cm <sup>2</sup> )	Inhibition (%)	Extraction efficiency (%)
1							
2							
3							
Mean							

## 6.6 Preparation of target viruses for the dilution series

In order to determine  $s_r$ , LOQ and LOD95, it is important to create a dilution series that covers some contamination levels that give 100% positive results and some contamination levels that give at least some negative results each. The ideal scenario is to get negative results in the two to four least concentrated dilutions. To find suitable target concentrations for these dilution levels, data generated from Poisson distributions can be used (Table 5). Note, however, that in order to use these data as a basis for determining dilution levels, it is important that the dsDNA quantification standards do not introduce significant quantification bias. For example, overestimation of the amount of virus will lead to an increased number of negative results in the most diluted test samples.

Table 5. Expected probabilities of detection at different target concentrations in real-time RT-PCR.

Dilution level	Average number of target copies/RT-PCR well (5 µl sample)	Expected probability of detection (from a Poisson distribution)	Number of copies in the RNA extraction (with an input volume of 500 µl and output volume of 100 µl)
Neat	128	1	2560
1/2	64	1	1280
1/4	32	1	640
1/8	16	1	320
1/16	8	1	160
1/32	4	0.98	80
1/64	2	0.86	40
1/128	1	0.63	20
1/256	0.5	0.39	10

For soft fruit, leaf, stem and bulb vegetables, surfaces and bottled water, the entire sample volume is used for the RNA extraction, which means that the number of copies per sample giving 128 copies/RT-

PCR well (i.e. the neat dilution level) is 2560. For bivalve molluscan shellfish, approximately  $\frac{1}{4}$  of the supernatant volume goes into RNA extraction, meaning that the copy number per sample yielding 128 copies/ RT-PCR well is 10,240.

### 6.6.1 Laboratory procedure

Taking into account the results of the calibration extractions described in section 6.5, adjust the concentration of each target virus so that spiking 10  $\mu$ l of the target virus solution gives values comparable to 2560 copies/sample for soft fruit, leaf, stem and bulb vegetables, surfaces and bottled water and 10,240 copies/sample for bivalve molluscan shellfish.

Prepare several single-use aliquots containing at least 100  $\mu$ l each of this concentration for each target virus and store at  $-70^{\circ}\text{C}$ . These aliquots will be used as the neat (the most concentrated) dilution level in the subsequent experiments.

## 6.7 Determination of LOD95, LOQ and $s_r$

### 6.7.1 Laboratory procedure

#### 6.7.1.1 Dilution series

Defrost an aliquot of each target virus and prepare a twofold dilution series, e.g. as suggested in Figure 5. Prepare a separate dilution series of each target virus.

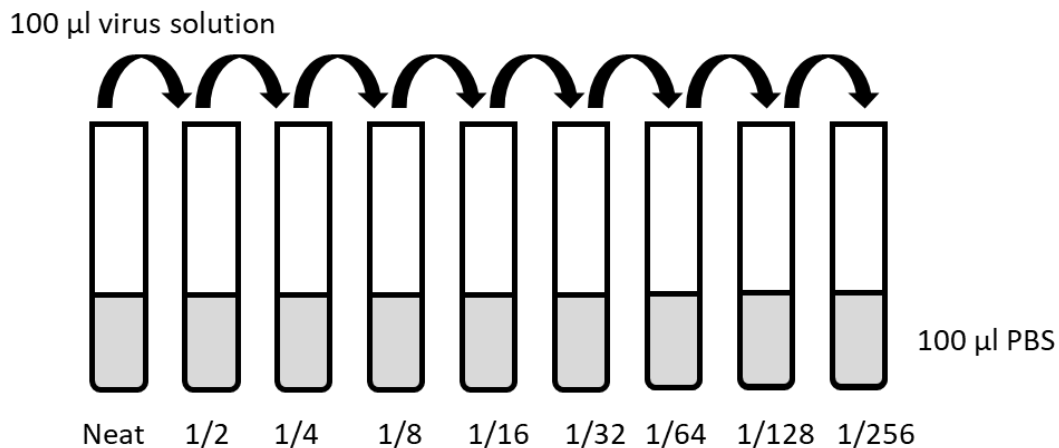


Figure 5. Preparation of a twofold dilution series (example).

#### 6.7.1.2 Preparation of foodstuff

Prepare nine subsamples from the selected batch of the foodstuff of interest (e.g.  $9 \times 25$  g raspberries or  $9 \times 2$  g homogenised digestive glands for oysters). Label the subsamples as follows: neat, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256. If the subsamples are frozen, allow them to thaw at room temperature for at least 20 minutes before proceeding.

#### 6.7.1.3 Artificial contamination

Artificially contaminate the subsamples according to the following principle:

To the neat subsample, pipet 10  $\mu$ l of neat virus solution from each target virus. To the 1/2 subsample, pipet 10  $\mu$ l of 1/2 virus solution from each target virus, and so on, until all subsamples are artificially contaminated. Do not forget to add a process control virus to each subsample.

For **soft fruit, surface and leaf, stem and bulb vegetables**, pipet each target virus solution across the surface of the subsample, and let the sample air dry in room temperature for 20 min before proceeding with the analysis.

For **bivalve molluscan shellfish**, pipet each target virus solution across the surface of the homogenised digestive tissue subsample and mix by vortexing.

For **bottled water**, pipet each target virus solution directly to the subsample and mix by inversion or vortexing.

6.7.1.4 Laboratory analysis of the subsamples

Perform the subsequent analysis as described in ISO 15216-1:2017 (1). Figure 6 shows proposed plate layouts for the analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Neat	Neat	Neat + HAV EC RNA	Dil 1/2	Dil 1/2	Dil 1/2 + HAV EC RNA	Dil 1/4	Dil 1/4	Dil 1/4 + HAV EC RNA	Dil 1/8	Dil 1/8	Dil 1/8 + HAV EC RNA	HAV assay (42wells)
B	Dil 1/16	Dil 1/16	Dil 1/16 + HAV EC RNA	Dil 1/32	Dil 1/32	Dil 1/32 + HAV EC RNA	Dil 1/64	Dil 1/64	Dil 1/64 + HAV EC RNA	Dil 1/128	Dil 1/128	Dil 1/128 + HAV EC RNA	
C	Dil 1/256	Dil 1/256	Dil 1/256 + HAV EC RNA	H <sub>2</sub> O + HAV EC RNA	-ve extraction control	-ve extraction control							
D	HAV dsDNA (undiluted)	HAV dsDNA (undiluted)	HAV dsDNA (-1)	HAV dsDNA (-1)	HAV dsDNA (-2)	HAV dsDNA (-2)	HAV dsDNA (-3)	HAV dsDNA (-3)	HAV dsDNA (-4)	HAV dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
E	Neat	Neat	Neat + GI EC RNA	Dil 1/2	Dil 1/2	Dil 1/2 + GI EC RNA	Dil 1/4	Dil 1/4	Dil 1/4 + GI EC RNA	Dil 1/8	Dil 1/8	Dil 1/8 + GI EC RNA	GI assay (42 wells)
F	Dil 1/16	Dil 1/16	Dil 1/16 + GI EC RNA	Dil 1/32	Dil 1/32	Dil 1/32 + GI EC RNA	Dil 1/64	Dil 1/64	Dil 1/64 + GI EC RNA	Dil 1/128	Dil 1/128	Dil 1/128 + GI EC RNA	
G	Dil 1/256	Dil 1/256	Dil 1/256 + GI EC RNA	H <sub>2</sub> O + GI EC RNA	-ve extraction control	-ve extraction control							
H	GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Neat	Neat	Neat + GI EC RNA	Dil 1/2	Dil 1/2	Dil 1/2 + GI EC RNA	Dil 1/4	Dil 1/4	Dil 1/4 + GI EC RNA	Dil 1/8	Dil 1/8	Dil 1/8 + GI EC RNA	GI assay (42 wells)
B	Dil 1/16	Dil 1/16	Dil 1/16 + GI EC RNA	Dil 1/32	Dil 1/32	Dil 1/32 + GI EC RNA	Dil 1/64	Dil 1/64	Dil 1/64 + GI EC RNA	Dil 1/128	Dil 1/128	Dil 1/128 + GI EC RNA	
C	Dil 1/256	Dil 1/256	Dil 1/256 + GI EC RNA	H <sub>2</sub> O + GI EC RNA	-ve extraction control	-ve extraction control							
D	GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)	H <sub>2</sub> O	H <sub>2</sub> O	
E	Neat	Dil 1/2	Dil 1/4	Dil 1/8	Dil 1/16	Dil 1/32	Dil 1/64	Dil 1/128	Dil 1/256				Process control virus assay (15 wells)
F	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H <sub>2</sub> O							
G													
H													

Figure 6. Proposed plate layouts for determination of LOD95, LOQ and s<sub>r</sub>.

6.7.1.5 Repetition

After the first round, make sure that the concentrations obtained are close to the expected values (**Fel! Hittar inte referenskölla.** and **Fel! Hittar inte referenskölla.**) (± around 50%). Adjust the dilution series if necessary (if adjusted repeat the first round – do not include the initial data in calculations of the method characteristics). Repeat the procedure from section 6.7.1.1 five more times to generate results for a total of 54 subsamples. Prepare a new dilution series of the target virus each time to minimise the risk of bias in the dilutions.

6.7.2 Data collection and quality control

Collect the data from the experiments described above. For each subsample, calculate RT-PCR inhibition and extraction efficiency. Record the obtained concentration, expressed in

copies per g, ml or cm<sup>2</sup>, depending on foodstuff. If only one of two wells in a subsample is positive, that subsample shall be considered positive. Table 6 provides a template for summarising the results.

Table 6. Reporting template for quantification of HAV, norovirus GI and norovirus GII in artificially contaminated foodstuff.

Dilution	Round	HAV		Norovirus GI		Norovirus GII		Process control virus	Valid (yes/no)
		Obtained concentration (copies per g, ml or cm <sup>2</sup> )	Inhibition (%)	Obtained concentration (copies per g, ml or cm <sup>2</sup> )	Inhibition (%)	Obtained concentration (copies per g, ml or cm <sup>2</sup> )	Inhibition (%)	Extraction efficiency (%)	
Neat	1								
½	1								
¼	1								
1/8	1								
1/16	1								
1/32	1								
1/64	1								
1/128	1								
1/256	1								
Neat	2								
½	2								
¼	2								
1/8	2								
1/16	2								
1/32	2								
1/64	2								
1/128	2								
1/256	2								
Continue according to above for all 6 subsamples									

Exclude any subsample with more than 75% inhibition and/or less than 1% extraction efficiency. Subsamples with no more than 75% inhibition and at least 1% extraction efficiency are considered valid, and should be used for determination of LOQ and *s<sub>r</sub>*. However, a positive subsample showing unacceptable values for inhibition or extraction efficiency can still be reported as positive according to ISO 15216-1:2017 and thus all positive subsamples can be considered for the determination of LOD95.

### 6.7.3 Determination of LOD95, LOQ and $s_r$

#### 6.7.3.1 Note

The following calculations should be done separately for each target and foodstuff, if simultaneous verification of the method in multiple foodstuffs is carried out.

#### 6.7.3.2 Calculation of anticipated values

Record the obtained value of all valid, detected neat subsamples, expressed as copies per g, ml or cm<sup>2</sup>, depending on type of foodstuff.

Calculate the geometric mean of the neat subsamples. For example, if there are six neat subsamples with obtained values of 1167, 968, 1129, 1186, 1182 and 1067 copies/g respectively, the geometric mean is  $(1167 \cdot 968 \cdot 1129 \cdot 1186 \cdot 1182 \cdot 1067)^{\frac{1}{6}} = 1113.66$ . Geometric mean values can be obtained, for instance, by using the GEOMEAN-function in Excel 2016.

Calculate the anticipated values of the other dilution levels by multiplying the value of the neat dilution with the dilution level as illustrated in Table 7 below.

Table 7. Illustrative example of how to calculate anticipated values at each dilution level.

Dilution level	Anticipated value (copies/g)
1	$1113.66 \cdot 1 = 1113.66$
1/2	$1113.66 \cdot 1/2 = 556.83$
1/4	$1113.66 \cdot 1/4 = 278.41$
1/8	$1113.66 \cdot 1/8 = 139.21$
1/16	$1113.66 \cdot 1/16 = 70.16$
1/32	$1113.66 \cdot 1/32 = 34.52$
1/64	$1113.66 \cdot 1/64 = 17.82$
1/128	$1113.66 \cdot 1/128 = 8.91$
1/256	$1113.66 \cdot 1/256 = 4.45$

6.7.3.3 Determination of LOD95

Calculate the number of total and positive observations at each dilution level, and summarise the results as indicated in Table 8.

Table 8. Example data sheet with anticipated values together with the number of total and detected subsamples.

Anticipated value (copies/g)	Total number of subsamples (n)	Number of detected subsamples (n)
1113.66	6	6
556.83	6	6
278.41	6	6
139.21	6	6
70.16	6	6
34.52	6	5
17.82	6	4
8.91	6	2
4.45	6	1

Estimate LOD95 by importing the data as described in Table 8 to the PODLOD program for Excel (5). The program is openly available at <http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html>. Note that LOD95 can only be determined if there are negative (non-detected) observations in the dataset.

The data from Table 8 generates a LOD95 of 55 copies/g (Figure 7).

Input data			Results of the PODLOD calculations												
Matrix 1	Designation:	Inoculation level in cfu/g or cfu/ml	No. of inoculated tubes	No. of positive tubes	Matrix	Matrix effect	Log matrix effect	SD of log matrix effect	LOD <sub>50%</sub> = 50% limit of detection in cfu/g or cfu/ml			LOD <sub>95%</sub> = 95% limit of detection in cfu/g or cfu/ml			Test statistic matrix effect
		$d$	$n$	$p$		$F_i$	$f_i$	$s_B$	Detection limit	Lower conf. limit	Upper conf. limit	Detection limit	Lower conf. limit	Upper conf. limit	$ z_j $
	1113.66		6	6											
	556.83		6	6											
	278.41		6	6											
	139.21		6	6											
	70.16		6	6											
	34.52		6	5											
	17.82		6	4											
	8.91		6	2											
	4.45		6	1											
Combined results		not calculated because results for at least 2 matrices would have to be available													

Figure 7. Example output from the PODLOD Excel program.

6.7.3.4 Determination of LOQ and  $s_r$

The determination of LOQ is based on the procedure described by Cefas (9), with some modifications to better agree with the validation of ISO 15216-1.

First, prepare a dataset with anticipated and obtained values as shown in the example in Table 9. Include only the data points belonging to dilution levels where anticipated values are above or immediately below LOD95 (i.e. all the values above the LOD95 should be included, and the value immediately below). For example, with the sample dataset, if LOD95 is 55 copies/g, include all data points belonging to dilution levels with anticipated concentrations of 34.52 copies/g and above (see Table 8 and Table 9).



Table 9. Example datasheet with anticipated and obtained values.

Anticipated value (copies/g)	Obtained value (copies/g)
1113.66	968
1113.66	1129
1113.66	1186
1113.66	1182
1113.66	1067
1113.66	1167
556.83	629
556.83	739
556.83	426
556.83	476
556.83	312
556.83	427
...	...

Use the dataset to perform a linear regression analysis by using  $\log_{10}$  obtained value as a response variable (i.e. on the y-axis) and  $\log_{10}$  anticipated value as an explanatory variable (on the x-axis), as illustrated in Figure 8 below.

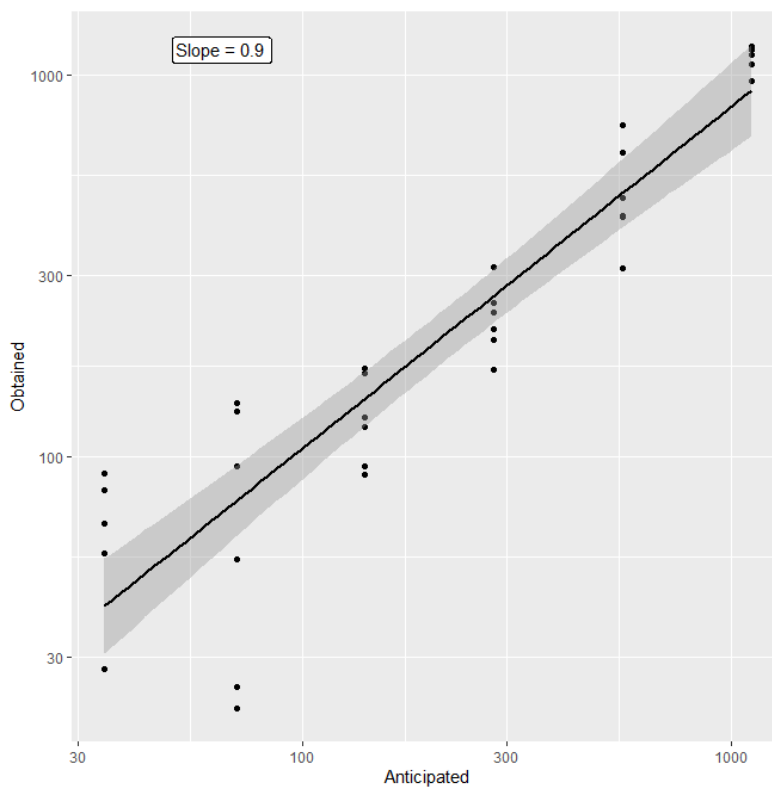


Figure 8. Linear regression analysis using example data.

Check by visual inspection that the relationship is linear, i.e. that the data points fits the regression line well. Record the slope of the regression line. Proceed to the next step if the slope is between 0.75 and 1.25. If the slope value lies outside this range, exclude the data points associated with the least concentrated dilution level and repeat the analysis. If the slope value is still outside this range, make a further exclusion of the least concentrated dilution level. If the problem remains, contact the EURL for advice.

Calculate the SD of the  $\log_{10}$  obtained values at each dilution level. If the analyses were carried out under repeatability conditions, the SD value also represents the  $s_r$ .

The SD on the  $\log_{10}$  scale can be converted into a coefficient of variation (%CV) on the normal scale using the following formula:

$$\sqrt{e^{\log(10)^2 \cdot SD^2} - 1} \cdot 100$$

A sample dataset is provided in Table 10.

Table 10. Sample dataset with SD and %CV at each dilution level.

Anticipated value (copies/g)	$s_r$ : SD (on $\log_{10}$ scale)	%CV
1113.66	0.03	7.63
556.83	0.13	30.95
278.41	0.09	21.28
139.21	0.12	26.78
70.16	0.35	66.63
34.52	0.20	37.95

Determine LOQ as the anticipated value from which the SD from are always below  $0.50 \log_{10}$ . However, do not set LOQ lower than the LOD95. This means that, with the sample dataset in Table 10 and a LOD95 of 55 copies/g (Figure 7), the LOQ would be 55 copies/g.

## 7 Support for calculation of anticipated values and LOQ

If required, the EURL can support NRLs in calculation of anticipated values and LOQ.

## 8 Acceptance criteria

As there are no microbiological criteria for viruses in food, there are currently no formal acceptance criteria for LOD95 and LOQ. It is therefore up to each user laboratory to determine its acceptance criteria on these performance parameters. However, if the values of LOD95 and/or LOQ differ substantially from those available from the validation of ISO 15216-1:2017 (1, 3) (Appendix I), or if LOQ is much higher than LOD95, it is recommended to perform a root cause analysis to find an explanation.

### 8.1 A special note on LOQ in oysters

The EU is currently discussing the introduction of microbiological criteria for norovirus GI and GII in raw oysters. A specific threshold has not yet been set (as of 2023), but there are discussions about using thresholds between around 200-500 viral RNA copies/g digestive tissue. Therefore, in the event

that ISO 15216-1:2017 is used in conjunction with microbiological criteria in oysters, it is recommended that LOQ for norovirus GI and GII in oysters is no more than 1/2 of the established microbiological threshold.

## Appendix I

Summarised results from validation of ISO 15216-1:2017.

The  $s_r$  values are from ISO 15216-1:2017, Annex J (1) and the LOD95 and LOQ values are from a publication by Lowther *et al.* (3), describing the validation procedure of ISO 15216-1:2017. Note that the LOD95 and LOQ values are not described in ISO 15216-1:2017. In the validation of ISO 15216-1:2017, the  $s_r$  values were determined at three different contamination levels, referred to as low, medium and high. Note that the contamination levels indicate the average concentrations detected in the inoculated samples during the validation study, i.e. it is not the concentration used during the inoculation stage.

The following tables describe the values of the individual food types included in the validation study.

Table A1. Surface (bell pepper).  $s_r$  values are given as  $\log_{10}$  copies/cm<sup>2</sup>. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/cm<sup>2</sup>.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.13 (0.69)	0.30 (0.79)	0.32 (0.36)
$s_r$ , medium level	0.31 (1.39)	0.20 (1.47)	0.13 (1.03)
$s_r$ , high level	0.16 (2.17)	0.21 (2.03)	0.18 (1.57)
LOD95	Not determined	Not determined	Not determined
LOQ	Not determined	Not determined	Not determined

Table A2. Soft fruit (raspberries).  $s_r$  values are given as  $\log_{10}$  copies/g. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/g. LOD95 and LOQ values are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.25 (1.09)	0.41 (0.32)	0.68 (0.18)
$s_r$ , medium level	0.15 (1.88)	0.22 (1.04)	0.12 (1.16)
$s_r$ , high level	0.18 (2.57)	0.23 (1.65)	0.28 (1.68)
LOD95	0.60	0.65	0.79
LOQ	10	10	31.6

Table A3. Leaf, stem and bulb vegetables (lettuce).  $s_r$  values are given as  $\log_{10}$  copies/g. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/g. LOD95 and LOQ values are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.16 (1.00)	0.13 (1.26)	0.24 (0.88)
$s_r$ , medium level	0.27 (1.47)	0.26 (1.89)	0.27 (1.65)
$s_r$ , high level	0.26 (2.40)	0.30(2.62)	0.23 (2.42)
LOD95	3.18	0.46	0.88
LOQ	31.6	0.46	0.88

Table A4. Leaf, stem and bulb vegetables (green onions).  $s_r$  values are given as  $\log_{10}$  copies/g. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.21 (1.28)	0.24 (1.49)	0.31 (0.57)
$s_r$ , medium level	0.27 (1.90)	0.28 (2.05)	0.28 (1.27)
$s_r$ , high level	0.18 (2.57)	0.29 (2.62)	0.14 (1.94)
LOD95	Not determined	Not determined	Not determined
LOQ	Not determined	Not determined	Not determined

Table A5. Bottled water.  $s_r$  values are given as  $\log_{10}$  copies/ml. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/ml. LOD95 and LOQ values are given as copies/ml.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.23 (0.77)	0.19 (-0.36)	0.22 (-0.26)
$s_r$ , medium level	0.17 (1.38)	0.27 (0.32)	0.21 (0.21)
$s_r$ , high level	0.09 (1.81)	0.10 (0.97)	0.95 (0.95)
LOD95	0.40	0.18	0.07
LOQ	1.00	0.18	0.10

Table A6. Bivalve molluscan shellfish (pacific oysters).  $s_r$  values are given as  $\log_{10}$  copies/g. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/g. LOD95 and LOQ values are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.12 (1.83)	0.21 (2.35)	0.35 (2.13)
$s_r$ , medium level	0.37 (2.99)	0.18 (3.53)	0.14 (3.31)
$s_r$ , high level	0.08 (3.44)	0.15 (4.02)	0.18 (3.85)
LOD95	198	34	53
LOQ	198	34	53

Table A7. Bivalve molluscan shellfish (common mussels).  $s_r$  values are given as  $\log_{10}$  copies/g. The values in parentheses describe the contamination level used for determination of  $s_r$ , given in  $\log_{10}$  copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
$s_r$ , low level	0.19 (4.55)	0.22 (2.89)	0.34 (2.82)
$s_r$ , medium level	0.20 (4.57)	0.15 (3.12)	0.21 (3.80)
$s_r$ , high level	0.41 (4.91)	0.25 (4.54)	0.20 (4.76)
LOD95	Not determined	Not determined	Not determined
LOQ	Not determined	Not determined	Not determined

## References

1. ISO. Microbiology of the food chain - Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR - Part 1:Method for quantification (ISO 15216-1:2017). 2017.
2. ISO. Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification — Amendment 1. 2021.
3. Lowther J, Bosch A, Butot S, Ollivier J, Mäde D, Rutjes S, et al. Validation of EN ISO method 15216-Part 1—Quantification of hepatitis A virus and norovirus in food matrices. *International journal of food microbiology*. 2019;288:82-90.
4. ISO. Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. 1994.
5. Wilrich C, Wilrich P-T. Estimation of the POD function and the LOD of a qualitative microbiological measurement method. *Journal of AOAC International*. 2009;92(6):1763-72.
6. Hartnell R, Lowther J, Avant J, Dancer D, Lees D, Russell J. The development of LENTICULES™ as reference materials for noroviruses. *Journal of applied microbiology*. 2012;112(2):338-45.
7. EFSA. Analysis of the European baseline survey of norovirus in oysters. *EFSA Journal*. 2019;17(7).
8. EFSA. Technical specifications for a European baseline survey of norovirus in oysters. *EFSA Journal*. 2016;14(3):4414.
9. Lowther J. Guidance note - Determination of limit of detection (LOD95) and limit of quantification (LOQ) characteristics for the method for quantification of viruses in bivalve molluscan shellfish. Food and Agriculture Organization of the United Nations and Centre for Environment Fisheries & Aquaculture Science  
2020; Available from: <https://www.cefas.co.uk/icoe/seafood-safety/designations/fao-reference-centre/protocols-and-guidance/technical-guidance-and-calculation-spreadsheets/>.