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

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

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Foreword

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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.

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ISO 22119 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) has been shown to be a fast, sensitive, and specific method for detection of food-borne pathogens. Further developments of the technology allow the detection of specific PCR products generated by the amplification process. The principle relies on the excitation of fluorescent markers during the PCR process.

This International Standard is part of a series of documents under the general title *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens*:

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*

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 22118, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection and quantification of food-borne pathogens — Performance characteristics*

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*

The following Technical Specification is in preparation:

ISO/TS 13136, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Shiga toxin-producing* Escherichia coli (*STEC*) *belonging to O157, O111, O26, O103 and O145 serogroups — Qualitative real-time polymerase chain reaction (PCR)-based method*

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

1 Scope

This International Standard defines terms for the detection of food-borne pathogens in foodstuffs, and isolates obtained from them, using the polymerase chain reaction (PCR). This International Standard also specifies requirements for the amplification and detection of nucleic acid sequences (DNA or RNA after reverse transcription) by real-time PCR.

The minimum requirements laid down in this International Standard provide the basis for comparable and reproducible results within individual and between different laboratories.

This International Standard is also applicable, for example, to the detection of food-borne pathogens in environmental samples and in animal feeding stuffs.

NOTE Because of the rapid progress in this field, the examples given are those most frequently in use at the time of development of this International Standard.

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 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 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 following terms and definitions apply.

3.1
real-time polymerase chain reaction
real-time PCR
enzymatic procedure which combines the *in vitro* amplification of specific DNA segments by a process of denaturation, annealing of specific primers, and synthesis of DNA with the detection of specific PCR products during the amplification process

NOTE 1 Generally, the amplification reaction mixture contains one or more specific DNA probes coupled with one or more fluorescent dyes. Using this technology, the signal is generated after specific hybridization of the probes to the target nucleic acid sequence and excitation with light of a definite wavelength.

NOTE 2 The use of non-specific DNA-binding fluorescent dyes can be applied if positive results are verified in accordance with ISO 20838.

3.2
PCR product
DNA amplified by PCR

[ISO 22174:2005, 3.4.5]

3.3
fluorescence resonance energy transfer
FRET
⟨food-borne pathogen detection by PCR⟩ distance-dependent energy transfer from a donor molecule to an acceptor molecule resulting in enhanced fluorescence of the acceptor molecule after excitation with electromagnetic radiation of a definite wavelength

NOTE Taken from Reference [2].

3.4
reporter
⟨food-borne pathogen detection by PCR⟩ fluorescent molecule used to detect the hybridization of specific probes by excitation with electromagnetic radiation of an appropriate wavelength

3.5
quencher
⟨food-borne pathogen detection by PCR⟩ fluorescent molecule serving as an energy acceptor and thus quenching the fluorescence signal of the reporter (donor)

3.6
dark quencher
molecule serving as an acceptor, which does not emit energy in a spectral range detected by the optical detection system of the real-time PCR instrument

3.7
5′-3′-exonuclease activity
ability of an enzyme, e.g. a nucleic acid polymerase, to cleave a hybridized nucleic acid molecule in the 5′-3′-direction

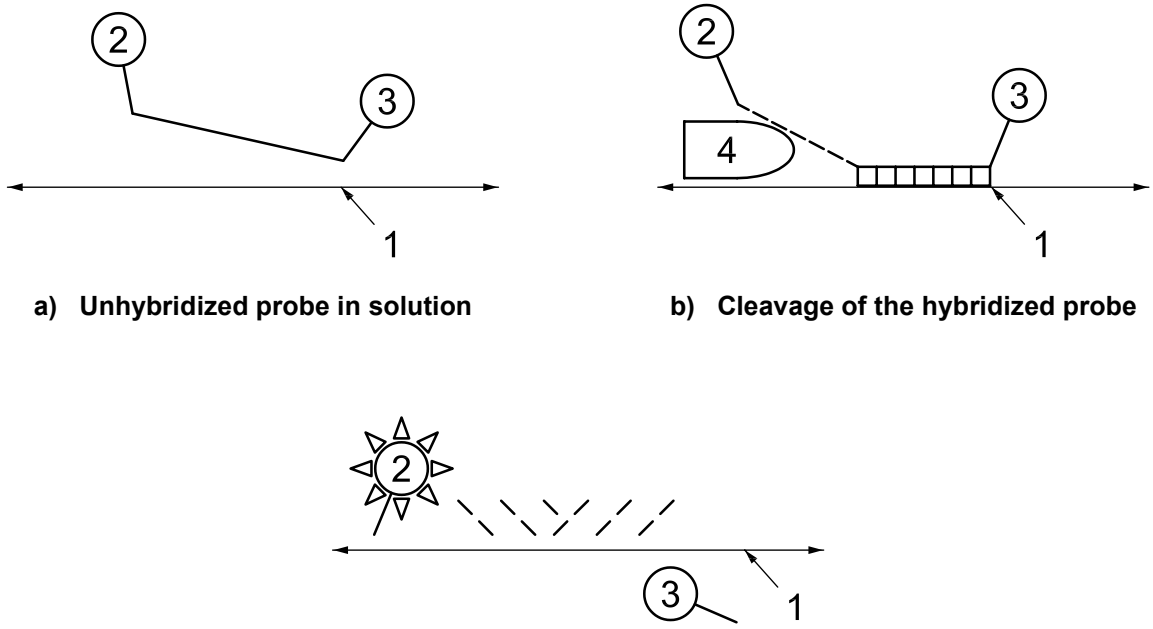
NOTE The activity of 5′-3′-exonuclease is double stranded DNA specific. It is dependent on the type of enzyme and can be present, for example, in Taq-, Tth- and Tfl-polymerase.

3.8
fluorescent probe
oligonucleotide or oligonucleotide analogon of defined sequence coupled with one or more fluorescent molecules

NOTE Any system emitting a fluorescence signal after specific hybridization to the target nucleic acid sequence which can be detected by the specific equipment can be used as a fluorescent probe.

3.9
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

NOTE The principle of a hydrolysis probe is illustrated in Figure 1.



a) Unhybridized probe in solution

b) Cleavage of the hybridized probe

c) Cleaved probe resulting in reporter fluorescence after excitation

Key

1 DNA substrate
2 fluorescent molecule (reporter)
3 quenching molecule
4 enzyme

Figure 1 — Principle of a hydrolysis probe

3.10
hybridization probe
system of two fluorescent probes coupled with one fluorescent molecule each, where one molecule serves as donor and the other serves as acceptor

NOTE The principle of a hybridization probe is illustrated in Figure 2.

a) Unhybridized probes in solution

b) Hybridized probes resulting in acceptor fluorescence

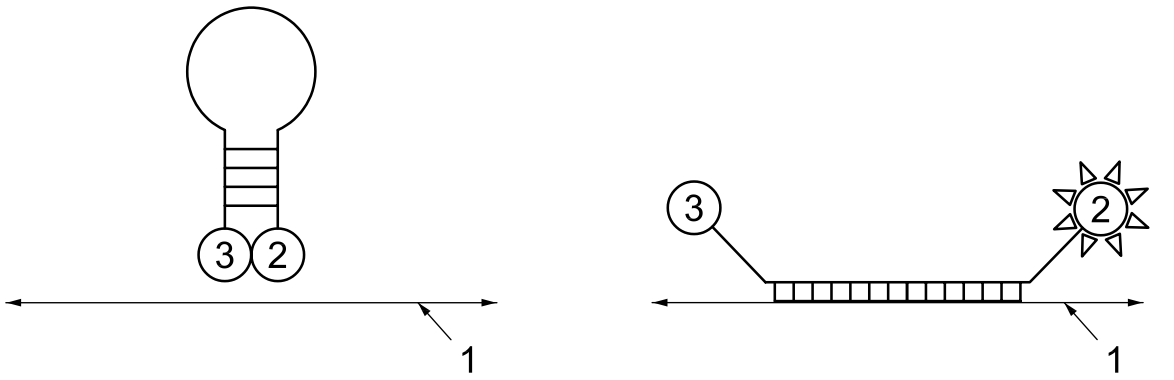
Key

1 DNA substrate
2 acceptor molecule
3 donor molecule

Figure 2 — Principle of a hybridization probe

3.11
molecular beacon
fluorescent probe consisting of three different parts: a central part complementary to the target nucleic acid sequence, plus a 5′-part and a 3′-part which are complementary; the reporter is attached to one arm of the molecule, while the end of the other carries a quencher

NOTE The principle of a molecular beacon is illustrated in Figure 3.



a) Unhybridized molecular beacon in solution

b) Hybridized molecular beacon resulting in reporter fluorescence

Key
1 DNA substrate
2 fluorescent molecule (reporter)
3 quenching molecule

Figure 3 — Principle of a molecular beacon

3.12
probe for detection of a specific pathogen DNA sequence
probe with a sequence complementary to the DNA of a pathogen with a reporter emitting a signal of a definite wavelength which can be detected by the optical detection system

3.13
probe for detection of an internal control nucleic acid sequence
probe with a reporter designed to confirm amplification performance

NOTE 1 The probe emits a signal clearly distinguishable from the signal of the probe designed for the detection of the specific pathogen.

NOTE 2 The application of internal controls requires the use of an instrument able to detect signals of different wavelength.

3.14
passive reference
fluorescent molecules present in the reaction mix used to normalize the signal

NOTE These may be coupled nucleic acid sequences or other molecules not taking part in the reaction.

3.15
baseline fluorescence detection level
“baseline”
point at which a reaction reaches a fluorescence intensity above the background

3.16
background fluorescence
"background"
intrinsic level of fluorescence resulting from the reagents and consumables used

3.17
threshold cycle crossing point
point of the amplification curve at which the fluorescence signal rises above the baseline or crosses a predefined threshold setting

4 Principle

4.1 General

Real-time PCR analysis generally consists of:

a) amplification of specific target sequences by PCR in the presence of fluorescent probes;

b) binding of fluorescent probes during each amplification cycle;

c) generation of a fluorescent signal by excitation during each cycle;

d) monitoring of the fluorescence signals by the optical detection system;

e) data analysis.

NOTE For screening purposes, fluorescence signals from DNA double strand binding dyes can also be used.

4.2 Probes for real-time PCR

4.2.1 Hydrolysis probes

The hydrolysis probe is a specific oligonucleotide present in the PCR assay together with the PCR primers. One end of the probe bears a fluorescent reporter molecule with an emission spectrum which is quenched by a second molecule located at the other end.

The probe hybridizes to the target nucleic acid sequence. During the extension step, the 5′-3′-exonuclease activity of the DNA polymerase cleaves the hybridized probe. After cleavage, the reporter is separated from the quencher, resulting in an increase in the fluorescence intensity of the reporter. The resulting fluorescence signal is proportional to the production of specific PCR product.

The 3′-end of the probe should be blocked to prevent its extension during the PCR.

4.2.2 Hybridization probes

Two hybridization probes are present in the PCR assay as specific oligonucleotides in addition to the PCR primers. These probes, each containing a fluorescent molecule, one of which serves as a donor, the other as an acceptor, hybridize to the target nucleic acid sequences. After hybridization, both dyes are in a close proximity so that fluorescence resonance energy transfer occurs on excitation and the acceptor molecule generates a detectable signal. The resulting fluorescence signal is proportional to the production of a specific PCR product.

The 3′-end of the probes should be blocked to prevent its extension during the PCR.