

Australian/New Zealand Standard®

Food microbiology

Method 2.16.1: Examination for specific organisms—Food and animal feeding stuffs—Horizontal method for the detection and enumeration of *Listeria monocytogenes*—Detection method

PREFACE

This Standard was prepared by the Joint Standards Australia/Standards New Zealand Committee FT/4, Food Microbiology.

This Standard is technically equivalent to and reproduced from ISO 11290-1:1996, *Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of Listeria monocytogenes*, Part 1: *Detection method*, with additional requirements for Australian/New Zealand conditions listed in Annex ZZ.

As this Standard is reproduced from an International Standard, the following applies:

- (a) Its number does not appear on each page of text and its identity is shown only on the cover and title page.
- (b) In the source text, 'this International Standard' should read 'this Australian/New Zealand Standard'.
- (c) A full point substitutes for a comma when referring to a decimal marker.
- (d) References to International Standards should be replaced by references to the following Australian Standards:

Reference to International Standard or other publication

Australian Standard

ISO		AS	
6887	Microbiology—General guidance for the preparation of dilutions for microbiological examination	1766	Food microbiology
		1766.3	Part 3: Examination of specific products
7218	Microbiology of food and animal feeding stuffs—General rules for microbiological examination	1766.1	Part 1: General procedures and techniques

The terms 'normative' and 'informative' have been used in this Standard to define the application of the annex to which they apply. A 'normative' annex is an integral part of a Standard, whereas an 'informative' annex is only for information and guidance.

STANDARDS NEW ZEALAND

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METHOD

WARNING — In order to safeguard the health of laboratory personnel, it is strongly recommended that tests for detecting *Listeria monocytogenes* are 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. In particular, it is strongly recommended that pregnant personnel do not manipulate cultures of *L. monocytogenes*.

1 Scope

This part of ISO 11290 specifies a horizontal method for the detection of *Listeria monocytogenes*.

Subject to the limitations discussed in the introduction, this part of ISO 11290 is applicable to products intended for human consumption or animal feeding.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 11290. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 11290 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*.

3 Definitions

For the purposes of this part of ISO 11290, the following definitions apply.

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 part of ISO 11290.

3.2 detection of *Listeria monocytogenes*: Determination of the presence or absence of these microorganisms, in a given mass or volume of product, when tests are carried out in accordance with this part of ISO 11290.

4 Principle

Within the limits of this part of ISO 11290, the detection of *L. monocytogenes* necessitates four successive stages (see annex A for a flowchart).

NOTE 1 *Listeria* spp. may be present in small numbers and are often accompanied by considerably larger numbers of other genera, therefore selective enrichment is necessary. It is also necessary to detect injured *Listeria* spp. and the primary selective enrichment medium, with reduced inhibitor concentration, fulfils at least part of this function.

4.1 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 one volume of lithium chloride and half a volume of both acriflavine and nalidixic acid (half Fraser broth), which is also used as a dilution fluid for the test portion (9.1).

Incubation of the test portion at 30 °C for 24 h.

4.2 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 4.1.

Incubation of the Fraser broth at 35 °C or 37 °C for 48 h.

4.3 Plating out and identification

From the cultures obtained in 4.1 and in 4.2, plating out on the two selective solid media:

- a) Oxford agar;
- b) PALCAM agar.

Incubation at 30 °C, 35 °C or 37 °C and examination after 24 h and, if necessary, after 48 h to check for the presence of characteristic colonies which are presumed to be *L. monocytogenes*.

4.4 Confirmation

Subculturing of the colonies of presumptive *L. monocytogenes*, plated out as described in 4.3, and confirmation by means of appropriate morphological, physiological and biochemical tests.

5 Culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218.

NOTE 2 Because of the large number of culture media and reagents, it has been considered preferable, for clarity of the text, to give their composition and preparation in annex B.

5.2 Selective primary enrichment medium: Fraser broth with reduced concentration of selective agents (half Fraser broth)

See clause B.1.

5.3 Selective secondary enrichment medium with full concentration of selective agents (Fraser broth)

See clause B.2.

5.4 Selective solid plating-out media

5.4.1 First medium: Oxford agar

See clause B.3.

5.4.2 Second medium: PALCAM agar

See clause B.4.

5.5 Solid culture medium: Tryptone soya yeast extract agar (TSYEA)

See clause B.5.

5.6 Liquid culture medium: Tryptone soya yeast extract broth (TSYEB)

See clause B.6.

5.7 Sheep blood agar

See clause B.7.

5.8 Carbohydrate utilization broth (rhamnose and xylose)

See clause B.8.

5.9 Motility agar (optional)

See clause B.9.

5.10 CAMP (Christie, Atkins, Munch-Petersen) medium and test strains

See clause B.10.

5.11 Hydrogen peroxide solution

See clause B.11.

5.12 Phosphate-buffered saline (PBS)

See clause B.12.

6 Apparatus and glassware

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

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

See ISO 7218.

6.2 Drying cabinet or incubator, capable of being maintained at between $25\text{ °C} \pm 1\text{ °C}$ and $50\text{ °C} \pm 1\text{ °C}$.

6.3 Incubators, for maintaining the inoculated media, plates and tubes within the following temperature ranges:

- a) $25\text{ °C} \pm 1\text{ °C}$;
- b) $30\text{ °C} \pm 1\text{ °C}$; and
- c) $35\text{ °C} \pm 1\text{ °C}$ or $37\text{ °C} \pm 1\text{ °C}$.

6.4 Water bath, capable of being maintained at $47\text{ °C} \pm 2\text{ °C}$.

6.5 Loops, of platinum/iridium or nickel/chromium, approximately 3 mm in diameter, and **wires** of the same material, or **hockey-stick-shaped glass rods** or **single-use loops**.

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 $\pm 0,1$ pH unit.

6.7 Test tubes or flasks, of appropriate capacity, for sterilization and storage of culture media and incubation of liquid media.

6.8 Measuring cylinders, of capacity 50 ml to 1 000 ml, for preparation of dilutions and complete media.

6.9 Total-delivery graduated pipettes, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.10 Petri dishes, of diameter 90 mm to 100 mm.

6.11 Jars, suitable for microaerobic incubation (optional).

6.12 Gas mixture (optional), of specified composition for microaerobic incubation:

5 % to 12 % CO_2 , 5 % to 15 % O_2 , and 75 % N_2 .

6.13 Equipment for the Henry illumination test (optional).

See annex C.

6.14 Microscope, preferably with phase-contrast, and with slides and coverslips.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 11290. 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.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. 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

See ISO 6887 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 5.2.

In general, to prepare the initial suspension, add a test portion of x g or x ml to $9x$ ml or $9x$ g of the selective primary enrichment medium (5.2), to obtain a ratio of test portion to selective primary enrichment medium of 1/10 (mass to volume or volume to volume).

9.2 Primary enrichment

Incubate [6.3 b)] the initial suspension, prepared in accordance with 9.1, at 30 °C for 24 h $\pm$ 2 h.

NOTE 3 A black coloration may develop during the incubation.

9.3 Secondary enrichment

9.3.1 After incubation of the initial suspension (primary enrichment) for 24 h $\pm$ 2 h (9.2), transfer 0,1 ml of the culture obtained in 9.2 (regardless of its colour) to a tube (6.7) containing 10 ml of secondary enrichment medium (Fraser broth) (5.3).

9.3.2 Incubate the inoculated medium (9.3.1) for 48 h $\pm$ 2 h at 35 °C or 37 °C.

NOTE 4 The temperature of the inoculated medium should be agreed upon between the parties concerned and recorded in the test report.

9.4 Plating out and identification

9.4.1 From the primary enrichment culture incubated for 24 h $\pm$ 2 h at 30 °C (9.2), take, by means of a loop or glass rod (6.5), a portion of the culture and inoculate the surface of the first selective plating medium (Oxford agar) (5.4.1) so that well-separated colonies are obtained.

Proceed in the same way with the second selective plating-out medium (PALCAM agar) (5.4.2).

NOTE 5 This plating out is carried out regardless of the colour of the medium.

9.4.2 From the secondary enrichment medium incubated for 48 h $\pm$ 2 h at 35 °C or 37 °C (9.3.2), repeat the procedure described in 9.4.1 with the two selective plating-out media.

9.4.3 Invert the dishes obtained in 9.4.1 and 9.4.2 and place them in an incubator (6.3) set at 30 °C, 35 °C or 37 °C. PALCAM agar plates are incubated either microaerobically in a jar (6.11) containing the gas mixture (6.12) or aerobically.

NOTE 6 Incubation of the Oxford agar at 30 °C is suitable for foodstuffs only lightly contaminated by a supplementary flora. For products heavily contaminated by a supplementary flora, it is preferable to incubate the Oxford agar at

35 °C or 37 °C because *Listeria* spp. tend to develop at the same time as the supplementary flora. In all cases, the incubation temperature of the agar should be agreed upon between the parties concerned and recorded in the test report.

9.4.4 After incubation for 24 h and for an additional 18 h to 24 h (if growth is slight or if no colonies are observed after 24 h of incubation), examine the dishes (9.4.3) for the presence of colonies presumed to be *Listeria* spp.

9.4.4.1 Oxford agar: Typical colonies of *Listeria* spp. grown on Oxford agar for 24 h are small (1 mm) greyish colonies surrounded by black halos. After 48 h colonies become darker, with a possible greenish sheen, and are about 2 mm in diameter, with black halos and sunken centres.

9.4.4.2 PALCAM agar: For plates incubated microaerobically, after incubation expose the PALCAM agar plates to air for 1 h to allow the medium to regain its pink to purple colour. After 24 h *Listeria* spp. grow as small or very small greyish green or olive green colonies, 1,5 mm to 2 mm in diameter, sometimes with black centres, but always with black halos. After 48 h *Listeria* spp. appear in the form of green colonies about 1,5 mm to 2 mm in diameter, with a central depression and surrounded by a black halo.

9.5 Confirmation of *Listeria* spp.

9.5.1 Selection of colonies for confirmation

9.5.1.1 For confirmation, take from each plate of each selective medium (see 9.4.4.1 and 9.4.4.2), five colonies presumed to be *Listeria* spp.

If on one plate there are fewer than five presumed colonies, take for confirmation all of them.

9.5.1.2 Streak the selected colonies onto the surface of pre-dried plates of tryptone soya yeast extract agar (TSYEA) (5.5) in a manner which will allow well-separated colonies to develop.

Place the plates in the incubator [6.3 c)] set at 35 °C or 37 °C for 18 h to 24 h or until growth is satisfactory.

NOTE 7 The temperature of the inoculated medium should be agreed upon between the parties concerned and recorded in the test report.

Typical colonies are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. If the colonies are not well separated, pick a typical *Listeria* spp. colony onto another TSYEA plate. Carry out the following tests from colonies of a pure culture on the TSYEA.