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**Microbiology of food and animal feed —
Horizontal method for determination
of hepatitis A virus and norovirus in
food using real-time RT-PCR —**

**Part 2:
Method for qualitative detection**

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

Partie 2: Méthode de détection qualitative



Reference number
ISO/TS 15216-2:2013(E)

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
- an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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.

ISO/TS 15216-2 was prepared by the European Committee for Standardization (CEN), in collaboration with Technical committee ISO/TC 34, *Food products*, Subcommittee SC 9 *Microbiology*.

This corrected version of ISO/TS 15216-2:2013 incorporates the following corrections.

- Throughout, textual references have been updated to take reordering of the annexes into account. [Annex B](#) was formerly Annex E; [Annex C](#) was formerly Annex D; [Annex D](#) was formerly Annex G; [Annex E](#) was formerly Annex C; [Annex F](#) was formerly Annex B; [Annex G](#) was formerly Annex H; [Annex H](#) was formerly Annex F.
- Where units of shaking operations are mentioned, “oscillations min⁻¹” replaces “min⁻¹”.
- Many cross-references to reagents or apparatus subclauses are added.
- A phrase citing [Annex A](#) is added to the end of the introduction.
- The definitions for “food surface” (formerly 3.2 and 3.3) are combined and expanded in a redrafted [3.2](#); in consequence, the following terms in [Clause 3](#) are renumbered.
- In [3.4](#), Note 2, “There is only one serotype” is transposed to the end of Note 1. Also, “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” replaces “UK Advisory Committee on Dangerous Pathogens (ACDP) hazard group 2 pathogen”.

- In G.5, “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” replaces “mastermix, the”.

ISO/TS 15216 consists of the following parts, under the general title *Microbiology of food and animal feed — Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR*:

- *Part 1: Method for quantification*
- *Part 2: Method for qualitative detection*

Introduction

Hepatitis A virus (HAV) and norovirus (NoV) are important agents of food-borne human viral illness. No routine methods exist to culture these viruses from 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 and salad 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, viruses are extracted from the tissues of the digestive glands using treatment with a proteinase K solution. For all matrices which are not covered by this Technical Specification, 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 PCR cycle by measuring the excitation of fluorescently labelled molecules. In the 5' fluorogenic nuclease real-time RT-PCR assay, the fluorescent labels are attached to a sequence-specific nucleotide probe (hydrolysis probe) that also enables simultaneous confirmation of target template. These modifications increase the sensitivity and specificity of the PCR method, and obviate the need for additional amplification product confirmation steps post PCR. Due to the complexity of the method, it is necessary to include a comprehensive suite of controls. The method described in this part of ISO/TS 15216 enables qualitative detection of virus RNA in the test sample. A schematic diagram of the testing procedure is shown in [Annex A](#).

Microbiology of food and animal feed — Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR —

Part 2: Method for qualitative detection

1 Scope

This part of ISO/TS 15216 describes a method for qualitative detection of HAV and NoV genogroups I (GI) and II (GII), from test samples of foodstuffs 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 approach is also relevant for detection of the target viruses on fomites, or of other human viruses in foodstuffs, on food surfaces or on fomites following appropriate validation and using target-specific primer and probe sets.

2 Normative references

The following referenced documents are indispensable for the application 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 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 and the following apply.

3.1

foodstuff

substance used or prepared for use as food

Note 1 to entry: For the purposes of this part of ISO/TS 15216, this definition includes bottled water.

3.2

food surface

surface of food, food preparation surface or food contact surface

3.3

fomite

inanimate object or material on which infectious agents can be conveyed

3.4
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. Hepatitis A virus 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.

3.5
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 five separate genogroups.

Note 2 to entry: 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. 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. Genogroup I and II 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.

3.6
detection of hepatitis A virus

detection of HAV RNA in a predetermined mass or volume of foodstuff, or area of food surface

3.7
detection of norovirus

detection of norovirus RNA in a predetermined mass or volume of foodstuff, or area of food surface

3.8
process control virus

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

3.9
process control virus RNA

RNA released from the process control virus in order to produce standard curve data for the estimation of extraction efficiency

3.10
negative RNA extraction control

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

3.11
negative process control

target pathogen-free sample of the food matrix which is run through all stages of the analytical process

3.12
hydrolysis probe

fluorescent probe coupled with two fluorescent molecules which are sterically separated by the 5'-3'-exonuclease activity of the enzyme during the amplification process

3.13**negative 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

3.14**external control RNA**

reference RNA that can serve as target for the real-time PCR assay of relevance, e.g. RNA synthesized by *in-vitro* transcription from a plasmid carrying a copy of the target gene, which is added to an aliquot of sample RNA in a defined amount to serve as a control for amplification in a separate reaction

3.15 **C_q value**

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

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

3.16**theoretical limit of detection****tLOD**

level that constitutes the smallest quantity of target that can in theory be detected

Note 1 to entry: This corresponds to one genome copy per volume of RNA tested in the target assay, but varies according to the test matrix and the quantity of starting material.

3.17**practical limit of detection****pLOD**

lowest concentration of target in a test sample that can be reproducibly detected (95 % confidence interval) under the experimental conditions specified in the method, as demonstrated by a collaborative trial or other validation

Note 1 to entry: The pLOD is related to the test portion, the quality or quantity of the template RNA, and the tLOD of the method.

4 Principle**4.1 Virus extraction**

The foodstuffs and food surfaces covered by this part of ISO/TS 15216 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 clean RNA preparations to reduce the effect of PCR inhibitors. In this part of ISO/TS 15216 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 reverse transcription polymerase chain reaction (real-time RT-PCR)

This Technical Specification 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 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 broken down and the fluorescent signal from the label increases proportionately.

Due to the low levels of virus template often present in foodstuffs 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 [5.2.17](#) and [5.2.18](#). Illustrative details of reagents, primers, and probes (used in the development of this part of ISO/TS 15216) are provided in [Annexes B](#) and [C](#).

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 control for these losses, samples are spiked prior to processing 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 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 under test.

An example of the preparation of process control virus (used in the development of this part of ISO/TS 15216) is provided in [Annex D](#).

4.4.2 External amplification control (EC) RNA control

Many foodstuffs 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, external control (EC) RNA (an RNA species carrying the target sequence of interest, 5.3.8) is added to an aliquot of sample RNA and tested using the 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 an RT-PCR positive control.

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

4.5 Test results

For food surfaces, this method provides a result 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.^[10]

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 (Na₂HPO₄).

5.2.6 Potassium dihydrogenphosphate (KH₂PO₄).

5.2.7 Tris base.

5.2.8 Glycine.

5.2.9 Beef extract powder.

5.2.10 Proteinase K (30 U/mg).

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

5.2.12 Chloroform.

5.2.13 Butanol.

5.2.14 Sodium hydroxide (NaOH).

5.2.15 Hydrochloric acid (HCl).

5.2.16 Silica, lysis, wash, and elution buffers for extraction of viral RNA. Reagents shall enable processing of 500 µl of extracted virus, using lysis with a chaotropic buffer containing guanidine thiocyanate (Reference [1]) 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 E](#) for illustrative details of RNA extraction reagents (used in the development of the method described in this part of ISO/TS 15216).

5.2.17 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 RT-PCR using hydrolysis probes (the DNA polymerase used shall possess 5'-3' exonuclease activity) and sufficiently sensitive for the detection of levels of virus RNA as typically found in virus-contaminated foodstuffs. See [Annex B](#) for illustrative details of one step real-time RT-PCR reagents (used in the development of this part of ISO/TS 15216).

5.2.18 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 C](#) for illustrative details of primers and hydrolysis probes (used in the development of this part of ISO/TS 15216).

5.2.19 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 F](#).

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

5.3.2 Chloroform/butanol mixture. See F.2.

5.3.3 Proteinase K solution. See F.3.

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

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

5.3.6 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 $(-80 \pm 5) ^\circ\text{C}$. See [Annex D](#) for illustrative details of the preparation of process control virus (used in the development of the method described in this part of ISO/TS 15216).

5.3.7 Real-time RT-PCR mastermixes for target and process control virus. Reagents shall be added in quantities as specified by the manufacturers ([5.2.17](#)) 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 B](#) for illustrative details of real-time RT-PCR mastermixes (used in the development of this part of ISO/TS 15216).

5.3.8 External control (EC) RNA control material. Separate 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 and stock shall be diluted to a concentration of 1×10^6 to 1×10^8 template copies per microlitre. Split the diluted EC RNA preparation (EC RNA control material) into single use aliquots and store frozen at $-15 ^\circ\text{C}$ or below. See [Annex G](#) for illustrative details of the preparation of EC RNA (used in the development of this part of ISO/TS 15216).

6 Apparatus and materials

Standard microbiological laboratory equipment (ISO 7218)^[10] 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.

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 500 oscillations min^{-1} .

6.5 Shaking incubator operating at $(37 \pm 1,0) ^\circ\text{C}$ and (320 ± 20) oscillations min^{-1} or equivalent.

6.6 Rocking platform(s) or equivalent for use at room temperature and $(4 \pm 2) ^\circ\text{C}$ at (60 ± 5) oscillations min^{-1} .

- 6.7 Aspirator** or equivalent apparatus for removing supernatant.
- 6.8 Heating block** capable of operating at $(95 \pm 1,0) ^\circ\text{C}$ or equivalent.
- 6.9 Water bath** capable of operating at $(60 \pm 2,0) ^\circ\text{C}$ or equivalent.
- 6.10 Centrifuge(s) and rotor(s)** capable of the following run speeds, run temperatures, and rotor capacities:
- a) $10\,000 \times g$ at $(5 \pm 3) ^\circ\text{C}$ with capacity for tubes of at least 35 ml volume;
 - b) $10\,000 \times g$ at $(5 \pm 3) ^\circ\text{C}$ with capacity for narrow gauge (15 mm is too large) chloroform-resistant tubes of at least 1 ml volume;
 - c) $4\,000 \times g$ at room temperature with capacity for centrifugal filter concentration devices ([6.17](#)).
- 6.11 Microcentrifuge.**
- 6.12 Centrifuge and microcentrifuge tubes and bottles** of a range of sizes, 1,5 ml, 5 ml, 15 ml, 50 ml, etc. Narrow gauge (15 mm is too large) chloroform-resistant tubes with 1 ml capacity are necessary.
- 6.13 pH meter** (or pH testing strips).
- 6.14 Sterile cotton swabs.**
- 6.15 Mesh filter bags** (400 ml).
- 6.16 Positively charged membrane filters** with $0,45 \mu\text{m}$ pore size (47 mm diameter).
- 6.17 Centrifugal filter concentration devices** with 15 ml capacity and 100 kDa relative molecular mass cutoff.
- 6.18 Vacuum source** or equivalent positive pressure apparatus for filtering and filtration tower with aperture for 47 mm diameter membrane.
- 6.19 Sterile shucking knife** for opening bivalve molluscan shellfish (BMS).
- 6.20 Rubber block** for opening BMS.
- 6.21 Scissors.**
- 6.22 Forceps.**
- 6.23 Sterile Petri dishes.**
- 6.24 Razor blades** or equivalent homogenizer.
- 6.25 Heavy duty safety glove.**
- 6.26 RNA extraction equipment** suitable for extraction methods using silica and associated reagents ([5.2.16](#)). See [Annex E](#) for illustrative details of RNA extraction apparatus (used in the development of this part of ISO/TS 15216).

6.27 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 PCR with hydrolysis probe chemistry.

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

8 Procedure

8.1 General laboratory requirements

Unfrozen samples arriving at the laboratory shall not be frozen prior to testing. Samples arriving already frozen should be defrosted prior to testing. Sample extraction and 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 (5.3.6) for all individual test samples (allow 10 µl per test sample plus 25 µl excess).

Dilute a $(20 \pm 0,5)$ µl portion of pooled process control virus material to 10^{-1} using water (5.2.1) and store at (5 ± 3) °C for a maximum of 24 h or in single-use aliquots at -15 °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 premoistened in PBS (5.3.4), intensively swab the surface (maximum area, 100 cm²) under test, applying a little pressure to detach virus particles. Record the approximate area swabbed in square centimetres.

Add $(10 \pm 0,1)$ 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 ± 5) 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.

For rough surfaces that may cause deterioration of the swab, more than one swab may be required to completely treat the target surface.

Retain for RNA extraction.

8.2.4 Soft fruit and salad vegetables

Coarsely chop ($25 \pm 0,3$) g of soft fruits or salad vegetables into pieces of approximately $2,5 \text{ cm} \times 2,5 \text{ cm} \times 2,5 \text{ cm}$ (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 (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) and ($10 \pm 0,1$) μl of process control virus material (8.2.1).

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 $9,5 \pm 0,1$ with NaOH. Extend the period of incubation by 10 min for every time the pH is adjusted. Decant the eluate from the filtered compartment into a centrifuge tube (use two tubes if necessary to accommodate volume).

Clarify by centrifugation at $10\,000 \times g$ for (30 ± 5) min at (5 ± 3) °C.

Decant the supernatant into a single clean tube or bottle and adjust to pH $7,0 \pm 0,5$ with HCl.

Add 0,25 volumes of $5 \times \text{PEG/NaCl}$ 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 around 60 oscillations min^{-1} at (5 ± 3) °C for (60 ± 5) min.

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

Decant and discard the supernatant, then centrifuge at $10\,000 \times g$ 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). If a single sample has been split across two tubes, resuspend both pellets stepwise in the same aliquot of PBS.

For extraction from salad vegetables, transfer the suspension to a suitable tube and retain for RNA extraction.

For extraction from soft fruits, a further clarification step is required. Transfer the suspension to a narrow gauge chloroform-resistant centrifuge tube (6.12). Add (500 ± 10) μl chloroform/butanol mixture (5.3.2), vortex to mix, then incubate at room temperature for 5 min.

Centrifuge at $10\,000 \times g$ for (15 ± 1) min at (5 ± 3) °C. Carefully transfer the aqueous phase to a fresh tube and retain for RNA extraction.

8.2.5 Bottled water

This part of ISO/TS 15216 is appropriate for volumes between 0,3 l and 5 l. For each sample, record the volume tested.

Add ($10 \pm 0,1$) μl of process control virus material (8.2.1) to the sample under test. Shake to mix.

Using a vacuum or positive pressure source (6.18), filter entire sample through a positively charged 47 mm membrane (6.16) using aseptic techniques. Transfer the filter into a sterile tube, then add ($4 \pm 0,1$) ml of TGBE buffer (5.3.5).

Add ($10 \pm 0,2$) ml TGBE buffer to the empty bottle. Shake both tube and bottle at approximately 500 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 ($2 \pm 0,1$) ml TGBE buffer by gentle shaking and inversion by hand, and add to the tube.

Adjust the eluates to pH $7,0 \pm 0,5$ with 0,1 mol/l HCl and transfer to a centrifugal filter concentration device (6.17).

Centrifuge at $4\,000 \times g$ 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.

8.2.6 Bivalve molluscan shellfish (BMS)

BMS for analysis shall be live, or if frozen, undamaged. 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. 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.

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

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

Add $(10 \pm 0,1)$ μl of process control virus material (8.2.1).

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

Carry out a secondary incubation by placing the tube in a water bath or equivalent at $(60 \pm 2,0)$ °C for (15 ± 1) min.

Centrifuge at $3\,000 \times g$ for $(5,0 \pm 0,5)$ min at room temperature, decant the supernatant into a clean tube, measure and record the volume of supernatant, in millilitres, and retain for RNA extraction.

8.3 RNA extraction

Extract RNA from (500 ± 10) μl of each sample 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, stored at (5 ± 3) °C for <8 h or at -15 °C or below for up to 6 months.

For long-term storage, a temperature of (-80 ± 5) °C is recommended.

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 E for illustrative details of an RNA extraction method (used in the development of this part of ISO/TS 15216).

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 PCR are specified in ISO 22174.

8.4.2 Real-time RT-PCR

This part of ISO/TS 15216 describes methods for the detection of HAV, norovirus genogroup I and norovirus genogroup II.

Under certain circumstances, testing for all three viruses in a single sample is not necessary. The procedure described in the following enables a test sample to be analysed for one virus (i.e. HAV, norovirus

genogroup I or norovirus genogroup II) 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](#). Alternative approaches for RT-PCR inhibition control that can be demonstrated to provide equivalent performance to the use of EC RNA are permitted.

8.4.2.1 Analysis of target virus

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

For each sample prepare:

- one well of an optical plate with $(5 \pm 0,1)$ μl of undiluted sample RNA;
- one well with $(5 \pm 0,1)$ μl of 10^{-1} sample RNA;
- one well with $(5 \pm 0,1)$ μl of undiluted sample RNA and $(1 \pm 0,05)$ μl of undiluted EC RNA ([5.3.8](#));
- one well with $(5 \pm 0,1)$ μl of 10^{-1} sample RNA and $(1 \pm 0,05)$ μl of undiluted EC RNA.

For the EC RNA control prepare:

- one well with $(5 \pm 0,1)$ μl of water ([5.2.1](#)) and $(1 \pm 0,05)$ μl of undiluted EC RNA;

For negative controls prepare:

- one well with $(5 \pm 0,1)$ μl of water ([5.2.1](#));
- one well with $(5 \pm 0,1)$ μl of negative extraction control or negative process control RNA.

Add $(20 \pm 0,5)$ μl of the relevant real-time RT-PCR mastermix ([5.3.7](#)) to each well (mastermix may also be added to all relevant wells before addition of template material).

8.4.2.2 Analysis of process control virus

Defrost if necessary one aliquot of diluted (10^{-1}) process control virus material ([8.2.1](#)) for the batch used with the samples under test.

Heat at (95 ± 2) °C for $(5,0 \pm 0,5)$ min using a heating block or equivalent to release RNA.

Chill tubes rapidly, centrifuge at $\geq 3\,000 \times g$ for 1 min, then transfer the supernatant ("process control virus RNA") to a fresh tube.

Prepare 10^{-1} , 10^{-2} and 10^{-3} dilutions of process control virus RNA in water ([5.2.1](#)) for each batch of process control virus material.

For each sample prepare:

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

For the process control virus RNA standard curve prepare:

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

For negative controls prepare:

- one well with $(5 \pm 0,1)$ μl of water ([5.2.1](#));

— one well with $(5 \pm 0,1)$ μl of negative extraction control or negative process control RNA.

Add $(20 \pm 0,5)$ μl of process control virus real-time RT-PCR mastermix ([5.3.7](#)) to each well (mastermix may also be added to all relevant wells before addition of template material).

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 ([6.27](#)). 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 B](#) for illustrative details of an amplification method (used in the development of this part of ISO/TS 15216).

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 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 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 process control virus RNA standard curve

Check C_q values of the process control virus RNA standard curve dilution series for any points that do not fall close to the line of best fit. These C_q values shall not be incorporated into standard curve calculations.

Use the remaining C_q values of each dilution series to create a standard curve. Points from a minimum of three dilutions shall be included. Curves with r^2 values of $<0,98$, where r is Pearson's correlation coefficient, shall not be used for calculations.

9.3 Control for amplification efficiency

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 $\geq 2,00$ 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 $\geq 2,00$ greater than the C_q value of the water + EC RNA well, it is possible that results are not valid and the sample can require retesting.

NOTE 1 A sample showing an unacceptable amplification efficiency, but producing an otherwise valid positive result can, if appropriate, be reported as positive as described in [Clause 10](#).

NOTE 2 If alternative methods for determining amplification efficiency 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 amplification efficiency results ([9.3](#)) to estimate process control virus recovery by reference to the process control virus RNA standard curve (if 10^{-1} sample RNA results are used, multiply by 10 to correct for the dilution factor).

For BMS samples, calculate the extraction efficiency by dividing the recovery by 0,5 and multiplying by the total measured homogenate volume.

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.

NOTE 1 A sample producing the same C_q value as undiluted process control virus RNA has a process control virus recovery (equal to the extraction efficiency in matrices other than BMS) of 100 %. For a process control virus RNA standard curve with an idealized slope of $-3,32$, if the C_q value of an undiluted sample RNA well is $< 6,64$ greater than the C_q value of the undiluted process control virus RNA, the process control virus recovery for that sample is $> 1\%$ and therefore acceptable.

NOTE 2 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 Theoretical limit of detection

The theoretical limit of detection (tLOD) is the level that constitutes the smallest quantity of target that is in theory be detected. This corresponds to one genome copy per volume of RNA tested in the target assay, but varies according to the test matrix and the quantity of starting material.

For each hard surface, soft fruit, salad vegetable or bottled water sample, the minimum quantity of target in the entire sample that is detectable in theory is 20 (results using undiluted sample RNA) or 200 genome copies (10^{-1} RNA).

For BMS samples, the theoretical minimum quantity detectable is obtained by dividing the above values by 0,5 and multiplying by the total homogenate volume.

To obtain the tLOD of each sample in detectable virus genome copies per millilitre, per gram or per square centimetre, divide the number of theoretically detectable genome copies in the entire sample by the starting volume (bottled water), mass (BMS, soft fruits, salad vegetables) or area (hard surfaces) of the sample.

10 Expression of results

Positive results for each target virus for food surfaces shall be expressed as “virus genome detected”; 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 food surfaces shall be expressed as “virus genome not detected”; 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 “no result”. If however, an otherwise valid positive result is obtained from a sample showing an unacceptable amplification or extraction efficiency, results may be expressed as detailed in the preceding. Details shall be included in the test report.

11 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 part of ISO/TS 15216 (ISO/TS 15216-2:2013);
- d) all operating details not specified in this part of ISO/TS 15216, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- e) pLOD ([3.17](#)) of the method (adjusted to account for use of 10^{-1} RNA if appropriate) and the matrix it was established in;
- f) the tLOD ([3.16](#)) of the sample;
- g) the extraction efficiency of the sample ([9.4](#));
- h) the test result(s) obtained, expressed according to [Clause 10](#).

Annex A (normative)

Diagram of procedure

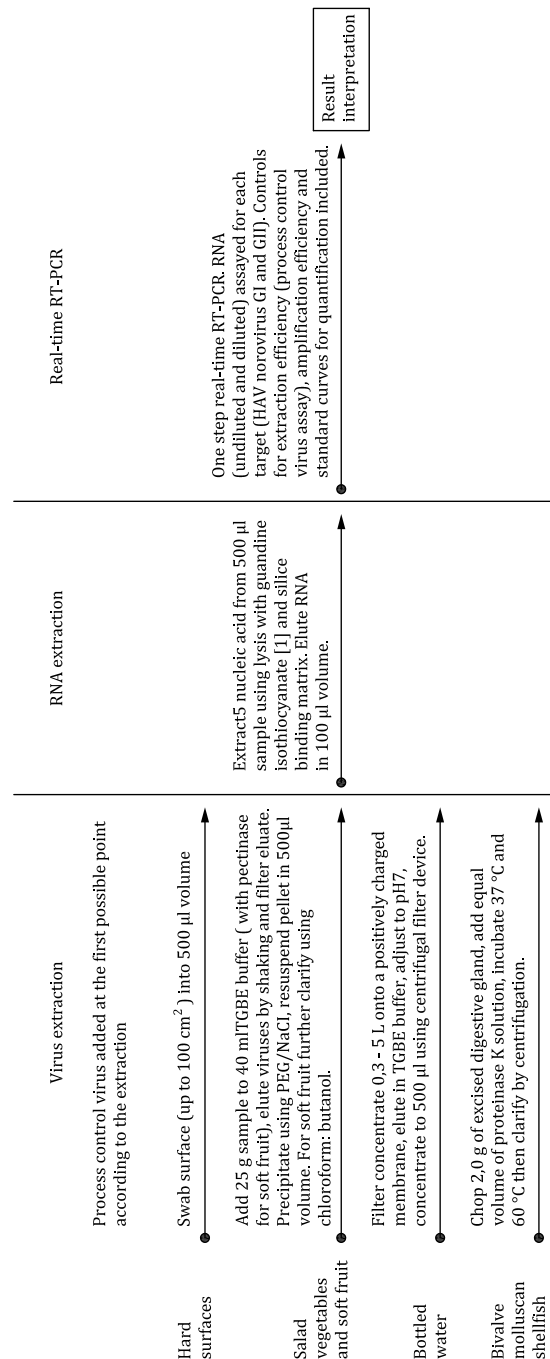


Figure A.1

Annex B (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™¹⁾ one step qRT-PCR system, see Table B.1, and for cycling parameters, see Table B.2.

Table B.1 — Mastermix

Reagent	Final concentration (in 25 µl)	Volume per reaction (µl)
5× Ultrasense reaction mix	1×	5 ± 0,1
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 ± 0,05
Water (5.2.1)	—	as required
Total volume	—	20 ± 0,2
^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 B.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 C

(informative)

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

C.1 HAV

HAV68 (FW):	TCA CCG CCG TTT GCC TAG	Reference [2]
HAV240 (REV):	GGA GAG CCC TGG AAG AAA G	Reference [2]
HAV150(-) (PROBE):	CCT GAA CCT GCA GGA ATT AA	Reference [2]

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 173 bp corresponding to nucleotides 68–240 of HAV isolate HM174 43c (GenBank accession number M59809).

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 (Reference [2]).

C.2 Norovirus GI

QNIF4 (FW):	CGC TGG ATG CGN TTC CAT	Reference [3]
NV1LCR (REV):	CCT TAG ACG CCA TCA TCA TTT AC	Reference [4]
NVGG1p (PROBE):	TGG ACA GGA GAY CGC RAT CT	Reference [4]

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 86 bp corresponding to nucleotides 5291–5376 of Norwalk virus (GenBank accession number M87661).

C.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 [6]
QNIFs (PROBE):	AGC ACG TGG GAG GGC GAT CG	Reference [5]

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 (Reference [6]). 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 NoV respectively. In addition, the efficacy and sensitivity of the primers and probes was verified using 18 NoV 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); hepatitis A virus; 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 paraheamolyticus* 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 NoV) (References [5][7]).

C.4 Mengo virus

Mengo 110 (FW):	GCG GGT CCT GCC GAA AGT	Reference [8]
Mengo 209 (REV):	GAA GTA ACA TAT AGA CAG ACG CAC AC	Reference [8]
Mengo 147 (PROBE):	ATC ACA TTA CTG GCC GAA GC	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 100 bp corresponding to nucleotides 110–209 of the deletant mengo virus strain MC₀ used in the development of this part of ISO/TS 15216. This corresponds to nucleotides 110–270 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 (Reference [2]). The primer sequences do not align with any other sequences available in GenBank.

Annex D (informative)

Growth of mengo virus strain MC₀ for use as a process control

D.1 General

Mengo virus is a murine virus of the *Picornaviridae* family. Mengo virus strain MC₀ (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 (Reference [9]). This strain has been used as a process control virus in detection methods for HAV and noroviruses (References [2][7]) and was used as the process control in the development of this part of ISO/TS 15216.

Mengo virus strain MC₀ is a genetically modified organism (GMO); for laboratories where use of a GMO is prohibited or problematic a different process control shall be used.

D.2 Reagents and apparatus

D.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.

D.2.2 For preparation of cell cultures and growth of virus, cell culture facilities including incubator(s) with controllable CO₂ levels, and cell culture consumables (flasks etc.) are required.

D.3 Procedure

Mengo virus shall be grown in a (50 ± 10) ml/l CO₂ atmosphere (with open vessels) or an uncontrolled atmosphere (closed vessels) on 80 % to 90 % confluent monolayers of HeLa cells (ATCC® CCL-2™)³⁾ 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 (5.3.6).

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 E (informative)

RNA extraction using the BioMerieux NucliSens^{®3)} system

E.1 Reagents

E.1.1 BioMerieux NucliSens[®] lysis buffer.

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

E.2 Apparatus

E.2.1 BioMerieux NucliSens[®] miniMAG or easyMAG instrument.

E.2.2 BioMerieux NucliSens[®] magnetic rack.

E.2.3 Thermoshaker or equivalent apparatus for shaking 1,5 ml tubes at $(60 \pm 2)^\circ\text{C}$ and approximately 1 400 oscillations min^{-1} .

E.2.4 Tubes with screw caps, capacity 1,5 ml, suitable for use with the NucliSens[®] instrument.

E.3 Procedure

Add $(2 \pm 0,1)$ 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) 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 $1\,500 \times g$ 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 miniMAG or easyMAG extraction systems. After washing, allow silica to sediment using the magnet of the miniMAG or 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.

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.

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.

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 ± 2) μl elution buffer. Cap tubes and transfer to thermoshaker or equivalent and incubate for $(5,0 \pm 0,5)$ min at (60 ± 1) °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 F (normative)

Composition and preparation of reagents and buffers

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

F.1.1 Composition

Polyethylene (PEG) 8 000	(500 ± 2) g
NaCl	(87 ± 1) g
Water (5.2.1)	as required

F.1.2 Preparation

Dissolve the solids in (450 ± 5) ml water ([5.2.1](#)), heating gently if necessary. Adjust volume to (1 000 ± 10) ml with water and mix well. Sterilize by autoclaving.

F.2 Chloroform/butanol mixture

F.2.1 Composition

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

F.2.2 Preparation

Mix the components together.

F.3 Proteinase K solution

F.3.1 Composition

Proteinase K (30 U/mg)	(20 ± 0,1) mg
Water (5.2.1)	(200 ± 2) ml

F.3.2 Preparation

Dissolve the proteinase K in the water. Mix thoroughly.

Store in working volumes at (−20 ± 5) °C for a maximum of 6 months. Once defrosted, store at (4 ± 2) °C and use within 1 week.

F.4 Phosphate-buffered saline (PBS)**F.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 (5.2.1)	(1 000 ± 2) ml

F.4.2 Preparation

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

F.5 Tris/glycine/beef extract (TGBE) buffer**F.5.1 Composition**

Tris base [<i>tris</i> (hydroxymethyl)aminomethane]	(12,1 ± 0,2) g
Glycine	(3,8 ± 0,1) g
Beef extract	(10 ± 1,0) g
Water (5.2.1)	(1 000 ± 1) ml

F.5.2 Preparation

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

Annex G **(informative)**

Generation of external control RNA (EC RNA) stocks

G.1 General

Control plasmids shall be constructed by ligating the target DNA sequence into a suitable plasmid vector such that the target sequence is downstream of a promoter sequence for RNA polymerase.

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 One step real-time RT-PCR (using hydrolysis probes) reagents and equipment.

G.2.8 Incubator, capable of operating at $(37 \pm 1) ^\circ\text{C}$.

G.3 Linearization of plasmid DNA

Add 100 ng to 500 ng of purified control 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) and buffers as recommended by the manufacturer of the enzyme.

Incubate at $(37 \pm 1) ^\circ\text{C}$ for (120 ± 5) min.

Purify DNA from the mastermix using PCR purification reagents, eluting in $(50 \pm 0,5)$ μl elution buffer.

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

G.4 *In vitro* RNA transcription

Add 100 ng to 500 ng of purified linearized plasmid DNA to an *in vitro* RNA transcription reaction mix prepared as recommended by the manufacturer of the RNA polymerase enzyme.

Incubate at $(37 \pm 1) ^\circ\text{C}$ for (120 ± 5) min.

Add RNase-free DNase to the reaction and incubate at $(37 \pm 1) ^\circ\text{C}$ for (15 ± 1) min.

Purify the RNA using RNA purification reagents, eluting in (100 ± 1) μl water (5.2.1).

G.5 DNA contamination check

Prepare target-specific real-time RT-PCR mastermix (5.3.7), split into two and deactivate the RT enzyme in one portion by heating at (95 ± 2) °C for $(5,0 \pm 0,5)$ min.

Use both portions of the mastermix subject EC RNA stock to real-time RT-PCR alongside a dsDNA dilution series as a standard curve (8.4).

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 (-20 ± 5) °C until required for preparation of the EC RNA control material (5.3.8).

G.6 Quantification of EC RNA

Determine the absorbance at 260 nm of the DNase-treated EC RNA stock using spectrophotometry.

Multiply the absorbance reading by 4×10^{-8} 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.

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Table H.1

5 μ l RNA ($\pm 1 \mu$ l EC RNA) and 20 μ l MasterMix per well

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