INTERNATIONAL STANDARD

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of *Listeria monocytogenes* —

Part 2:
Enumeration method

Microbiologie des aliments — Méthode horizontale pour la recherche et le dénombrement de Listeria monocytogenes —

Partie 2: Méthode de dénombrement

Reference number
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International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet iso@iso.ch

Printed in Switzerland

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.

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.

International Standard ISO 11290-2 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 9, *Microbiology*.

ISO 11290 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of* Listeria monocytogenes:

— *Part 1: Detection method*

— *Part 2: Enumeration method*

Annexes A and B form an integral part of this part of ISO 11290. Annex C is for information only.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this part of ISO 11290 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 this method 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 part of ISO 11290 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of *Listeria monocytogenes* —

Part 2:
Enumeration 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 contaminated materials. In particular, 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 *Listeria monocytogenes*. National legislation may involve more specific demands.

1 Scope

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

NOTE — The method also allows enumeration of other *Listeria* species which may be used as indicators of the hygienic quality of food or feed products.

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

In general (see note in 9.2.1), the lower limit of enumeration of this method is 10 *L. monocytogenes* per millilitre of sample for liquid products, or 100 *L. monocytogenes* per gram of sample for other products.

2 Normative references

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

ISO 6887-1:—[1)], *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and of decimal dilutions*.

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

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*.

1) To be published. (Revision of ISO 6887:1993)

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 the solid selective medium described and which display the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this part of ISO 11290

3.2
enumeration of *Listeria monocytogenes*
determination of the number of colony-forming units (CFU) of *Listeria monocytogenes* (see 3.1), in a given quantity of product, when the analysis is carried out in accordance with this part of ISO 11290

4 Principle

Within the limits of this part of ISO 11290, the enumeration of *Listeria monocytogenes* requires six successive steps (see annex A for a flowchart).

4.1 Preparation of the initial suspension in one of the two diluents described, as necessary.

4.2 Resuscitation for 1 h at 20 °C.

4.3 Surface plating, on the solid selective culture medium contained in two Petri dishes, of a specified quantity of the test sample for liquid products or the initial suspension for other products.

Preparation of other dishes, under the same conditions, using decimal dilutions of the test sample or initial suspension.

4.4 Incubation of the dishes at 35 °C or 37 °C and examination after 24 h and 48 h.

4.5 Confirmation of presumptive colonies of *Listeria monocytogenes* with the tests described.

4.6 From the number of confirmed colonies, calculation of the number of *Listeria monocytogenes* per gram or per millilitre of the test sample.

5 Culture media and reagents

For current laboratory practice, see ISO 7218.

NOTE — Because of the large number of culture media and reagents, it has been considered preferable, for clarity of the text, to describe them in annex B.

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 between 25 °C ± 1 °C and 50 °C ± 1 °C.

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

a) 20 °C ± 1 °C (optional);

b) 25 °C ± 1 °C (optional);

c) 35 °C ± 1 °C or 37 °C ± 1 °C.

6.4 **Water bath,** capable of being maintained at 47 °C ± 2 °C.

6.5 **Loops** and **wires** of platinum/iridium or nickel/chromium, or **Pasteur pipettes** or **single-use loops**.

6.6 **Glass** or **plastic spreaders,** sterile.

6.7 **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.

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

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

6.10 **Petri dishes**, of diameter 90 mm and 140 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 N_2 up to 100 %.

6.13 **Equipment for the Henry illumination test** (optional)

See annex B.

6.14 **Microscope**, preferably with phase-contrast.

7 Sampling

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.

It is important that the laboratory receive 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 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

WARNING — Whenever a choice is given between 35 °C or 37 °C for the incubation temperature, this temperature shall be agreed between the parties concerned and recorded in the test report.

9.1 Test portion, initial suspension and dilutions

See ISO 6887-1 and any specific International Standard appropriate to the product concerned.

Use as diluent for preparing the initial suspension either buffered peptone water (B.1), or half-Fraser broth base medium (B.2).

Half-Fraser broth base **without the addition of selective agents** may be used as a diluent for the food or feed sample when both the detection method (see ISO 11290-1) and this enumeration method are carried out on the same test sample. This procedure is to avoid the need to prepare two initial suspensions; the selective agents are added to the suspension once the test portion for enumeration has been used. Use of this procedure should be noted in the test report.

Let the initial suspension stand for 1 h ± 5 min at 20 °C ± 2 °C [by using, if necessary, the incubator 6.3 a)], in order to resuscitate the stressed microorganisms.

If a dilution range is used, prepare it after resuscitation.

9.2 Inoculation and incubation

9.2.1 Transfer, by means of a sterile pipette (6.9), 0,1 ml of the initial suspension (9.1) to each of two dishes of PALCAM agar (B.3), dried beforehand if necessary in the incubator (6.2).

Repeat the procedure using further decimal dilutions if necessary.

NOTE — When, for certain products, it is necessary to estimate low numbers of *Listeria monocytogenes*, the limit of enumeration can be lowered by a factor of 10 by examining 1,0 ml of the initial suspension. Distribute the 1 ml of inoculum either on the surface of the agar medium in a large Petri dish (140 mm) or over the surface of the agar medium in three small dishes (90 mm) using a sterile spreader (6.6). In both cases, prepare duplicates by using two large dishes or six small dishes.

9.2.2 Carefully spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader. Use a fresh sterile spreader for each plate.[2)] Leave the plates closed for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.

9.2.3 Invert the dishes prepared in 9.2.2 and place them in an incubator [6.3 c)] set at 35 °C or 37 °C. Incubate PALCAM agar dishes either microaerobically in a jar (6.11) containing the gas mixture (6.12) or aerobically.

9.3 Enumeration of characteristic colonies

9.3.1 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 (9.2.3) for the presence of colonies presumed to be *Listeria* spp. (see 9.3.3).

2) It is possible to use the same spreader for a given sample, by beginning with the higher dilution.

9.3.2 For dishes incubated microaerobically, after incubation leave the PALCAM agar dishes (9.3.1) to air for 1 h to allow the agar to regain its pink to purple colour.

9.3.3 After 24 h the characteristic colonies of *Listeria* spp. grow as small or very small greyish green or olive green colonies, 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.

9.3.4 Count all the colonies presumed to be *Listeria* spp. (9.3.3) on each dish containing less than 150 characteristic or non-characteristic colonies.

9.4 Confirmation of *Listeria* spp.

9.4.1 Selection of colonies for confirmation

9.4.1.1 After the period of incubation (9.3.1), keep the dishes containing less than 150 presumptive *Listeria* spp. colonies, at all dilutions and, if possible, at two successive dilutions.

Select five of the presumptive colonies on each plate retained. If there are fewer than five presumptive colonies on a dish, select for confirmation all presumptive colonies.

9.4.1.2 Streak the selected colonies onto the surface of predried plates of tryptone soya yeast extract agar (TSYEA) (B.4) 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.

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.

NOTE — The Henry illumination test may be conducted if necessary (see annex C and note in B.4.2). The colonies then appear bluish with a granular surface.

9.4.2 Catalase reaction

Take a colony separated in 9.4.1.2 and suspend it in a drop of hydrogen peroxide solution (B.10) on a slide. The immediate formation of gas bubbles indicates a positive reaction (see ISO 7218).

9.4.3 Gram staining

Perform the Gram stain on a colony separated in 9.4.1.2 (see ISO 7218). *Listeria* spp. are revealed as Gram-positive slim, short rods (of approximately 0,4 µm to 0,5 µm diameter, and 1 µm to 2 µm length).

9.4.4 Motility test (if necessary)[3)]

Take a colony separated in 9.4.1.2 and suspend it in a tube containing TSYEB (B.5).

Incubate in the incubator [6.3 b)] set at 25 °C for 8 h to 24 h until a cloudy medium is observed.

Deposit a drop of the above culture using a loop (6.5) onto a clean glass microscope slide. Place a coverslip on top and examine it with the microscope (6.14). *Listeria* spp. appear as slim, short rods with tumbling motility.

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

3) This examination is not necessary in all cases if the analysis is carried out by a microbiologist who regularly works on the detection of *L. monocytogenes*.

As an alternative test for motility, using an inoculating needle (6.5), stab the motility agar (B.8) with a typical colony on TSYEA (9.4.1.2). Incubate it for 48 h in the incubator [6.3 b)] set at 25 °C.

Examine for growth around the stab. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern immediately below the surface of the agar. If growth is not sufficient, incubate for up to an additional 5 days and examine the culture during this time.

9.5 Confirmation of *L. monocytogenes*

9.5.1 Haemolysis test

9.5.1.1 If the morphological and physiological characteristics and catalase reaction are indicative of *Listeria* spp., determine the haemolytic reaction on sheep blood agar dishes (B.6).

Before use, thoroughly dry the blood agar surface, then mark the agar into squares. For each culture, take a colony separated in 9.4.1.2 and stab one labelled square using a wire (6.5). Also stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures.

After incubation at 35 °C or 37 °C for 24 h ± 2 h, examine the test strains and controls. *L. monocytogenes* show narrow, clear, light zones of ß-haemolysis[4]; *L. innocua* show no clear zone around the stab. *L. seeligeri* show a weak zone of ß-haemolysis. *L. ivanovii* usually show wide, clearly delineated zones of ß-haemolysis. Examine the plates by transparency to compare test cultures with controls.

9.5.1.2 The haemolytic reaction may also be carried out by using the CAMP test (9.5.3), or by using red cells in suspension as follows. Disperse the colony in 150 µl of TSYEB (B.5); incubate at 35 °C or 37 °C for 2 h. Add 150 µl of sheep blood red cell suspension (B.12). Incubate at 35 °C or 37 °C for between 15 min and 60 min, then refrigerate at +3 °C ± 2 °C for about 2 h. Examine for the presence or absence of ß-haemolysis. If the reaction is not definite, leave the culture at +3 °C ± 2 °C for up to 24 h.

9.5.2 Carbohydrate utilization

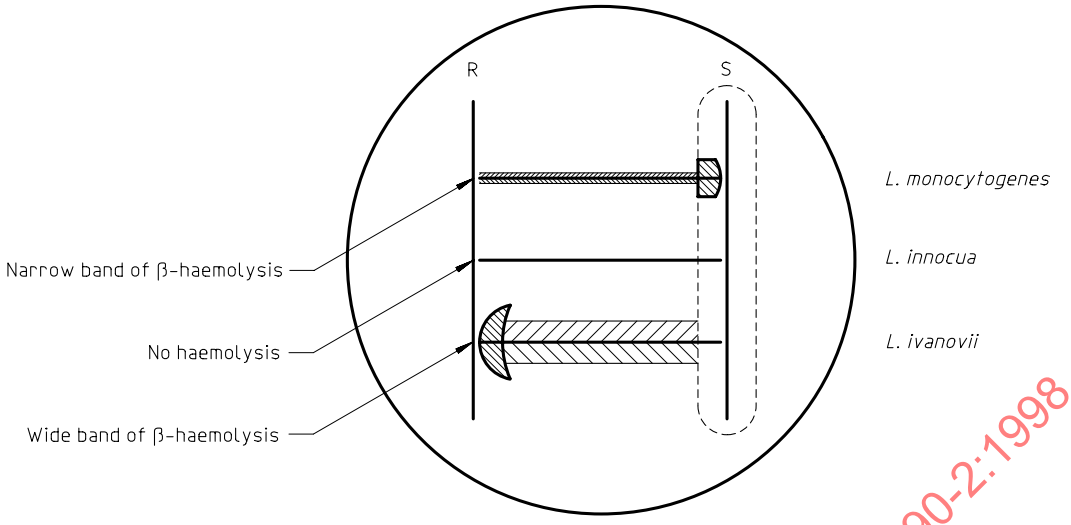
Using a loop (6.5), inoculate each of the carbohydrate utilization broths (B.7) with the culture from TSYEB (9.4.4). Incubate at 35 °C or 37 °C for up to 5 days. Positive reactions [formation of acid(s)] are indicated by the evolution of the colour from purple to yellow and occur mostly within 24 h to 48 h.

9.5.3 CAMP test

Streak each of the *Staphylococcus aureus* and *Rhodococcus equi* (B.9.4) cultures in single lines across the sheep blood agar plate (B.6 or B.9.3) so that the two cultures are parallel and diametrically opposite (see figure 1). A thin, even inoculum is required. This can be obtained by using an inoculation loop or a wire (6.5) held at right angles to the agar.

Streak the test strain separated in 9.4.1.2 in a similar fashion at right angles to these cultures so that the test culture and *S. aureus* and *R. equi* cultures do not touch but at their closest are about 1 mm to 2 mm apart. Several test strains may be streaked on the same plate.

4) The zone of β-haemolysis is more readily seen by removing any colony grown on the surface of agar around the inoculum mark.



NOTES

1 Inoculate thin blood agar plates (B.6 or B.9.3) as shown in the diagram. Vertical lines represent streaks of *S. aureus* (S) and *R. equi* (R). Horizontal lines represent streaks of the test cultures. Hatched areas indicate the locations of enhanced haemolysis.

2 The dotted area indicates the zone of influence of the *S. aureus* culture.

Figure 1 — Inoculation and interpretation of CAMP test plates

Simultaneously, streak control cultures of *L. monocytogenes*, *L. innocua* and *L. ivanovii.* If blood agar (B.6) is used, incubate the plates at 35 °C or 37 °C for 18 h to 24 h. If double-layer plates (B.9.3) are used, incubate at 35 °C or 37 °C for 12 h to 18 h.

An enhanced zone of ß-haemolysis at the intersection of the test strain with the cultures of *S. aureus* and *R. equi* is considered to be a positive reaction.

The positive reaction with *R. equi* is seen as a wide (5 mm to 10 mm) “arrow-head” of haemolysis. The reaction is considered as negative if a small zone of weak haemolysis extends only about 1 mm at the intersection of the test strain with the diffusion zone of the *R. equi* culture.

A positive reaction with *S. aureus* appears as a small zone of enhanced haemolysis extending only about 3 mm to 4 mm from the test strain and within the weakly haemolytic zone due to growth of the *S. aureus* culture. Large zones of ß-haemolysis do not occur in the proximity area between *S. aureus* and *L. monocytogenes*.

9.6 Interpretation of morphological and physiological properties and of the biochemical reactions

All *Listeria* spp. are small, Gram-positive rods that demonstrate motility (see 9.4.4). If they are observed by Henry illumination, they appear bluish with a granular surface. They are generally catalase positive.

L. monocytogenes are distinguished from other species by the characteristics listed in table 1.

Table 1 — Reactions for the identification of *Listeria* spp.

Species	Haemolysis	Production of acid		CAMP test	
		Rhamnose	Xylose	*S. aureus*	*R. equi*
L. monocytogenes	+	+	–	+	–
L. innocua	–	V	–	–	–
L. ivanovii	+	–	+	–	+
L. seeligeri	(+)	–	+	(+)	–
L. welshimeri	–	V	+	–	–
L. grayi	–	–	–	–	–
V: variable reaction (+): weak reaction +: > 90 % of positive reactions –: no reaction					
NOTE — There exist rare strains of *L. monocytogenes* which do not show ß-haemolysis or a positive reaction to the CAMP test under the conditions described in this part of ISO 11290.					

9.7 Definitive confirmation

Strains which are considered to be *L. monocytogenes* (9.6) may be sent to a recognized *Listeria* reference laboratory for serological or, possibly, lysogenic typing, or an alternative reliable molecular typing method. The dispatch shall be accompanied by all possible information concerning the strain(s).

10 Expression of results (see ISO 7218)

10.1 Counting of colonies of *L. monocytogenes*

Calculate for each of the plates the number a of colonies of *L. monocytogenes* present, using the following formula:

$$a = \frac{b}{A} \times C$$

where

b is the number of colonies conforming to the identification criteria (9.6);

A is the number of colonies plated out for confirmation (9.4.1.1);

C is the total number of characteristic colonies enumerated on the dish (9.3.4).

Round off a to a whole number.

10.2 Method of calculation

10.2.1 Plates containing less than 150 colonies of *L. monocytogenes*, one of which contains at least 15 *L. monocytogenes*

Calculate the number N of *L. monocytogenes* present in 1 ml or 1 g of product, using the following formula:

$$N = \frac{\sum a}{V(n_1 + 0{,}1\, n_2)\, d}$$

where

Σa is the sum of the colonies of *L. monocytogenes* calculated after confirmation, on all the dishes retained at two consecutive dilutions, one of which at least contains at least 15 identified colonies;

V is the volume of the inoculum applied to each dish in millilitres;

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained.

Round the results obtained to two significant figures (see ISO 7218).

Take as the result the number of *L. monocytogenes* per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 multiplied by 10 to the appropriate power.

NOTE — See ISO 7218 for an example.

10.2.2 Estimation of small numbers

10.2.2.1 If the two dishes, at the level of the initial suspension, contain less than 15 colonies of *L. monocytogenes*, calculate the number of confirmed colonies on each dish, using the formula given in 10.1. Calculate the arithmetical mean y of the colonies counted on two dishes.

Express the result as follows:

— estimated number of *L. monocytogenes* per gram or per millilitre $N_E = \frac{y}{d \times V}$

where

d is the dilution factor of the initial suspension;

V is the volume of inoculum on each dish.

10.2.2.2 If the two dishes at the level of the initial suspension do not contain any colonies, express the result as follows:

— less than $\frac{1}{d \times V}$ *L. monocytogenes* per gram or per millilitre

where

d is the dilution factor of the initial suspension;

V is the volume of inoculum on each dish.

11 Precision

See ISO 7218.

12 Quality control of culture media

Since there is yet no general standard on this topic, check the ability of the culture media to support the selective growth of *L. monocytogenes* as follows. Introduce a dilution of the reference culture of recently isolated strains of *L. monocytogenes* and negative control strains (e.g. lactobacilli, *Streptococcus*) in a control flask of the selective primary enrichment medium (see 9.2). Add 10 to 100 *L. monocytogenes* cells or negative control strains per flask.

Proceed with the control flasks as for the test cultures to demonstrate that the positive control culture is recovered.

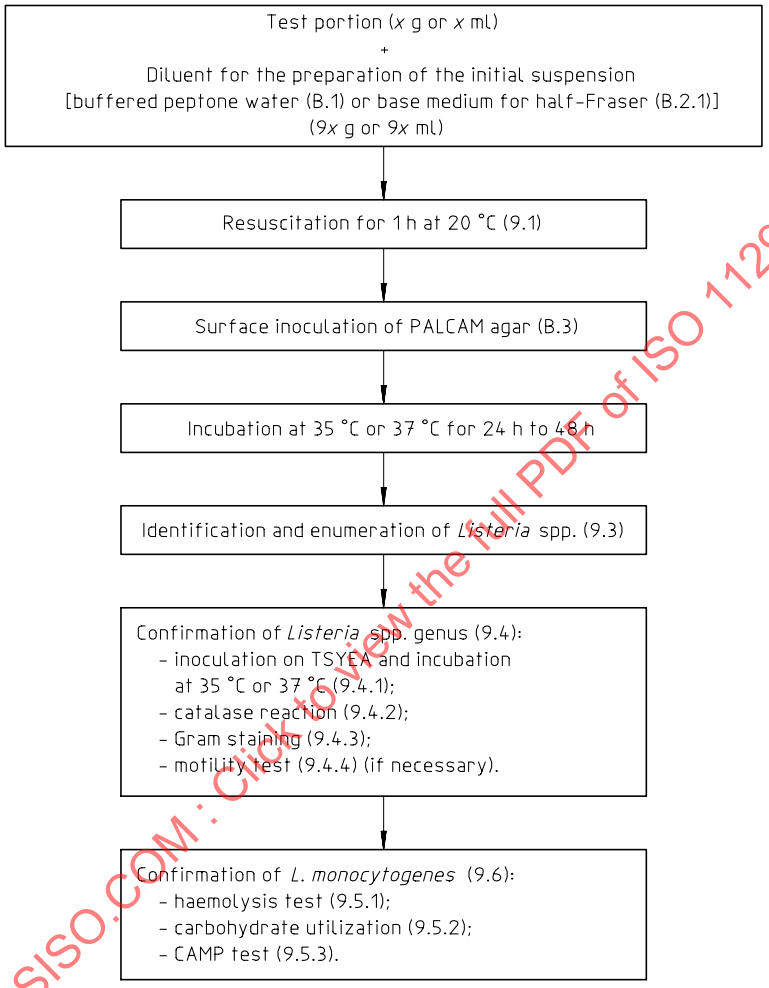
13 Test report

The test report shall specify the method used, the temperature of incubation and the results obtained. It shall also mention all operating details not specified in this part of ISO 11290, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report shall contain all information necessary for the complete identification of the sample.

Annex A
(normative)

Diagram of procedure



Annex B
(normative)

Composition and preparation of media and reagents

B.1 Buffered peptone water

B.1.1 Composition

Enzymatic digest of animal tissues	10,0 g
Sodium chloride (NaCl)	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

B.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH [measured with the pH-meter (6.7)], if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium in flasks (6.8) of suitable capacity to obtain portions appropriate for the test (see 9.1).

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.2 Base medium for half-Fraser broth with ammonium iron(III) citrate (see ISO 11290-1)

B.2.1 Base medium for half-Fraser broth

B.2.1.1 Composition

Enzymatic digest of animal tissues	5,0 g
Enzymatic digest of casein	5,0 g
Meat extract	5,0 g
Yeast extract	5,0 g
Sodium chloride (NaCl)	20,0 g
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	12,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,35 g
Aesculin	1,0 g
Water	1 000 ml

B.2.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

Dispense the base in flasks (6.8) of suitable capacity to obtain portions appropriate for the test (see 9.1).

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.2.2 Ammonium iron(III) citrate solution

B.2.2.1 Composition

Ammonium iron(III) citrate	5,0 g
Water	100 ml

B.2.2.2 Preparation

Dissolve the ammonium iron(III) citrate in the water.

Sterilize by filtration.

B.2.3 Preparation of the medium

Immediately before use, add 1,0 ml of the ammonium iron(III) citrate solution (B.2.2) to each 100 ml portion of the base medium for half-Fraser broth (B.2.1).

B.3 Selective plating-out medium: PALCAM agar[5)]

B.3.1 Agar base

B.3.1.1 Composition

Peptones	23,0 g
Starch	1,0 g
Sodium chloride (NaCl)	5,0 g
Yeast extract	3,0 g
Agar	9 g to 18 g[1)]
D-Glucose	0,5 g
D-Mannitol	10,0 g
Aesculin	0,8 g
Ammonium iron(III) citrate	0,5 g
Phenol red	0,08 g
Lithium chloride (LiCl)	15,0 g
Water	960 ml

1) Depending on the gel strength of the agar.

5) Details are given in: VAN NETTEN P., PERALES I., VAN DE MOOSDIJK A., CURTIS G.D.W. and MOSSEL D.A.A. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. *Int. J. Food Microbiol.*, **8**, 1989, pp. 299-316.

B.3.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water, by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

Sterilize for 15 min in the autoclave set at 121 °C.

B.3.2 Polymyxin B sulfate solution

B.3.2.1 Composition

Polymyxin B sulfate (100 000 IU)	0,1 g
Water	100 ml

B.3.2.2 Preparation

Dissolve the polymyxin B sulfate in the water.

Sterilize by filtration.

B.3.3 Acriflavine hydrochloride solution

B.3.3.1 Composition

Acriflavine hydrochloride	0,05 g
Water	100 ml

B.3.3.2 Preparation

Dissolve the acriflavine hydrochloride in the water.

Sterilize by filtration.

B.3.4 Sodium ceftazidime pentahydrate solution

B.3.4.1 Composition

Sodium ceftazidime pentahydrate	0,116 g
Water	100 ml

B.3.4.2 Preparation

Dissolve the sodium ceftazidime in the water.

Sterilize by filtration.

B.3.5 Complete medium

B.3.5.1 Composition

Agar base (B.3.1)	960 ml
Polymyxin B sulfate solution (B.3.2)	10 ml
Acriflavine hydrochloride solution (B.3.3)	10 ml
Sodium ceftazidime pentahydrate solution (B.3.4)	20 ml

B.3.5.2 Preparation

Add solutions B.3.2, B.3.3 and B.3.4 to the molten base (B.3.1) at 47 °C, mixing thoroughly between each addition.

The pH of the complete medium shall be 7,2 ± 0,2 at 25 °C.

B.3.6 Preparation of the agar plates

Place in each of an appropriate number of Petri dishes (6.10) appropriate quantities of the freshly prepared complete medium (B.3.5). Allow to solidify.

Store the medium away from light.

B.4 Solid culture medium: Tryptone soya yeast extract agar (TSYEA)

B.4.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of soya meal	3,0 g
Sodium chloride (NaCl)	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2,5 g
D-Glucose	2,5 g
Yeast extract	6 g
Agar	9 g to 18 g[1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

B.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the medium into tubes of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave set at 121 °C.

Allow to set in a sloping position.

For the preparation of agar plates, dispense the medium into sterile Petri dishes in portions appropriate for the test. Allow to solidify.

NOTE — If the Henry illumination test is conducted, it is important that the agar medium layer is thin (approximately 12 ml per dish of 90 mm diameter).

B.5 Liquid culture medium: Tryptone soya yeast extract broth (TSYEB)

B.5.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of soya meal	3,0 g
Sodium chloride (NaCl)	5,0 g
Dipotassium hydrogen phosphate(K_2HPO_4)	2,5 g
D-Glucose	2,5 g
Yeast extract	6 g
Water	1 000 ml

B.5.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the medium into flasks or tubes of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave set at 121 °C.

B.6 Sheep blood agar

B.6.1 Base

B.6.1.1 Composition

Enzymatic digest of animal tissues	15 g
Enzymatic liver digest	2,5 g
Yeast extract	5 g
Sodium chloride (NaCl)	5 g
Agar	9 g to 18 g[1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

B.6.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

Dispense the medium into flasks of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave set at 121 °C.