

EURL Guidance document for verification of ISO 15216-2:2019

Version history

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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 2: Method for detection (ISO 15216-2:2019) (1).

2 Validation of ISO 15216-2:2019

The methods described in ISO 15216-1:2019 (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. The following performance parameters were determined:

Sensitivity, which according to ISO 15216-2:2019 is defined as: *“the number of samples found positive divided by the number of true positive samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample”*

Specificity, which according to ISO 15216-2:2019 is defined as: *“the number of samples found negative divided by the number of true negative (or blank) samples tested”*

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

50% limit of detection (LOD50), which according to ISO 15216-2:2019 is defined as: *“the concentration (in copies/cm², copies/g or copies/ml) for which the probability of detection is 50%”*

The results from the validation study are given in Annex I of ISO 15216-2:2019 (1). For convenience, all performance parameters relevant to this verification guide 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 15216-2:2019 (Annex I) (1). The user laboratory verification should be performed for all relevant targets and foodstuffs and should include the determination of sensitivity and LOD50. The determination of specificity is not included as there is no certified reference material for viruses in food (as it is impossible for the user laboratory to know if a selected food sample is indeed a true negative).

4 Terms and definitions

This document uses, as far as possible, the same terms and definitions as described in section 3 of ISO 15216-2:2019 (1).

5 Notes

- The method implemented in the user laboratory shall be consistent with the method described in ISO 15216-2:2019 (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.
- The results from the user laboratory verification may not be applicable to foodstuffs other than those included in the validation of ISO 15216-2:2019 (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 15216-2:2019 (1).
- 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 important that the user laboratory participate 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 fivefold 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. The results are used to determine the LOD50 and sensitivity. Figure 1 shows an overview of the procedure. The completion of the verification procedures described in this document is estimated to take approximately two working weeks.

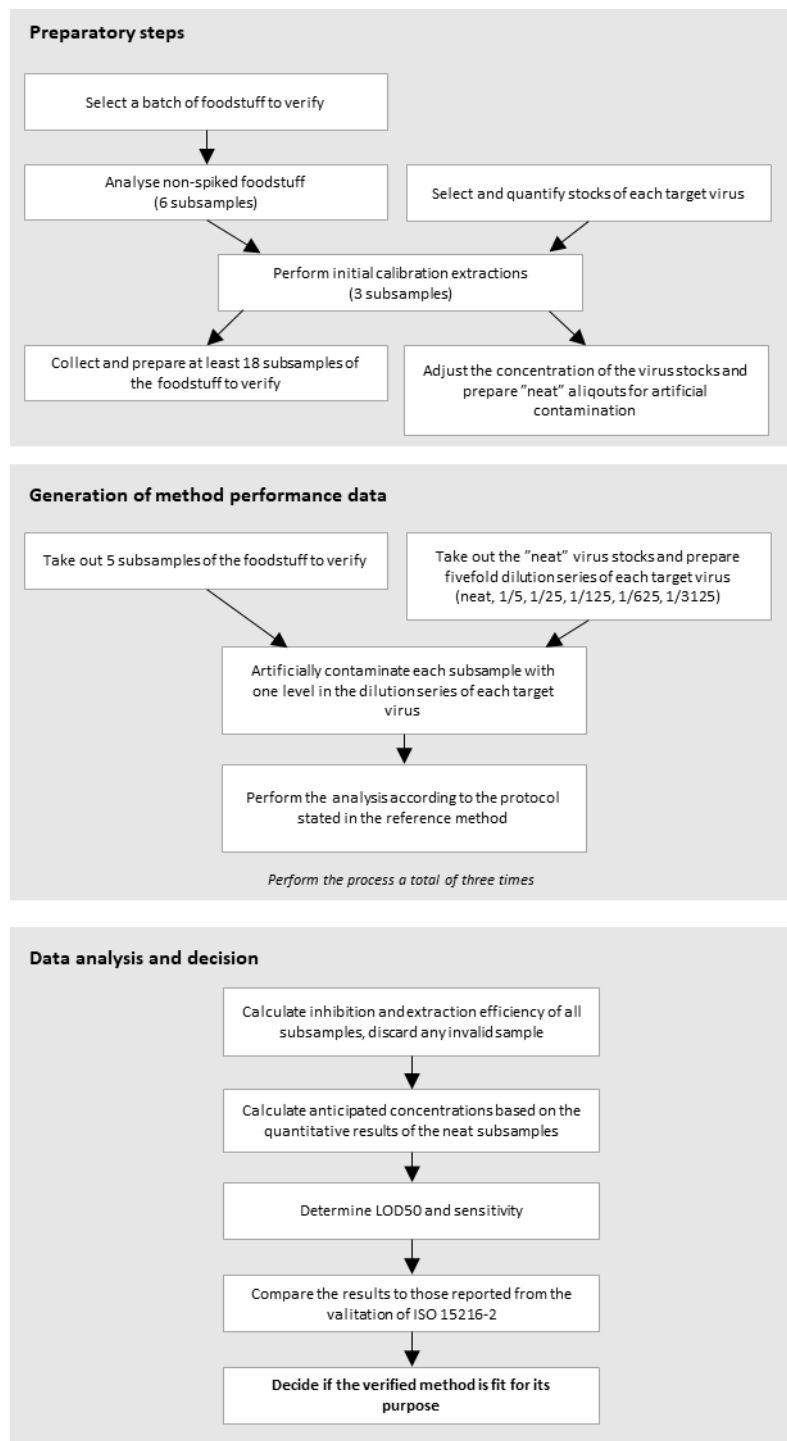


Figure 1. Schematic procedure for user laboratory verification.

6.2 Number of contamination levels and subsamples

At least six contamination levels with at least three 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 LOD50 and sensitivity.

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 15216-2:2019 (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 (23 wells)
B	Subsample 5	Subsample 5	Subsample 5 + HAV EC RNA	Subsample 5	Subsample 5	Subsample 6 + HAV EC RNA	H ₂ O + HAV EC RNA	-ve extraction control	-ve extraction control	H ₂ O	H ₂ 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 (23 wells)
D	Subsample 5	Subsample 5	Subsample 5 + GI EC RNA	Subsample 6	Subsample 6	Subsample 6 + GI EC RNA	H ₂ O + GI EC RNA	-ve extraction control	-ve extraction control	H ₂ O	H ₂ 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 (23 wells)
F	Subsample 5	Subsample 5	Subsample 5 + GII EC RNA	Subsample 6	Subsample 6	Subsample 6 + GII EC RNA	H ₂ O + GII EC RNA	-ve extraction control	-ve extraction control	H ₂ O	H ₂ 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 ₂ 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 15216-2:2019 (10⁻¹) 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 <2,00 greater than the Cq value of the water + EC RNA well, the user laboratory can make the exception to remove (10⁻¹) dilutions 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 (Cq shift)	Real-time PCR (pos/neg)	RT-result (pos/neg)	Inhibition Cq shift)	Real-time RT-PCR result (pos/neg)	Inhibition (Cq shift)	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 LOD50 and sensitivity. 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	21	51 g
Bottled water	Up to 2000 ml (i.e. one water bottle)	21	Up to 25 water bottles
Leaf, stem and bulb vegetables	25 g	21	630 g
Soft fruit	25 g	21	630 g
Surface	5 × 5 cm (25 cm ²)	21	126 × 126 cm (15,876 cm ²)

*This includes estimation of the matrix effect (n = 3) and determination of LOD50 and sensitivity (n = 18)

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 (2). If the virus concentrations are not known in advance, the virus stocks must be quantified as described below. Therefore, although ISO 15216-2:2019 is a qualitative method, it is necessary to perform quantitative real-time RT-PCR analysis as a part of the verification. If required, the EURL can provide dsDNA quantification standards and additional guidance on these issues.

6.4.1 Laboratory procedure

Prepare the stock sample(s) using a procedure appropriate for the sample type¹. Extract the RNA and perform real-time RT-PCR analysis according to the protocol in ISO 15216-2:2019 (1), but with additional dsDNA standards. Include both undiluted and 10⁻¹ and 10⁻² 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 10⁻¹ or 10⁻² 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 ₂ 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 ₂ O	H ₂ 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 ₂ 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 ₂ O	H ₂ 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 ₂ 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 ₂ O	H ₂ 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. 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.

¹If required, the EURL can provide specific protocols for sample preparation.

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 15216-2:2019 (1). Note that bioaccumulated bivalve molluscan shellfish were used for validation of ISO 15216-2:2019 (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 LOD for each participating laboratory (3, 4). 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 15216-2:2019 (1). Again, although ISO 15216-2:2019 is a qualitative method, please note that this step requires a quantitative real-time RT-PCR analysis. 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 ₂ 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 ₂ O	H ₂ 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 ₂ 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 ₂ O	H ₂ 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 ₂ 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 ₂ O	H ₂ 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 ₂ 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 cm², depending on foodstuff. Instructions on how to calculate obtained values are given in Appendix II.

Calculate the RT-PCR inhibition and extraction efficiency as described in ISO 15216-2:2019 (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 ²)	Inhibition (Cq shift)	Obtained concentration (copies per g, ml or cm ²)	Inhibition (Cq shift)	Obtained concentration (copies per g, ml or cm ²)	Inhibition (Cq shift)	Extraction efficiency (%)
1							
2							
3							
Mean							

6.6 Preparation of target viruses for the dilution series

In order to determine LOD₅₀ and sensitivity, 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. 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 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	125	1.00	2500
1/5	25	1.00	500
1/25	5	0.99	100
1/125	1	0.63	20
1/625	0.2	0.18	4.0
1/3125	0.04	0.04	0.8

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 125 copies/RT-PCR well (i.e. the neat dilution level) is 2500. For bivalve molluscan shellfish, approximately ¼ of the supernatant volume goes into RNA extraction, meaning that the copy number per sample yielding 125 copies/ RT-PCR well is 10,000.

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 µl of the target virus solution gives values comparable to 2500 copies/sample for soft fruit, leaf, stem and bulb vegetables, surfaces and bottled water and 10,000 copies/sample for bivalve molluscan shellfish.

Prepare several single-use aliquots containing at least 100 µl each of this concentration for each target virus and store at -70°C. These aliquots will be used as the neat (the most concentrated) dilution level in the subsequent experiments.

6.7 Determination of LOD50 and sensitivity

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.

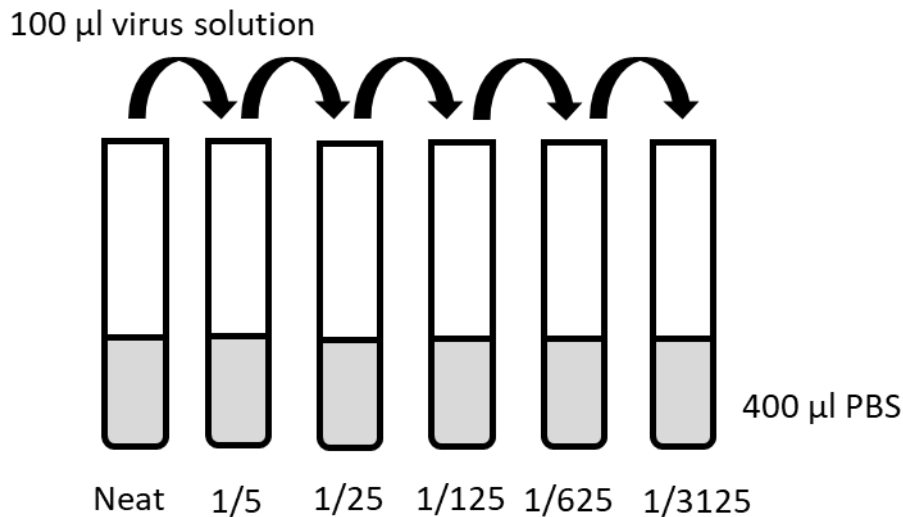


Figure 5. Preparation of a twofold dilution series (example).

6.7.1.2 Preparation of foodstuff

Prepare six subsamples from the selected batch of the foodstuff of interest (e.g. 6 × 25 g of raspberries or 6 × 2 g of homogenised digestive glands for oysters). If frozen, let the material thaw in room temperature for at least 20 min before proceeding. Label the subsamples as neat, 1/5, 1/25, 1/125, 1/625 and 1/3125.

6.7.1.3 Artificial contamination

Artificially contaminate the subsamples according to the following principle:

To the neat subsample, pipet 10 µl of neat virus solution from each target virus. To the 1/5 subsample, pipet 10 µl of 1/5 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 15216-2:2019 (1). Figure 6 shows proposed plate layouts for the analysis. Again, although ISO 15216-2:2019 is a qualitative method, please note that a quantitative real-time RT-PCR analysis is required for this step.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Neat	Neat	Neat + HAV EC RNA	Dil 1/5	Dil 1/5	Dil 1/5 + HAV EC RNA	Dil 1/25	Dil 1/25	Dil 1/25 + HAV EC RNA	Dil 1/125	Dil 1/125	Dil 1/125 + HAV EC RNA	HAV assay (33 wells)
B	Dil 1/625	Dil 1/625	Dil 1/625 + HAV EC RNA	Dil 1/3125	Dil 1/3125	Dil 1/3125 + HAV EC RNA	H ₂ O + HAV EC RNA	-ve extraction control	-ve extraction control				
C	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 ₂ O	H ₂ O	
D	Neat	Neat	Neat + GI EC RNA	Dil 1/5	Dil 1/5	Dil 1/5 + GI EC RNA	Dil 1/25	Dil 1/25	Dil 1/25 + GI EC RNA	Dil 1/125	Dil 1/125	Dil 1/125 + GI EC RNA	GI assay (33 wells)
E	Dil 1/625	Dil 1/625	Dil 1/625 + GI EC RNA	Dil 1/3125	Dil 1/3125	Dil 1/3125 + GI EC RNA	H ₂ O + GI EC RNA	-ve extraction control	-ve extraction control				
F	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 ₂ O	H ₂ O	
G													
H													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	Neat	Neat	Neat + GII EC RNA	Dil 1/5	Dil 1/5	Dil 1/5 + GII EC RNA	Dil 1/25	Dil 1/25	Dil 1/25 + GII EC RNA	Dil 1/125	Dil 1/125	Dil 1/125 + GII EC RNA	GI assay (33 wells)
B	Dil 1/625	Dil 1/625	Dil 1/625 + GII EC RNA	Dil 1/3125	Dil 1/3125	Dil 1/3125 + GII EC RNA	H ₂ O + GII EC RNA	-ve extraction control	-ve extraction control				
C	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 ₂ O	H ₂ O	
D	Neat	Dil 1/5	Dil 1/25	Dil 1/125	Dil 1/625	Dil 1/3125	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H ₂ O	Process control virus assay (12 wells)
E													
F													
G													
H													

Figure 6. Proposed plate layouts for determination of LOD50 and sensitivity.

6.7.1.5 Repetition

After the first round, make sure that the concentrations obtained are close to the expected values (**Fel! Hittar inte referenskälla.**) (± 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 four more times to generate results for a total of 18 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 whether each subsample was detected or not. If only one of two wells in a subsample is detected, that subsample shall be considered detected. 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)
		Detected (yes/no)	Inhibition (Cq shift)	Detected (yes/no)	Inhibition (Cq shift)	Detected (yes/no)	Inhibition (Cq shift)	Extraction efficiency (%)	
Neat	1								
1/5	1								
1/25	1								
1/125	1								
1/625	1								
1/3125	1								
Neat	2								
1/5	2								
1/25	2								
1/125	2								
1/625	2								
1/3125	2								
Neat	3								
1/5	3								
1/25	3								
1/125	3								
1/625	3								
1/3125	3								

Exclude any negative subsample with more than 2 Cq inhibition and/or less than 1% extraction efficiency. However, it is not necessary to exclude positive subsamples with unacceptable values for inhibition or extraction efficiency, as such subsamples can still be reported as positive according to ISO 15216-2:2019.

6.7.3 Determination of LOD50 and sensitivity

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

Calculate and record the obtained value of all valid, detected neat subsamples (expressed as copies per g, ml or cm², depending on foodstuff). Instructions on how to calculate obtained values are given in Appendix II.

Calculate the anticipated value of the neat dilution level by taking the geometric mean of the obtained values above. For example, if there are three neat subsamples with obtained values of 120, 130 and 110 copies/g respectively, the geometric mean is $(120 \cdot 130 \cdot 110)^{\frac{1}{3}} = 119.72$. Geometric mean values can be obtained 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	$119.72 \cdot 1 = 119.72$
1/5	$119.72 \cdot 1/5 = 23.94$
1/25	$119.72 \cdot 1/25 = 4.79$
1/125	$119.72 \cdot 1/125 = 0.96$
1/625	$119.72 \cdot 1/625 = 0.19$
1/3125	$119.72 \cdot 1/3125 = 0.04$

6.7.3.3 Determination of LOD50

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)	Subsamples (n)	Detected samples (n)
119.72	3	3
23.94	3	3
4.79	3	3
0.96	3	2
0.19	3	1
0.04	3	0

Estimate LOD50 by importing the data as described in Table 8 to the PODLOD program for Excel (5). The program is openly available at <http://www.wiwiwss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html>. Note that LOD50 can only be determined if there are negative (non-detected) observations in the dataset.

The data from Table 8 generates a LOD50 of 0.53 copies/g (Figure 7).

Input data				Results of the PODLOD calculations										
Matrix 1	Designation:	No. of inoculated tubes	No. of positive tubes	Matrix	Matrix effect	Log matrix effect	SD of log matrix effect	LOD _{50%} = 50% limit of detection in cfu/g or cfu/ml			LOD _{95%} = 95% limit of detection in cfu/g or cfu/ml			Test statistic matrix effect
Inoculation level in cfu/g or cfu/ml	<i>d</i>	<i>n</i>	<i>y</i>					Detection limit	Lower conf. limit	Upper conf. limit	Detection limit	Lower conf. limit	Upper conf. limit	
119.72		3	3	1	0.656	-0.422	0.585	0.529	0.164	1.704	2.285	0.709	7.366	0.742
23.94		3	3											
4.79		3	3											
0.96		3	2											
0.19		3	1											
0.04		3	0											
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 sensitivity

Determine the sensitivity at each dilution level by dividing the number of detected subsamples with the total number of subsamples. Record the results as shown in the example in Table 9.

Table 9. An example data sheet with anticipated value together with the number of total and detected subsamples and sensitivity (%).

Anticipated value (copies/g)	Subsamples (n)	Detected subsamples (n)	Sensitivity (%)
119.72	3	3	100
23.94	3	3	100
4.79	3	3	100
0.96	3	2	67
0.19	3	1	33
0.04	3	0	0

7 Acceptance criteria

Compare the results obtained from the verification study with those that are available from the validation of ISO 15216-2:2019 (1) (see tables in Appendix I). As there are no microbiological criteria for viruses in food, there are currently no acceptance criteria for LOD or other performance parameters. It is therefore up to each user laboratory to determine their requirements on method performance, and decide whether the implemented method is fit for its intended purpose. If the performance of the method differs substantially from the data of the validation study (e.g. if the estimated LOD50 values differ by more than four times), it is recommended to perform a root cause analysis to explain the deviations in the results. The EURL can provide assistance in these matters.

Appendix I

Summarised results from the validation of ISO 15216-2:2019 (1), shown in Annex I of ISO 15216-2:2019.

In the validation of ISO 15216-2:2019, the sensitivities 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), The values in parentheses describe the contamination level used, concentrations are given as copies/cm².

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	100 (4.9)	95 (6.2)	80 (2.3)
Sensitivity (%) (medium, contamination level used)	95 (25)	95 (30)	90 (11)
Sensitivity (%) (high, contamination level used)	100 (150)	100 (110)	100 (37)
Specificity (%)	95.0	100	100
LOD50 (95% CI)	Not determined	Not determined	Not determined

Table A2. Soft fruit (raspberries), concentrations are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	89.5 (12)	73.7 (2.1)	61.1 (1.5)
Sensitivity (%) (medium, contamination level used)	100 (76)	100 (11)	89.5 (15)
Sensitivity (%) (high, contamination level used)	100 (370)	100 (45)	100 (48)
Specificity (%)	100	94.4	100
LOD50 (95% CI)	0.92 (0.41-2.04)	0.15 (0.06-0.35)	0.18 (0.07-0.45)

Table A3. Leaf, stem and bulb vegetables (lettuce), concentrations are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	88.9 (10)	88.9 (18)	88.9 (7.6)
Sensitivity (%) (medium, contamination level used)	100 (30)	100 (78)	100 (45)
Sensitivity (%) (high, contamination level used)	94.7 (250)	94.7 (420)	100 (260)
Specificity (%)	81.3	94.1	100
LOD50 (95% CI)	0.74 (0.36-1.49)	0.11 (0.05-0.22)	0.20 (0.10-0.41)

Table A4. Leaf, stem and bulb vegetables (green onions), concentrations are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	100 (19)	100 (31)	87.5 (3.7)
Sensitivity (%) (medium, contamination level used)	100 (79)	100 (110)	94.1 (19)
Sensitivity (%) (high, contamination level used)	94.4 (370)	94.4 (420)	94.1 (87)
Specificity (%)	94.4	95.0	94.7
LOD50 (95% CI)	Not determined	Not determined	Not determined

Table A5. Bottled water, concentrations are given as copies/ml.

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	100 (5.9)	95.0 (0.47)	90 (0.55)
Sensitivity (%) (medium, contamination level used)	100 (24)	100 (2.1)	100 (1.6)
Sensitivity (%) (high, contamination level used)	100 (65)	100 (9.3)	100 (8.9)
Specificity (%)	100	100	100
LOD50	0.093 (0.046-0.189)	0.043 (0.021-0.087)	0.016 (0.008-0.033)

Table A6. Bivalve molluscan shellfish (pacific oysters), concentrations are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	73.3 (68)	93.3 (220)	80 (140)
Sensitivity (%) (medium, contamination level used)	100 (980)	100 (3400)	100 (2000)
Sensitivity (%) (high, contamination level used)	94.7 (2800)	100 (11,000)	94.7 (7100)
Specificity (%)	94.7	89.5	84.2
LOD50 (95% CI)	45.9 (22.8-92.3)	7.8 (3.9-15.7)	12.4 (6.2-24.7)

Table A7. Bivalve molluscan shellfish (common mussels), concentrations are given as copies/g.

Performance parameter	HAV	Norovirus GI	Norovirus GII
Sensitivity (%) (low, contamination level used)	100 (35,000)	100 (780)	92.3 (660)
Sensitivity (%) (medium, contamination level used)	100 (37,000)	100 (1300)	100 (6300)
Sensitivity (%) (high, contamination level used)	100 (81,000)	100 (35,000)	100 (58,000)
Specificity (%)	93.3	87.5	93.3
LOD50 (95% CI)	Not determined	Not determined	Not determined

Appendix II

Instructions on how to calculate obtained values in different foodstuff (based on the procedures described in ISO 15216-1:2017 (6).

More detailed instructions are included in the standard operating procedures (SOPs) produced by the EURL for foodborne viruses, available at: <https://www.livsmedelsverket.se/en/business-legislation-and-control/eurl-foodborne-viruses/public-documents/laboratories3>

Soft fruit, surface and leaf, stem and bulb vegetables

From the dsDNA wells, create a standard curve with \log_{10} number of copies/ μl in the well and Cq value on the y-axis. Note the slope and the intercept of the standard curve.

Take the Cq values of the sample without EC RNA wells (undiluted or 10^{-1} diluted depending on extraction efficiency and inhibition results) and calculate the concentration for each replicate as follows:

$$\text{Concentration} = 10^{(\text{Cq-value of the sample} - \text{intercept})/\text{slope}}$$

Multiply this concentration by 100 (if undiluted samples were used) or 1000 (if diluted samples were used) to calculate the number of detectable genome copies in the entire sample.

Bivalve molluscan shellfish

From the the dsDNA wells, create a standard curve with \log_{10} number of copies/ μl in the well and Cq value on the y-axis. Note the slope and the intercept of the standard curve.

Take the Cq values of the sample without EC RNA wells (undiluted or 10^{-1} diluted depending on extraction efficiency and inhibition results) and calculate the concentration for each replicate as follows:

$$\text{Concentration} = 10^{(\text{Cq-value of the sample} - \text{intercept})/\text{slope}}$$

Multiply this concentration by 200 (if undiluted samples were used) or 2000 (if diluted samples were used) and then multiply by the total volume of supernatant in ml, to calculate the number of detectable genome copies in the entire sample.

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