INTERNATIONAL STANDARD

ISO 20837

First edition
2006-04-15

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

Microbiologie des aliments — Réaction de polymérisation en chaîne (PCR) pour la détection des micro-organismes pathogènes dans les aliments — Exigences relatives à la préparation des échantillons pour la détection qualitative

Reference number
ISO 20837:2006(E)

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Foreword

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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 20837 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).

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Introduction

The detection of food-borne pathogens by PCR is usually performed by means of the following successive (or simultaneous) steps:

- homogenization of the sample;
- (cultural) enrichment of the pathogen under study and sample treatment;
- nucleic acid extraction (optional);
- amplification of nucleic acids from the pathogen under study;
- detection of the amplified DNA from the pathogen under study.

References to International Standards concerning enrichment of bacteria from food matrices are given in Annex A. An example of a specific method for sample preparation is given in Annex B.

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.

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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 sample preparation for qualitative detection

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 criteria and examples for sample preparation in order to obtain PCR-compatible samples or nucleic acids of suitable quality and quantity for PCR.

It provides a description of the general principles involved. References to standards concerning the enrichment of microorganisms are given in Annex A, and a detailed method for DNA extraction is given in Annex B.

This International Standard has been established for food matrices, but could also be applied to feed and agricultural/environmental matrices with some adaptations, if necessary.

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 22174:2005, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Principle

3.1 General

The objective of the sample preparation methods described is to obtain samples or nucleic acids of suitable quality and quantity for PCR.

NOTE The quality of nucleic acids depends for example on the chemical purity, the average length of the molecules and the structural integrity of the extracted nucleic acid molecules.

Enrichment and sample treatment should allow the detection of low numbers of target microorganisms and the reduction of PCR inhibitory substances. Physical, chemical or biochemical procedures, least destructive to the nucleic acid integrity, should render the sample or the nucleic acid solution compatible with PCR amplification.

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3.2 Enrichment and sample treatment

Sample treatment may start directly from the sample or after enrichment as described in the standards listed in Annex A or other appropriate standards.

3.3 Nucleic acid extraction

The basic principles of nucleic acid extraction consist of releasing DNA present in the bacteria and concurrent or subsequent removal of PCR inhibitors.

An example of a DNA extraction/purification method is provided in Annex B. This method is only an example and should be further modified by the users to meet the purpose of each laboratory.

4 General laboratory requirements

Sample treatment should be carried out in separate working areas/rooms as specified in ISO 22174:2005, 6.3.

5 Reagents, apparatus and equipment

See Annexes A and B of this International Standard and also ISO 22174.

6 Procedure

6.1 Enrichment and sample treatment for bacteria

Food samples should be enriched according to the corresponding International Standards or other appropriate standards. Other enrichment media found to be more PCR compatible could be used, if they have been shown, through validation, to have performance at least comparable to those described in International Standards.

Some enrichment media recommended in International Standards contain less PCR-inhibitory substances than others, which should be carefully considered in connection with the choice of sample preparation method.

For some products, special care should be taken to suppress the growth of competing background micro-organisms (e.g. by addition of selective chemicals or antibiotics).

Least destructive methods, such as a simple dilution, centrifugation, protein digestion, filtration, density centrifugation, immunomagnetic separation, etc. may be tried. In the case of lack of PCR response, more rigorous methods such as boiling, the use of chelating agents or harsh chemicals, such as chloroform and ethanol, or kits with similar actions may be tried. Simple physical methods may be used to reduce the fat content of high-fat samples. Chelating agents may be used to reduce the high calcium content of dairy products which can be inhibitory.

6.2 Nucleic acid extraction

6.2.1 DNA extraction

6.2.1.1 DNA release and purification

Several DNA extraction principles may be combined. For example, the following steps may be carried out:

a) degrade the proteins in the cell extract with proteases (e.g. Proteinase K) and RNA with ribonucleases;

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b) precipitate the resulting peptides with organic solvents (e.g. a mixture of phenol and chloroform) to leave the DNA in the aqueous phase;

c) purify the DNA solution and concentrate further by ethanol precipitation in the presence of monovalent cations;

d) collect the precipitated DNA by centrifugation;

e) wash the DNA with ethanol and resuspend in buffer [e.g. tris(hydroxymethyl)aminomethane/EDTA buffer (Tris-EDTA buffer) or Tris buffer].

A DNA co-precipitant such as glycogen, polyethylene glycol (PEG) or transfer RNA (t-RNA) may be used to improve the DNA recovery during the precipitation steps. Only co-precipitants without any nuclease activity and without PCR inhibitors/competitors, and without any sequence homology with potential PCR targets under study may be used.

NOTE Using vacuum freeze dryers to dry the DNA pellets obtained after a precipitation step can cause cross contamination.

DNA may be released by thermal cell disruption (e.g. by boiling for 10 min). After boiling, centrifuge the chilled sample and use the supernatant for PCR. Before boiling, to facilitate cell disruption, enzymatic treatment may be applied (e.g. lysozyme, mutanolysin, for use with Gram-positive bacteria) followed by a protease/proteinase incubation. Other methods such as vigorous agitation with beads may be required when the organism has a particularly tough cell wall (e.g. *Mycobacterium* spp.).

Any other method including commercial kits may be used for nucleic acid extraction provided the results are at least comparable.

6.2.1.2 DNA quality and quantity

The quality and yield of DNA extracted using a given method on a given matrix should be both repeatable and reproducible in terms of amplification by PCR, provided enough DNA is present in the matrix. In particular, the method used shall allow the recovery of DNA fragments with average size equal to or larger than the PCR products under study.

The concentration and purity of the DNA isolated may be estimated by fluorometric methods or by gel electrophoresis. Purified DNA may be quantified by spectrophotometric methods.

A rapid way to estimate the quality and amount of DNA is agarose gel electrophoresis, followed by ethidium bromide staining and fluorescence under ultraviolet (UV) light (see Reference [1]).

For some sample preparation methods (e.g. boiling), it is necessary to use the nucleic acid solution directly after the preparation, when the nucleic acids released are not stable.

In general, repeated freezing and thawing of nucleic acid solutions should be avoided.

Use appropriate plastic ware to store low copy numbers of nucleic acids.

NOTE Some tube materials can bind nucleic acids.

6.3 Controls

Controls shall be used according to ISO 22174:2005, 9.3 and Table 1.

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Annex A
(informative)

Standards concerning the enrichment of microorganisms (bacteria)

The standards listed in Table A.1 contain information concerning the enrichment of microorganisms (bacteria).

Table A.1 — Specific standards for some microorganisms (bacteria)

Bacteria	International Standard
Salmonella spp.	ISO 6579
Staphylococcus aureus	ISO 6888-3
Bacillus cereus	ISO 7932
Clostridium perfringens	ISO 7937
Campylobacter spp.	ISO 10272-1
Yersinia enterocolitica	ISO 10273
Listeria monocytogenes	ISO 11290-1
Escherichia coli O157	ISO 16654
Shigella spp.	ISO 21567

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Annex B
(informative)

Method for DNA extraction from Gram-negative bacteria

B.1 Applicability

This method is suitable for the extraction of DNA from Gram-negative bacteria by lysis or boiling.

B.2 Status

This method is well known and has been widely applied [1]. Furthermore this method was validated in a collaborative study for the detection of *Salmonella* strains in enriched broth samples from milk powder [2], [3] and in a collaborative study for the detection of verotoxin-forming *Escherichia coli* (VTEC) in minced meat [4], [5].

B.3 Principle

This method consists of centrifugation steps to harvest the bacteria. The bacterial cells are washed, lysed by an incubation step at 95 °C to 100 °C and then centifuged to precipitate cell wall debris, polysaccharides and proteins in order to obtain the nucleic acids extract.

The starting material is usually enriched broth.

B.4 Reagents

B.4.1 Washing buffer, physiological saline [ρ(NaCl) = 9 g/l] solution or phosphate-buffered saline, PBS [ρ(NaCl) = 8 g/l, ρ(KCl) = 0,2 g/l, $\rho(Na_2HPO_4)$ = 1,44 g/l, $\rho(KH_2PO_4)$ = 0,24 g/l; pH 7,4].

B.5 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

B.5.1 Microlitre pipettes.

B.5.2 Bench-top centrifuge, for microlitre reaction vessels having a capacity of 1,5 ml to 2 ml and an adjustable acceleration of up to 10 000 g.

B.5.3 Waterbath or **thermal block**, for microlitre reaction vessels, capable of being heated to 100 °C.

B.5.4 Agitator, e.g. vortex mixer.

B.6 Procedure

B.6.1 General

After enrichment, bacterial DNA is extracted as described in B.6.2.

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B.6.2 Extraction procedure

Transfer 1 ml of a microbial enrichment to a reaction tube. Centrifuge the cell suspension for ten minutes at 10 000 g (B.5.2). Discard the supernatant.

Re-suspend the sediment in 1 ml of washing buffer (B.4.1) or water. Centrifuge the cell suspension for ten minutes at 10 000 g (B.5.2). Discard the supernatant.

Re-suspend the sediment in 200 µl to 250 µl of water. Carry out a thermal cell digestion by heating for 20 min at 95 °C (*Salmonella*) or 15 min at 100 °C (STEC).

Transfer the reaction vessels to an ice-bath in order to cool them down quickly.

Mix the lysate vigorously [e.g. using an agitator (B.5.4)]. Centrifuge at a temperature of 20 °C to 25 °C for 3 min at 10 000 g (*Salmonella*) or 10 s at 10 000 g (STEC).

Transfer the supernatant to a fresh microlitre reaction vessel.

Nucleic acid extracts that have been shown to cause PCR inhibition, indicated by appropriate controls, shall be purified before use in the PCR.

Provided the manufacturer’s instructions are observed, commercially available extraction and purification systems may optionally be used instead of thermal cell digestion and any necessary purification.

B.7 Validation

This method is widely used and a basic method in molecular biology. Furthermore this method has been validated in collaborative trials.

In 1998, an interlaboratory test was organized by the DIN (German Institute for Standardization) working group “PCR for the detection of food-borne microorganisms”. Twelve laboratories took part in the test, each laboratory receiving 20 samples. Sample material was low fat milk powder. A part of this milk powder was artificially contaminated with 1 000 cfu of *Salmonella*/25 g. In the laboratories the samples were prepared as follows before the DNA extraction was performed: 25 g sample was added to 250 ml non-selective enrichment fluid (brilliant green 0,002 %) and incubated at 37 °C for 16 h. The nucleic acid extraction started with 1 ml of this enriched sample broth. The extracts were tested by PCR. A total of 240 samples were analysed; 1 sample was found to be false negative and 2 samples false positive; for 14 samples PCR inhibition was observed.

In 2001, this method was validated in a collaborative study organized by the working group “PCR for the detection of food-borne microorganisms” of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) under article 35 of the German Federal Foodstuffs Act. Eleven laboratories took part. Each laboratory received ten minced meat samples prepared from two basic matrices. In each case, five of the samples were contaminated uniformly with known STEC/EHEC strains in doses of between 54 cfu/25 g and 308 cfu/25 g of minced meat. The accompanying flora was between 10^5 cfu/g and 10^6 cfu/g and sometimes higher (as a result of transportation). Samples 1 to 9 (prepared from a basic material) were additionally naturally contaminated with 4 STEC strains. PCR yielded a 100 % detection rate for all the laboratories.

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[6] ISO 6579, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of* Salmonella *spp.*

[7] ISO 6888-3, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus *and other species) — Part 3: Detection and MPN technique for low numbers*

[8] ISO 7932, *Microbiology — General guidance for the enumeration of* Bacillus cereus *— Colony-count technique at 30 °C*

[9] ISO 7937, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of* Clostridium perfringens *— Colony-count technique*

[10] ISO 10272-1, *Microbiology of food and animal feeding stuffs — Horizontal method for detection and enumeration of* Campylobacter *spp. — Part 1: Detection method*

[11] ISO 10273, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of presumptive pathogenic* Yersinia enterocolitica

[12] ISO 11290-1, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of* Listeria monocytogenes *— Part 1: Detection method*

[13] ISO 16654, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of* Escherichia coli *O157*

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