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

Microbiologie des aliments — Réaction de polymérisation en chaîne (PCR) pour la recherche de micro-organismes pathogènes dans les aliments — Exigences générales et définitions

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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.

ISO 22174 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, 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).

Introduction

The polymerase chain reaction (PCR) is a fast, sensitive and specific method for the detection of food-borne pathogens. Although a relatively young technology, the application of PCR-based methods in food analysis is increasing.

In brief, existing protocols can be divided in two main groups, depending on the type of nucleic acid used as target for amplification:

— RNA-based amplification (RT-PCR);

— DNA-based amplification (PCR).

Numerous variations of both methods have been established and can be characterized by their degree of complexity and automation. The level of specificity of the methods varies from screening assays which detect nucleic acid sequences common to a microbiological genus, to specific assays which identify nucleic acid sequences unique to an individual strain- or type-specific nucleic acid sequence.

This International Standard presents a comprehensive list of requirements for PCR-based methods used for the detection of microorganisms in food samples. It contains terms and definitions used in reference to PCR and RT-PCR.

ISO 22174 is part of a series of International Standards and a Technical Specification under the general title *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens*:

— *General requirements and definitions* (ISO 22174);

— *Requirements for sample preparation for qualitative detection* (ISO 20837) [1)];

— *Requirements for amplification and detection for qualitative methods* (ISO 20838) [1)];

— *Performance testing for thermal cyclers* (ISO/TS 20836) [1)].

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of one or more patents concerning the PCR technology.

ISO takes no position concerning the evidence, validity and scope of these patent rights.

ISO has been informed that Applied Biosystems, Roche Molecular Systems, Inc. and F. Hoffman-La Roche Ltd. hold patent rights concerning the PCR technology. The companies have assured the ISO that they are willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statements of the holders of these patent rights are registered with ISO. Information may be obtained from:

Licensing Department
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1) To be published.

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

WARNING — The use of this standard may involve hazardous materials, operations and equipment. This standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determines the applicability of regulatory limitations prior to use.

1 Scope

This International Standard gives the general requirements for the *in vitro* amplification of nucleic acid sequences (DNA or RNA). It is applicable to the testing of foodstuffs and isolates obtained from foodstuffs for food-borne pathogens using the polymerase chain reaction (PCR).

The minimum requirements laid down in this International Standard are intended to ensure that comparable and reproducible results are obtained in different laboratories.

This International Standard has been established for food-borne pathogens in or isolated from food and feed matrices, but is also applicable to other matrices (e.g. environmental samples) and for the detection of non-pathogenic microorganisms.

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 3534-1, *Statistics — Vocabulary and symbols — Part 1: Probability and general statistical terms*

ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

ISO 20837, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection*

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/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply. For definitions concerning validation, see ISO 3534-1 and ISO 5725-1.

3.1 General terms

3.1.1
nucleic acid
macromolecule that is the medium for genetic information or acts as an agent in expressing the information

NOTE There are two types of nucleic acid, DNA and RNA.

3.1.2
DNA
deoxyribonucleic acid
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

3.1.3
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

3.1.4
matrix
products submitted for analysis, which might have differences in chemical composition and physical state

3.1.5
repeatability conditions
conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time

[ISO 3534-1]

3.1.6
reproducibility conditions
conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment

[ISO 3534-1]

3.1.7
detection
recognition of the presence of the target nucleic acid

3.1.8
detection limit
limit of detection
lowest concentration or content of the target organism per defined amount of matrix that can be consistently detected under the experimental conditions specified in the method

3.1.9
identification
process for determining that an isolate belongs to one of the established taxa

3.2 Terms related to the extraction and purification of DNA/RNA

3.2.1
nucleic acid extraction
sample treatment for the liberation of target nucleic acid

3.2.2
nucleic acid purification
method resulting in a more purified DNA

NOTE In this context, purity refers to the reduction of observable effects of PCR inhibitors on PCR inhibition controls.

3.2.3
PCR quality DNA
DNA template of sufficient length and quantity for PCR

3.2.4
RT-PCR quality RNA
RNA template of sufficient length and quantity suitable for reverse transcription and PCR

3.3 Terms related to reverse transcription (RT) of RNA to DNA

3.3.1
RT
reverse transcription
synthesis of DNA from an RNA template using a reverse transcriptase enzyme combined with an RT-primer in the presence of deoxyribonucleoside triphosphate

3.3.2
reverse transcriptase
enzyme which catalyses the reverse transcription of RNA to DNA using RT-primers

3.3.3
ribonuclease
enzyme which degrades RNA

3.3.4
ribonuclease inhibitor
substance which blocks ribonuclease activity

3.3.5
RT-primer
primer used in reverse transcription

3.3.6
RT mix
mixture of reagents needed for reverse transcription

3.3.7
deoxyribonucleoside triphosphate
dNTP
solution containing dATP, dCTP, dGTP, dTTP and/or dUTP

3.4 Terms related to DNA amplification by PCR/RT-PCR

3.4.1
polymerase chain reaction
PCR
enzymatic procedure which allows *in vitro* amplification of DNA

3.4.2
RT-PCR
method consisting of two reactions, a reverse transcription (RT) of RNA to DNA and a subsequent PCR

3.4.3
one-step RT-PCR
method combining reverse transcription (RT) of RNA to DNA and PCR in a single reaction

3.4.4
two-step RT-PCR
method composed of a reverse transcription (RT) and PCR in two separate reactions

NOTE Two-step RT-PCR can be performed sequentially in a single tube or in two different tubes.

3.4.5
PCR product
DNA amplified by PCR

3.4.6
detection of PCR product
process which signals the presence of a PCR product

3.4.7
confirmation of PCR product
process which demonstrates that the PCR product originates from the target sequence

3.4.8
PCR-ELISA
method of detecting PCR products in liquid phase after their retention on a solid phase, such as in the wells of a microtitre plate

NOTE The presence of the PCR product is visualized by hybridization and subsequent immunoenzymatic detection.

3.4.9
hot-start PCR
activation of thermostable DNA polymerase by an initial heating step to avoid non-specific amplification

3.4.10
nested PCR
PCR which amplifies a sequence within the product of the first PCR

3.4.11
multiplex PCR
PCR reaction that uses multiple pairs of primers

3.4.12
primer
oligonucleotide of defined length and sequence complementary to a segment of an analytically relevant DNA sequence

NOTE A primer borders the target DNA sequence.

3.4.13
DNA target
DNA sequence selected for amplification

3.4.14
denaturation
process which results in the separation of the double-stranded DNA into single-stranded DNA

3.4.15
annealing
binding of a primer to the complementary nucleic acid sequence under specific conditions

3.4.16
primer extension
enzymatic reaction which leads to the synthesis of a new DNA strand by the addition of single deoxyribonucleotides to the 3′-end of the primer sequence

3.4.17
DNA polymerase for PCR
thermostable enzyme which catalyses repeated DNA synthesis

3.4.18
mastermix
mixture of reagents needed for PCR, except for the target DNA and the controls

3.4.19
UNG
uracil *N*-glycosylase
enzyme which can cleave any sequence of nucleic acid containing deoxyuridine (dUTP) at the location of that nucleotide

3.4.20
thermal cycler
automatic device which performs defined heating and cooling cycles necessary for PCR

3.4.21
endpoint analysis
qualitative analysis to detect PCR products

3.4.22
real-time analysis
method to detect PCR products during amplification

3.5 Terms related to controls

3.5.1
positive process control
sample, spiked with the target microorganism, which should be treated in the same way as the test samples

3.5.2
negative process control
target pathogen-free sample of the food matrix which is run through all stages of the analytical process

NOTE The process can include sample preparation, enrichment, DNA extraction and target amplification.

3.5.3 Amplification controls

3.5.3.1
internal amplification control
DNA added to each reaction in a defined amount or copy number which serves as an internal control for amplification

3.5.3.2
external amplification control
control DNA added to an aliquot of the extracted nucleic acid in a defined amount or copy number serving as a control for amplification in a separate reaction

3.5.4
negative extraction control
extraction blank
control carried through all steps of the DNA extraction procedure in the absence of a test sample

3.5.5
positive PCR control
reaction containing the target DNA in a defined amount or copy number

3.5.6
negative PCR control
reaction performed with DNA-free water without any PCR inhibitors

3.6 Terms related to DNA probes

3.6.1
DNA probe
labelled nucleic acid molecule with a defined sequence used to detect target DNA by hybridization

3.6.2
blocking reagent
compound used to saturate the residual unspecific binding sites of a solid phase prior and during hybridization with a DNA probe

3.6.3
hybridization
specific binding of complementary nucleic acid sequences under suitable reaction conditions

3.6.4
specificity
capacity to exclusively recognise the target to be detected, distinguishing it from similar substances and impurities

4 Principle

4.1 General

The examination comprises the following consecutive steps:

a) preliminary microbial enrichment of the food-borne pathogen from the test material, if required (see 4.2);

b) nucleic acid extraction and purification, if required (see 4.3);

c) amplification of the target nucleic acid sequence by PCR using specific primers (see 4.4);

d) detection of the specific PCR products (see 4.5).

4.2 Preliminary microbial enrichment

If required, the number of cells of the food-borne pathogen to be detected is increased by encouraging growth of the target microorganisms in the sample in selective or non-selective liquid nutrient media.

NOTE For viruses, other techniques are available such as filtration and/or concentration.

4.3 Nucleic acid preparation

The microbial cells in the test material or enriched culture are lysed to liberate their DNA. If required, a separation stage is added prior to lysis and/or a purification step is carried out following lysis.

4.4 PCR amplification

Specific nucleic acid sequences are amplified using PCR. The reaction is a cyclic process consisting of three steps:

a) denaturation of the double-stranded nucleic acid (dsDNA);

b) annealing of the primers to the complementary target sequence;

c) extension of the attached primers by means of a thermostable DNA polymerase.

RNA can be detected using PCR if the target has first been transcribed into a copy DNA (cDNA) by reverse transcription.

NOTE 1 Following denaturation of double-stranded DNA, two oligonucleotide primers anneal (hybridize) to the target DNA segment to be amplified. The primers are directed opposite to each other regarding their orientation to the target sequence.

NOTE 2 Double-stranded regions are formed as a result of specific base-pairing between the primers and the target sequence bordering the DNA segment to be amplified and serve as start positions for DNA synthesis by means of a heat-stable DNA polymerase.

NOTE 3 The repeated process of heat denaturation, primer annealing and DNA synthesis (cycles) results in the near exponential amplification of the DNA segment bordered by the primers.

4.5 Detection and confirmation of PCR products

PCR products are detected by gel electrophoresis or an appropriate alternative.

The identity of the PCR products is confirmed by any appropriate method, if required.

5 Test material

Any food or feeding stuff is suitable as a test material provided it has been established that the nucleic acid solution prepared from the sample does not inhibit the PCR.

6 General laboratory requirements

6.1 General

Accidental DNA contamination can originate from dust and spreading aerosols. As a consequence, the organization of the work area in the laboratory and good practices shall be based on

a) the systematic containment of the methodological steps involved in the production of results, and

b) a “forward flow” principle for sample handling.

These measures ensure that DNA in the test material and amplified DNA generated by PCR remain physically separated.

6.2 Personnel

All personnel who perform aspects of the testing procedures shall be trained to work with PCR and microbiology as appropriate.

Different sets of laboratory coats shall be worn pre- and post-PCR. Disposable gloves should be worn at sample preparation and when setting up PCR. Laboratory coats and gloves shall be changed at appropriate frequencies.

6.3 Laboratory setup

6.3.1 General

To prevent contamination of the reaction mixture by previously amplified target sequences, it shall be ensured that separate work areas with their own apparatus are available.

6.3.2 Work areas and work facilities

A minimum of four separate dedicated work areas and working facilities are required:

a) a work area for preparation of a nucleic acid solution from the test material;

b) a work area for the preparation of mastermix;

c) a work area for the addition of the nucleic acid solution prepared from the test material;

d) a work area for detection and confirmation of PCR products.

The amplification may be carried out in work area c) or in work area d).

If the thermal cycler is placed in work area c), tubes containing amplification reaction products shall not be opened within work area c).

A different set of pipettes shall be used for sample preparation and mastermix preparation.

The experiments should be run under appropriate environmental conditions.

Physical separation through the use of different rooms is the most effective and preferable way of ensuring separate work areas and working facilities.

NOTE PCR products can be destroyed using a 3 % (mass fraction) hypochlorite solution.

6.4 Waste management

Appropriate waste management and decontamination procedures shall be used.

7 Reagents

Reagents shall be as given in ISO 20837 and ISO 20838.

8 Apparatus and equipment

8.1 General

The laboratory shall use properly maintained equipment according to the manufacturers' instructions and the requirements given in ISO/IEC 17025. In addition to standard laboratory equipment, specific apparatus is described in the individual standards.

8.2 Special considerations

Where available, calibration should be routinely performed on equipment where performance may impact the data produced.

9 Procedure

9.1 Sample preparation

Cell lysis, nucleic acid preparation and/or purification of the test sample, if required, should be carried out according to the method described in ISO 20837.