TECHNICAL SPECIFICATION

ISO/TS 15216-1

First edition

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

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 1: Méthode de quantification

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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Reference number
ISO/TS 15216-1:2012(E)

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Foreword

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ISO/TS 15216-1 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*

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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 quantification of levels of virus RNA in the test sample.

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

1 Scope

This part of ISO/TS 15216 describes a method for quantification of levels of HAV and NoV genogroup I (GI) and II (GII) RNA, 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
quantification of hepatitis A virus
quantification of HAV RNA in a predetermined mass or volume of foodstuff, or area of food surface

3.8
quantification of norovirus
quantification 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.

3.19
limit of quantification
LOQ
lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy under the experimental conditions specified in the method, as demonstrated by a collaborative trial or other validation

NOTE The LOQ is related to the test portion and the quality or quantity of the template RNA.

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 part of ISO/TS 15216 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. Fluorescence can be measured at each stage throughout the cycle. The first point in the PCR cycle at which amplification can be detected for any reaction is proportional to the quantity of template, therefore analysis of the fluorescence plots enables determination of the quantity of target sequence in the sample.

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 Double-stranded DNA (dsDNA) control

For quantification of a target virus, results shall be related to a standard of known concentration. A dilution series of double-stranded DNA carrying the target sequence of interest (5.3.8) and quantified using spectrophotometry shall be used to produce a standard curve in template copies per microlitre. Reference to the standard curve enables quantification of the sample in detectable virus genome copies per microlitre.

4.4.3 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, 5.3.9) 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.

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

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

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 (Na_2HPO_4).

5.2.6 Potassium dihydrogenphosphate (KH_2PO_4).

5.2.7 Tris base.

5.2.8 Glycine.

5.2.9 Beef extract powder.

5.2.10 Proteinase K (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.

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. See B.2.

5.3.3 Proteinase K solution. 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 Process control virus material. Process control virus stock shall be diluted by a minimum factor of 10 in a suitable buffer, e.g. PBS. 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 ± 5) °C. See Annex G 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 E for illustrative details of real-time RT-PCR mastermixes (used in the development of this part of ISO/TS 15216).

5.3.8 Double-stranded DNA (dsDNA) control material. Separate purified plasmids carrying the target sequence for each target virus shall be used. The preparations shall not cause RT-PCR inhibition. The concentrations of each dsDNA stock in template copies per microlitre shall be determined then the stock shall be diluted to a concentration of 1×10^4 to 1×10^5 template copies per microlitre. Split the diluted dsDNA preparation (dsDNA control material) into single use aliquots and store frozen at −15 °C or below. See Annex H for illustrative details of the preparation of dsDNA (used in the development of this part of ISO/TS 15216).

5.3.9 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

