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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

Microbiologie dans la chaine alimentaire — Méthode horizontale pour la recherche des virus de l'hépatite A et norovirus par la technique RT-PCR en temps réel —

Partie 2: Méthode de détection

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition cancels and replaces ISO/TS 15216-2:2013, which has been technically revised with the following changes:

- a requirement to use a suitable buffer for the dilution of control materials has been added;
- the method for generating process control virus RNA for the standard curve has been changed;
- breakpoints with a defined temperature and time parameters in the extraction methods have been added;
- the terminology has been changed from amplification efficiency to RT-PCR inhibition;
- extra real-time RT-PCR reactions for sample RNA and negative controls have been added;
- method characteristics and the results of method validation studies have been added.

A list of all parts in the ISO 15216 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Hepatitis A virus (HAV) and norovirus are important agents of food-borne human viral illness. No routine methods exist for culture of norovirus, and HAV culture methods are not appropriate for routine application to food matrices. Detection is therefore reliant on molecular methods using the reverse-transcriptase polymerase chain reaction (RT-PCR). As many food matrices contain substances that are inhibitory to RT-PCR, it is necessary to use an extraction method that produces highly clean RNA preparations that are fit for purpose. For surfaces, viruses are removed by swabbing. For soft fruit and leaf, stem and bulb vegetables, virus extraction is by elution with agitation followed by precipitation with PEG/NaCl. For bottled water, adsorption and elution using positively charged membranes followed by concentration by ultrafiltration is used. For bivalve molluscan shellfish (BMS), viruses are extracted from the tissues of the digestive glands using treatment with a proteinase K solution. For all matrices that are not covered by this document, it is necessary to validate this method. All matrices share a common RNA extraction method based on virus capsid disruption with chaotropic reagents followed by adsorption of RNA to silica particles. Real-time RT-PCR monitors amplification throughout the real-time RT-PCR cycle by measuring the excitation of fluorescently labelled molecules. In real-time RT-PCR with hydrolysis probes, the fluorescent label is attached to a sequence-specific nucleotide probe that also enables simultaneous confirmation of target template. These modifications increase the sensitivity and specificity of the real-time RT-PCR method, and obviate the need for additional amplification product confirmation steps post real-time RT-PCR. Due to the complexity of the method, it is necessary to include a comprehensive suite of controls. The method described in this document enables detection of virus RNA in the test sample. A schematic diagram of the testing procedure is shown in Annex A.

The main changes, listed in the Foreword, introduced in this document compared to ISO/TS 15216-2:2013, are considered as minor (see ISO 17468).

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

1 Scope

This document specifies a method for detection of hepatitis A virus (HAV) and norovirus genogroups I (GI) and II (GII), from test samples of foodstuffs [(soft fruit, leaf, stem and bulb vegetables, bottled water, bivalve molluscan shellfish (BMS)] or surfaces using real-time RT-PCR.

This method is not validated for detection of the target viruses in other foodstuffs (including multi-component foodstuffs), or any other matrices, nor for the detection of other viruses in foodstuffs, surfaces or other matrices.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 20838, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22119, *Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 20838, ISO 22119, ISO 22174 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
foodstuff
substance used or prepared for use as food

Note 1 to entry: For the purposes of this document, this definition includes bottled water.

3.2
surface
surface of food, food preparation surface or food contact surface

3.3
soft fruit
small edible stoneless fruit

EXAMPLE Strawberries, raspberries, currants.

3.4
leaf, stem and bulb vegetables
leaves, stems and bulbs of plants, eaten as a vegetable

EXAMPLE Lettuce, green onions.

3.5
hepatitis A virus
HAV
member of the *Picornaviridae* family responsible for infectious hepatitis

Note 1 to entry: Genetically, HAV can be subdivided into six genotypes on the basis of the VP1/2A region (genotypes 1, 2 and 3 have been found in humans, while genotypes 4, 5, and 6 are of simian origin). There is only one serotype.

Note 2 to entry: Transmission occurs via the faecal-oral route by person-to-person contact, through the consumption of contaminated *foodstuffs* (3.1), contact with contaminated water or *surfaces* (3.2), or contact with contaminated fomites. HAV is classified as a group 2 biological agent by the European Union and as a risk group 2 human aetiological agent by the United States National Institutes of Health.

3.6
norovirus
member of the *Caliciviridae* family responsible for sporadic cases and outbreaks of acute gastroenteritis

Note 1 to entry: Genetically, norovirus can be subdivided into seven separate genogroups. Three of these genogroups, GI, GII and GIV have been implicated in human gastrointestinal disease. GI and GII are responsible for the vast majority of clinical cases.

Note 2 to entry: Transmission occurs via the faecal-oral route by person-to-person contact, through the consumption of contaminated *foodstuffs* (3.1), through contact with contaminated water or *surfaces* (3.2), or contact with contaminated fomites. GI and GII noroviruses are classified as group 2 biological agents by the European Union and as risk group 2 human aetiological agents by the United States National Institutes of Health.

3.7
detection of HAV
detection of *HAV* (3.5) RNA in a predetermined mass or volume of *foodstuff* (3.1), or on the area of a *surface* (3.2)

3.8
detection of norovirus
detection of *norovirus* (3.6) RNA in a predetermined mass or volume of *foodstuff* (3.1), or on the area of a *surface* (3.2)

3.9
process control virus
virus added to the sample portion at the earliest opportunity prior to virus extraction to control for extraction efficiency

3.10
process control virus RNA
RNA extracted from the *process control virus* (3.9) in order to produce standard curve data for the estimation of extraction efficiency

3.11
negative RNA extraction control
control free of target RNA carried through all steps of the RNA extraction and detection procedure to monitor any contamination events

3.12
negative process control

target pathogen-free sample of the food matrix, or target pathogen-free non-matrix sample, that is run through all stages of the analytical process

3.13
hydrolysis probe

fluorescent probe coupled with a fluorescent reporter molecule and a quencher molecule, which are sterically separated by the 5′-3′-exonuclease activity of the enzyme during the amplification process

3.14
negative real-time RT-PCR control

aliquot of highly pure water used in a real-time RT-PCR reaction to assess contamination in the real-time RT-PCR reagents

3.15
external control RNA

EC RNA

reference RNA that can be used to assess inhibition of amplification for the real-time RT-PCR assay of relevance by being added in a defined amount to an aliquot of sample RNA in a separate reaction

EXAMPLE RNA synthesized by *in vitro* transcription from a plasmid carrying a copy of the target gene.

3.16
C_q value

quantification cycle, which is the cycle at which the target is quantified in a given real-time RT-PCR reaction

Note 1 to entry: This corresponds to the cycle at which reaction fluorescence rises above a threshold level.

4 Principle

4.1 Virus extraction

The foodstuffs and surfaces covered by this document are often highly complex matrices and the target viruses can be present at low concentrations. It is therefore necessary to carry out matrix-specific virus extraction and/or concentration in order to provide a substrate for subsequent common parts of the process. The choice of method depends upon the matrix.

4.2 RNA extraction

It is necessary to extract RNA using a method that yields RNA preparations of suitable purity to reduce the effect of RT-PCR inhibitors. In this document, the chaotropic agent guanidine thiocyanate is used to disrupt the viral capsid. RNA is then adsorbed to silica to assist purification through several washing stages. Purified viral RNA is released from the silica into a buffer prior to real-time RT-PCR.

4.3 Real-time RT-PCR

This document uses one-step real-time RT-PCR using hydrolysis probes. In one-step real-time RT-PCR, reverse transcription and PCR amplification are carried out consecutively in the same tube.

Real-time RT-PCR using hydrolysis probes utilizes a short DNA probe with a fluorescent label and a fluorescence quencher attached at the 5’ and 3’ ends, respectively. The assay chemistry ensures that as the quantity of amplified product increases, the probe is hydrolysed and the fluorescent signal from the label increases proportionately.

Due to the low levels of virus template often present in foodstuffs or surfaces and the strain diversity in the target viruses, the selection of fit-for-purpose one step real-time RT-PCR reagents and PCR primers and hydrolysis probes for the target viruses is important. Guidelines for their selection are given in

5.2.19 and 5.2.20. Illustrative details of reagents, primers, and probes (used in the development of this document) are provided in Annexes C and D.

4.4 Control materials

4.4.1 Process control virus

Losses of target virus can occur at several stages during sample virus extraction and RNA extraction. To account for these losses, samples are spiked at the earliest opportunity prior to virus extraction with a defined amount of a process control virus. The level of recovery of the process control virus shall be determined for each sample.

The virus selected for use as a process control shall be a culturable non-enveloped positive-sense ssRNA virus of a similar size to the target viruses to provide a good morphological and physicochemical model. The process control virus shall exhibit similar persistence in the environment to the targets. The virus shall be sufficiently distinct genetically from the target viruses that real-time RT-PCR assays for the target and process control viruses do not cross-react, and shall not normally be expected to occur naturally in the foodstuffs or surfaces under test.

An example of the preparation of process control virus (used in the development of this document) is provided in Annex E.

4.4.2 EC RNA control

Many food matrices contain substances inhibitory to RT-PCR, and there is also a possibility of carryover of further inhibitory substances from upstream processing. In order to evaluate RT-PCR inhibition in individual samples, EC RNA (an RNA species carrying the target sequence of interest, 5.3.11) is added to an aliquot of sample RNA and tested using the real-time RT-PCR method. Comparison of the results of this with the results of EC RNA in the absence of sample RNA enables determination of the level of RT-PCR inhibition in each sample under test. In addition, in this method, the EC RNA control acts as a positive control for real-time RT-PCR for the target viruses.

Alternative approaches for the assessment of RT-PCR inhibition are permitted, provided that the alternative approach can be demonstrated to provide equivalent performance to the use of EC RNA control as described in this document.

4.5 Test results

For surfaces, this method provides a result expressed either as “virus genome detected” or “virus genome not detected” followed by “in x cm^2”, where x is the approximate surface area swabbed. Where it is not possible to record the surface area swabbed, results are expressed either as “virus genome detected” or “virus genome not detected”. For other sample types, results are expressed as “virus genome detected” or “virus genome not detected” followed by “in x ml” or “in x g”, where x is the amount of sample tested.

5 Reagents

5.1 General

Use only reagents of recognized analytical grade, unless otherwise specified.

For current laboratory practice, see ISO 7218.

5.2 Reagents used as supplied

5.2.1 Molecular biology grade water.

5.2.2 Polyethylene glycol (PEG), mean relative molecular mass 8 000.

5.2.3 Sodium chloride (NaCl).

5.2.4 Potassium chloride (KCl).

5.2.5 Disodium hydrogenphosphate (Na_2HPO_4).

5.2.6 Potassium dihydrogenphosphate (KH_2PO_4).

5.2.7 Tris base.

5.2.8 Glycine.

5.2.9 Beef extract powder.

5.2.10 Proteinase K.

5.2.11 Pectinase from *Aspergillus niger* or *A. aculeatus*.

5.2.12 Chloroform.

5.2.13 n-Butanol.

5.2.14 Sodium hydroxide (NaOH) (≥ 10 mol/l).

5.2.15 Hydrochloric acid (HCl) (≥ 5 mol/l).

5.2.16 Ethylenediaminetetraacetic acid (EDTA) **disodium dihydrate**.

5.2.17 Silica, lysis, wash and elution buffers for extraction of viral RNA. Reagents shall enable processing of 500 µl of sample extract, using lysis with a chaotropic buffer containing guanidine thiocyanate[4] and using silica as the RNA-binding matrix. Following treatment of silica-bound RNA with wash buffer(s) to remove impurities, RNA shall be eluted in 100 µl elution buffer.

The RNA preparation shall be of a quality and concentration suitable for the intended purpose. See Annex F for illustrative details of RNA extraction reagents (used in the development of the method described in this document).

5.2.18 Reagents for one step real-time RT-PCR. Reagents shall allow processing of 5 µl RNA in 25 µl total volume. They shall be suitable for one step real-time RT-PCR using hydrolysis probes (the DNA polymerase used shall possess 5′-3′ exonuclease activity) and sufficiently sensitive for the detection of virus RNA as expected in virus-contaminated foodstuffs and surfaces. See Annex C for illustrative details of one step real-time RT-PCR reagents (used in the development of this document).

5.2.19 Primers and hydrolysis probes for detection of HAV and norovirus GI and GII. Primer and hydrolysis probe sequences shall be published in a peer-reviewed journal and be verified for use against a broad range of strains of target virus. Primers for detection of HAV shall target the 5′ non-coding region of the genome. Primers for detection of norovirus GI and GII shall target the ORF1/ORF2 junction of the genome. See Annex D for illustrative details of primers and hydrolysis probes for detection of HAV and norovirus GI and GII (used in the development of this document).

5.2.20 Primers and hydrolysis probes for detection of the process control virus. Primer and hydrolysis probe sequences shall be published in a peer-reviewed journal and be verified for use against the strain of process virus used. They shall demonstrate no cross-reactivity with the target virus. See Annex D for illustrative details of primers and hydrolysis probes for detection of the process control virus (used in the development of this document).

5.3 Reagents requiring preparation

Due to the large number of reagents requiring individual preparation, details of composition and preparation are given in Annex B. The instructions in Annex B shall be followed when preparing reagents listed under 5.3.1 to 5.3.8.

5.3.1 5 × PEG/NaCl solution (500 g/l PEG 8 000, 1,5 mol/l NaCl). See B.1.

5.3.2 Chloroform/butanol mixture (1:1 volume fraction). See B.2.

5.3.3 Proteinase K solution (3 000 U/l). See B.3.

5.3.4 Phosphate-buffered saline (PBS). See B.4.

5.3.5 Tris/glycine/beef extract (TGBE) buffer. See B.5.

5.3.6 Tris solution (1 mol/l). See B.6.

5.3.7 EDTA solution (0,5 mol/l). See B.7.

5.3.8 Tris EDTA (TE) buffer (10 mmol/l Tris, 1 mmol/l EDTA). See B.8.

5.3.9 Process control virus material. Process control virus stock shall be diluted by a minimum factor of 10 in a suitable buffer, e.g. PBS (5.3.4). This dilution shall allow for inhibition-free detection of the process control virus genome using real-time RT-PCR, but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve (see 8.4.2.2). Split the diluted process control virus material into single use aliquots and store at −15 °C or below. See Annex E for illustrative details of the preparation of process control virus (used in the development of the method described in this document).

5.3.10 Real-time RT-PCR mastermixes for target and process control virus. Reagents shall be added in quantities as specified by the manufacturers (5.2.18) to allow 20 µl mastermix per reaction in a 25 µl total volume. Optimal primer and probe concentrations shall be used after determination following the recommendations of the reagent manufacturers. See Annex C for illustrative details of real-time RT-PCR mastermixes (used in the development of this document).

5.3.11 EC RNA control material. Purified ssRNA carrying the target sequence for each target virus shall be used. They shall contain levels of contaminating target DNA no higher than 0,1 % and shall not cause RT-PCR inhibition. The concentrations of each EC RNA stock in copies per microlitre shall be determined, then the stock shall be diluted in a suitable buffer, e.g. TE buffer (5.3.8), to a concentration of 1×10^2 to 1×10^5 template copies per microlitre. The concentration used shall be appropriate for the types of samples under test and shall ensure that RT-PCR inhibition calculations are not affected by the presence of endogenous target RNA in the samples. As EDTA can act as an inhibitor of RT-PCR, buffers used to dilute EC RNA shall not contain concentrations of EDTA greater than 1 mmol/l. Split the diluted EC RNA preparation (EC RNA control material) into single use aliquots and store at 5 °C (6.4) for up to 24 h, at

−15 °C or below for up to 6 months, or at −70 °C or below for longer periods. See Annex G for illustrative details of the preparation of EC RNA (used in the development of this document).

Alternative approaches for the assessment of RT-PCR inhibition are permitted, provided that the alternative approach can be demonstrated to provide equivalent performance to the use of EC RNA control as described in this document.

6 Equipment and consumables

Standard microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Micropipettes and **tips** of a range of sizes, e.g. 1 000 µl, 200 µl, 20 µl, 10 µl. Aerosol resistant tips shall be used unless unobstructed tips are required, e.g. for aspiration (as in 6.8 and F.3).

6.2 Pipette filler and **pipettes** of a range of sizes, e.g. 25 ml, 10 ml, 5 ml.

6.3 Vortex mixer.

6.4 Refrigerator, capable of operating at (5 ± 3) °C

6.5 Shaker, capable of operating at approximately 50 oscillations min^{-1}.

6.6 Shaking incubator, operating at (37 ± 2) °C and approximately 320 oscillations min^{-1} or equivalent.

6.7 Rocking platform(s) or equivalent for use at room temperature and (5 ± 3) °C at approximately 60 oscillations min^{-1}.

6.8 Aspirator or equivalent apparatus for removing supernatant.

6.9 Water bath, capable of operating at (60 ± 2) °C or equivalent.

6.10 Centrifuge(s) and **rotor(s)**, capable of the following run speeds, run temperatures and rotor capacities:

a) 10 000g at (5 ± 3) °C with capacity for tubes of at least 35 ml volume;

b) 10 000g at (5 ± 3) °C with capacity for chloroform-resistant tubes with 2 ml volume;

c) 4 000g at room temperature with capacity for centrifugal filter concentration devices (6.16).

6.11 Centrifuge tubes and **bottles** of a range of sizes, 1,5 ml, 5 ml, 15 ml, 50 ml, etc. Chloroform-resistant tubes with 2 ml capacity are necessary.

6.12 pH meter (or pH testing strips with demarcations of 0,5 pH units or lower).

6.13 Sterile cotton swabs.

6.14 Mesh filter bags (400 ml).

6.15 Positively charged membrane filters, with 0,45 µm pore size (47 mm diameter).

6.16 Centrifugal filter concentration devices, with 15 ml capacity and 100 kDa relative molecular mass cut-off.

6.17 Vacuum source or equivalent positive pressure apparatus for filtering and filtration tower with aperture for 47 mm diameter membrane.

6.18 Sterile shucking knife or equivalent tools for opening BMS.

6.19 Rubber block or equivalent apparatus for holding BMS during opening.

6.20 Scissors and forceps or equivalent tools for dissecting BMS.

6.21 Sterile Petri dishes.

6.22 Razor blades or equivalent tools for chopping BMS digestive glands.

6.23 Heavy duty safety glove.

6.24 RNA extraction equipment, suitable for extraction methods using silica and associated reagents (5.2.17). See Annex F for illustrative details of RNA extraction apparatus (used in the development of this document).

6.25 Real-time PCR machine(s), i.e. thermal cycler(s), equipped with an energy source suitable for the excitation of fluorescent molecules, and an optical detection system for real-time detection of fluorescence signals generated during real-time RT-PCR with hydrolysis probe chemistry.

6.26 Associated consumables for real-time RT-PCR, e.g. optical plates and caps, suitable for use with the selected real-time RT-PCR machine.

7 Sampling

If there is no specific International Standard dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on the subject.

It is important the laboratory receive a sample that is representative and that has not been damaged or changed during transport or storage, e.g. samples that were frozen on collection shall not be allowed to defrost prior to receipt at the laboratory, samples that were not frozen upon collection shall not be frozen prior to receipt at the laboratory.

8 Procedure

8.1 General laboratory requirements

The testing procedure shall be as shown in the schematic diagram in Annex A.

Sample extraction and real-time RT-PCR shall be carried out in separate working areas or rooms as specified in ISO 22174.

8.2 Virus extraction

8.2.1 General

The selection of method is dependent upon the food matrix under test.

8.2.2 Process control virus material

Immediately before a batch of test samples is processed, pool together sufficient aliquots of process control virus material (5.3.9) for all individual test samples (allow 10 µl per test sample plus 25 µl excess).

Retain a (20 ± 1) µl portion of pooled process control virus material for RNA extraction and preparation of the standard curve of process control virus RNA (see 8.4.2.2). Store at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

8.2.3 Negative process control

A negative process control sample shall be run in parallel to test samples at a frequency determined as part of the laboratory quality assurance programme.

8.2.4 Surfaces

Using a sterile cotton swab premoistened in PBS (5.3.4), intensively swab the surface (maximum area, 100 cm^2) under test, applying a little pressure to detach virus particles. Where practical, record the approximate area swabbed in square centimetres.

Process the swab immediately, or place in a suitable container and store at 5 °C (6.4) for a maximum of 72 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

Add (10 ± 0,5) µl of process control virus material (see 8.2.2) to the swab.

Immediately after the addition of process control virus material, immerse the swab in a tube containing (490 ± 10) µl lysis buffer as used for RNA extraction (5.2.17), then press against the side of the tube to release liquid. Repeat the immersion and pressing cycle three or four times to ensure maximum yield of virus.

Proceed immediately to RNA extraction (see 8.3).

8.2.5 Soft fruit and leaf, stem and bulb vegetables

Soft fruit and leaf, stem and bulb vegetables for analysis shall be fresh or frozen. Samples shall not have been subject to any processing other than chopping, trimming, washing, decontamination, conditioning, etc. as for pre-cut and packaged soft fruit, leaf, stem and bulb vegetables, etc. Mud adhering to the surface shall be removed prior to analysis by gentle scrubbing, but without immersing the samples in water.

Coarsely chop (25 ± 0,3) g of soft fruits or leaf, stem or bulb vegetables into pieces of approximately 2,5 cm × 2,5 cm × 2,5 cm (it is not necessary to chop if, for example, individual fruits are smaller than this size) and transfer to the sample compartment of a 400 ml mesh filter bag. Add (10 ± 0,5) µl of process control virus material (see 8.2.2) to the sample. Add (40 ± 1) ml TGBE (5.3.5) (for soft fruit samples, add ≥ 30 units pectinase from *A. niger*, or ≥ 1 140 units pectinase from *A. aculeatus* to the buffer [5.2.11]).

Incubate at room temperature with constant rocking at approximately 60 oscillations min^{-1} for (20 ± 1) min. For acidic soft fruits, the pH of the eluate shall be monitored at 10 min intervals during incubation. If the pH falls below 9,0, it shall be adjusted to 9,5 ± 0,5 with NaOH (≥ 10 mol/l). Extend the period of incubation by 10 min for every time the pH is adjusted. Do not make more than three such pH adjustments per sample. Decant the eluate from the filtered compartment into a centrifuge tube (use two tubes if necessary to accommodate volume).

Clarify by centrifugation at 10 000*g* for (30 ± 5) min at 5 °C (6.10).

Decant the supernatant into a single clean tube or bottle and adjust to pH 7,0 ± 0,5 with HCl (≥ 5 mol/l).

Add 0,25 volumes of 5 × PEG/NaCl solution (5.3.1) (to produce a final concentration of 100 g/l PEG 0,3 mol/l NaCl), homogenize by shaking for (60 ± 5) s then incubate with constant rocking at approximately 60 oscillations min^{-1} at 5 °C for (60 ± 5) min (6.7).

Centrifuge at 10 000*g* for (30 ± 5) min at 5 °C (6.10). Split the volume across two centrifuge tubes if necessary.

Decant and discard the supernatant, then centrifuge at 10 000*g* for (5 ± 1) min at 5 °C (6.10) to compact the pellet.

Discard the supernatant and resuspend the pellet in (500 ± 10) µl PBS (5.3.4). For samples that produce large pellets after centrifugation, a larger volume up to (1 000 ± 20) µl of PBS may be necessary in order to completely resuspend the pellet. If a single sample has been split across two tubes, resuspend both pellets stepwise in the same aliquot of PBS using the same pipette tip.

For extraction from leaf, stem or bulb vegetables, transfer the suspension to a suitable tube and retain for RNA extraction (see 8.3). Sample extract shall be processed immediately, or stored at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

For extraction from soft fruit, a further extraction step is required. Transfer the suspension to a chloroform-resistant centrifuge tube (6.11). Add (500 ± 10) µl chloroform/butanol mixture (5.3.2), vortex to mix, then incubate at room temperature for 5 min. If more than 500 µl PBS was used to resuspend the pellet, add an equal volume of chloroform/butanol mixture.

Centrifuge at 10 000*g* for (15 ± 1) min at 5 °C (6.10). Carefully transfer the aqueous (upper) phase to a fresh tube and retain for RNA extraction (see 8.3).

Sample extract shall be processed immediately, or stored at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

8.2.6 Bottled water

This document is appropriate for volumes up to 2 l. For each sample, record the volume tested.

Add (10 ± 0,5) µl of process control virus material (see 8.2.2) to the sample under test. Shake to mix.

Using a vacuum or positive pressure source (6.17), filter entire sample through a positively charged 47 mm membrane (6.15). Transfer the filter into a sterile tube, then add (4 ± 0,1) ml of TGBE buffer (5.3.5).

Add (10 ± 0,2) ml TGBE buffer to the empty sample bottle. Shake both tube and bottle at approximately 50 oscillations min^{-1} for (20 ± 5) min.

Pool the eluates from the tube and bottle together in a single clean tube.

Rinse the interior walls of the bottle with an additional (2 ± 0,1) ml TGBE buffer by gentle shaking and inversion by hand, and add to the tube.

Adjust the eluates to pH 7,0 ± 0,5 with HCl (≥ 5 mol/l) and transfer to a centrifugal filter concentration device (6.16).

Centrifuge at 4 000*g* for (15 ± 1) min. Transfer the concentrate to a clean tube.

Adjust the volume to (500 ± 10) µl with PBS (5.3.4). Retain for RNA extraction (see 8.3).

Sample extract shall be processed immediately, or stored at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

8.2.7 Bivalve molluscan shellfish (BMS)

BMS for analysis shall be live, or if frozen, undamaged, and shall not be cooked or otherwise thermally treated. Mud adhering to the shell shall be removed. BMS shall not be reimmersed in water.

Open the shells of a minimum of 10 BMS with a sterile shucking knife or equivalent tool. When opening, ensure that the hand holding the animal is protected with a heavy-duty safety glove and the animal is supported with a rubber block or equivalent apparatus.

Dissect out the digestive glands from all animals using scissors and forceps or equivalent tools and transfer to a clean Petri dish. A minimum combined gland mass of (2,0 ± 0,2) g is required.

Finely chop the digestive glands with a razor blade or equivalent tools to a paste-like consistency, then transfer a (2,0 ± 0,2) g portion into a centrifuge tube.

Digestive glands shall be processed immediately, or stored at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods [any digestive glands remaining after taking the (2,0 ± 0,2) g portion can be stored at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.]

Add (10 ± 0,5) µl of process control virus material (see 8.2.2) directly onto the (2,0 ± 0,2) g portion.

Add (2,0 ± 0,2) ml of proteinase K solution (5.3.3) and mix. Incubate at 37 °C with shaking at approximately 320 oscillations min^{-1} in a shaking incubator (6.6) or equivalent for (60 ± 5) min.

Carry out a secondary incubation by placing the tube in a water bath or equivalent at 60 °C (6.9) for (15 ± 1) min.

Centrifuge at 3 000*g* for (5,0 ± 0,5) min at room temperature, decant the supernatant into a clean tube, measure and record the volume of supernatant, in millilitres (typically volumes of 2,0 ml to 3,0 ml will be recovered) and retain for RNA extraction (see 8.3).

Supernatant shall be processed immediately, or stored at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

8.3 RNA extraction

Extract RNA from (500 ± 10) µl of each sample (BMS) or entire sample (other matrices) using an appropriate guanidine thiocyanate disruption and silica adsorption-based method. Elute purified RNA into (100 ± 2) µl of elution buffer and retain for real-time RT-PCR analysis.

Extracted RNA shall be processed immediately, or stored at 5 °C (6.4) for a maximum of 24 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods. For each batch of samples tested, a negative RNA extraction control shall be included unless the batch includes a negative process control (see 8.2.3). RNA extraction shall be carried out using the same method in parallel on (500 ± 10) µl of water (5.2.1).

See Annex F for illustrative details of an RNA extraction method (used in the development of this document).

8.4 Real-time RT-PCR

8.4.1 General requirements

The minimum requirements for the amplification and detection of nucleic acid sequences by real-time RT-PCR are specified in ISO 20838, ISO 22119 and ISO 22174.

This document specifies methods for the detection of HAV, norovirus GI and norovirus GII.

Under certain circumstances, testing for all three viruses in a single sample is not necessary. The procedure described in the following clauses enables a test sample to be analysed for one virus (i.e. HAV, norovirus GI or norovirus GII) and includes a full set of recommended controls. Laboratories wishing to test for more than one target shall adjust the reaction format to accommodate additional tests. A typical plate layout is included as Annex H.

Results generated using 10^{-1} sample RNA are used only in the event that RT-PCR inhibition is outside the acceptable parameters for undiluted sample RNA (C_q value of the undiluted sample RNA + EC RNA well is ≥ 2,00 greater than the C_q value of the water + EC RNA well) (see 9.3). 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).

Alternative approaches for the assessment of RT-PCR inhibition are permitted, provided that the alternative approach can be demonstrated to provide equivalent performance to the use of EC RNA as described in this document.

8.4.2 Real-time RT-PCR analysis

8.4.2.1 Analysis of target virus

Prepare 10^{-1} dilutions of each sample RNA in water (5.2.1).

For each sample prepare:

- two wells of an optical plate with (5 ± 0,5) µl of undiluted sample RNA;
- two wells with (5 ± 0,5) µl of 10^{-1} sample RNA;
- one well with (5 ± 0,5) µl of undiluted sample RNA and (1 ± 0,2) µl of undiluted EC RNA (5.3.11);
- one well with (5 ± 0,5) µl of 10^{-1} sample RNA and (1 ± 0,2) µl of undiluted EC RNA.

For the EC RNA control prepare:

- one well with (5 ± 0,5) µl of water (5.2.1) and (1 ± 0,2) µl of undiluted EC RNA.

For negative controls prepare:

- two wells with (5 ± 0,5) µl of water (5.2.1) to act as a negative real-time RT-PCR control;
- two wells with (5 ± 0,5) µl of negative RNA extraction control or negative process control RNA.

Add (20 ± 1) µl of the relevant real-time RT-PCR mastermix (5.3.10) to each well. The mastermix may also be added to all relevant wells before the addition of template material.

The above describes the case where undiluted and 10^{-1} sample RNA are tested simultaneously. Where RNA is only tested at one concentration (see 8.4.1), wells including the other concentration shall be omitted.

8.4.2.2 Analysis of process control virus

For each batch used with the samples under test, add (10 ± 0,5) µl of process control virus material (see 8.2.2) to a separate (500 ± 10) µl portion of water. Extract and store RNA for each batch using the same method and conditions applied to the test samples (see 8.3).

Prepare 10^{-1} dilutions of each sample RNA in water (5.2.1).

Prepare 10^{-1}, 10^{-2} and 10^{-3} dilutions of process control virus RNA in water (5.2.1) or a suitable buffer, e.g. TE buffer (5.3.8) for each batch of process control virus material. As EDTA can act as an inhibitor of RT-PCR, buffers used to dilute process control virus RNA shall not contain concentrations of EDTA greater than 1 mmol/l.

For each sample prepare:

- one well with (5 ± 0,5) µl of undiluted sample RNA;
- one well with (5 ± 0,5) µl of 10^{-1} sample RNA.

For the process control virus RNA standard curve prepare:

- one well with (5 ± 0,5) µl of undiluted process control virus RNA;
- one well with (5 ± 0,5) µl of 10^{-1} process control virus RNA;
- one well with (5 ± 0,5) µl of 10^{-2} process control virus RNA;
- one well with (5 ± 0,5) µl of 10^{-3} process control virus RNA.

For negative controls prepare:

- one well with (5 ± 0,5) µl of water (5.2.1) to act as a negative real-time RT-PCR control;
- one well with (5 ± 0,5) µl of negative RNA extraction control or negative process control RNA.

Add (20 ± 1) µl of process control virus real-time RT-PCR mastermix (5.3.10) to each well. The mastermix may also be added to all relevant wells before the addition of template material.

The above describes the case where undiluted and 10^{-1} sample RNA are tested simultaneously. Where RNA is only tested at one concentration (see 8.4.1), wells including the other concentration shall be omitted.

8.4.2.3 Amplification

Subject the plate to a reaction cycle including an initial stage for reverse transcription and at least 45 cycles of PCR using a real-time PCR machine (6.25). The duration and temperatures of each stage (reverse transcription, RT deactivation, denaturation, annealing, extension) depends on the reagents used. They shall be based on the manufacturer's recommendations, but can be further optimized.

For real-time PCR machines where the user can set the point of fluorescence data collection, this shall be set at the end of the extension stage.

See Annex C for illustrative details of an amplification method (used in the development of this document).

8.4.2.4 Analysis of fluorescence data

The minimum requirements for the analysis of amplification data are specified in ISO 22174. Amplification plots shall be analysed using the approach recommended by the manufacturer of the real-time PCR machine. The threshold shall be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase).

All amplification plots shall be checked to identify false-positive results (reactions with C_q values not associated with exponential amplification) caused by a high or uneven background signal. This shall be noted and results for any reactions affected in this way shall be regarded as negative. In addition, all true positive fluorescent plots shall be checked to ensure that the C_q value generated by the analysis software corresponds to the exponential phase of amplification for that reaction (and is not distorted by a high or uneven background signal). Where C_q values are distorted, corrected C_q values shall be recorded in addition to the value generated by the software. Corrected C_q values shall be used for efficiency calculations.

9 Interpretation of results

9.1 General

Each control (EC RNA, process control virus RNA) has an expected valid value or range of values. If the observed result for any control is different from the expected value, samples may require retesting.

Negative controls [water (5.2.1) and negative RNA extraction or process control] shall always be negative; if positive results occur in these controls, then any samples giving positive results shall be retested.

Where a sample provides one positive and one negative result in duplicate reactions, the overall result for that sample shall be considered as positive.

9.2 Construction of process control virus RNA standard curve

Use the results for the process control virus RNA dilution series to create a standard curve by plotting the C_q values obtained against $\log_{10}$ concentration to determine r^2 (where r is Pearson's correlation coefficient), slope and intercept parameters.

Curves with r^2 values of < 0,980, or where the slope is not between −3,10 and −3,60 (corresponding to amplification efficiencies of ~90 % to 110 %), shall not be used for calculations. In these cases, check C_q values of the standard curve for any outlying values and remove these from the series. No more than one such outlying C_q value shall be removed per series.

Repeat the calculations to determine r^2, slope and intercept parameters. Where the modified curve has an r^2 value of < 0,980, or where the slope is not between −3,10 and −3,60, the modified slope shall not be used for calculations.

9.3 Control for RT-PCR inhibition

If the C_q value of the undiluted sample RNA + EC RNA well is < 2,00 greater than the C_q value of the water + EC RNA well, results for the undiluted RNA shall be used for that sample. If the C_q value of the undiluted sample RNA + EC RNA well is ≥ 2,00 greater than the C_q value of the water + EC RNA well, repeat the comparison with the 10^{-1} sample RNA + EC RNA well.

If the C_q value of the 10^{-1} sample RNA + EC RNA well is < 2,00 greater than the C_q value of the water + EC RNA well, results for the 10^{-1} RNA shall be used for that sample. If the C_q value of the 10^{-1} sample RNA + EC RNA well is ≥ 2,00 greater than the C_q value of the water + EC RNA well, it is possible that results are not valid and the sample may require retesting.

The above describes the case where undiluted and 10^{-1} sample RNA are tested simultaneously. Where only undiluted sample RNA is tested in the initial round (see 8.4.1), and where the C_q value of the undiluted sample RNA + EC RNA well is ≥ 2,00 greater than the C_q value of the water + EC RNA well, real-time RT-PCR shall be repeated using 10^{-1} sample RNA and the calculation repeated using the results of the repeat analysis.

A sample showing an unacceptable RT-PCR inhibition level, but producing an otherwise valid positive result may, if appropriate, be reported as positive as described in Clause 10.

In the case that the C_q value of the undiluted sample RNA + EC RNA well is < 2,00 greater than the C_q value of the water + EC RNA well, but where the sample provides positive results only using 10^{-1} sample RNA, results for the 10^{-1} RNA shall be used for that sample.

If alternative methods for determining RT-PCR inhibition are used, this procedure requires adaptation to provide the same level of stringency.

9.4 Calculation of extraction efficiency

Use the C_q value for the process control virus assay from the test sample RNA well (undiluted or 10^{-1} dependent on the RT-PCR inhibition results, see 9.3) to estimate process control virus recovery by reference to the process control virus RNA standard curve as follows (if 10^{-1} sample RNA results are used, multiply by 10 to correct for the dilution factor):

$$\text{Process control virus recovery} = 10^{(\Delta Cq/m)} \times 100\ \%$$

where

ΔC_q is the C_q value [sample RNA] – C_q value [undiluted process control virus RNA];

m is the slope of the process control virus RNA standard curve.

For BMS samples, calculate the extraction efficiency by reference to the process control virus recovery as follows:

$$\text{Extraction efficiency} = (\text{p}/0{,}5) \times v$$

where

p is the process control virus recovery;

v is the total measured volume of supernatant in ml (see 8.2.7).

For other sample matrices, the extraction efficiency is equal to the process control virus recovery.

Where the extraction efficiency is < 1 %, sample results are not valid and the sample may need to be retested.

Under the condition that the process control virus RNA standard curve has an idealized slope of −3,32, a (non-BMS) test sample with a ΔC_q of 0,00 has an extraction efficiency of 100 % and a test sample with a ΔC_q of 6,64 has an extraction efficiency of 1 %. Where the ΔC_q is ≤ 6,64, the extraction efficiency is ≥ 1 % and therefore acceptable. If the ΔC_q is > 6,64, the extraction efficiency is < 1 % and therefore not acceptable.

For specific types of soft fruit, leaf, stem or bulb vegetables or BMS where formal validation data does not exist, alternative extraction efficiency thresholds may be applied subject to appropriate evaluation.

A sample showing an unacceptable extraction efficiency, but producing an otherwise valid positive result may, if appropriate, be reported as positive as described in Clause 10.

10 Expression of results

Positive results for each target virus for surfaces shall be expressed as “virus genome detected in x cm²” where the approximate surface area can be recorded, or as “virus genome detected” where the approximate surface area cannot be recorded. For other sample types, positive results shall be expressed as “virus genome detected in x ml” or “virus genome detected in x g”, where x is the amount of sample tested.

If target virus is not detected, results for surfaces shall be expressed as “virus genome not detected in x cm²”, where the approximate surface area can be recorded, or as “virus genome not detected”, where the approximate surface area cannot be recorded. For other sample types, results shall be expressed as “virus genome not detected in x ml” or “virus genome not detected in x g”.

If a valid result is not obtained, results shall normally be expressed as “invalid”. If, however, an otherwise valid positive result is obtained from a sample showing an unacceptable RT-PCR inhibition or extraction efficiency, results may be expressed as detailed above. Details shall be included in the test report.

11 Performance characteristics of the method

11.1 Validation study

The performance characteristics of the method were determined in a validation study including both single laboratory and interlaboratory elements to determine the specificity, sensitivity and, where available, the LOD_{50} of the method. The data are summarized in Annex I. The values derived from the validation study may not be applicable to food types other than those given in Annex I.

11.2 Sensitivity

The sensitivity 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.

11.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of true negative (or blank) samples tested.

11.4 LOD_{50}

The LOD_{50} is the concentration (in copies/cm^2, copies/g or copies/ml) for which the probability of detection is 50 % (limit of detection at 50 %).

12 Test report

The test report shall contain at least the following information:

- the test method used, with reference to this document, i.e. ISO 15216-2;
- the sampling method used, if known;
- the size of the test portion;
- all operating conditions not specified in this document, or regarded as optional, together with details of any incidents that may have influenced the test result(s);
- any deviations from this document;
- all information necessary for the complete identification of the sample;
- the test result(s) obtained, expressed according to Clause 10;
- the extraction efficiency of the sample (see 9.4);
- the date of completion of the test.

Annex A
(normative)

Diagram of procedure

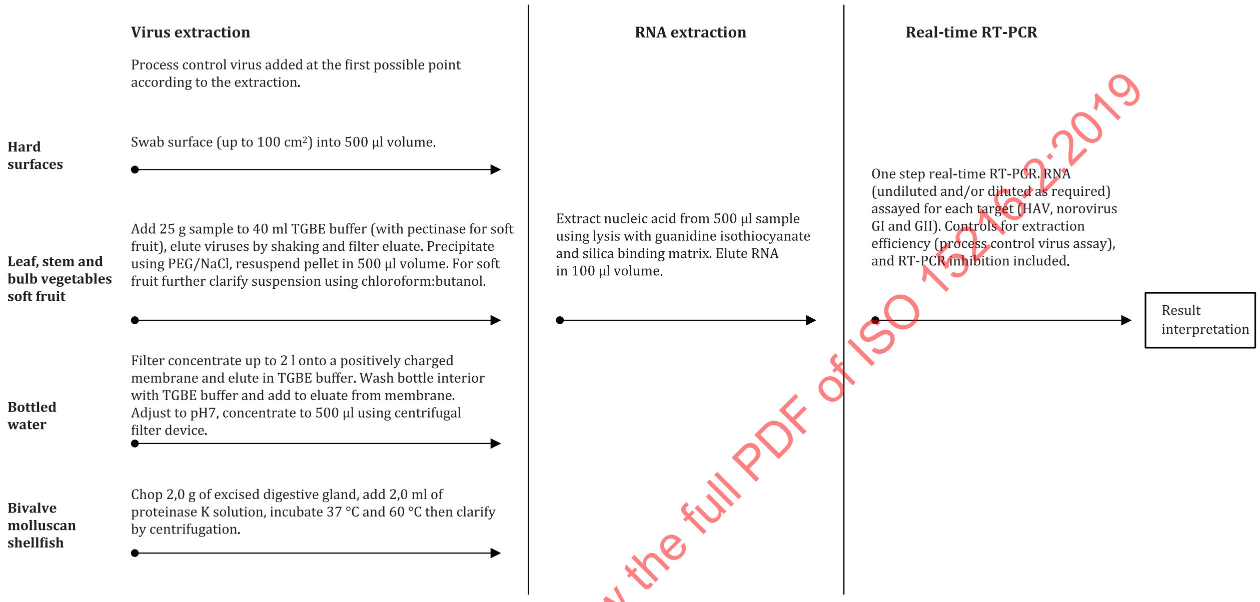

Figure A.1 — Diagram of procedure

Annex B
(normative)

Composition and preparation of reagents and buffers

B.1 5 × PEG/NaCl solution (500 g/l PEG 8 000, 1,5 mol/l NaCl)

B.1.1 Composition

Polyethylene glycol (PEG) 8 000	(CAS No. 25322-68-3; M_r 8 000)	(500 ± 2) g
Sodium chloride (NaCl)	(CAS No. 7647-14-5; M_r 58,44)	(87 ± 1) g
Water		as required in B.1.2

B.1.2 Preparation

Dissolve the solids in (450 ± 5) ml water, heating gently if necessary. Adjust the volume to (1 000 ± 10) ml with water and mix well. Sterilize by autoclaving.

Store at room temperature for a maximum of 6 months.

B.2 Chloroform/butanol mixture (1:1 volume fraction)

B.2.1 Composition

Chloroform	(CAS No. 67-66-3; M_r 119,38)	(10 ± 0,1) ml
n-Butanol	(CAS No. 71-36-3; M_r 74,12)	(10 ± 0,1) ml

B.2.2 Preparation

Mix the components together.

Store at room temperature in a dark glass bottle for a maximum of 12 months.

B.3 Proteinase K solution (3000 U/l)

B.3.1 Composition

Proteinase K	as necessary (see below)
Sterile water	(200 ± 2) ml

B.3.2 Preparation

Calculate from the specific activity of the proteinase K as provided by the manufacturer the quantity required to provide 600 units of the enzyme (e.g. for a specific activity of 30 U/mg, the quantity required is 20 mg).

Dissolve this quantity of proteinase K in the water. Mix thoroughly.

Store in working volumes at −15 °C or below for a maximum of 6 months. Once defrosted, store at 5 °C (6.4) and use within 1 week.

B.4 Phosphate-buffered saline (PBS)

B.4.1 Composition

Sodium chloride	(CAS No. 7647-14-5; M_r 58,44)	(8,0 ± 0,1) g
Potassium chloride	(CAS No. 7447-40-7; M_r 74,55)	(0,2 ± 0,01) g
Disodium hydrogenphosphate	(CAS No. 7558-79-4; M_r 141,96)	(1,15 ± 0,01) g
Potassium dihydrogenphosphate	(CAS No. 7778-77-0; M_r 136,09)	(0,2 ± 0,01) g
Water		(1 000 ± 10) ml

B.4.2 Preparation

Dissolve the solids in the water. Adjust, if necessary, to pH 7,3 ± 0,2 at 25 °C. Sterilize by autoclaving.

Store at 5 °C (6.4) for a maximum of 6 months.

B.5 Tris/glycine/beef extract (TGBE) buffer

B.5.1 Composition

Tris base [*tris*(hydroxymethyl) aminomethane]	(CAS No. 77-86-1; M_r 121,14)	(12,1 ± 0,2) g
Glycine	(CAS No. 56-40-6; M_r 75,07)	(3,8 ± 0,1) g
Beef extract powder	(CAS No. 68990-09-0)	(10 ± 1,0) g
Water		(1 000 ± 10) ml

B.5.2 Preparation

Dissolve the solids in the water. Adjust, if necessary, to pH 9,5 ± 0,2 at 25 °C. Sterilize by autoclaving.

Store at 5 °C (6.4) for a maximum of 6 months.

B.6 Tris solution (1 mol/l)

B.6.1 Composition

Tris base [*tris*(hydroxymethyl) aminomethane]	(CAS No. 77-86-1; M_r 121,14)	(12,1 ± 0,2) g
Water (5.2.1)		as required in B.6.2

B.6.2 Preparation

Dissolve the tris base in (90 ± 1) ml water. Adjust, if necessary, to pH 8,0 ± 0,2 at 25 °C. Adjust the volume to (100 ± 1) ml with water. Sterilize by autoclaving.

Store at 5 °C (6.4) for a maximum of 6 months.

B.7 EDTA solution (0,5 mol/l)

B.7.1 Composition

Ethylenediaminetetraacetic acid (EDTA) disodium dihydrate	(CAS No. 6381-92-6; M_r 372,24)	(18,6 ± 0,2) g
Water (5.2.1)		as required in B.7.2

B.7.2 Preparation

Dissolve the EDTA disodium dihydrate in (90 ± 1) ml water. Adjust, if necessary, to pH 8,0 ± 0,2 at 25 °C. Adjust the volume to (100 ± 1) ml with water. Sterilize by autoclaving.

Store at 5 °C (6.4) for a maximum of 6 months.

B.8 Tris EDTA (TE) buffer (10 mmol/l Tris, 1 mmol/l EDTA)

B.8.1 Composition

Tris solution (1 mol/l) (B.6)	(1 000 ± 10) µl
EDTA solution (0,5 mol/l) (B.7)	(200 ± 10) µl
Water (5.2.1)	(100 ± 1) ml

B.8.2 Preparation

Mix the components together.

Store at 5 °C (6.4) for a maximum of 6 months.

Annex C
(informative)

Real-time RT-PCR mastermixes and cycling parameters

For the composition of one step real-time RT-PCR mastermixes using the Invitrogen RNA Ultrasense™ one step qRT-PCR system[1], see Table C.1. For cycling parameters, see Table C.2.

Table C.1 — Mastermix

Reagent	**Final concentration** (in 25 µl reaction volume)	**Volume per reaction** µl
5 × Ultrasense reaction mix	1 ×	5 ± 0,25
FW Primer	0,5 pmol/µl	as required
REV Primer	0,9 pmol/µl	as required
Probe	0,25 pmol/µl	as required
ROX reference dye (50 ×)	as required[a]	as required
RNA Ultrasense enzyme mix	—	1,25 ± 0,1
Water (5.2.1)	—	as required
Total volume	—	20 ± 0,5

[a] With Applied Biosystems real-time PCR machines, ROX shall be used at 1 × concentration; for the Stratagene MX3000, ROX can be either used at 0,1 × concentration, or omitted from the mastermix. For other machines, consult the manufacturer's instructions.

Applied Biosystems real-time PCR machines and Stratagene MX3000 are products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named.

Table C.2 — Cycling parameters

Step description		**Temperature and time**	**Number of cycles**
RT		55 °C for 1 h	1
Preheating		95 °C for 5 min	1
Amplification	Denaturation	95 °C for 15 s	45
	Annealing-extension	60 °C for 1 min	
		65 °C for 1 min[a]	

[a] For real-time PCR machines where the user can set the point of fluorescence data collection, this shall be set at the end of the incubation at 65 °C.

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[1] Invitrogen RNA Ultrasense™ is the trademark of a product supplied by Invitrogen. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Annex D
(informative)

Real-time RT-PCR primers and hydrolysis probes for the detection of HAV, norovirus GI and GII and mengo virus (process control)

D.1 HAV

HAV68 (FW): `TCA CCG CCG TTT GCC TAG` Reference [5]

HAV240 (REV): `GGA GAG CCC TGG AAG AAA G` Reference [5]

HAV150(-) (PROBE): `CCT GAA CCT GCA GGA ATT AA` Reference [5]

Probe labelled: at the 5′-end with 6-carboxyfluorescein (FAM); at the 3′-end with MGBNFQ (minor groove binder/non-fluorescent quencher)

This primer set amplifies a product of variable length (157 to 188 base pairs [bp]) depending on the virus strain; for example, a product of 174 bp corresponding to nucleotides 68 to 241 of HAV strain HM175 (GenBank accession number M14707).

Sequence alignment using all sequences available in GenBank of the assay target region demonstrates that this primer and probe set is adequate for the detection of all HAV genotypes. In addition, quasispecies analysis of the mutant spectrum indicates that this region is not prone to variability, and this assay shall therefore provide long-term robustness. The specificity of the primers was verified with 10 different picornaviruses: poliovirus (serotype 1 vaccine strain); human enterovirus B (echovirus 1); human enterovirus B (echovirus 11); human enterovirus B (echovirus 30); human enterovirus B (Coxsackie virus-B5); human enterovirus C (Coxsackie virus-A24); human enterovirus D (enterovirus 70); bovine enterovirus; porcine teschovirus (porcine enterovirus 1); and encephalomyocarditis virus. Other enteric viruses such as hepatitis E virus, human and porcine rotavirus (group A), norovirus, mamastrovirus (human astrovirus type 1), and human adenovirus F (enteric adenovirus type 40) were also employed. None of the viruses tested gave positive results either at high concentration (10^6 to 10^8 TCID50/ml or undiluted 0,1 g/ml faecal suspensions) or low concentration (10^4 TCID50/ml or 10^{-1} dilutions of 0,1 g/ml faecal suspensions). The LOD of the assay is 10 ssRNA molecules, 1 viral RNA molecule and 0,05 infectious viruses per reaction[5].

D.2 Norovirus GI

QNIF4 (FW): `CGC TGG ATG CGN TTC CAT` Reference [6]

NV1LCR (REV): `CCT TAG ACG CCA TCA TCA TTT AC` Reference [7]

During the development and validation of this document, two different probes for norovirus GI were used. Either can be used with the FW and REV primers detailed here.

NVGG1p (PROBE): `TGG ACA GGA GAY CGC RAT CT` Reference [7]

Probe labelled: at the 5′-end with 6-carboxyfluorescein (FAM); at the 3′-end with 6-carboxytetramethylrhodamine (TAMRA)

TM9 (PROBE):	`TGG ACA GGA GAT CGC`	Reference [8]

Probe labelled: at the 5′-end with 6-carboxyfluorescein (FAM); at the 3′-end with MGBNFQ (minor groove binder/non-fluorescent quencher)

This primer set amplifies a product of 86 bp corresponding to nucleotides 5291 to 5376 of Norwalk virus (GenBank accession number M87661).

D.3 Norovirus GII

QNIF2 (FW):	`ATG TTC AGR TGG ATG AGR TTC TCW GA`	Reference [5]
COG2R (REV):	`TCG ACG CCA TCT TCA TTC ACA`	Reference [10]
QNIFs (PROBE):	`AGC ACG TGG GAG GGC GAT CG`	Reference [9]

Probe labelled: at the 5′-end with 6-carboxyfluorescein (FAM); at the 3′-end with 6-carboxytetramethylrhodamine (TAMRA)

This primer set amplifies a product of 89 bp corresponding to nucleotides 5012 to 5100 of Lordsdale virus (GenBank accession number X86557).

The area selected for norovirus detection is the well-conserved region at the 5′ end of ORF2[10]. Sequence alignments using all sequences available in GenBank of the assay target region demonstrate that these primer and probe sets are adequate for the detection of all GI and GII norovirus strains respectively. In addition, the efficacy and sensitivity of the primers and probes was verified using 18 norovirus reference strains: GI.1 (Norwalk virus); GI.2 (Whiterose); GI.3 (Southampton); GI.4 (Malta); GI.5 (Musgrove); GI.6 (Mikkeli); GI.7 (Winchester); GI.10 (Boxer); GII.1 (Hawaii); GII.2 (Melksham); GII.3 (Toronto); GII.4 (Grismby); GII.6 (Seacroft); GII.7 (Leeds); GII.10 (Erfurt); GIIb variants; GIIc variants; and GIV (Alphatron).

The specificity of the primers was verified with six different human enteric viruses: poliovirus (serotype 1 vaccine strain); HAV; hepatitis E virus; Aichi virus; astrovirus; and rotavirus. The specificity was also tested on seven bacteria that could be detected in BMS: *Escherichia coli, Shewenella putrefaciens, Chromobacterium violaceum, Aeromonas sobria, Vibrio alginolyticus, Vibrio parahaemolyticus* and *Vibrio cholerae.* None of the tested viruses or bacteria gave positive results. The LODs of the assays are 1 to 10 viral RNA molecules (dependent on the strain of norovirus)[9][11].

D.4 Mengo virus

Mengo 110 (FW):	`GCG GGT CCT GCC GAA AGT`	Reference [12]
Mengo 209 (REV):	`GAA GTA ACA TAT AGA CAG ACG CAC AC`	Reference [12]
Mengo 147 (PROBE):	`ATC ACA TTA CTG GCC GAA GC`	Reference [12]

Probe labelled: at the 5′-end with 6-carboxyfluorescein (FAM); at the 3′-end with MGBNFQ (minor groove binder/non-fluorescent quencher)

This primer set amplifies a product of 100 bp corresponding to nucleotides 110 to 209 of the deletant mengo virus strain MC_0 used in the development of this document. This corresponds to nucleotides 110 to 270 of the non-deletant mengo virus isolate M (GenBank accession number L22089).

The target region selected for the detection of mengo virus is as similar as possible to that of HAV in terms of structure, length and base composition[5]. The primer sequences do not align with any other sequences available in GenBank. The specificity of the primers was verified with 10 different human enteric viruses: hepatitis A virus; hepatitis E virus; norovirus GI; norovirus GII; human enterovirus B (echovirus 1); human enterovirus B (echovirus 11); human enterovirus B (echovirus 30); human enterovirus C (Coxsackie virus-A24); human enterovirus D (enterovirus 70); and human astrovirus type 2. None of the viruses tested gave positive results either at 10^6 TCID50/ml or 10^5 TCID50/ml (or at 10^6 genome copies/ml or 10^5 genome copies/ml in the case of norovirus GI and GII).

Annex E
(informative)

Growth of mengo virus strain MC_0 for use as a process control

E.1 General

Mengo virus is a murine virus of the *Picornaviridae* family. Mengo virus strain MC_0 (CECT 10000)2) is a recombinant (deletant) virus which lacks the poly(C) tract in comparison to the wild-type mengo virus, with identical growth properties to those of the wild-type virus but with an avirulent phenotype[13]. This strain has been used as a process control virus in detection methods for HAV and noroviruses[5][11] and was used as the process control in the development of this document.

Mengo virus strain MC_0 is a genetically modified organism (GMO). For laboratories where use of a GMO is prohibited or problematic, a different process control shall be used.

E.2 Reagents and apparatus

E.2.1 The recommended cell culture medium for HeLa cells is Eagle's minimum essential medium with 2 mmol/l L-glutamine and Earle's BSS, adjusted to 1,5 g/l sodium hydrogencarbonate, 0,1 mmol/l non-essential amino acids, 1,0 mmol/l sodium pyruvate, 1 × streptomycin/penicillin solution, 100 ml/l (growth) or 20 ml/l (maintenance) foetal bovine serum.

E.2.2 For preparation of cell cultures and growth of virus, cell culture facilities including incubator(s) with controllable CO_2 levels, and cell culture consumables (flasks, etc.) are required.

E.3 Procedure

Mengo virus shall be grown in a (50 ± 10) ml/l CO_2 atmosphere (with open vessels) or an uncontrolled atmosphere (closed vessels) on 80 % to 90 % confluent monolayers of HeLa cells (ATCC® CCL-2™)2 until at least 75 % cytopathic effect has been reached.

Subject the cell culture vessel to a single freeze–thaw cycle, then centrifuge the contents at 3 000*g* for (10 ± 1) min.

Retain the (cell culture) supernatant for preparation of the process control virus material (5.3.9).

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[2] CECT 10000 and ATCC® CCL-2™ are trademarks of products supplied by the Spanish Type Culture Collection and American Type Culture Collection, respectively. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named. Equivalent products may be used if they can be demonstrated to meet the requirements of the procedure.

Annex F
(informative)

RNA extraction using the BioMerieux NucliSens®[3] system

F.1 Reagents

F.1.1 NucliSens®[3] lysis buffer.

F.1.2 NucliSens®[3] magnetic extraction reagents (comprising magnetic silica solution, wash buffers 1, 2 and 3, and elution buffer).

F.2 Apparatus

F.2.1 Magnetic rack for 1,5 ml tubes.

F.2.2 Thermoshaker or equivalent apparatus for shaking 1,5 ml tubes at (60 ± 2) °C and approximately 1 400 oscillations min^{-1}.

F.3 Procedure

Add (2 ± 0,1) ml of NucliSens®[3] lysis buffer to a tube. Add (500 ± 10) µl of sample (BMS) or entire sample (other matrices) and mix by vortexing briefly.

Incubate for (10 ± 1) min at room temperature.

Add (50 ± 2,5) µl of well-mixed magnetic silica solution to the tube and mix by vortexing briefly.

Incubate for (10 ± 1) min at room temperature.

Centrifuge for (120 ± 10) s at 1 500*g* or allow silica to sediment using a magnetic rack then carefully discard supernatant by, for example, aspiration.

Add (400 ± 10) µl wash buffer 1 and resuspend the pellet by pipetting or vortexing, taking care to avoid foaming.

Transfer suspension to a clean 1,5 ml tube. Cap tube and wash silica for (30 ± 2) s by vortexing. After washing, allow silica to sediment using the magnetic rack. Discard supernatant by, for example, aspiration.

Add (400 ± 10) µl wash buffer 1. Cap tube and wash silica for (30 ± 2) s by vortexing, allow silica to sediment using magnetic rack then discard supernatant.

Add (500 ± 10) µl wash buffer 2. Cap tube and wash silica for (30 ± 2) s by vortexing, allow silica to sediment using magnetic rack then discard supernatant. Repeat.

Add (500 ± 10) µl wash buffer 3 (samples shall not be left in wash buffer 3 for more time than necessary). Cap tube and wash silica for (15 ± 1) s, allow silica to sediment using magnetic rack then discard supernatant.

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[3] BioMerieux NucliSens® is the trade name of a product supplied by BioMerieux. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Add (100 ± 5) µl elution buffer. Cap tube and transfer to thermoshaker or equivalent and incubate for (5,0 ± 0,5) min at 60 °C with shaking at approximately 1 400 oscillations min^{-1}.

Place tube in magnetic rack and allow silica to sediment, then transfer eluate to a clean tube.

Automated platforms for RNA extraction using the NucliSens®[3)] magnetic extraction reagents may also be used.

Annex G
(informative)

Generation of external control RNA (EC RNA) stocks

G.1 General

Special care shall be taken to separate work areas used for generation of EC RNA control stocks from those used for sample extraction.

In the development of this document, linearized plasmids carrying copies of the target genes were used for the production of EC RNA. For HAV, a control plasmid was constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector at a HincII restriction site such that the target sequence was downstream of a promoter sequence for the SP6 RNA polymerase. For norovirus GI and GII, control plasmids were separately constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector at a SmaI restriction site such that in each case the target sequence was downstream of a promoter sequence for the T7 RNA polymerase. For all three plasmids, unique BamHI restriction sites were introduced into the target sequence inserts by insertion into, or replacement of, wild-type sequences to act as a contamination check measure. Where contamination of samples with EC RNA is suspected, the presence of the BamHI site can be checked either by digestion of the PCR products with BamHI restriction enzyme, or by sequencing of the PCR products. Complete insert target sequences for these plasmids are as follows:

HAV

```
1      TCACCGCCGT   TTGCCTAGGC   TATAGGCTAA   ATTTTCCCTT   TCGGATCCCC   CTTTCCTATT

61     CCCTTTGTTT   TGCTTGTAAA   TATTGATTTG   TAAATATTGA   TTCCTGCAGG   TTCAGGGTTC

121    TTAAATCTGT   TTCTCTATAA   GAACACTCAT   TTCACGCTTT   CTGTCTTCTT   TCTTCCAGGG

181    CTCTCC
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norovirus GI

```
1    CGCTGGATGC   GCTTCCATGA   CCTCGGATTG   TGGACAGGAG   ATCGCGATCT   TCTGCGGATC

61   CGAATTCGTA   AATGATGATG   GCGTCTAAGG
```

norovirus GII

```
1    ATGTTCAGAT   GGATGAGATT   CTCAGATCTG   AGCACGTGGG   AGGGCGATCG   CAATCTGGCT

61   CGGATCCCCA   GCTTTGTGAA   TGAAGATGGC   GTCGA
```

G.2 Reagents and apparatus

G.2.1 Restriction enzymes for linearization and associated buffers.

G.2.2 PCR purification reagents.

G.2.3 *In vitro* RNA transcription reagents (RNA polymerase, NTPs, buffer, etc.).

G.2.4 RNase-free DNase.

G.2.5 RNA purification reagents.

G.2.6 DNA gel electrophoresis reagents and **equipment**.

G.2.7 Reagents and **equipment** for **one-step real-time RT-PCR with hydrolysis probes**.

G.2.8 Incubator, capable of operating at (37 ± 2) °C.

G.2.9 Heating block or equivalent for incubating 1,5 ml tubes at (95 ± 2) °C.

G.3 Linearization of plasmid DNA

Add 1 µg of purified plasmid DNA to a reaction mix containing a suitable restriction enzyme (to enable linearization of the plasmid at a point shortly downstream of the target sequence)[4] and buffers as recommended by the manufacturer of the enzyme.

Incubate at 37 °C for (120 ± 5) min.

Purify DNA from the mastermix using PCR purification reagents, eluting in (50 ± 2,5) µl elution buffer.

Check for linearization using gel electrophoresis (compare an aliquot of purified linearized with nonlinearized plasmid).

G.4 *In vitro* RNA transcription

Add 1 µg of purified linearized plasmid DNA to an *in vitro* RNA transcription reaction mix prepared as recommended by the manufacturer of the RNA polymerase enzyme[5].

Incubate at 37 °C for (120 ± 5) min.

Add RNase-free DNase to the reaction and incubate at 37 °C for (15 ± 1) min.

Purify the RNA using RNA purification reagents, eluting in (100 ± 5) µl water (5.2.1).

G.5 DNA contamination check

Prepare target-specific real-time RT-PCR mastermix (5.3.10), split into two and deactivate the RT enzyme in one portion by heating at 95 °C for (5,0 ± 0,5) min.

Subject EC RNA stock or an appropriate dilution thereof to real-time RT-PCR using both untreated and heat-treated mastermixes in parallel.

If detectable levels in the portion of EC RNA stock tested with the heat-treated mastermix are > 0,1 % of those in the portion tested with untreated mastermix (if the C_q difference between EC RNA stock tested with heat-treated and untreated mastermix is < 10), the stock is contaminated with DNA and shall be retreated with DNase (see G.4). If levels are < 0,1 %, store at −15 °C or below until required for preparation of the EC RNA control material (5.3.11).

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[4] For the plasmids used in the development of this document, suitable restriction enzymes are EcoRI for HAV and XbaI for norovirus GI and GII plasmids.

[5] For the plasmids used in the development of this document, suitable RNA polymerase enzymes for *in vitro* RNA transcription are SP6 for HAV and T7 for norovirus GI and GII plasmids.

G.6 Quantification of EC RNA

Determine the absorbance at 260 nm of the DNase-treated EC RNA stock (see G.4) using spectrophotometry.

Multiply the absorbance reading by 4 × 10^{-8} (and by the dilution factor used if diluted EC RNA stock is measured) to give the concentration of RNA in grams per microlitre.

Divide this number by the mass in grams of a single EC RNA molecule to calculate the concentration of RNA in copies per microlitre.

The mass of an individual RNA molecule can be calculated by multiplying the RNA length in ribonucleotides by 320,5 (the relative molecular mass of an average ribonucleotide) and dividing by the Avogadro constant (6,02 × 10^{23}), e.g. an RNA molecule of 200 ribonucleotides has a mass of 1,06 × 10^{-19} g.6)

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6) For the EC RNA controls used in the development of this document, the respective lengths and masses are as follows: HAV – 250 ribonucleotides, 1,33 × 10^{-19} g; Norovirus GI – 126 ribonucleotides, 6,73 × 10^{-20} g; Norovirus GII – 131 ribonucleotides, 7,00 × 10^{-20} g.

Annex H
(informative)

Typical optical plate layout

Table H.1 — Typical optical plate layout

HAV assay	Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + HAV EC RNA	Test sample (-1) + HAV EC RNA	H2O + HAV EC RNA	-ve extraction control	-ve extraction control	H_2O	H_2O	
Norovirus GI assay	Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GI EC RNA	Test sample (-1) + GI EC RNA	H2O + GI EC RNA	-ve extraction control	-ve extraction control	H_2O	H_2O	
Norovirus GII assay	Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GII EC RNA	Test sample (-1) + GII EC RNA	H2O + GII EC RNA	-ve extraction control	-ve extraction control	H_2O	H_2O	
Process control virus assay	Test sample (undiluted)	Test sample (-1)	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H_2O				

5 µl RNA (±1 µl EC RNA) and 20 µl mastermix per well

Annex I

(informative)

Method validation studies and performance characteristics

A validation study, including a series of single laboratory studies (for determination of LOD_{50}) and an international interlaboratory study (for determination of sensitivity and specificity) involving 18 laboratories in 11 countries, was carried out on surfaces (the exterior surfaces of bell peppers), raspberries, lettuce, green onions, bottled water, Pacific oysters and common mussels. In the single laboratory studies multiple $10^{0,5}$-fold dilution series of contaminated food samples were tested, while in the interlaboratory studies the food samples were each tested at three different levels of contamination, with “high” and “medium” levels designed to be approximately 25 × and 5 × “low” levels, respectively, plus a negative control. The genotypes of the virus stocks used for contamination were HM175/43c (HAV tissue culture supernatant), GI.4 (norovirus GI faecal suspension) and GII.4 (norovirus GII faecal suspension) and the mode of contamination of test samples was bioaccumulation (Pacific oysters and common mussels) or direct contamination (other matrices). The study was organized in 2013 to 2014 by the Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom.

The method submitted to the validation study included the specific methods for real-time RT-PCR and RNA extraction detailed in Annexes C and F, respectively. The values of the performance characteristics derived from this validation study are shown for each virus target and matrix combination in Tables I.1 to I.21.

LOD_{50} characteristics were determined using the data generated in the single laboratory studies. For these data sets, reference levels for the test samples with the highest level of contamination within each prepared dilution series were calculated directly using the method for quantification described in ISO 15216-1. For the other samples within the dilution series, reference levels were calculated by multiplying the reference level for the most highly contaminated sample by the dilution factor[14]. The LOD_{50} values were then calculated by counting positive and negative results at different reference levels and using the method for estimation of the POD (probability of detection) function and the LOD of a qualitative microbiological measurement method as described in Reference [15]. For some matrices, practical problems experienced during the single laboratory studies meant that it was not possible to determine LOD_{50} values.

Sensitivity and specificity characteristics were determined using the data generated in the interlaboratory studies. Reference levels for the different contamination levels were determined as the geometric mean of the results obtained by the participating laboratories, using the method for quantification described in ISO 15216-1.

The method used for determination of reference levels in both the single laboratory and interlaboratory studies (quantification of positive matrix samples after contamination) may have resulted in different method characteristics than if an alternative method for determination of reference levels (e.g. quantification of virus stocks prior to contamination) had been used.