
**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

ISO/CEN PARALLEL PROCESSING

This draft Technical Specification has been developed within the European Committee for Standardization (CEN), and processed under the **CEN-lead** mode of collaboration as defined in the Vienna Agreement.

This draft Technical Specification is hereby submitted to a parallel three-month P member vote in the ISO/TC concerned and three-month vote in CEN.

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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*.

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.

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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

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 For the purposes of this part of ISO/TS 15216, this definition includes bottled water.

3.2

food surface

<1> surface of food

3.3

food surface

<2> food preparation surface

3.4

fomite

inanimate object or material on which infectious agents can be conveyed

3.5
hepatitis A virus
HAV

member of the *Picornaviridae* family responsible for infectious hepatitis

NOTE 1 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).

NOTE 2 There is only one serotype. 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 UK Advisory Committee on Dangerous Pathogens (ACDP) hazard group 2 pathogen.

3.6
norovirus

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

NOTE 1 Genetically, norovirus can be subdivided into five separate genogroups

NOTE 2 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 ACDP Hazard Group 2 pathogens.

3.7
detection of hepatitis A virus

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

3.8
detection of norovirus

detection 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 released 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 cross-contamination events

3.12
negative process control

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

3.13
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

3.14
negative RT-PCR control

aliquot of highly pure water used as template in a real-time RT-PCR reaction to control for contamination in the real-time RT-PCR reagents

3.15**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

3.16 **C_q value**

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

NOTE This corresponds to the point at which reaction fluorescence rises above a threshold level.

3.17**theoretical limit of detection****tLOD**

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

NOTE 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.

3.18**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 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 D and E.

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 G.

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.**
- 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 C 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 E 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 D 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 B.