
**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*



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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.

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.

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:

Licensing Department
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
USA

and

Roche Molecular Systems, Inc.
Licensing Department
1145 Atlantic Avenue
Alameda, CA 94501
USA

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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.

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;

- 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.

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

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 centrifuged 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 [$\rho(\text{NaCl}) = 9 \text{ g/l}$] solution or phosphate-buffered saline, PBS [$\rho(\text{NaCl}) = 8 \text{ g/l}$, $\rho(\text{KCl}) = 0,2 \text{ g/l}$, $\rho(\text{Na}_2\text{HPO}_4) = 1,44 \text{ g/l}$, $\rho(\text{KH}_2\text{PO}_4) = 0,24 \text{ g/l}$; 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.