



BSI Standards Publication

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

Part 1: Method for quantification

National foreword

This British Standard is the UK implementation of EN ISO 15216-1:2017+A1:2021. It is identical to ISO 15216-1:2017, incorporating amendment 1:2021. It supersedes [BS EN ISO 15216-1:2017](#), which is withdrawn.

The start and finish of text introduced or altered by amendment is indicated in the text by tags. Tags indicating changes to ISO text carry the number of the ISO amendment. For example, text altered by ISO amendment 1 is indicated by **A1** **A1**.

The UK participation in its preparation was entrusted to Technical Committee AW/9, Microbiology.

A list of organizations represented on this committee can be obtained on request to its committee manager.

Contractual and legal considerations

This publication has been prepared in good faith, however no representation, warranty, assurance or undertaking (express or implied) is or will be made, and no responsibility or liability is or will be accepted by BSI in relation to the adequacy, accuracy, completeness or reasonableness of this publication. All and any such responsibility and liability is expressly disclaimed to the full extent permitted by the law.

This publication is provided as is, and is to be used at the recipient's own risk.

The recipient is advised to consider seeking professional guidance with respect to its use of this publication.

This publication is not intended to constitute a contract. Users are responsible for its correct application.

© The British Standards Institution 2021
Published by BSI Standards Limited 2021

ISBN 978 0 539 13468 1

ICS 07.100.30

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 April 2017.

Amendments/corrigenda issued since publication

Date	Text affected
30 April 2021	Implementation of ISO amendment 1:2021 with CEN endorsement A1:2021

English Version

Microbiology of the food chain - Horizontal method for
determination of hepatitis A virus and norovirus using
real-time RT-PCR - Part 1: Method for quantification (ISO
15216-1:2017)

Microbiologie de 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 1: Méthode
de quantification (ISO 15216-1:2017)

Mikrobiologie der Lebensmittelkette - Horizontales
Verfahren zur Bestimmung von Hepatitis-A-Virus
und Norovirus mittels Real-time-RT-PCR - Teil 1:
Verfahren zur Quantifizierung (ISO 15216-1:2017)

This European Standard was approved by CEN on 23 February 2017.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.



EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

European foreword

This document (EN ISO 15216-1:2017) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN, in collaboration with Technical Committee ISO/TC 34 "Food products".

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by September 2017 and conflicting national standards shall be withdrawn at the latest by September 2017.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes CEN ISO/TS 15216-1:2013.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Endorsement notice

The text of ISO 15216-1:2017 has been approved by CEN as EN ISO 15216-1:2017 without any modification.

Foreword to amendment A1

This document (EN ISO 15216-1:2017/A1:2021) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 463 "Microbiology of the food chain" the secretariat of which is held by AFNOR.

This Amendment to the European Standard EN ISO 15216-1:2017 shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2021, and conflicting national standards shall be withdrawn at the latest by October 2021.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Endorsement notice

The text of ISO 15216-1:2017/Amd 1:2021 has been approved by CEN as EN ISO 15216-1:2017/A1:2021 without any modification.

Contents

Page

Foreword	v
Introduction	vi
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 Principle	3
4.1 Virus extraction	3
4.2 RNA extraction	4
4.3 Real-time RT-PCR	4
4.4 Control materials	4
4.4.1 Process control virus	4
4.4.2 Double-stranded DNA (dsDNA) control	4
4.4.3 EC RNA control	4
4.5 Test results	5
5 Reagents	5
5.1 General	5
5.2 Reagents used as supplied	5
5.3 Prepared reagents	6
6 Equipment and consumables	7
6.10 Microcentrifuge	8
7 Sampling	9
8 Procedure	9
8.1 General laboratory requirements	9
8.2 Virus extraction	9
8.2.1 Process control virus material	9
8.2.2 Negative process control	9
8.2.3 Food surfaces	9
8.2.4 Soft fruit, leaf, stem and bulb vegetables	9
8.2.5 Bottled water	10
8.2.6 Bivalve molluscan shellfish	11
8.3 RNA extraction	11
8.4 Real-time RT-PCR	12
8.4.1 General requirements	12
8.4.2 Real-time RT-PCR analysis	12
9 Interpretation of results	14
9.1 General	14
9.2 Construction of standard curves	14
9.3 Calculation of RT-PCR inhibition	15
9.4 Calculation of extraction efficiency	15
9.5 Sample quantification	16
10 Expression of results	17
11 Precision	17
11.1 Interlaboratory study	17
11.2 Repeatability	17
11.3 Reproducibility limit	17
12 Test report	18
Annex A (normative) Diagram of procedure	19
Annex B (normative) Composition and preparation of reagents and buffers	20

Annex C (informative) Real-time RT-PCR mastermixes and cycling parameters	23
Annex D (informative) Real-time RT-PCR primers and hydrolysis probes for the detection of HAV, norovirus GI and GII and mengo virus (process control)	24
Annex E (informative) Growth of mengo virus strain MC₀ for use as a process control	26
Annex F (informative) RNA extraction using the NucliSENS® system	27
Annex G (informative) Generation of dsDNA control stocks	29
Annex H (informative) Generation of EC RNA stocks	32
Annex I (informative) Typical optical plate layout	34
Annex J (informative) Method validation studies and performance characteristics	36
Bibliography	45

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).

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).

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

For an explanation on 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 the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, in collaboration with ISO 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).

This first edition cancels and replaces ISO/TS 15216-1:2013, which has been technically revised with the following changes:

- 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 with defined temperature and time parameters 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;
- addition of precision data and results of interlaboratory study.

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

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 food surfaces, viruses are removed by swabbing. For soft fruit, 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 and 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 quantification of levels 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 15216-1:2013 are considered as minor (see [ISO 17468](#)).

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

Part 1: Method for quantification

1 Scope

This document specifies a method for the quantification of levels of HAV and norovirus genogroup I (GI) and II (GII) RNA, from test samples of foodstuffs (soft fruit, leaf, stem and bulb vegetables, bottled water, BMS) 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 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 7218](#), *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

[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 22174](#), [ISO 22119](#) and [ISO 20838](#) and the following apply.

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

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <http://www.iso.org/obp>

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

food surface

surface of food, food preparation surface or food contact surface

3.3

soft fruit

small edible stoneless fruit

EXAMPLE Strawberries, raspberries or currants

3.4

leaf, stem and bulb vegetables

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

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, contact with contaminated water or food surfaces, 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 or through contact with contaminated water or food surfaces 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

quantification of HAV

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

3.8

quantification of norovirus

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

3.9

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

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

3.17

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

Note 1 to entry: The LOD is related to the test portion and the quality of the template RNA.

3.18

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

Note 1 to entry: 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 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 opposite ends. 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. Fluorescence can be measured at each stage throughout the cycle. The first cycle in the real-time RT-PCR 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 or food surfaces 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 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 food 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 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 dsDNA 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 RNA in detectable virus genome copies per microlitre.

4.4.3 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 control for RT-PCR inhibition in individual samples, 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 the assessment of inhibition of RT-PCR that can be demonstrated to provide equivalent performance to the use of EC RNA control 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 LOD for the sample.

5 Reagents

5.1 General

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

Follow current laboratory practice, as specified in [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 (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.

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^[3] 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' to 3' exonuclease activity) and sufficiently sensitive for the detection of virus RNA as expected in virus-contaminated foodstuffs and food 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 (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.

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 (1:1 v/v); 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 ([8.4.2.2](#)). Split the diluted process control virus material into single use aliquots and store at -15 °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 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 C](#) for illustrative details of real-time RT-PCR mastermixes (used in the development of this document).

5.3.11 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 first 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 1 mmol/l. Split the diluted dsDNA preparation (dsDNA control material) into single use aliquots and store at $(5 \pm 3)^\circ\text{C}$ for up to 24 h, at -15°C or below for up to six months, or at -70°C or below for longer periods. See [Annex G](#) for illustrative details of the preparation of dsDNA (used in the development of this document).

5.3.12 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 1×10^2 to 1×10^5 template copies per microlitre. The concentration used shall be appropriate for the types of samples under test and 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 $(5 \pm 3)^\circ\text{C}$ for up to 24 h, at -15°C or below for up to six months, or at -70°C or below for longer periods. See [Annex H](#) for illustrative details of the preparation of EC RNA (used in the development of this document).

6 Equipment and consumables

Standard microbiological laboratory equipment ([ISO 7218](#)) 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 (as in [6.7](#) and [E.3](#)).

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 50 oscillations min^{-1} .

6.5 Shaking incubator operating at $(37 \pm 2)^\circ\text{C}$ and approximately 320 oscillations min^{-1} or equivalent.

6.6 Rocking platform(s) or equivalent for use at room temperature and $(5 \pm 3)^\circ\text{C}$ at approximately 60 oscillations min^{-1} .

6.7 Aspirator or equivalent apparatus for removing supernatant.

6.8 Water bath capable of operating at $(60 \pm 2)^\circ\text{C}$ 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 chloroform-resistant tubes with 2 ml volume;
- c) 4 000*g* at room temperature with capacity for centrifugal filter concentration devices ([6.16](#)).

6.10 Microcentrifuge.

6.11 Centrifuge and microcentrifuge tubes and bottles of a range of sizes, 1,5 ml, 5 ml, 15 ml, 50 ml, etc. Chloroform-resistant tubes with 2 ml capacity are necessary.

6.12 pH meter (or pH testing strips with demarcations of 0,5 pH units or lower).

6.13 Sterile cotton swabs.

6.14 Mesh filter bags (400 ml).

6.15 Positively charged membrane filters with 0,45 µm pore size (47 mm diameter).

6.16 Centrifugal filter concentration devices with 15 ml capacity and 100 kDa relative molecular mass cutoff.

6.17 Vacuum source or equivalent positive pressure apparatus for filtering and filtration tower with aperture for 47 mm diameter membrane.

6.18 Sterile shucking knife or equivalent tools for opening BMS.

6.19 Rubber block or equivalent apparatus for holding BMS during opening.

6.20 Scissors and forceps or equivalent tools for dissecting BMS.

6.21 Sterile Petri dishes.

6.22 Razor blades or equivalent tools for chopping BMS digestive glands.

6.23 Heavy duty safety glove.

6.24 RNA extraction equipment suitable for extraction methods using silica and associated reagents ([5.2.17](#)). See [Annex F](#) for illustrative details of RNA extraction apparatus (used in the development of this document).

6.25 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 real-time RT-PCR with hydrolysis probe chemistry.

6.26 Associated consumables for real-time RT-PCR, e.g. optical plates and caps, suitable for use with the selected real-time RT-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, e.g. samples that were frozen on collection should not have been allowed to defrost prior to receipt at the laboratory, samples that were not frozen on collection should not have been frozen prior to receipt at the laboratory.

8 Procedure

8.1 General laboratory requirements

Sample extraction and real-time RT-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 ([5.3.10](#)) for all individual samples (allow 10 µl per test sample plus 25 µl excess).

Retain a (20 ± 1) µl portion of pooled process control virus material for RNA extraction and preparation of the standard curve of process control virus RNA ([8.4.2.2](#)). Store at (5 ± 3) °C for a maximum of 24 h or at -15 °C or below for up to six months, or at -70 °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 pre-moistened 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.

Process the swab immediately, or place in a suitable container and store at (5 ± 3) °C for a maximum of 72 h or at -15 °C or below for up to six months, or at -70 °C or below for longer periods.

Add $(10 \pm 0,5)$ 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 ± 10) 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.

Proceed immediately to RNA extraction.

8.2.4 Soft fruit, leaf, stem and bulb vegetables

Soft fruit, leaf, stem and bulb vegetables for analysis shall be fresh or frozen. Samples shall not have been subject to any processing other than chopping, trimming, washing, decontamination, conditioning etc. as for pre-cut and packaged soft fruit, leaf, stem and bulb vegetables etc. Mud adhering to the surface shall be removed prior to analysis by gentle scrubbing, but without immersing the samples in water.