SLOVENSKI STANDARD
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Mikrobiologija v prehranski verigi - Horizontalna metoda za ugotavljanje virusa hepatitisa A in norovirusov v živilih z RT-PCR v realnem času - 1. del: Metoda za kvantifikacijo (ISO/DIS 15216-1:2015)

Microbiology of the food chain - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification (ISO/DIS 15216-1:2015)

Mikrobiologie der Lebensmittelkette - Horizontales Verfahren zur bestimmung von Hepatitis A-Virus und Norovirus in Lebensmitteln mittels Real-time-RT-PCR - Teil 1: Verfahren zur Quantifizierung (ISO/DIS 15216-1:2015)

Microbiologie dans la chaine alimentaire - 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/DIS 15216-1:2015)

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

Part 1:
Method for quantification

Microbiologie dans la chaine alimentaire — 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

ICS: 07.100.30

ISO/CEN PARALLEL PROCESSING

This draft 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 is hereby submitted to the ISO member bodies and to the CEN member bodies for a parallel five month enquiry.

Should this draft be accepted, a final draft, established on the basis of comments received, will be submitted to a parallel two-month approval vote in ISO and formal vote in CEN.

To expedite distribution, this document is circulated as received from the committee secretariat. ISO Central Secretariat work of editing and text composition will be undertaken at publication stage.

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Contents

Page

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.

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 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* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

ISO 15216 consists of the following parts, under the general title *Microbiology of the food chain — 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* (Technical Specification)

This edition cancels and replaces ISO/TS 15216-1:2013, which has been technically revised.

Main changes in this edition:

- Use of linear dsDNA molecules for quantification prescribed;
- Use of a suitable buffer for dilution of control materials prescribed;
- Change to the method for generating process control virus RNA for the standard curve;
- Addition of breakpoints in the extraction methods;
- Change in terminology from amplification efficiency to RT-PCR inhibition;
- Addition of extra real-time RT-PCR reactions for negative controls;
- Precision data and results of interlaboratory study.

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 real-time RT-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 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 part of ISO 15216 enables quantification of levels of virus RNA in the test sample. A schematic diagram of the testing procedure is shown in Annex A.

Microbiology of the food chain — 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 15216 describes a method for quantification of levels of HAV and NoV genogroup I (GI) and II (GII) RNA, from test samples of foodstuffs (soft fruit, salad vegetables, bottled water, bivalve molluscan shellfish) or food surfaces. Following liberation of viruses from the test sample, viral RNA is then extracted by lysis with guanidine thiocyanate and adsorption on silica. Target sequences within the viral RNA are amplified and detected by real-time RT-PCR.

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

2 Normative references

The following 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 15216, this definition includes bottled water.

3.2
food surface
surface of food, food preparation surface or food contact surface

3.3
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). There is only one serotype.

NOTE 2 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.

3.4
norovirus
member of the *Caliciviridae* family responsible for sporadic cases and outbreaks of acute gastroenteritis

NOTE 1 Genetically, norovirus can be subdivided into seven 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 group 2 biological agents by the European Union and as risk group 2 human aetiological agents by the United States National Institutes of Health.

3.5
quantification of hepatitis A virus
estimation of number of copies of HAV RNA in a predetermined mass or volume of foodstuff, or area of food surface

3.6
quantification of norovirus
estimation of number of copies of norovirus RNA in a predetermined mass or volume of foodstuff, or area of food surface

3.7
process control virus
virus added to the sample portion at the earliest opportunity prior to virus extraction to control for extraction efficiency

3.8
process control virus RNA
RNA extracted from the process control virus in order to produce standard curve data for the estimation of extraction efficiency

3.9
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

3.10
negative process control
target pathogen-free sample of the food matrix which is run through all stages of the analytical process

3.11
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

3.12
negative real-time RT-PCR control

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

3.13
external control RNA
reference RNA that can serve as target for the real-time RT-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

3.14
C_q value
quantification cycle; the cycle at which the target is quantified in a given real-time RT-PCR reaction

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

3.15
limit of detection
LOD
lowest concentration of target in a test sample that can be reproducibly detected (95 % confidence interval) under the experimental conditions specified in the method, as demonstrated by a collaborative trial or other validation

NOTE The LOD is related to the test portion and the quality of the template RNA.

3.16
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 of the template RNA.

4 Principle

4.1 Virus extraction

The foodstuffs and food surfaces covered by this part of ISO 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 RT-PCR inhibitors. In this part of ISO 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 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 RT-PCR using hydrolysis probes utilizes a short DNA probe with a fluorescent label and a fluorescence quencher attached at opposite ends. The assay chemistry ensures that as the quantity of amplified product increases, the probe is 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 real-time RT-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 concentration 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 5.2.18 and 5.2.19. Illustrative details of reagents, primers, and probes (used in the development of this part of ISO 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 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 under test.

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

4.4.2 Double-stranded DNA (dsDNA) control

For quantification of a target virus, results shall be related to a standard of known concentration. A dilution series of linear double-stranded DNA carrying the target sequence of interest (5.3.11) and quantified using an appropriate method, e.g. spectrophotometry, fluorimetry, digital PCR etc. shall be used to produce a standard curve in template copies per microlitre. Reference to the standard curve enables quantification of the sample 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, 5.3.12) is added to an aliquot of sample RNA and tested using the real-time RT-PCR method. Comparison of the results of this with the results of EC RNA in the absence of sample RNA enables determination of the level of RT-PCR inhibition in each sample under test.

Alternative approaches for RT-PCR inhibition control that provide equivalent stringency 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.[1]

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 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 Ethylenediaminetetraacetic acid (EDTA).

5.2.17 Silica, lysis, **wash**, and **elution buffers** for extraction of viral RNA. Reagents shall enable processing of 500 µl of extracted virus, using lysis with a chaotropic buffer containing guanidine thiocyanate (Reference [4]) 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 15216).

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 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 15216).

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

5.2.20 Primers and hydrolysis probes for detection of the process control virus. Primer and hydrolysis probe sequences shall be published in a peer-reviewed journal and be verified for use against the strain of process virus used. They shall demonstrate no cross-reactivity with the target virus.

5.3 Prepared reagents

Because of the large number of reagents requiring individual preparation, details of composition and preparation are given in Annex 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 1M Tris solution. See F.6.

5.3.7 0.5M EDTA solution. See F.7.

5.3.8 Tris EDTA (TE) buffer. See F.8.

5.3.9 Process control virus material. Process control virus stock shall be diluted by a minimum factor of 10 in a suitable buffer, e.g. PBS (5.3.4). This dilution shall allow for inhibition-free detection of the process control virus genome using real-time RT-PCR, but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve (8.4.2.2). Split the diluted process control virus material into single use aliquots and store at -15 °C or below. See Annex D for illustrative details of the preparation of process control virus (used in the development of the method described in this part of ISO 15216).

5.3.10 Real-time RT-PCR mastermixes for target and process control virus. Reagents shall be added in quantities as specified by the manufacturers (5.2.18) to allow 20 µl mastermix per reaction in a 25 µl total volume. Optimal primer and probe concentrations shall be used after determination

following the recommendations of the reagent manufacturers. See Annex B for illustrative details of real-time RT-PCR mastermixes (used in the development of this part of ISO 15216).

5.3.11 Double-stranded DNA (dsDNA) control material. Purified linear DNA molecules carrying the target sequence for each target virus shall be used. The sequence of the DNA molecules shall be verified prior to use. The preparations shall not cause RT-PCR inhibition. The concentrations of each dsDNA stock in template copies per microlitre shall be determined then the stock shall be diluted in a suitable buffer e.g. TE buffer (5.3.8), to a concentration of 1×10^4 to 1×10^5 template copies per microlitre. As EDTA can act as an inhibitor of RT-PCR, buffers used to dilute dsDNA shall not contain concentrations of EDTA greater than 1mM. Split the diluted dsDNA preparation (dsDNA control material) into single use aliquots and store frozen at −15 °C or below. See Annex G for illustrative details of the preparation of dsDNA (used in the development of this part of ISO 15216).

5.3.12 External control (EC) RNA control material. Purified ssRNA carrying the target sequence for each target virus shall be used. They shall contain levels of contaminating target DNA no higher than 0,1 % and shall not cause RT-PCR inhibition. The concentrations of each EC RNA stock in copies per microlitre shall be determined then the stock shall be diluted in a suitable buffer e.g. TE buffer (5.3.8), to a concentration of 1×10^4 to 1×10^5 template copies per microlitre. As EDTA can act as an inhibitor of RT-PCR, buffers used to dilute EC RNA shall not contain concentrations of EDTA greater than 1mM. Split the diluted EC RNA preparation (EC RNA control material) into single use aliquots and store frozen at −15 °C or below. See Annex H for illustrative details of the preparation of EC RNA (used in the development of this part of ISO 15216).

6 Apparatus and materials

Standard microbiological laboratory equipment (ISO 7218)[1] 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 ± 2) °C and (320 ± 20) oscillations min^{-1} or equivalent.

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

6.7 Aspirator or equivalent apparatus for removing supernatant.

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

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

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

b) 10 000 × *g* at (5 ± 3) °C with capacity for narrow gauge (15 mm is too large) chloroform-resistant tubes of at least 1 ml volume;

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