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Microbiology — General guidance for the detection of *Enterobacteriaceae* with pre-enrichment

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Microbiologie — Directives générales pour la recherche des Enterobacteriaceae *avec pré-enrichissement*

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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.

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.

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International Standard ISO 8523 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 9, *Microbiology*.

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Annex A forms an integral part of this International Standard.

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Introduction

This International Standard is intended to provide general guidance for the microbiological examination of food products not dealt with by existing International Standards and for reference for bodies preparing microbiological methods of test for application to foods or to animal feeding stuffs.

Because of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and for some other products it may be necessary to use different methods.

Nevertheless, it is hoped that in all cases every attempt will be made to apply the provided guidelines as far as possible, and that deviations from them will only be made if absolutely necessary for technical reasons.

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When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

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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 these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed[1]. However, it is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well-established technical reasons.

To allow for national practice, the test method given here may be carried out at an incubation temperature of either 35 °C or 37 °C.

NOTE 1 The technique described in this International Standard can be applied for carrying out an enumeration using the most probable number (MPN) technique. For this it is only necessary to carry out the presence/absence test, described in this International Standard, in replicate at several dilutions (as prescribed, for example, in the product specification). For the interpretation of the results obtained, reference should be made to the MPN tables given in ISO 7218.

1) For meat and meat products, see ISO 5552:1979, *Meat and meat products — Detection and enumeration of Enterobacteriaceae (Reference methods).*

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Microbiology — General guidance for the detection of *Enterobacteriaceae* with pre-enrichment

1 Scope

This International Standard gives general guidelines for the detection of *Enterobacteriaceae* with pre-enrichment in products intended for human consumption or feeding of animals.

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2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was 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 edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

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

3 Definitions

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

3.1 *Enterobacteriaceae*: Micro-organisms which ferment glucose and show a negative oxidase reaction when the test is carried out in accordance with the method specified in this International Standard.

3.2 detection of *Enterobacteriaceae*: Determination of the presence or absence of *Enterobacteriaceae* in a particular mass of product, when tests are carried out in accordance with the method specified in this International Standard.

4 Principle

In general, the detection of *Enterobacteriaceae* requires four successive stages (see also annex A).

4.1 Pre-enrichment in non-selective liquid medium

Inoculation of buffered peptone water (also used as diluent) with the test portion, and incubation at 35 °C or 37 °C for 16 h to 20 h.

The temperature shall be agreed by the parties concerned and shall be indicated in the test report.

4.2 Enrichment in selective liquid medium

Inoculation of enrichment broth with the culture obtained in 4.1.

Incubation at 35 °C or 37 °C (as agreed) for 24 h.

4.3 Plating out and identification

From the cultures obtained in 4.2, inoculation of a selective solid medium (violet red bile glucose agar).

Incubation at 35 °C or 37 °C (as agreed) and examination after 24 h to check for the presence of colonies presumed to be *Enterobacteriaceae* by their characteristics.

4.4 Confirmation

Subculturing of colonies of presumptive *Enterobacteriaceae* (4.3) on non-selective medium, and confirmation by means of appropriate biochemical tests.

5 Culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Culture media

5.2.1 Buffered peptone water (non-selective pre-enrichment medium).

5.2.1.1 Components

Peptone	10,0 g
Sodium chloride	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

5.2.1.2 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 7,0 at 25 °C.

Dispense the medium aseptically into flasks of suitable capacity (6.5) to obtain the portions necessary for the test.

Sterilize for 20 min in an autoclave (6.1) set at 121 °C.

5.2.2 Buffered brilliant green bile glucose broth (EE broth) (enrichment medium).

5.2.2.1 Components

Peptone	10,0 g
Glucose	5,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	6,45 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Ox bile, dehydrated	20,0 g
Brilliant green	0,015 g
Water	1 000 ml

5.2.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling. Do not heat the medium for longer than 30 min. Cool the medium rapidly.

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

Dispense the medium, in quantities of 10 ml, into sterile tubes (6.5).

Do not sterilize the medium.

The medium may be stored for up to 1 week at 0 °C to + 5 °C.

5.2.3 Violet red bile glucose agar.

5.2.3.1 Components

Peptone	7,0 g
Yeast extract	3,0 g
Bile salts	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Neutral red	0,03 g
Crystal violet	0,002 g
Agar	8 g to 18 g[1)]
Water	1 000 ml

1) According to the gel strength of the agar.

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5.2.3.2 Preparation

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

Adjust the pH, if necessary, so that after boiling it is 7,4 at 25 °C.

Dispense the culture medium into sterile tubes or flasks (6.5) of capacity not more than 500 ml.

Do not sterilize the medium.

Prepare the medium just before use.

5.2.3.3 Preparation of agar plates

Transfer immediately approximately 15 ml of the culture medium, cooled to approximately 45 °C, to Petri dishes (6.7) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in an oven (6.3) until the agar is dry.

If prepared in advance, the undried plates shall not be kept for longer than 4 h at room temperature or 1 day at 0 °C to + 5 °C.

5.2.4 Nutrient agar.

5.2.4.1 Components

Beef extract	3,0 g
Peptone	5,0 g
Agar	8 g to 18 g[1)]
Water	1 000 ml

1) According to the gel strength of the agar.

5.2.4.2 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 7,0 at 25 °C.

Dispense the culture medium into tubes or flasks (6.5) of capacity not more than 500 ml.

Sterilize for 20 min in an autoclave (6.1) set at 121 °C.

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5.2.4.3 Preparation of agar plates

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Transfer portions of about 15 ml of the culture medium, melted and cooled to approximately 45 °C, to Petri dishes (6.7) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in an oven (6.3) until the agar is dry.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or 1 day at 0 °C to + 5 °C.

5.2.5 Glucose agar.

5.2.5.1 Components

Tryptone	10,0 g
Yeast extract	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Bromocresol purple	0,015 g
Agar	8 g to 18 g[1)]
Water	1 000 ml

1) According to the gel strength of the agar.

5.2.5.2 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 7,0 at 25 °C.

Dispense the culture medium in quantities of 15 ml into tubes or flasks (6.5).

Sterilize for 20 min in an autoclave (6.1) set at 121 °C.

Leave the tubes or flasks in a vertical position.

The medium may be stored for up to 1 week at 0 °C to + 5 °C.

Just before use, heat in boiling water or flowing steam for 15 min, then cool rapidly to the incubation temperature.

5.3 Oxidase reagent

5.3.1 Components

N,N,N′,N′ -Tetramethyl-*p* -phenylene-diamine dihydrochloride	1,0 g
Water	1 000 ml

5.3.2 Preparation

Dissolve the component in the cold water.

Prepare the reagent just before use.

NOTE 2 Ready-to-use discs may be used.

6 Apparatus and glassware

NOTE 3 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

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

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

See ISO 7218.

6.2 Incubator, capable of operating at 35 °C ± 1 °C or 37 °C ± 1 °C, depending on the temperature agreed.

6.3 Drying cabinet, oven (ventilated by convection) or **incubator**, capable of operating between 37 °C ± 1 °C and 55 °C ± 1 °C.

6.4 Water-bath, capable of operating at 45 °C ± 1 °C.

6.5 Containers, e.g. bottles, tubes or flasks, suitable for the sterilization and storage of culture media.

6.6 Test tubes, of dimensions 16 mm × 160 mm and 20 mm × 200 mm, or **flasks** or **bottles** of equivalent capacity.

6.7 Petri dishes, made of glass or plastics, of 90 mm to 100 mm diameter.

6.8 Loop (of diameter approximately 3 mm) and **wire**, made of platinum/iridium or nickel/chromium, and/or a **glass rod**.

NOTE 4 A nickel/chromium loop or wire is not suitable for use in the oxidase test (see 9.5.4.1).

6.9 Total delivery graduated pipettes, of 1 ml nominal capacity, graduated in divisions of 0,1 ml and with an outflow opening of 2 mm to 3 mm diameter.

6.10 pH meter, accurate to ± 0,1 pH unit at 25 °C.

7 Sampling

Sampling shall have been carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of the 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 agreement be reached on this subject by the parties concerned.

9 Procedure (see figure A.1, annex A)

9.1 General

For guidance in carrying out the procedure, see ISO 7218.

9.2 Test portion and initial suspension

For preparation of the initial suspension, use as diluent the pre-enrichment medium (5.2.1).

In general, to prepare the initial suspension, take a test portion of 1 g and place it in a tube containing 10 ml of pre-enrichment medium (5.2.1); this corresponds to the test portion/pre-enrichment medium ratio specified in this method. A 10^{-1} dilution is thus obtained.

If the test portion used is not 1 g, use the quantity of pre-enrichment medium required to yield an approximately 10^{-1} dilution (mass to volume). For example, if 0,1 g of the product has to be examined, transfer 1 ml of a 10^{-1} dilution of the test sample to a tube containing 10 ml of pre-enrichment medium.

NOTE 5 Dried, powdered food products may need a special rehydration procedure to enhance the recovery of *Enterobacteriaceae*. Refer for this purpose to the International Standard appropriate to the product under examination. If such a standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

9.3 Non-selective pre-enrichment

Incubate the initial suspension (9.2) at 35 °C or 37 °C for not less than 16 h and not more than 20 h. The temperature shall be agreed by the parties concerned and shall be indicated in the test report.

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9.4 Selective enrichment

9.4.1 Transfer 1 ml of the culture obtained in 9.3 to a tube containing 10 ml of the enrichment medium (5.2.2).

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9.4.2 Incubate the inoculated medium (9.4.1) at 35 °C or 37°C (as agreed) for 18 h to 24 h.

9.5 Plating out and selection for confirmation

9.5.1 Plating out

Using a loop (6.8), streak the incubated enrichment medium (9.4.2) onto the surface of a plate containing the selective medium (5.2.3) and incubate the plate for 24 h at 35 °C or 37 °C (as agreed).

9.5.2 Selection of colonies for confirmation

From each of the incubated plates (9.5.1) on which characteristic deep-red colonies (with deep-red precipitate haloes) have developed, choose at random five such colonies for subculturing (9.5.3) for the biochemical confirmation tests (9.5.4).

NOTE 6 Certain *Enterobacteriaceae* may cause decoloration of their colonies and the medium. Therefore, when no characteristic colonies are present, choose five whitish colonies for confirmation.

9.5.3 Subculturing selected colonies

Streak onto nutrient agar plates (5.2.5) each of the colonies selected for confirmation (9.5.2).

Incubate these plates for 24 h at 35 °C or 37 °C (as agreed).

Select a well-isolated colony from each of the incubated plates for the biochemical confirmation tests (9.5.4).

9.5.4 Biochemical confirmation tests

9.5.4.1 Oxidase reaction

Using a platinum/iridium loop or wire or a glass rod (6.8), take a portion of each well-isolated colony (9.5.3) and streak onto a filter paper moistened with the oxidase reagent (5.3) or onto a commercially available disc. A nickel/chromium loop or wire shall not be used.

Consider the test to be negative when the colour of the filter paper has not turned dark within 10 s.

Consult the manufacturer's instructions for ready-to-use discs.

9.5.4.2 Fermentation test

Stab, using a wire (6.8), the same colonies selected in 9.5.4.1 into tubes containing glucose agar (5.2.4).

Incubate these tubes for 24 h at 35 °C or 37 °C (as agreed).

If a yellow colour develops throughout the contents of the tube, the reaction is regarded as positive.

10 Expression of results

If one of the selected characteristic colonies (9.5.2) is oxidase-negative and glucose-positive, the test portion shall be regarded as being positive for *Enterobacteriaceae*.

According to the results obtained in the confirmation tests, indicate the presence or absence of *Enterobacteriaceae* in x g of product (i.e. according to the quantity of product examined).

11 Test report

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

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

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