INTERNATIONAL STANDARD

ISO 11290-1

Second edition
2017-05

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. —

Part 1: Detection method

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Listeria monocytogenes *et de* Listeria *spp.* —

Partie 1: Méthode de recherche

Reference number
ISO 11290-1:2017(E)

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

This second edition cancels and replaces the first edition (ISO 11290-1:1996), which has been technically revised. It also incorporates the amendment ISO 11290-1:1996/Amd.1:2004.

The main changes, compared to ISO 11290-1:1996, are the following.

— The detection of *Listeria monocytogenes* has been modified as listed below.

— Primary enrichment in half-Fraser broth: incubation for 25 h ± 1 h.

— Secondary enrichment in Fraser broth: incubation for 24 h ± 2 h.[29]

— Half-Fraser and Fraser broths may be refrigerated before transfer or isolation on selective agar for a maximum of 72 h.

— Storage of isolation plates: incubated plates can be refrigerated for a maximum of two days before reading.

— Microscopic aspect for confirmation is optional if the isolation agar allows distinction between pathogenic and non-pathogenic *Listeria* spp.

— CAMP test and catalase test are optional.

— Inclusion of new performance characteristics.

— Moreover, detection of *Listeria* spp. has been included in the scope and the title changed accordingly.

A list of parts in the ISO 11290 series can be found on the ISO website.

Introduction

The main changes, listed in the foreword, introduced in this document compared to ISO 11290-1:1996 are considered as major (see ISO 17468[28]). The technical changes were assessed and were considered to have no significant effect on the method performance characteristics or test results.

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products for which it may be necessary to use different or specific methods. Nevertheless, in all cases, this horizontal method is intended to be applied as far as possible and deviations from this only be made for justified technical reasons.

When this document is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from it in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed, they will be changed to comply with this document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. —

Part 1: Detection method

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *L. monocytogenes* and *Listeria* spp. are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices. In particular, it is strongly recommended that tests for detecting *L. monocytogenes* are undertaken in laboratories providing biosafety level 2 conditions. It is strongly recommended that female laboratory staff are made aware of the particular risk to the developing foetus presented by infection of the mother through exposure to *L. monocytogenes* and *Listeria* spp., and that pregnant personnel and persons with recognized underlying conditions or diseases that impair cell-mediated immunity do not manipulate cultures of *L. monocytogenes* and *Listeria* spp.

1 Scope

This document specifies a horizontal method for

— the detection of *L. monocytogenes*, and

— the detection of *Listeria* spp. (including *L. monocytogenes*).

This document is applicable to

— products intended for human consumption and for the feeding of animals, and

— environmental samples in the area of food production and food handling.

It is possible that certain additionally described *Listeria* species may not be detected or confirmed by this method.[5],[10],[12],[14],[25],[26],[27]

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
Listeria monocytogenes
microorganisms which form typical colonies on solid selective media and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this document

3.2
detection of *Listeria monocytogenes*
determination of the detection/non detection of *Listeria monocytogenes* (3.1), in a given mass or volume of product or a specified surface, when tests are carried out in accordance with this document

3.3
***Listeria* spp.**
microorganisms which form typical colonies on solid selective media and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this document

3.4
detection of *Listeria* spp.
determination of the detection/non detection of *Listeria* spp. (3.3), in a given mass or volume of product or a specified surface, when tests are carried out in accordance with this document

4 Principle

4.1 General

Listeria spp. may be present in small numbers and are often accompanied by considerably larger numbers of other microorganisms, therefore selective enrichment is necessary. It is also necessary to detect injured and stressed *Listeria* spp. and the primary selective enrichment medium, with reduced inhibitor concentration, fulfils at least part of this function.

NOTE Presence of *L. monocytogenes* can be masked by the presence of other *Listeria* species, in particular *L. innocua* or *L. ivanovii*.

Within the limits of this document, the detection of *L. monocytogenes* and of *Listeria* spp. necessitates four successive stages, as described in the flowchart in Annex A.

4.2 Primary enrichment in a selective liquid enrichment medium with reduced concentration of selective agents (half-Fraser broth)

Inoculation of a selective primary enrichment medium containing half the concentrations of acriflavine and nalidixic acid (half-Fraser broth, see B.1), which is also used as a dilution fluid for the test portion (9.1).

Incubation of the initial suspension at 30 °C for 24 h to 26 h.

4.3 Secondary enrichment with a selective liquid enrichment medium with full concentration of selective agents (Fraser broth)

Inoculation of full-strength secondary liquid enrichment medium (Fraser broth) with a culture obtained from 4.2.

Incubation of the Fraser broth at 37 °C for 24 h.[29]

4.4 Plating out and identification

From the cultures obtained in 4.2 and 4.3, plating out on the two selective solid media:

— Agar *Listeria* according to Ottaviani and Agosti (see References [16] and [17] and B.3);

— any other solid selective medium at the choice of the laboratory complementary to Agar *Listeria* according to Ottaviani and Agosti, using a different substrate and/or principle than the one used in *Listeria* agar according to Ottaviani and Agosti (see B.4). See Annex E for information about media.

Incubation of the Agar *Listeria* according to Ottaviani and Agosti at 37 °C for a total of 48 h. If colonies of presumptive *L. monocytogenes* or *Listeria* spp. are evident at 24 h the incubation may be stopped at this stage. Incubation of the second selective medium at the appropriate temperature and examination after the appropriate time.

4.5 Confirmation

Subculturing of the colonies of presumptive *L. monocytogenes* or *Listeria* spp., plated out as described in 4.4, and confirmation by means of appropriate morphological and/or biochemical tests.

5 Culture media and reagents

For current laboratory practices, refer to ISO 11133.

Composition and performance testing of culture media and reagents and their preparation are described in Annex B.

6 Equipment and consumables

Usual microbiological equipment (as specified in ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) **or wet sterilization** (autoclave).

As specified in ISO 7218.

6.2 Drying cabinet or incubator, capable of being maintained between 25 °C and 50 °C.

6.3 Incubators, capable of operating at 30 °C ± 1 °C, 37 °C ± 1 °C and at 25 °C ± 1 °C (optional).

6.4 Water bath, capable of operating at 47 °C to 50 °C.

6.5 Sterile loops approximately 3 mm in diameter or 10 µl, and inoculating **needle** or **wire.**

6.6 pH meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ± 0,1 pH unit.

6.7 Graduated pipettes or automatic pipettes, of nominal capacities 1 ml and 10 ml.

6.8 Petri dishes, for example of diameter 90 mm.

6.9 Microscope, preferably with phase-contrast, and with slides and cover slips.

6.10 Refrigerator, capable of operating at 5 °C ± 3 °C.

7 Sampling

Sampling is not part of the method specified in this document. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject. For food and feed samples, refer to ISO/TS 17728[3]. For environmental samples, use ISO 18593[2] and see Reference [24].

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage (see ISO 7218).

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned [see ISO 6887 (all parts) and ISO 18593[2]]. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion and initial suspension

Refer to ISO 6887 (all parts) and any specific International Standard appropriate to the product concerned.

For preparation of the initial suspension, use as dilution fluid the selective primary enrichment medium specified in B.1 (half-Fraser broth).

In general, to prepare the initial suspension, add a test portion of 25 g or 25 ml to 225 g or 225 ml of the selective primary enrichment medium (B.1), to obtain a tenfold dilution, and homogenize. Pre-warm the selective primary enrichment medium to room temperature before use.

This document has been validated for test portions of 25 g or ml. A smaller test portion may be used, without the need for additional validation/verification, providing that the same ratio between primary enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, if a validation/verification study has shown that there are no adverse effects on the detection of *L. monocytogenes* or *Listeria* spp.

NOTE 1 Validation can be conducted in accordance with the appropriate document of ISO 16140 (all parts)[1]. Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests). See References [21] and [22] as they provide information on the particular case of *Listeria* pooling samples.

NOTE 2 For large quantities, it is recommended to pre-warm the selective primary enrichment medium to 30 °C ± 1 °C before mixing it with the test portion.

9.2 Primary enrichment

Incubate the primary enrichment medium (half-Fraser broth, see B.1), prepared in accordance with 9.1, at 30 °C (6.3) for 25 h ± 1 h.

NOTE 1 A black coloration can develop during the incubation.

NOTE 2 It is possible to store at 5 °C (6.10) the pre-enriched sample after incubation before transfer to Fraser broth for a maximum of 72 h.

(See Reference [20].)

9.3 Secondary enrichment

9.3.1 After incubation of the initial suspension (primary enrichment) for 25 h ± 1 h (9.2), transfer 0,1 ml of the culture obtained in 9.2 (regardless of its colour) to a tube or bottle containing 10 ml of secondary enrichment medium (Fraser broth) (B.2).

9.3.2 Incubate the inoculated medium (9.3.1) for 24 h ± 2 h at 37 °C (6.3).

NOTE In the case of *Listeria* spp. other than *Listeria monocytogenes* detection, additional 24 h incubation can allow for recovery of more species.

9.4 Plating out and identification

9.4.1 General

9.4.1.1 From the primary enrichment culture (9.2) incubated for 25 h ± 1 h at 30 °C (6.3), inoculate, by means of a loop (6.5), the surface of the first selective plating medium, Agar *Listeria* according to Ottaviani and Agosti (B.3), to obtain well-separated colonies.

Proceed in the same way with the second selective plating-out medium of choice (B.4).

NOTE Half-Fraser broth and Fraser broth can be refrigerated at 5 °C (6.10) before isolation on selective agar for a maximum of 72 h.[20]

9.4.1.2 From the secondary enrichment medium incubated for 24 h ± 2 h at 37 °C (6.3) (9.3.2), repeat the procedure described in 9.4.1.1 with the two selective plating-out media.

9.4.1.3 Invert the Petri dishes obtained in 9.4.1.1 and 9.4.1.2 and place them in an incubator set at 37 °C (6.3) for Agar *Listeria* according to Ottaviani and Agosti (B.3). For the second selective medium (B.4), follow the manufacturer's instructions.

9.4.1.4 For Agar *Listeria* according to Ottaviani and Agosti incubate for a total of 48 h ± 2 h. If colonies of presumptive *L. monocytogenes* or *Listeria* spp. are evident at 24 h ± 2 h the incubation may be stopped at this stage. For second selective agar incubate for the appropriate time. Examine the dishes (9.4.1.3) for the presence of presumptive colonies of *L. monocytogenes* or *Listeria* spp.

NOTE After incubation plates can be refrigerated at 5 °C (6.10) for a maximum of 48 h before reading.

9.4.2 Agar *Listeria* according to Ottaviani and Agosti

Consider as presumptive *L. monocytogenes* the blue-green colonies surrounded by an opaque halo (typical colonies). Colonies of *L. ivanovii* are also blue-green and surrounded by an opaque halo.

Consider as presumptive *Listeria* spp. the blue-green colonies with or without opaque halo.

NOTE 1 Some strains of *L. monocytogenes* exposed to stress conditions, particularly acid stress, can show a very weak halo (or even no halo).

NOTE 2 Some rare *L. monocytogenes* are characterized by a slow PIPLC (phosphatidyl inositol phospholipase C) activity. Such bacteria are detected when the total duration of incubation is more than, for example, four days. Some of these strains could be pathogenic.[13] No *L. monocytogenes* strains have been described as PIPLC negative.

NOTE 3 Some organisms other than *Listeria* spp. can produce blue colonies on this medium. See Annex C and Reference [23].

9.4.3 Second selective medium

After the appropriate time, examine the plates for the presence of colonies which are considered to be presumptive *Listeria* spp. or *L. monocytogenes*, based on their characteristics for the type of medium used (B.4). See Annex E for more information.

9.5 Confirmation of *Listeria monocytogenes* or *Listeria* spp.

9.5.1 Selection of colonies for confirmation

9.5.1.1 For confirmation of presumptive *L. monocytogenes*, take at least one colony presumed to be *L. monocytogenes* (see 9.4.2 and 9.4.3). One confirmed isolate per sample is sufficient. If the first colony is negative take further colonies presumed to be *L. monocytogenes* from selective medium (up to a maximum of five colonies from each plate of each selective medium).

Streak the selected colonies onto the surface of pre-dried plates of a non-selective agar, for example blood agar, nutrient agar, tryptone soya yeast extract agar (TSYEA) (B.14), in a manner which will allow isolated colonies to develop.

Use of blood agar for pure culture enables interpretation of haemolysis, when positive, already at that stage (see 9.5.2.5.2 and Annex D). If streaking on blood agar does not show haemolysis, then the haemolysis test shall be done by stabbing (9.5.2.5.2) or in liquid medium (9.5.2.5.3).

Place the plates in the incubator set at 37 °C (6.3) for 18 h to 24 h or until growth is satisfactory.

If the colonies are not isolated, pick a typical *L. monocytogenes* colony onto another non-selective agar plate. Carry out the following tests (9.5.2) from colonies of a pure culture on the non-selective agar.

9.5.1.2 For confirmation of presumptive *Listeria* spp., take at least one colony presumed to be *Listeria* spp. (see 9.4.2 and 9.4.3). One confirmed isolate per sample is sufficient. If the first colony is negative take further colonies presumed to be *Listeria* spp. from selective medium (up to a maximum of five colonies from each plate of each selective medium).

For confirmation of *Listeria* spp. use plates of TSYEA.

Streak the selected colonies onto the surface of pre-dried plates of TSYEA (B.14), in a manner which will allow isolated colonies to develop.

Place the plates in the incubator set at 37 °C (6.3) for 18 h to 24 h or until growth is satisfactory.

Typical colonies of *Listeria* spp. on TSYEA are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. When the plates are held to the light (artificial or natural) at about 45 degree angle, colonies exhibit a blue-grey colour and a granular surface.

If the colonies are not isolated, pick a typical *Listeria* spp. colony onto another non-selective agar plate.

Carry out the following tests (9.5.3) from typical colonies of a pure culture on TSYEA.

9.5.2 Confirmation of *L. monocytogenes*

9.5.2.1 General

Carry out the confirmation tests for *L. monocytogenes*. Appropriate positive and negative control strains for each of the confirmation tests shall be used.

Perform at minimum the mandatory tests as listed (in bold) in Table 1.

Table 1 — Confirmation tests for *L. monocytogenes*

Tests	*L. monocytogenes* confirmation tests	Results
Mandatory	**Microscopic aspect[a] (9.5.2.4)**	**Slim short rods or coccobacilli**
	Beta-haemolysis (9.5.2.5)	**+**
	L-Rhamnose (9.5.2.7)	**+**
	D-Xylose (9.5.2.7)	**-**
Optional	Catalase (9.5.2.2)	+
	Motility at 25°C (9.5.2.3)	+
	CAMP test (9.5.2.6)	+

[a] Microscopic aspect is optional for Agar *Listeria* according to Ottaviani and Agosti and for the second medium if it allows distinction between pathogenic and non-pathogenic *Listeria* spp.

Details on results for confirmation tests can be found in Annex D.

NOTE An alternative procedure as mentioned in ISO 7218 can be used to confirm the isolate as *Listeria monocytogenes*, providing the suitability of the relevant procedure is verified.

If shown to be reliable, miniaturized galleries for the biochemical identification of *Listeria monocytogenes* may be used (see ISO 7218).

Rare strains of *L. monocytogenes* do not show beta-haemolysis or a positive reaction to the CAMP test under the conditions described in this document. If typical colonies on Agar *Listeria* according to Ottaviani and Agosti with PIPLC activity even if it is low, are negative for haemolysis, it is recommended to perform additional tests (e.g. Gram stain, catalase, motility, CAMP test, PCR), in order to determine whether this isolate is a non-haemolytic *L. monocytogenes*.

9.5.2.2 Catalase reaction (optional)

Take an isolated colony obtained in 9.5.1.1 and suspend it in a drop of hydrogen peroxide solution (B.6) on a slide. The immediate formation of gas bubbles indicates a positive reaction.

NOTE A catalase reaction performed from a colony originating from a blood agar can sometimes lead to false-positive results.

9.5.2.3 Motility test (optional)

Take an isolated colony obtained in 9.5.1.1 and suspend it in a tube containing a non-selective nutrient liquid medium.

Incubate in the incubator (6.3) set at 25 °C for 8 h to 24 h until the medium turns cloudy.

Take a drop of the above culture using a loop (6.5) onto a clean glass microscope slide. Place a cover slip on top and examine it under a microscope (6.9).

Listeria spp. (including *L. monocytogenes*) appear as slim, short rods with tumbling motility.

Cultures grown at temperatures above 25 °C may fail to exhibit this motion. Always compare them to a known *Listeria* culture. Cocci, large rods, or rods with rapid swimming motility are not *Listeria* spp.

As an alternative test for motility, using an inoculating needle (6.5), dilute in sterile water (or other appropriate diluent) a fragment of isolated colony obtained on non-selective agar. *Listeria* spp. (including *L. monocytogenes*) appear as slim, short rods with tumbling motility.

As another alternative test for motility, using an inoculating needle (6.5), stab the motility agar (B.7) with a culture taken from a typical colony obtained in 9.5.1.1. Incubate at 25 °C for 48 h ± 2 h.

