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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

Microbiologie des aliments — Réaction de polymérisation en chaîne (PCR) pour la détection des micro-organismes pathogènes dans les aliments — Exigences relatives à l'amplification et à la détection pour les méthodes qualitatives

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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 20838 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 amplification and detection of target nucleic acid sequences is performed to determine whether certain nucleic acid sequences are present or not in the test portion. This determination is relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

This International Standard describes the procedure used to detect food-borne microorganisms, including pathogens, by analysing nucleic acids extracted from foodstuffs, feed and environmental samples, or from cultures or cell suspensions prepared from the foodstuff. Appropriate procedures for sample preparation, culturing of microorganisms and extraction of nucleic acids are described in ISO 20837.

The main focus of this International Standard is on PCR-based amplification methods. However, because of the rapid rate of technological change in this area, other amplification technologies and detection methods may be considered.

This International Standard is related to a series of 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)

— *Performance testing for thermal cyclers* (ISO/TS 20836)

— *Requirements for amplification and detection for qualitative methods* (ISO 20838)

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 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:

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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

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 determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard provides the overall framework for qualitative methods for the detection of food-borne pathogens using the polymerase chain reaction (PCR).

It covers the general requirements for the specific amplification of target nucleic acid sequences and the detection and confirmation of the identity of the amplified nucleic acid sequence.

Guidelines, minimum requirements and performance characteristics described 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 can also be applied to other matrices, for example environmental samples, or to the detection of other microorganisms under investigation.

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 16140, *Microbiology of food and animal feeding stuffs — Protocol for the validation of alternative methods*

ISO 22174:2005, *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 apply.

4 Principle

For the purposes of this International Standard, qualitative analysis consists of screening and/or specific detection of target nucleic acid sequences in the test samples. Specificity can be at genus, species or a lower taxonomic level.

A qualitative result shall clearly demonstrate the presence or absence of the target sequence under study, relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

The analysis generally consists of

a) amplification by PCR of specific target sequences,

b) detection of the PCR product,

c) confirmation of the identity of the PCR product, and/or

d) confirmation by a standardized microbiological cultural method (e.g. an International Standard).

5 Reagents

In all cases, analytically pure reagents suitable for molecular biological applications shall be used. It is generally advisable to take aliquots of the reaction solutions required for a PCR method and to store them under appropriate conditions, for example at –20 °C.

5.1 DNA polymerase

A thermostable polymerase (possibly including reverse transcriptase activity) is used for PCR. This may be a purified, native enzyme, or a purified, genetically engineered recombinant form of the enzyme.

It should be used according to the manufacturer's instructions.

Each DNA polymerase may need different experimental conditions, e.g. buffer, temperature.

5.2 Reverse transcriptase

This enzyme is used for transcription of RNA in complementary single-stranded DNA (cDNA) able to be amplified by a subsequent PCR.

It should be used according to the manufacturer's instructions.

5.3 Reaction buffer

The appropriate buffer should be used according to the enzyme manufacturer's instructions. Ready to use reagents are commercially available. Materials used for preparation of a PCR buffer shall be stable with respect to storage and cycling conditions.

It should be used according to the manufacturer's instructions.

5.4 **Deoxyribonucleoside triphosphates** (dNTP), for PCR

Solutions containing molecular biology grade dATP, dCTP, dGTP, dTTP and/or dUTP, as appropriate, shall be used. They shall be stable during storage and under PCR conditions. They are commercially available.

5.5 Primers

The primers should be selected based on a sequence specifically to detect the DNA of the target microorganism.

5.6 Water

For the amplification reaction water that is DNase- and RNase-free should be used at all times. Suitable ultra pure water is available commercially.

5.7 Magnesium chloride ($MgCl_2$)

This is supplied either as a component of the reaction buffer or as a separate solution.

5.8 Chemicals for detection of PCR products

The chemicals used for the detection system described in a PCR method should be of appropriate quality.

5.9 Optional additional reagents

5.9.1 Mineral oil

This is dispensed onto the reaction mix to minimize evaporation during thermal cycling.

5.9.2 Facilitators

These are substances, such as polyethylene glycol or bovine serum albumin, which can be added to the PCR reaction to reduce inhibition by matrix-derived substances [1].

5.9.3 RNase inhibitors

These are added to an RT-PCR to prevent degradation of target RNA by RNase enzymes, which may have contaminated the reagents or plastic ware used in the extraction procedure. RNase inhibitors are commercially available.

5.9.4 Reagents to prevent carry over of PCR products

As a further guard against contamination, a decontamination system (based on psoralene, dUTP and UNG, for example) may be included in the PCR system to minimize the risk of carryover contamination from PCR products produced during previous PCR reactions.

6 Apparatus and equipment

This shall be in accordance with ISO 22174.

In addition to standard laboratory equipment, the following apparatus and equipment shall be used.

6.1 Thermal cycler apparatus, capable of reproducibly and accurately performing the temperature and time cycles described in a PCR method.

6.2 Pipettes, with filter tips.

At least three sets of pipettes are required, one of each dedicated to

— sample preparation,

— master mix preparation,

— post-amplification steps.

NOTE The use of filter tips is not necessary in the post-amplification steps.

6.3 Reaction vessels, suitable for use in thermal cyclers, and which can be repeatedly heated to 100 °C and cooled to 4 °C without damage.

6.4 System for detection of PCR products, comprising

a) apparatus for agarose or polyacrylamide gel electrophoresis and, if necessary, a UV radiation source for recording visualization of amplified DNA, or

b) apparatus for nucleic acid column chromatography and the appropriate detection system, or

c) solid phases loaded with a specific probe and apparatus for detecting PCR products, or

d) other equally suitable systems.

7 Procedure

7.1 PCR amplification

Amplification of specific nucleic acid sequences can occur *in vitro* through a reaction catalysed by a DNA polymerase in the presence of oligonucleotide primers and deoxyribonucleoside triphosphates in a defined reaction buffer. An important prerequisite for amplification of the target sequence is that there is no inhibition of the DNA polymerase in the reaction. Amplification of DNA is a cyclical process consisting of

a) denaturation of double-stranded DNA into single-stranded nucleic acid by means of heating,

b) annealing of oligonucleotide primers to the complementary target sequence on both of the single strands of DNA at a suitable temperature, and

c) extension of the primers with deoxyribonucleoside triphosphates by a DNA polymerase at a suitable temperature.

RNA can be detected using PCR if the sequence has first been transcribed into a complementary DNA sequence by a reverse transcriptase.

7.2 Detection and/or confirmation of PCR products

PCR products may be detected by gel electrophoresis, or an appropriate alternative. The size of the PCR products may be estimated by comparison against PCR products of the positive control and a suitable DNA length standard.

For real time PCR analysis, the detection occurs simultaneously with amplification.

Confirmation of the identity of the PCR product should be undertaken by an appropriate method other than size determination, for example by the following:

a) by DNA sequencing of the PCR product;

b) by hybridization of the PCR product with specific DNA probes;

c) by carrying out restriction analysis of the PCR product; the length of the fragments after restriction shall correspond to the expected length of the target DNA sequence after restriction.

A positive result may also or alternatively be confirmed by using a standardized microbiological cultural and confirmatory method as described by appropriate International Standards.

7.3 Controls

The quality, integrity and amount of the DNA template influences the outcome of the PCR, and hence the analytical results obtained.

Because of the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each PCR run. The frequency of use shall be determined as part of the laboratory quality assurance programme. An internal or external amplification control shall be used in every PCR run.

If available and appropriate, reference materials and reference cultures should be used as positive and negative controls.

For the purposes of this International Standard, the controls to be used are given in ISO 22174:2005, 9.3 and Table 1.

7.4 General considerations for amplification reactions

The reaction conditions and the thermocycling conditions should be optimized for every primer pair and/or system. When any PCR is used for the first time on an extract or suspension derived from a particular matrix, it is necessary to demonstrate that the reaction conditions are suitable to achieve the desired detection limit. The detection limit of PCR is the minimum amount of target DNA needed to obtain a signal. In an optimal reaction, less than 40 cycles are required to amplify target molecules to produce a product that is readily detectable by standard methods. The optimized PCR should be able to amplify, in 40 PCR cycles or less, sufficient copies of the PCR product to be detectable. If available this would be from approximately 100 copies of pure template DNA. The absence of PCR inhibition shall be demonstrated using appropriate controls as described in ISO 22174.

In general, the specificity of the reaction should be enhanced as much as possible, for example by using a hot-start PCR. Hot-start PCR is a method which increases specificity by reducing side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer oligomerization.

7.5 Aspects of primer design

Because the performance of each specific PCR should be comparable with other specific PCRs, several aspects of design shall be taken into account.

The primer sequences should preferably have the following characteristics wherever practical:

a) length of each primer of between 18 nucleotides and 30 nucleotides;

b) GC:AT ratio of 50:50 if possible, or as close to this ratio as possible;

c) no concentration of Gs and Cs in short segments of primers (high internal stability);

d) no 3′ end complementarity to avoid primer-dimer formation;

e) no internal secondary structure;

f) no possible dimer formation with primers or probes used in the PCR.

Software packages are available to aid in primer design.

7.6 Verification of primer specificity

7.6.1 General

The ability of the primers to detect the target sequence shall be demonstrated.

This is ideally done in two steps: a first theoretical evaluation, and a second empirical evaluation.

7.6.2 Theoretical evaluation

Theoretical evaluation should be done by performing a sequence similarity search (e.g. FastA, Blast) against one of the major nucleic acid sequence databases (e.g. EMBL, GenBank).

7.6.3 Empirical evaluation

Irrespective of the design criteria used, the specificity of primers shall always be empirically evaluated to confirm the primers' ability to discriminate between the target sequence and closely related species/strains of microorganism sequences and negative controls. ISO 16140 specifies points to consider when selecting strains.

8 Interpretation

The results obtained, including the controls specified in ISO 22174, should be unambiguous and should yield expected results, otherwise the procedure should be repeated.

The PCR result will be either

a) positive if a specific PCR product has been detected and all the controls give expected results, or

b) negative within the limits of detection if a specific PCR product has not been detected, and all the controls give expected results.

9 Performance

The terms are defined in ISO 22174.

The PCR method should be subject to an interlaboratory or intralaboratory test to determine the performance characteristics.

The detection limit of PCR methods for microorganisms other than viruses should be determined by an appropriate method, see Reference [2].

10 Test report

The test report shall be written in accordance with ISO 22174.

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[3] ISO/TS 20836, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Performance testing for thermal cyclers*

[4] 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*

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