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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 1: Method for quantification

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

Partie 1: Méthode de quantification

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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 on 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 the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, in collaboration with ISO Technical Committee ISO/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 15216-1:2013, which has been technically revised with the following changes:

— use of linear dsDNA molecules for quantification prescribed;

— use of a suitable buffer for dilution of control materials prescribed;

— change to the method for generating process control virus RNA for the standard curve;

— addition of breakpoints with defined temperature and time parameters in the extraction methods;

— change in terminology from amplification efficiency to RT-PCR inhibition;

— addition of extra real-time RT-PCR reactions for negative controls;

— addition of precision data and results of interlaboratory study.

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

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 food surfaces, viruses are removed by swabbing. For soft fruit, 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 and 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 quantification of levels 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 15216-1:2013 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 1:
Method for quantification

1 Scope

This document specifies a method for the quantification of levels of HAV and norovirus genogroup I (GI) and II (GII) RNA, from test samples of foodstuffs (soft fruit, leaf, stem and bulb vegetables, bottled water, BMS) or food surfaces. Following liberation of viruses from the test sample, viral RNA is then extracted by lysis with guanidine thiocyanate and adsorption on silica. Target sequences within the viral RNA are amplified and detected by 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, food 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 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

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 22174, ISO 22119 and ISO 20838 and the following apply.

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

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

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

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

3.3
soft fruit
small edible stoneless fruit

EXAMPLE Strawberries, raspberries or currants

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

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, contact with contaminated water or food surfaces, 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 or through contact with contaminated water or food surfaces 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
quantification of HAV
estimation of number of copies of HAV RNA in a predetermined mass or volume of foodstuff, or area of food surface

3.8
quantification of norovirus
estimation of number of copies of norovirus RNA in a predetermined mass or volume of foodstuff, or area of food surface

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 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 control for 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; the cycle at which the target is quantified in a given real-time RT-PCR reaction

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

3.17
limit of detection
LOD
lowest concentration of target in a test sample that can be reproducibly detected (95 % confidence interval) under the experimental conditions specified in the method

Note 1 to entry: The LOD is related to the test portion and the quality of the template RNA.

3.18
limit of quantification
LOQ
lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy under the experimental conditions specified in the method

Note 1 to entry: The LOQ is related to the test portion and the quality of the template RNA.

4 Principle

4.1 Virus extraction

The foodstuffs and food 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 opposite ends. 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. Fluorescence can be measured at each stage throughout the cycle. The first cycle in the real-time RT-PCR at which amplification can be detected for any reaction is proportional to the quantity of template; therefore, analysis of the fluorescence plots enables determination of the concentration of target sequence in the sample.

Due to the low levels of virus template often present in foodstuffs or food surfaces and the strain diversity in the target viruses, 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 5.2.18 and 5.2.19. 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 food 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 Double-stranded DNA (dsDNA) control

For quantification of a target virus, results shall be related to a standard of known concentration. A dilution series of linear dsDNA carrying the target sequence of interest (5.3.11) and quantified using an appropriate method, e.g. spectrophotometry, fluorimetry, digital PCR etc. shall be used to produce a standard curve in template copies per microlitre. Reference to the standard curve enables quantification of the sample RNA in detectable virus genome copies per microlitre.

4.4.3 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 control for RT-PCR inhibition in individual samples, EC RNA (an RNA species carrying the target sequence of interest, 5.3.12) 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.

Alternative approaches for the assessment of inhibition of RT-PCR that can be demonstrated to provide equivalent performance to the use of EC RNA control are permitted.

4.5 Test results

This method provides a result expressed in detectable virus genome copies per millilitre, per gram or per square centimetre. In samples where virus is not detected, results shall be reported as "not detected; <*z* detectable virus genome copies per millilitre, per gram or per square centimetre" where *z* is the LOD for the sample.

5 Reagents

5.1 General

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

Follow current laboratory practice, as specified in 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).

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[3] 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' to 3' exonuclease activity) and sufficiently sensitive for the detection of virus RNA as expected in virus-contaminated foodstuffs and food 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 (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.

5.3 Prepared reagents

Because of the large number of reagents requiring individual preparation, details of composition and preparation are given in Annex B.

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 v/v); 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 (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 dsDNA control material. Purified linear DNA molecules carrying the target sequence for each target virus shall be used. The sequence of the DNA molecules shall be verified prior to first use. The preparations shall not cause RT-PCR inhibition. The concentrations of each dsDNA stock in template 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 1×10^4 to 1×10^5 template copies per microlitre. As EDTA can act as an inhibitor of RT-PCR, buffers used to dilute dsDNA shall not contain concentrations of EDTA greater than 1 mmol/l. Split the diluted dsDNA preparation (dsDNA control material) into single use aliquots and store at (5 ± 3) °C for up to 24 h, at −15 °C or below for up to six months, or at −70 °C or below for longer periods. See Annex G for illustrative details of the preparation of dsDNA (used in the development of this document).

5.3.12 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 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 ± 3) °C for up to 24 h, at −15 °C or below for up to six months, or at −70 °C or below for longer periods. See Annex H for illustrative details of the preparation of EC RNA (used in the development of this document).

6 Equipment and consumables

Standard microbiological laboratory equipment (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 should be used unless unobstructed tips are required, e.g. for aspiration (as in 6.7 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 Shaker capable of operating at approximately 50 oscillations min^{-1}.

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

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

6.7 Aspirator or equivalent apparatus for removing supernatant.

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

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

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

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

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

6.10 Microcentrifuge.

6.11 Centrifuge and microcentrifuge 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 cutoff.

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 truly representative sample which has not been damaged or changed during transport or storage, e.g. samples that were frozen on collection should not have been allowed to defrost prior to receipt at the laboratory, samples that were not frozen on collection should not have been frozen prior to receipt at the laboratory.

8 Procedure

8.1 General laboratory requirements

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

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

8.2.1 Process control virus material

Immediately before a batch of test samples is processed, pool together sufficient aliquots of process control virus material (5.3.10) for all individual 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 (8.4.2.2). Store at (5 ± 3) °C for a maximum of 24 h or at –15 °C or below for up to six months, or at –70 °C or below for longer periods.

8.2.2 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.

8.2.3 Food surfaces

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

Process the swab immediately, or place in a suitable container and store at (5 ± 3) °C for a maximum of 72 h or at –15 °C or below for up to six months, or at –70 °C or below for longer periods.

Add (10 ± 0,5) µl of process control virus material (8.2.1) to the swab. Immediately after the addition of process control virus material, immerse the swab in a tube containing (490 ± 10) µl lysis buffer, 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.

8.2.4 Soft fruit, leaf, stem and bulb vegetables

Soft fruit, 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 fruit, 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) and transfer to the sample compartment of a 400 ml mesh filter bag. Add (10 ± 0,5) µl of process control virus material (8.2.1) to the sample.

Add (40 ± 1) ml TGBE buffer (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).

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 ± 3) °C.

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 ± 3) °C for (60 ± 5) min.

Centrifuge at 10 000*g* for (30 ± 5) min at (5 ± 3) °C (split volume across two centrifuge tubes if necessary).

Decant and discard the supernatant, then centrifuge at 10 000*g* for (5 ± 1) min at (5 ± 3) °C 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.

For extraction from leaf, stem or bulb vegetables, transfer the suspension to a suitable tube and retain for RNA extraction.

Sample extract shall be processed immediately, or stored at (5 ± 3) °C for a maximum of 24 h or at –15 °C or below for up to six 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 ± 3) °C. Carefully transfer the aqueous (upper) phase to a fresh tube and retain for RNA extraction.

Sample extract shall be processed immediately, or stored at (5 ± 3) °C for a maximum of 24 h or at –15 °C or below for up to six months, or at –70 °C or below for longer periods.

8.2.5 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 (8.2.1) to the sample under test. Shake to mix.

Using a vacuum or positive pressure source (6.17), filter the 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 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 eluate 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.

Sample extract shall be processed immediately, or stored at (5 ± 3) °C for a maximum of 24 h or at –15 °C or below for up to six months, or at –70 °C or below for longer periods.

8.2.6 Bivalve molluscan shellfish

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 re-immersed 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 ± 3) °C for a maximum of 24 h at –15 °C or below for up to six 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 six months, or at –70 °C or below for longer periods.)

Add (10 ± 0,5) µl of process control virus material (8.2.1) 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 ± 2) °C with shaking at approximately 320 oscillations min^{-1} in a shaking incubator or equivalent (6.5) for (60 ± 5) min.

Carry out a secondary incubation by placing the tube in a water bath or equivalent at (60 ± 2) °C 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.

Supernatant shall be processed immediately, or stored at (5 ± 3) °C for a maximum of 24 h at –15 °C or below for up to six 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 ± 5) µl of elution buffer and retain for real-time RT-PCR analysis.

Extracted RNA shall be processed immediately, or stored at (5 ± 3) °C for a maximum of 24 h or at –15 °C or below for up to six months, or at –70 °C or below for longer periods.

For each batch of samples tested, a negative extraction control shall be included unless the batch includes a negative process control (8.2.2). 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 22174, ISO 22119 and ISO 20838.

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 I.

Results generated using 10^{-1} sample RNA are used only in the event that RT-PCR inhibition is >75 % for undiluted sample RNA (9.3); for matrices where RT-PCR inhibition is normally ≤75 % (food 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 >75 % 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, leaf, stem and bulb vegetables (matrices where RT-PCR inhibition is frequently >75 %).

Alternative approaches for RT-PCR inhibition control that can be demonstrated to provide equivalent performance to the use of EC RNA are permitted.

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).

Prepare 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of target dsDNA control material (5.3.11) in a suitable buffer, e.g. TE buffer (5.3.8). As EDTA can act as an inhibitor of RT-PCR, buffers used to dilute dsDNA shall not contain concentrations of EDTA greater than 1 mmol/l.

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 (5.3.12), and

— 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 the dsDNA standard curve, prepare

— two wells with (5 ± 0,5) µl of undiluted dsDNA,

— two wells with (5 ± 0,5) µl of 10^{-1} dsDNA,

— two wells with (5 ± 0,5) µl of 10^{-2} dsDNA,

— two wells with (5 ± 0,5) µl of 10^{-3} dsDNA, and

— two wells with (5 ± 0,5) µl of 10^{-4} dsDNA.

For negative controls, prepare

— two wells with (5 ± 0,5) µl of water (5.2.1), and

— two wells with (5 ± 0,5) µl of negative 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 (mastermix may also be added to all relevant wells before addition of template material).

NOTE The above describes the case where undiluted and 10^{-1} sample RNA are tested simultaneously; where RNA is only tested at one concentration (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 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 (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, and

— 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, and

— 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), and

— one well with (5 ± 0,5) µl of negative 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 (mastermix may also be added to all relevant wells before addition of template material).

NOTE The above describes the case where undiluted and 10^{-1} sample RNA are tested simultaneously; where RNA is only tested at one concentration (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 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 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 calculations.

9 Interpretation of results

9.1 General

Each control (dsDNA, 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 extraction or process control] shall always be negative; if positive results occur in these controls, then any samples giving positive results shall be retested.

9.2 Construction of standard curves

Use the results for each dilution series (process control virus RNA, target dsDNA) to create standard curves for each control by plotting the C_q values obtained against $\log_{10}$ concentration (e.g. $\log_{10}$ copies per microlitre target dsDNA) to determine r^2 (where r is Pearson's correlation coefficient), slope and intercept parameters. Do not average C_q values from duplicate reactions prior to plotting.

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 two such outlying C_q values shall be removed per series and values from a minimum of three (process control virus RNA) or four dilutions (dsDNA) shall be retained.

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 Calculation of RT-PCR inhibition

In this document, RT-PCR inhibition levels are used as quality assurance parameters only and are not used to adjust test results.

Use the undiluted sample RNA + EC RNA well C_q value to estimate RT-PCR inhibition by reference to the C_q value of the water + EC RNA well and the slope of the dsDNA standard curve as follows:

$$\text{RT-PCR inhibition} = (1 - 10^{(\Delta Cq/m)}) \times 100\ \%$$

where ΔC_q = C_q value (sample RNA + EC RNA) − C_q value (water + EC RNA) and m = slope of the dsDNA standard curve.

If the RT-PCR inhibition is ≤75 %, results for the undiluted RNA shall be used for that sample. If the RT-PCR inhibition is >75 %, repeat the calculation with the 10^{-1} sample RNA + EC RNA wells.

If the RT-PCR inhibition using the 10^{-1} RNA is ≤75 %, results for the 10^{-1} RNA shall be used for that sample. If RT-PCR inhibition levels for both undiluted and 10^{-1} sample RNA are >75 %, results are not valid and the sample shall be retested.

Under the condition that the dsDNA standard curve has an idealized slope of −3,32, a test sample with a ΔC_q of 0,00 has an RT-PCR inhibition level of 0% and a test sample with a ΔC_q of 2,00 has an RT-PCR inhibition level of 75 %. Where the ΔC_q is ≤2,00 the RT-PCR inhibition level is ≤75 % and therefore acceptable; if the ΔC_q is >2,00, the RT-PCR inhibition level is >75% and therefore not acceptable.

NOTE 1 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 (8.4.1), and where the RT-PCR inhibition for the undiluted sample RNA is >75 %, real-time RT-PCR shall be repeated using 10^{-1} sample RNA and the calculation repeated using the results of the repeat analysis.

NOTE 2 A sample showing an unacceptable RT-PCR inhibition level, but producing an otherwise valid positive result can, if appropriate, be reported as positive as described in Clause 10.

NOTE 3 In the case that the RT-PCR inhibition determined using undiluted sample RNA is ≤75 %, 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.

NOTE 4 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

In this document, extraction efficiencies are used as quality assurance parameters only and not used to adjust test results.

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; 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 = C_q value (sample RNA) − C_q value (undiluted process control virus RNA) and m = 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:

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

where p = the process control virus recovery and v = the total measured volume of supernatant in ml (8.2.6).

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 shall 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.

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

9.5 Sample quantification

For each target virus, take the C_q values for the sample RNA without EC RNA wells (undiluted or 10^{-1} dependent on the amplification efficiency results; 9.3) and use these to calculate target concentrations (in detectable virus genome copies per microlitre RNA) for each replicate by reference to the dsDNA standard curve as follows:

$$\text{concentration} = 10^{(\Delta Cq/m)}$$

where ΔC_q = C_q value (sample RNA) – standard curve intercept and m = slope of the dsDNA standard curve.

Negative replicates shall be given a concentration of zero copies per microlitre RNA. For each sample, calculate the average of the concentrations for both replicates.

For non-BMS samples, multiply this concentration by 100 (undiluted RNA) or 1 000 (10^{-1} RNA) to calculate the number of detectable virus genome copies in the entire sample.

For BMS samples, multiply this value by 200 (undiluted RNA) or 2 000 (10^{-1} RNA) then multiply by the total volume of supernatant in ml (8.2.6) to calculate the number of detectable virus genome copies in the entire sample.

To obtain the estimated concentration of target virus in detectable virus genome copies per millilitre, per gram or per square centimetre, divide the number of genome copies in the entire sample by the starting volume in ml (bottled water), mass in g (BMS digestive glands, soft fruits, leaf, stem or bulb vegetables) or area in cm^2 (hard surfaces).

EXAMPLE 1 A 300 ml bottled water sample that provides an average target concentration of 15 detectable virus genome copies per microlitre in its undiluted sample RNA will produce a calculated concentration of (15 × 100)/300 = 5 detectable virus genome copies per millilitre.

EXAMPLE 2 A BMS sample (comprising 2 g of digestive glands) that produces 2,5 ml of supernatant (8.2.6) and provides an average target concentration of 50 detectable virus genome copies per microlitre in its undiluted sample RNA will produce a calculated concentration of (50 × 200 × 2,5)/2 = 12 500 detectable virus genome copies per gram.

NOTE Where quantification calculations are generated by the software of the real-time PCR machine, these can be used provided that the user laboratory can demonstrate that any calculations made are accurate according to the formulae in this document.

10 Expression of results

Positive results for each target virus shall be expressed as "*x* detectable virus genome copies per millilitre", "*x* detectable virus genome copies per gram" or "*x* detectable virus genome copies per square centimetre" where *x* is the calculated concentration for that sample, provided that this level is above the LOQ of the method.

If target RNA is detected at levels <LOQ, results shall be expressed as "virus genome detected at levels below the limit of quantification" followed by "(*y* detectable virus genome copies per millilitre)", "(*y* detectable virus genome copies per gram)" or "(*y* detectable virus genome copies per square centimetre)" where *y* is the LOQ of the method.

If target virus is not detected results shall be expressed as "not detected" followed by "(<*z* detectable virus genome copies per millilitre)", "(<*z* detectable virus genome copies per gram)" or "(<*z* detectable virus genome copies per square centimetre)" where *z* is the LOD of the method.

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, if appropriate, be expressed as "virus genome detected in" followed by "*b* ml;" "*b* g;" or "*b* cm^2;" followed by "not quantifiable" where *b* is the amount of sample tested. Details shall be included in the test report.

If results from 10^{-1} RNA are used, LOD and LOQ values shall be adjusted upwards by multiplying by 10.

11 Precision

11.1 Interlaboratory study

Results of the interlaboratory study to determine the precision of the method are summarized in Annex J. Repeatability and reproducibility standard deviations and limits for HAV, norovirus GI and norovirus GII were determined using seven food matrices contaminated at various levels. The values derived from the interlaboratory study may not be applicable to concentration ranges and food matrices other than those given in Annex J.

11.2 Repeatability

The absolute difference between two independent single (log_{10}-transformed) test results (number of detectable virus genome copies per square centimetre, per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval will, in not more than 5 % of cases, exceed the repeatability limit *r*.

As a general indication of repeatability limit (*r*), the following values may be used when testing food samples in general:

— *r* = 0,60 (expressed as a difference between log_{10}-transformed test results; range for different matrices, target viruses and contamination levels 0,24 to 1,89), or

— *r* = 3,98 (expressed as a ratio between test results).

EXAMPLE A first test result of 10 000 or $1,0 \times 10^4$ detectable virus genome copies per gram of food product was observed in a laboratory. Under repeatability conditions, the ratio between the first test result and a second test result should not be greater than 3,98. So the second result should lie between 2 513 (10 000/3,98) and 39 800 (10 000 × 3,98) detectable virus genome copies per gram.

11.3 Reproducibility limit

The absolute difference between two single (log_{10}-transformed) test results (number of detectable virus genome copies per square centimetre, per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment will, in not more than 5 % of cases, exceed the reproducibility limit *R*.

As a general indication of reproducibility limit (*R*), the following values may be used when testing food samples in general:

— *R* = 1,35 (expressed as a difference between log_{10}-transformed test results, range 0,65 to 2,04), or

— *R* = 22,39 (expressed as a ratio between test results).

EXAMPLE A test result of 10 000 or $1,0 \times 10^4$ detectable virus genome copies per gram of food product was observed in a first laboratory. Under reproducibility conditions, the ratio between the test results from this first laboratory and a second laboratory should not be greater than 22,39. So the result from the second laboratory should be between 447 (10 000/22,39) and 223 900 (10 000 × 22,39) detectable virus genome copies per gram.

12 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this document, i.e. ISO 15216-1;

d) all operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

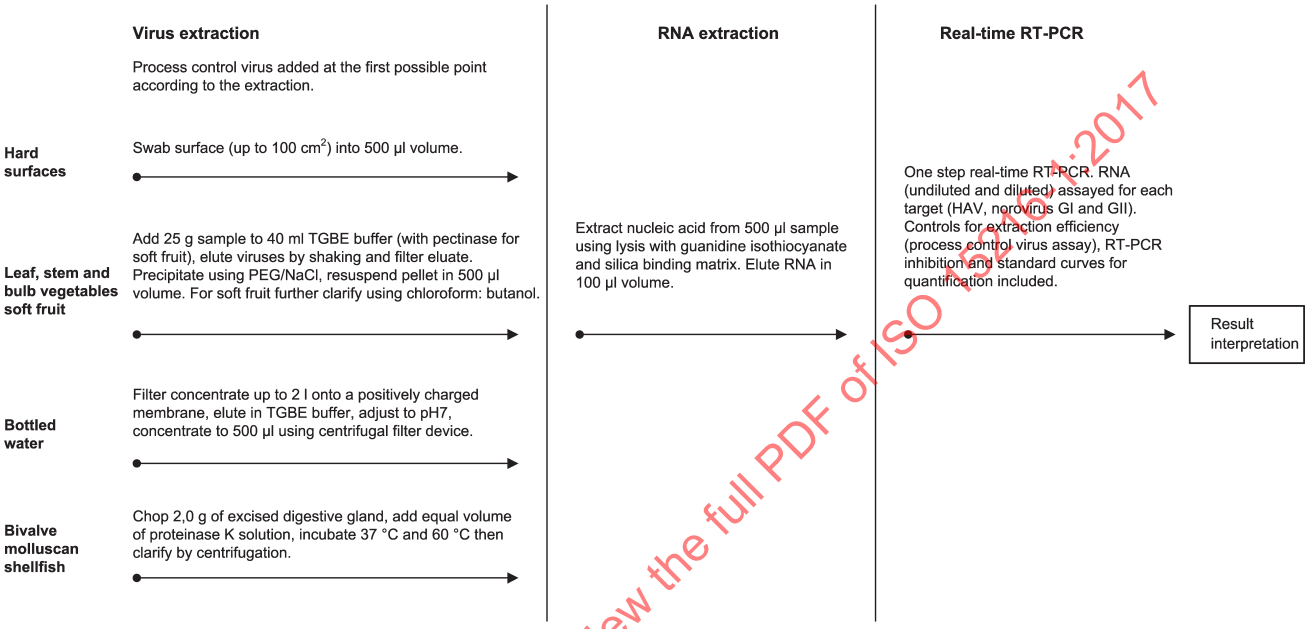
e) LOD (3.17) and LOQ (3.18) of the method (adjusted to account for use of 10^{-1} RNA if appropriate) and the matrix it was established in;

f) the extraction efficiency of the sample (9.4);

g) the test result(s) obtained, expressed according to Clause 10.

Annex A
(normative)

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	(500 ± 2) g
NaCl	(87 ± 1) g
Water	as required

B.1.2 Preparation

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

Store at (5 ± 3) °C for a maximum of six months.

B.2 Chloroform/butanol mixture (1:1 v/v)

B.2.1 Composition

Chloroform	(10 ± 0,1) ml
n-Butanol	(10 ± 0,1) ml

B.2.2 Preparation

Mix the components together.

Store at room temperature for a maximum of 12 months.

B.3 Proteinase K solution (3 000 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 water. Mix thoroughly.

Store in working volumes at –15 °C or below for a maximum of six months. Once defrosted, store at (5 ± 3) °C and use within 1 week.

B.4 Phosphate-buffered saline (PBS)

B.4.1 Composition

NaCl	(8,0 ± 0,1) g
Potassium chloride	(0,2 ± 0,01) g
Disodium hydrogenphosphate	(1,15 ± 0,01) g
Potassium dihydrogenphosphate	(0,2 ± 0,01) g
Water	(1 000 ± 10) ml

B.4.2 Preparation

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

Store at (5 ± 3) °C for a maximum of six months.

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

B.5.1 Composition

Tris base [*tris*(hydroxymethyl)aminomethane]	(12,1 ± 0,2) g
Glycine	(3,8 ± 0,1) g
Beef extract	(10 ± 1,0) g
Water	(1 000 ± 10) ml

B.5.2 Preparation

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

Store at (5 ± 3) °C for a maximum of six months.

B.6 Tris solution(1 mol/l)

B.6.1 Composition

Tris base [*tris*(hydroxymethyl)aminomethane]	(12,1 ± 0,2) g
Water (5.2.1)	as required

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 volume to (100 ± 1) ml with water. Sterilize by autoclaving.

Store at (5 ± 3) °C for a maximum of six months.

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

B.7.1 Composition

Ethylenediaminetetraacetic acid (EDTA)	(18,6 ± 0,2) g
Water (5.2.1)	as required

B.7.2 Preparation

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

Store at (5 ± 3) °C for a maximum of six 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 ± 3) °C for a maximum of six 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, 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.

NOTE Applied Biosystems real-time PCR machines and the 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	

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 [4]
HAV240 (REV):	GGA GAG CCC TGG AAG AAA G	Reference [4]
HAV150(-) (PROBE):	CCT GAA CCT GCA GGA ATT AA	Reference [4]

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 [157-188 base pairs (bp)] depending on the virus strain; for example a product of 174 bp corresponding to nucleotides 68-241 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 quantification 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.[4]

D.2 Norovirus GI

QNIF4 (FW):	CGC TGG ATG CGN TTC CAT	Reference [5]
NV1LCR (REV):	CCT TAG ACG CCA TCA TCA TTT AC	Reference [6]

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 [6]

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 [7]

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 5291–5376 of Norwalk virus (GenBank accession number M87661).

D.3 Norovirus GII

QNIF2 (FW):	ATG TTC AGR TGG ATG AGR TTC TCW GA	Reference [8]
COG2R (REV):	TCG ACG CCA TCT TCA TTC ACA	Reference [9]
QNIFs (PROBE):	AGC ACG TGG GAG GGC GAT CG	Reference [8]

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 5012–5100 of Lordsdale virus (GenBank accession number X86557).

The area selected for norovirus detection is the well-conserved region at the 5' end of ORF2.[9] Sequence alignments using all sequences available in GenBank of the assay target region demonstrate that these primer and probe sets are adequate for the quantification 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).[8][10]

D.4 Mengo virus

Mengo 110 (FW):	GCG GGT CCT GCC GAA AGT	Reference [11]
Mengo 209 (REV):	GAA GTA ACA TAT AGA CAG ACG CAC AC	Reference [11]
Mengo 147 (PROBE):	ATC ACA TTA CTG GCC GAA GC	Reference [11]

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 110–209 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 quantification of mengo virus is as similar as possible to that of HAV in terms of structure, length, and base composition.[4] The primer sequences do not align with any other sequences available in GenBank.

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 (ATCC® VR-1597™)[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. [12] This strain has been used as a process control virus in detection methods for HAV and norovirus[4] [10] 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 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).

2) ATCC® VR-1597™ and ATCC® CCL-2™ are trademarks of products supplied by the American Type Culture Collection. 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 NucliSENS® system

F.1 Reagents

F.1.1 NucliSENS®[3] lysis buffer.

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

F.2 Apparatus

F.2.1 NucliSENS® miniMAG®[3] or easyMAG®[3] instrument.

F.2.2 NucliSENS® magnetic rack.

F.2.3 Thermoshaker or equivalent apparatus for shaking 1,5 ml tubes at (60 ± 2) °C and approximately 1 400 oscillations min^{-1}.

F.2.4 Tubes with screw caps, capacity 1,5 ml, suitable for use with the NucliSENS® instrument.

F.3 Procedure

Add (2 ± 0,1) ml of NucliSENS® 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.

Transfer suspension to a 1,5 ml screw-cap tube. Wash for (30 ± 2) s using the automated wash steps of the NucliSENS® miniMAG® or NucliSENS® easyMAG® extraction systems. After washing, allow silica to sediment using the magnet of the NucliSENS® miniMAG® or NucliSENS® easyMAG® extraction systems. Discard supernatant by, for example, aspiration.

Separate tubes from magnet, then add (400 ± 10) µl wash buffer 1. Resuspend pellet, wash for (30 ± 2) s, allow silica to sediment using magnet then discard supernatant.

3) NucliSENS®, miniMAG® and easyMAG® are trade names of products 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 products named. Equivalent products may be used if they can be shown to lead to the same results.

Separate tubes from magnet, then add (500 ± 10) µl wash buffer 2. Resuspend pellet, wash for (30 ± 2) s, allow silica to sediment using magnet then discard supernatant. Repeat.

Separate tubes from magnet, then add (500 ± 10) µl wash buffer 3 (samples shall not be left in wash buffer 3 for more time than necessary). Wash for (15 ± 1) s, allow silica to sediment using magnet then discard supernatant.

Add (100 ± 5) µl elution buffer. Cap tubes and transfer to thermoshaker or equivalent and incubate for (5,0 ± 0,5) min at (60 ± 2) °C with shaking at approximately 1 400 oscillations min^{-1}.

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

Annex G
(informative)

Generation of dsDNA control stocks

G.1 General

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

In the development of this document linearized plasmids carrying copies of the target genes were used as dsDNA controls. 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. Complete insert target sequences for these plasmids are as follows:

HAV

```
   1   TCACCGCCGT TTGCCTAGGC TATAGGCTAA ATTTTCCCTT TCGGATCCCC CTTTCCTATT
  61   CCCTTTGTTT TGCTTGTAAA TATTGATTTC TAAATATTGA TTCCTGCAGG TTCAGGGTTC
 121   TTAAATCTGT TTCTCTATAA GAACACTCAT TTCACGCTTT CTGTCTTCTT TCTTCCAGGG
 181   CTCTCC
```

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 JM109 strain competent cells.

G.2.2 LB broth, 10 g/l Bacto tryptone,[4] 5 g/l yeast extract, 10 g/l NaCl, adjusted to pH 7,0.

4) Bacto tryptone is a product 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.

G.2.3 LB agar, LB broth plus 15 g/l agar.

G.2.4 Ampicillin.

G.2.5 Plasmid miniprep reagents.

G.2.6 Restriction enzymes for linearization and associated buffers.

G.2.7 PCR purification reagents.

G.2.8 DNA gel electrophoresis reagents and equipment.

G.2.9 Ice.

G.2.10 Incubator, capable of operating at (37 ± 2) °C.

G.2.11 Shaking incubator or equivalent operating at (37 ± 2) °C and approximately 160 oscillations min^{-1}.

G.3 Transformation

Add 100 ng to 1 µg of previously purified dsDNA control to (50 ± 2,5) µl of JM109 competent cells and mix gently. Chill on ice for (20 ± 1) min.

Incubate at (42 ± 1) °C for (45 ± 2) s then immediately chill on ice for (120 ± 10) s.

Add (950 ± 20) µl of LB broth to the cells then shake at (37 ± 2) °C at approximately 160 oscillations min^{-1} for (90 ± 10) min.

Spread (100 ± 5) µl of inoculated broth on to an LB agar plate supplemented with 50 µg/ml ampicillin.

Incubate plate at (37 ± 2) °C overnight, check for growth of colonies, then store at (5 ± 3) °C until required for purification of plasmid DNA.

G.4 Purification of plasmid DNA

Inoculate (5 ± 0,1) ml of LB broth supplemented with 100 µg/ml ampicillin with a single colony containing the plasmid of interest.

Incubate at (37 ± 2) °C overnight.

Purify plasmid DNA from the culture using miniprep reagents following the appropriate protocol.

G.5 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)[5)] and buffers as recommended by the manufacturer of the enzyme.

Incubate at (37 ± 2) °C for 2 h ± 5 min.

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

5) 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.

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

Store linearized plasmid DNA stock at −15 °C or below until required for preparation of the dsDNA control material (5.3.11).

G.6 Quantification of dsDNA control

Determine the absorbance at 260 nm of the linearized plasmid stock using spectrophotometry.

Multiply the absorbance reading by 5×10^{-8} (and by the dilution factor used if diluted linearized plasmid stock is measured) to give the concentration of DNA in grams per microlitre.

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

The mass of an individual plasmid molecule may be calculated by multiplying the plasmid length in bp by 607,4 (the relative molecular mass of an average bp) and dividing by the Avogadro constant ($6{,}02 \times 10^{23}$), e.g. a plasmid of 3 000 bp has a mass of $3{,}02 \times 10^{-18}$ g.[6)]

6) For the plasmids used in the development of this document, the respective lengths and masses are as follows: HAV – 3 383 bp, $3{,}41 \times 10^{-18}$ g; Norovirus GI – 3 287 bp, $3{,}32 \times 10^{-18}$ g; Norovirus GII – 3 292 bp, $3{,}32 \times 10^{-18}$ g.

Annex H
(informative)

Generation of EC RNA stocks

H.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.

Linearized dsDNA control plasmids as described in Annex G are used for the production of EC RNA.

H.2 Reagents and apparatus

H.2.1 ***In vitro*** **RNA transcription reagents** (RNA polymerase, NTPs, buffer, etc.).

H.2.2 **RNase-free DNase**.

H.2.3 **RNA purification reagents**.

H.2.4 **Reagents and equipment for one-step real-time RT-PCR with hydrolysis probes.**

H.2.5 **Incubator**, capable of operating at (37 ± 2) °C.

H.3 *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.[7)]

Incubate at (37 ± 2) °C for (120 ± 5) min.

Add RNase-free DNase to the reaction and incubate at (37 ± 2) °C for (15 ± 1) min.

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

H.4 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 ± 2) °C for (5,0 ± 0,5) min.

Subject EC RNA stock or an appropriate dilution thereof to real-time RT-PCR [alongside a dsDNA dilution series (8.4) as a standard curve] 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 for a dsDNA standard curve with an idealized slope of −3,32), the stock is contaminated with DNA and shall be retreated with DNase (H.3). If levels are <0,1 %, store at −15 °C or below until required for preparation of the EC RNA control material (5.3.12).

7) 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.

H.5 Quantification of EC RNA

Determine the absorbance at 260 nm of the DNase-treated EC RNA stock (H.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.

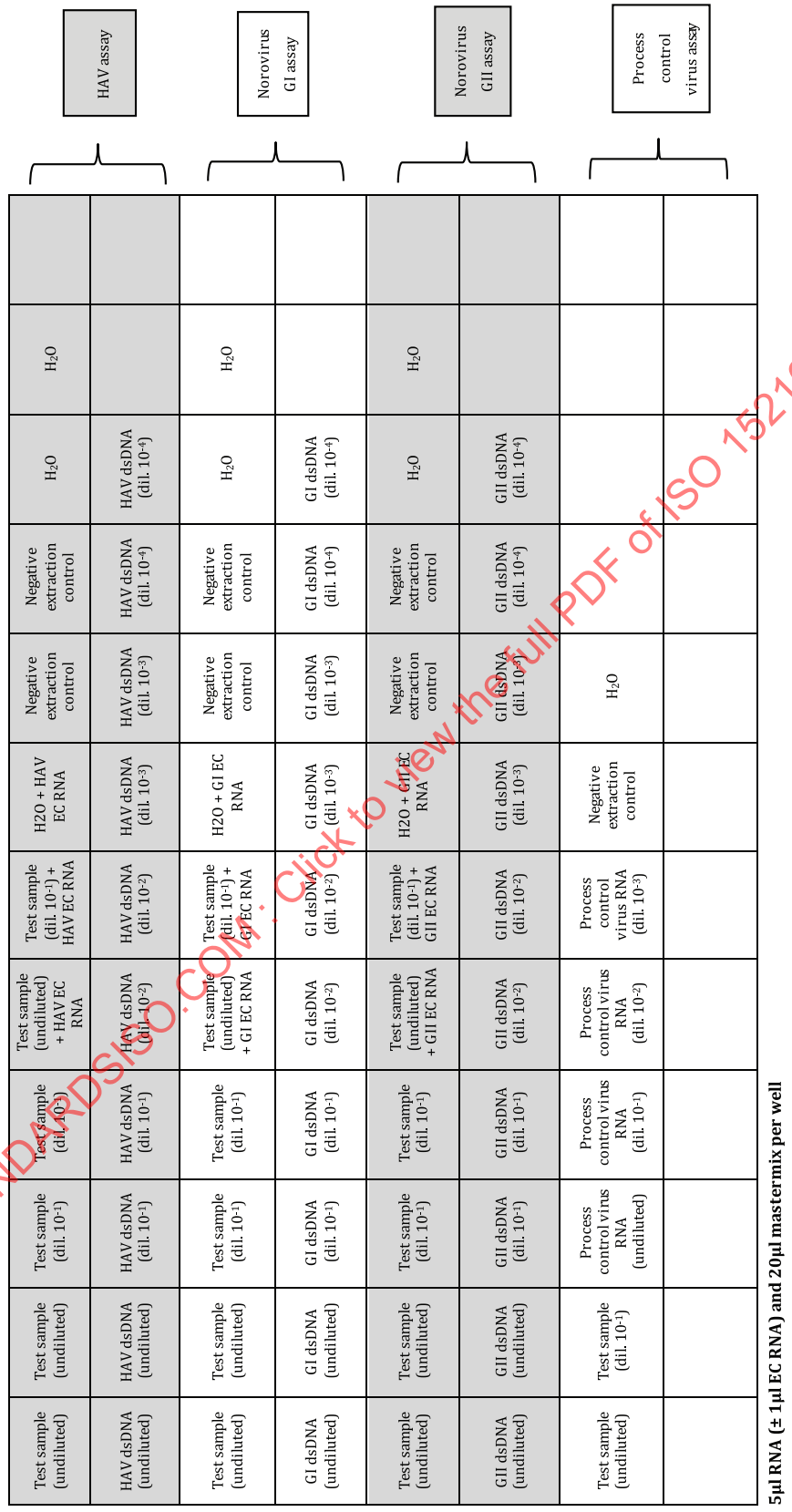
The mass of an individual RNA molecule may be calculated by multiplying the RNA length in ribonucleotides by 320,5 (the relative molecular mass of an average ribonucleotide) and dividing by the Avogadro constant ($6,02 \times 10^{23}$), e.g. an RNA molecule of 200 ribonucleotides has a mass of $1,06 \times 10^{-19}$ g.[8)]

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

Annex I
(informative)

Typical optical plate layout

Table I.1 — Typical optical plate layout



Annex J
(informative)

Method validation studies and performance characteristics

An international interlaboratory study involving 18 laboratories in 11 countries was carried out on food surfaces (the exterior surfaces of bell peppers), raspberries, lettuce, green onions, bottled water, Pacific oysters and common mussels. 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 (mean values of participants' results for "high", "medium" and "low" contamination levels for each matrix and virus are shown in Tables J.1 to J.21). 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 2013-2014 by the Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom.

The method submitted to the interlaboratory study included the specific methods for real-time RT-PCR and RNA extraction detailed in Annex C and Annex F, respectively. Precision data including repeatability and reproducibility limits were calculated in accordance with ISO 5725-2; these are shown in Tables J.1 to J.21.

Table J.1 — Results of data analysis obtained with HAV in food surfaces

	Sample type (contamination level)		
	(low level)	(medium level)	(high level)
Number of participating collaborators	10	10	10
Number of collaborators retained after evaluation of the data	8	10	10
Number of samples	20	20	20
Number of samples retained after evaluation of the data	16	19	20
Mean value Σa ($\log_{10}$ copies/cm^2)	0,69	1,39	2,17
Repeatability standard deviation s_r ($\log_{10}$ copies/cm^2)	0,13	0,31	0,16
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ copies/cm^2)	0,35	0,88	0,43
as ratio on normal scale (copies/cm^2)	2,26	7,52	2,72
Reproducibility standard deviation s_R ($\log_{10}$ copies/cm^2)	0,45	0,48	0,23
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ copies/cm^2)	1,26	1,33	0,65
as ratio on normal scale (copies/cm^2)	18,29	21,53	4,44

Table J.2 — Results of data analysis obtained with norovirus GI in food surfaces

	Sample type (contamination level)		
	(low level)	(medium level)	(high level)
Number of participating collaborators	10	10	10
Number of collaborators retained after evaluation of the data	10	9	10
Number of samples	20	20	20
Number of samples retained after evaluation of the data	19	17	20
Mean value Σa ($\log_{10}$ copies/cm^2)	0,79	1,47	2,03
Repeatability standard deviation s_r ($\log_{10}$ copies/cm^2)	0,30	0,20	0,21
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ copies/cm^2)	0,83	0,57	0,59
as ratio on normal scale (copies/cm^2)	6,77	3,72	3,90
Reproducibility standard deviation s_R ($\log_{10}$ copies/cm^2)	0,35	0,39	0,27
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ copies/cm^2)	0,97	1,09	0,77
as ratio on normal scale (copies/cm^2)	9,39	12,37	5,85

Table J.3 — Results of data analysis obtained with norovirus GII in food surfaces

	Sample type (contamination level)		
	(low level)	(medium level)	(high level)
Number of participating collaborators	10	10	10
Number of collaborators retained after evaluation of the data	8	8	10
Number of samples	20	20	20
Number of samples retained after evaluation of the data	16	16	20
Mean value Σa ($\log_{10}$ copies/cm^2)	0,36	1,03	1,57
Repeatability standard deviation s_r ($\log_{10}$ copies/cm^2)	0,32	0,13	0,18
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ copies/cm^2)	0,89	0,37	0,49
as ratio on normal scale (copies/cm^2)	7,70	2,37	3,10
Reproducibility standard deviation s_R ($\log_{10}$ copies/cm^2)	0,50	0,50	0,39
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ copies/cm^2)	1,39	1,40	1,09
as ratio on normal scale (copies/cm^2)	24,64	24,87	12,32