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

**Part 2:  
Method for detection**

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

*Partie 2: Méthode de détection*

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## Foreword

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

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

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

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

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

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

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

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

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

## Introduction

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

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

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# Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR —

## Part 2: Method for detection

### 1 Scope

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

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

### 2 Normative references

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

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

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

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

### 3 Terms and definitions

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

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

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

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

#### 3.1 foodstuff

substance used or prepared for use as food

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

#### 3.2 surface

surface of food, food preparation surface or food contact surface

### 3.3

#### **soft fruit**

small edible stoneless fruit

EXAMPLE Strawberries, raspberries, currants.

### 3.4

#### **leaf, stem and bulb vegetables**

leaves, stems and bulbs of plants, eaten as a vegetable

EXAMPLE Lettuce, green onions.

### 3.5

#### **hepatitis A virus**

##### **HAV**

member of the *Picornaviridae* family responsible for infectious hepatitis

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

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

### 3.6

#### **norovirus**

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

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

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

### 3.7

#### **detection of HAV**

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

### 3.8

#### **detection of norovirus**

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

### 3.9

#### **process control virus**

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

### 3.10

#### **process control virus RNA**

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

### 3.11

#### **negative RNA extraction control**

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



**3.12****negative process control**

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

**3.13****hydrolysis probe**

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

**3.14****negative real-time RT-PCR control**

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

**3.15****external control RNA****EC RNA**

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

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

**3.16** **$C_q$  value**

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

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

**4 Principle****4.1 Virus extraction**

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

**4.2 RNA extraction**

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

**4.3 Real-time RT-PCR**

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

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

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

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

## 4.4 Control materials

### 4.4.1 Process control virus

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

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

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

### 4.4.2 EC RNA control

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

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

## 4.5 Test results

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

## 5 Reagents

### 5.1 General

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

For current laboratory practice, see ISO 7218.

### 5.2 Reagents used as supplied

#### 5.2.1 Molecular biology grade water.

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

**5.2.3 Sodium chloride (NaCl)**.

**5.2.4 Potassium chloride (KCl)**.

**5.2.5 Disodium hydrogenphosphate (Na<sub>2</sub>HPO<sub>4</sub>)**.

**5.2.6 Potassium dihydrogenphosphate (KH<sub>2</sub>PO<sub>4</sub>)**.

**5.2.7 Tris base**.

**5.2.8 Glycine**.

**5.2.9 Beef extract powder**.

**5.2.10 Proteinase K**.

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

**5.2.12 Chloroform**.

**5.2.13 n-Butanol**.

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

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

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

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

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

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

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

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

### 5.3 Reagents requiring preparation

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

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

**5.3.2 Chloroform/butanol mixture** (1:1 volume fraction). See [B.2](#).

**5.3.3 Proteinase K solution** (3 000 U/l). See [B.3](#).

**5.3.4 Phosphate-buffered saline (PBS)**. See [B.4](#).

**5.3.5 Tris/glycine/beef extract (TGBE) buffer**. See [B.5](#).

**5.3.6 Tris solution** (1 mol/l). See [B.6](#).

**5.3.7 EDTA solution** (0,5 mol/l). See [B.7](#).

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

**5.3.9 Process control virus material.** Process control virus stock shall be diluted by a minimum factor of 10 in a suitable buffer, e.g. PBS ([5.3.4](#)). This dilution shall allow for inhibition-free detection of the process control virus genome using real-time RT-PCR, but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve (see [8.4.2.2](#)). Split the diluted process control virus material into single use aliquots and store at  $-15\text{ }^{\circ}\text{C}$  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  $\mu\text{l}$  mastermix per reaction in a 25  $\mu\text{l}$  total volume. Optimal primer and probe concentrations shall be used after determination following the recommendations of the reagent manufacturers. See [Annex C](#) for illustrative details of real-time RT-PCR mastermixes (used in the development of this document).

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