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Milk and milk products — Detection of *Listeria monocytogenes*

Lait et produits laitiers — Recherche de Listeria monocytogenes

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Foreword

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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 10560 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC), and will also be published by these organizations.

Annex A forms an integral part of this International Standard. Annex B is for information only.

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Milk and milk products — Detection of *Listeria monocytogenes*

1 Scope

This International Standard specifies methods for the detection of *Listeria monocytogenes* in milk and milk products.

2 Normative references

The following standards contain provisions which, through reference in this 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 the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations.*

ISO 8261:1989, *Milk and milk products — Preparation of test samples and dilutions for microbiological examination.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 *Listeria* **spp.:** Microorganisms which form typical colonies on a solid selective medium and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this International Standard.

3.1.1 *Listeria monocytogenes*: A *Listeria* species which is considered as pathogenic and which can be differentiated by specific biochemical characteristics from other, non-pathogenic species occurring in milk and milk products.

3.2 detection of *Listeria monocytogenes*: Determination of the presence or absence of this microorganism, in a specified mass or volume, when tests are carried out in accordance with this International Standard.

4 Principle

In general, the detection of *Listeria* spp. necessitates at least three successive stages as in 4.1 to 4.3. See also the diagram of procedure in figure 1.

Test portion (25 g or 25 ml)
+
Selective enrichment medium (225 ml)

Incubation at 30 °C for 48 h
↓
Streak-out on Oxford agar

Incubation at 37 °C for 48 h
↓
Selection of five characteristic colonies
(for each plate)
↓
Inoculation on TSYEA

Incubation at 37 °C for 24 h (or longer if necessary)
↓
Further examinations

Figure 1 — Diagram of procedure

4.1 Enrichment in selective liquid medium

Inoculation of the selective medium with the test portion of the sample and incubation at 30 °C for 48 h.

4.2 Isolation and presumptive identification

Inoculation of the isolation medium with the culture obtained in the enrichment medium (4.1), incubation at 37 °C and examination after 48 h to check for the presence of colonies which, from their appearance, are considered to be presumptive *Listeria* spp.

4.3 Confirmation

Subculturing of colonies of presumptive *Listeria* spp. (4.2) on a non-selective solid medium, and confirmation by means of appropriate morphological, physiological and biochemical tests.

5 Culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Culture media

5.2.1 Selective medium: Enrichment medium

5.2.1.1 Base

5.2.1.1.1 Composition

Tryptone soya broth[1)]	30 g
Yeast extract	6 g
Water	1 000 ml

1) *Composition of tryptone soya broth*

Tryptone (pancreatic digest of casein): 17 g
Soytone (papaic digest of soyabean meal): 3 g
Dextrose: 2,5 g
Sodium chloride: 5 g
Dipotassium phosphate: 2,5 g

5.2.1.1.2 Preparation

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

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

Dispense the base in quantities of 225 ml into flasks of 500 ml capacity (or multiples of 225 ml into flasks of suitable capacity). Sterilize for 15 min in the autoclave (6.1.1.2) set at 121 °C.

5.2.1.2 Supplement 1

5.2.1.2.1 Composition

Acriflavine hydrochloride	23 mg
Water	10 ml

5.2.1.2.2 Preparation

Dissolve the acriflavine hydrochloride in the water. Sterilize by filtration.

NOTE 1 For details of the technique of sterilization by filtration, reference may be made to any appropriate textbook on microbiology.

5.2.1.3 Supplement 2

5.2.1.3.1 Composition

Nalidixic acid (sodium salt)	46 mg
Sodium hydroxide (0,05 mol/l)	10 ml

5.2.1.3.2 Preparation

Dissolve the nalidixic acid in the sodium hydroxide solution. Sterilize by filtration.

5.2.1.4 Supplement 3

5.2.1.4.1 Composition

Cycloheximide	57,5 mg
Ethanol	4 ml
Water	6 ml

5.2.1.4.2 Preparation

Dissolve the cycloheximide in the ethanol/water mixture. Sterilize by filtration.

5.2.1.5 Complete medium

Store the base (5.2.1.1) and the prepared supplements (5.2.1.2 to 5.2.1.4) separately in the dark at a temperature between 2 °C and 5 °C. Prepare the complete medium by adding 1 ml of supplement 1, 2 ml of supplement 2 and 2 ml of supplement 3 to 225 ml of the base medium (5.2.1.1).

NOTE 2 Multiples of 1 ml, 2 ml and 225 ml, respectively, in flasks of suitable capacity may be used where appropriate.

5.2.2 Isolation medium (Oxford agar)

5.2.2.1 Agar base

5.2.2.1.1 Composition

Columbia agar base	39 g
Aesculin	1 g
Ammonium iron(III) citrate	0,5 g
Lithium chloride	15 g
Water	1 000 ml

5.2.2.1.2 Preparation

Dissolve the solid ingredients in the water by boiling.

5.2.2.2 Supplement for 500 ml medium

5.2.2.2.1 Composition

Cycloheximide	200 mg
Colistin sulfate	10 mg
Acriflavin	2,5 mg
Cefotetan	1 mg
Fosfomycin	5 mg
Ethanol	2,5 ml
Water	2,5 ml

5.2.2.2.2 Preparation

Dissolve the solid ingredients in the ethanol/water mixture. Sterilize by filtration.

5.2.2.3 Preparation of complete medium

Take 500 ml of the agar base (5.2.2.1). Sterilize for 15 min in the autoclave (6.1.1.2) set at 121 °C. Cool to 50 °C and aseptically add the supplement (5.2.2.2). The pH of the final medium should be 7,0 °C at 25 °C.

Dispense the medium into sterile Petri dishes in quantities of about 15 ml and allow to solidify.

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

5.2.3.1 Composition

Tryptone soya broth	30 g
Yeast extract	6 g
Agar[1)]	12 g to 18 g
Water	1 000 ml

1) According to the gel strength of the agar.

5.2.3.2 Preparation

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

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

Dispense quantities of about 6 ml of the solid culture medium into tubes (6.2.2).

Sterilize the tubes for 15 min in the autoclave (6.1.1.2) set at 121 °C.

Allow to set in a sloping position.

For the preparation of agar plates, sterilize the solid culture medium in flasks or bottles of suitable capacity. Dispense the medium while still liquid in quantities of about 15 ml into sterile Petri dishes and allow to solidify.

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

5.2.4.1 Composition

The formulation of the medium is described in 5.2.1.1.1. Use 6 g of yeast extract.

5.2.4.2 Preparation

Prepare the medium as described in 5.2.1.1.2.

Dispense quantities of about 6 ml into tubes before sterilizing in the autoclave.

5.2.5 Blood agar

5.2.5.1 Composition

Blood agar base No. 2[1)]	40 g
Water	1 000 ml
Horse or sheep defibrinated blood	70 ml

1) *Composition of blood agar base No. 2*

Proteose peptone: 15 g
Liver digest: 2,5 g
Yeast extract: 5 g
Sodium chloride: 5 g
Agar (according to the gel strength of the agar): 12 g to 18 g

5.2.5.2 Preparation

Dissolve the dehydrated blood agar base in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,0 °C at 25 °C. Dispense the medium into tubes or flasks of not more than 500 ml capacity.

Sterilize the blood agar base for 15 min in the autoclave (6.1.1.2) set at 121 °C.

Cool the medium to 45 °C. Add the defibrinated blood and mix well.

Dispense the medium in quantities of about 20 ml into sterile Petri dishes and allow to solidify.

5.2.6 Carbohydrate utilization broth

5.2.6.1 Base

5.2.6.1.1 Composition

Proteose peptone	10 g
Beef extract	1 g
Sodium chloride	5 g
Bromocresol purple	0,02 g
Water	1 000 ml

5.2.6.1.2 Preparation

Dissolve the components in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 6,8 °C at 25 °C.

Dispense the medium into tubes in quantities such that after sterilization 9 ml will remain.

Sterilize for 15 min in the autoclave (6.1.1.2) set at 121 °C.

5.2.6.2 Carbohydrate solutions

5.2.6.2.1 Composition

Carbohydrate[1)]	5 g
Water	100 ml

1) 100 ml of L-rhamnose solution and 100 ml of D-xylose solution are required.

5.2.6.2.2 Preparation

Dissolve separately each carbohydrate in 100 ml of water. Sterilize by filtration.

5.2.6.3 Complete media

For each carbohydrate, add aseptically 1 ml of solution (5.2.6.2) to 9 ml of the base medium (5.2.6.1). If smaller volumes of base medium are prepared, add accordingly smaller volumes of the carbohydrate solution.

5.2.7 Motility medium

5.2.7.1 Composition

Casein peptone	20,0 g
Meat peptone	6,1 g
Agar	3,5 g
Water	1 000 ml

5.2.7.2 Preparation

Dissolve the components in the water by boiling.

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

Dispense the medium into tubes in quantities of about 5 ml.

Sterilize for 15 min in the autoclave (6.1.1.2) set at 121 °C.

NOTE 3 Commercially available media for the examination of motility may be used.

5.2.8 CAMP (Christie, Atkins, Munch-Petersen) test

Blood agar plates (5.2.5) may be used for this test, but

it is preferable to use double-layered sheep blood agar plates with a very thin blood layer (5.2.8.3).

5.2.8.1 Base

5.2.8.1.1 Composition

Blood agar base No. 2 (see 5.2.5)	40 g
Water	1 000 ml

5.2.8.1.2 Preparation

Dissolve the dehydrated base in the water by boiling.

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

Dispense the base into bottles or flasks in quantities of 100 ml.

Sterilize for 15 min in the autoclave (6.1.1.2) set at 121 °C. Cool to 45 °C.

5.2.8.2 Blood medium

5.2.8.2.1 Composition

Basal layer medium (5.2.8.1)	100 ml
Sheep or horse defibrinated blood	7 ml

5.2.8.2.2 Preparation

Add the defibrinated blood to the sterilized, molten base (5.2.8.1).

5.2.8.3 Complete medium

Dispense the base (5.2.8.1) into sterile Petri dishes in quantities of about 10 ml and allow to solidify. Pour a very thin layer of the blood medium (5.2.8.2) using amounts not greater than 3 ml per plate.

Allow to solidify in an even layer. If the blood is added to dishes containing the base which have been prepared in advance, it may be necessary to warm the dishes for 20 min by placing them in an incubator set at 37 °C before pouring the blood layer.

Dry the plates before use.

5.2.8.4 CAMP reaction cultures

A weakly β-haemolytic strain of *Staphylococcus aureus* (e.g. NCTC 1803) and a strain of *Rhodococcus equi* (e.g. NCTC 1621) are required to undertake the CAMP test. Not all strains of *Staphylococcus aureus* are suitable for the CAMP test.

Maintain stock cultures of *S. aureus*, *R. equi*, *L. monocytogenes*, *L. innocua* and *L. ivanovii* by inoculating the TSYEA slopes (5.2.3), incubating them at 37 °C for 24 h to 28 h, or until growth has occurred, and storing in the refrigerator (6.1.10) at 4 °C. Subculture at least once per month.

5.3 Reagent

5.3.1 Hydrogen peroxide solution, 3% (*V/V*)

6 Apparatus and glassware

IMPORTANT — Sterilize all apparatus that will enter into contact with the culture media, the dilution fluid or the sample, except for apparatus that is supplied sterile (particularly plastic apparatus).

6.1 Apparatus

Usual microbiological laboratory apparatus and, in particular, the following.

6.1.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave) (see ISO 7218)

6.1.1.1 Oven, capable of operating at 173 °C ± 3 °C.

6.1.1.2 Autoclave, capable of operating at 121 °C ± 1 °C.

6.1.2 Incubator, capable of operating at 30 °C ± 1 °C.

6.1.3 Incubator, capable of operating at 37 °C ± 1 °C.

6.1.4 Incubator, capable of operating at 25 °C ± 1 °C.

6.1.5 Water baths, capable of being maintained at 45 °C ± 1 °C or at 37 °C ± 1 °C.

6.1.6 Blending equipment

Use one of the following:

a) **rotary blender**, operating at a rotational frequency between 8 000 min^{-1} and 45 000 min^{-1}, with glass or metal bowls fitted with lids, resistant to the conditions of sterilization; or

b) **peristaltic-type blender** (stomacher), with sterile plastic bags.

NOTE 4 The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the

volume of the container should be equal to about twice the volume of the sample plus diluent.

6.1.7 Loops, of platinum/iridium, nickel/chromium or plastic, of diameter approximately 3 mm.

6.1.8 Inoculating needle, of platinum/iridium, nickel/chromium or plastic.

6.1.9 Temperature-compensated pH-meter (for measuring the pH of prepared media and reagents), accurate to ± 0,1 pH units at 25 °C.

6.1.10 Refrigerator (for storage of prepared media and reagents), capable of operating at + 2 °C to + 5 °C.

6.1.11 Source of beamed white light

6.1.12 Mirror, flat or concave.

6.1.13 Tripod, for illuminating Petri dishes.

6.1.14 Phase-contrast microscope, with oil-immersion objective.

6.2 Glassware

The glassware shall be resistant to repeated sterilization.

6.2.1 Culture flasks, for sterilization and storage of culture media and incubation of liquid media.

6.2.2 Culture tubes[1], 16 mm in diameter and 125 mm in length.

6.2.3 Measuring cylinders, for preparation of the complete media.

6.2.4 Graduated pipettes, of capacities 25 ml, 10 ml and 1 ml, graduated respectively in divisions of 0,5 ml, 0,5 ml and 0,1 ml.

6.2.5 Sterile Petri dishes

6.2.6 Microscopic slides

6.2.7 Glass beads

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 International Standard. A recommended sampling method is given in ISO 707 [1].

The instructions for sampling for microbiological purposes should be followed.

8 Preparation of test sample

See ISO 8261.

8.1 Milk

Agitate the sample carefully so that the microorganisms are distributed as evenly as possible by rapidly inverting the sample container 25 times. Foaming should be avoided or foam allowed to disperse. The interval between mixing and removing the test portion should not exceed 3 min.

8.2 Dried milk, dried whey, dried buttermilk, lactose, casein and caseinate

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it. If the container is too full to permit thorough mixing, transfer contents to a larger container, then mix.

8.3 Butter

Melt the sample in a sterile container in a water bath (6.1.5) set at 45 °C. Agitate during melting and remove the container immediately from the water bath when the sample has just melted.

8.4 Cheese

Normally, the laboratory sample will consist of body and rind. Interested parties should agree on the parts of the cheese that will constitute the test sample.

Proceed as described in 9.1.5.

8.5 Edible ices

Proceed as in the case of butter (8.3) but use a water bath (6.1.5) maintained at less than 37 °C as the sample shall not be allowed to exceed this temperature.

8.6 Fermented milks, yoghurts, custards and desserts

Mix the contents of the closed container by repeatedly shaking and inverting, or open the container and mix the contents aseptically using a sterile spatula or spoon.

1) Tubes with metal caps may be used.

9 Procedure

For safety precautions, see annex A. Also see figure 1.

9.1 Inoculation of enrichment medium

NOTE 5 To reduce the workload when more than one 25 g test portion from a specified lot of milk or milk product has to be examined, and when evidence is available that compositing (pooling the test portions) does not affect the result for the milk or milk product, the test portions may be composited. For example, if 10 test portions of 25 g are to be examined, combine the 10 units to form a composite test portion of 250 g and dissolve or disperse in 2,25 l of enrichment medium.

Add a test portion to the enrichment medium (5.2.1) as described in 9.1.1 to 9.1.7.

9.1.1 Milk

Add 25 ml of the test sample to 225 ml of the enrichment medium (5.2.1) and mix.

9.1.2 Dried milk, dried whey, dried buttermilk and caseinate

Weigh 25 g of the test sample aseptically into a stoppered flask containing 225 ml of the enrichment medium (5.2.1). Dissolve by shaking.

9.1.3 Casein

Weigh 25 g of the test sample aseptically into the sterile container of the blender (6.1.6), and add 225 ml of the enrichment medium (5.2.1) at 45 °C. Dissolve the test portion carefully by blending (1 min to 3 min).

9.1.4 Butter

Shake the melted test sample and, with a pipette warmed to approximately 45 °C, transfer 25 ml into a flask containing 225 ml of the enrichment medium (5.2.1). Mix carefully.

9.1.5 Cheese

Weigh 25 g of the test sample into the sterile container of the blender (6.1.6). Add 225 ml of the enrichment medium (5.2.1) prewarmed to about 37 °C. Disperse the test portion by blending carefully.

9.1.6 Frozen milk products (including edible ices)

Transfer 25 g of the melted test sample using a pipette into a flask containing 225 ml of the enrichment medium (5.2.1) and mix.

9.1.7 Fermented milks, yoghurts, custards and desserts

Weigh 25 g of the test sample aseptically into a stoppered flask containing glass beads (6.2.7) and 225 ml of the enrichment medium (5.2.1). Disperse by shaking.

When examining samples of low pH value, aseptically check the pH of the suspension using indicator paper and adjust, if necessary, to 7,0 ± 0,5 at 25 °C.

9.2 Incubation

Incubate the inoculated enrichment medium for 48 h in the incubator (6.1.2) set at 30 °C.

9.3 Isolation and presumptive identification

9.3.1 Using a loop (6.1.7), streak the enrichment culture onto the surface of an Oxford agar plate (5.2.2) to obtain well-isolated colonies.

9.3.2 Invert the plate and place in the incubator (6.1.3) set at 37 °C for 48 h.

9.3.3 Examine the plate for the presence of colonies typical of *Listeria* spp. (colonies surrounded by dark brown or black haloes).

9.4 Confirmation

9.4.1 Selection of colonies for confirmation

From each plate of isolation medium (Oxford agar, 5.2.2), select five typical or suspect colonies or, if there are fewer than five such colonies, select all for confirmation.

9.4.2 Incubation

Streak the selected colonies onto the surface of the TSYEA plates (5.2.3) in a manner which will allow well-isolated colonies to develop. Place the plates in the incubator (6.1.3) set at 37 °C for 24 h or until growth is satisfactory. The thinness of the agar medium (15 ml/plate) is important for good Henry illumination (see below).

Examine the plates using a source of beamed white light (6.1.11), powerful enough to illuminate plates well, striking the bottom of the plate at a 45° angle (see figure 2). When examined in this obliquely transmitted light (Henry illumination) from directly above the plate, colonies of *Listeria* spp. exhibit a blue colour and a granular surface.

If TSYEA plates do not show ample, well-isolated, typical colonies, restreak a colony and proceed as described above.