INTERNATIONAL STANDARD

ISO 7402

Second edition
1993-09-15

Microbiology — General guidance for the enumeration of *Enterobacteriaceae* without resuscitation — MPN technique and colony-count technique

Microbiologie — Directives générales pour le dénombrement sans revivification des Enterobacteriaceae *— Technique NPP et méthode par comptage des colonies*

Reference number
ISO 7402:1993(E)

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 7402 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 9, *Microbiology*.

This second edition cancels and replaces the first edition (ISO 7402:1985), which has been technically revised.

Annexes A and B form an integral part of this International Standard.

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Case Postale 56 • CH-1211 Genève 20 • Switzerland
Printed in Switzerland

Introduction

This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and to be taken into account by organizations 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 in every detail for certain products, 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 guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

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.

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, but 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.

For any particular product, the method to be used will be specified in the International Standard dealing with that product.

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Microbiology — General guidance for the enumeration of *Enterobacteriaceae* without resuscitation — MPN technique and colony-count technique

1 Scope

This International Standard gives general guidance for the enumeration of *Enterobacteriaceae* present in products intended for human consumption or animal feeding stuffs:

— by calculation of the most probable number (MPN) after incubation at 35 °C or 37 °C in liquid medium, or

— by counting colonies in a solid medium after incubation at 35 °C or 37 °C.

The temperature used is to be the subject of agreement between the parties concerned, and is to be stated in the test report.

NOTE 1 In the case of frozen foods, an incubation temperature of 30 °C is preferred when the aim of the enumeration is technological.

For low numbers, the MPN method is preferable, otherwise the colony-count method is preferred.

This International Standard does not include resuscitation procedures and the results should not, therefore, be related to criteria or specifications based on the assumption that resuscitation has been carried out.

A limitation on the applicability of this International Standard is imposed by the susceptibility of the methods to a large degree of variability. The methods should be used and the results interpreted in the light of the information given in 10.3.

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

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*: Microorganisms which ferment glucose and show a negative oxidase reaction when the test is carried out according to the method specified.

3.2 count of *Enterobacteriaceae*: The number of *Enterobacteriaceae* found per millilitre or per gram of the test sample when the test is carried out according to the method specified.

4 Principle

4.1 Preparation of dilutions

Preparation of decimal dilutions from the test sample.

4.2 Enumeration of *Enterobacteriaceae*

4.2.1 Most probable number (MPN) technique

NOTE 2 This technique is recommended when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of the test sample.

Inoculation of three tubes of double-strength medium with a specified quantity of test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation of three tubes of single-strength medium with a specified quantity of the test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products. Then, under the same conditions, inoculation of three tubes of single-strength medium with the first decimal dilution of the test sample or of the initial suspension.

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

From the number of confirmed positive tubes, calculation of the most probable number of *Enterobacteriaceae* per millilitre or per gram of the test sample using the MPN table (see annex A).

4.2.2 Colony-count technique

NOTE 3 This technique is recommended when the number sought is expected to be greater than 100 per millilitre or per gram of the test sample.

Inoculation of violet red bile glucose agar contained in two Petri dishes (poured-plate technique) with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of the initial suspension in the case of other products. Covering with an overlayer of the same medium.

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

Incubation of the dishes at 35 °C or 37 °C (as agreed) for 24 h ± 2 h.

Calculation of the number of *Enterobacteriaceae* per millilitre or per gram of the test sample from the number of confirmed typical colonies per dish.

5 Dilution fluid, culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Dilution fluid

See ISO 6887 and any specific standard dealing with the product to be examined.

5.3 Culture media

5.3.1 Buffered brilliant green bile glucose broth (E.E. broth)

5.3.1.1 Composition

	a) Double-strength medium	b) Single-strength medium
Peptone	20,0 g	10,0 g
Glucose	10,0 g	5,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	12,90 g	6,45 g
Potassium dihydrogen phosphate (KH_2PO_4)	4,0 g	2,0 g
Ox bile, dehydrated	40,0 g	20,0 g
Brilliant green	0,030 g	0,015 g
Water	1 000 ml	1 000 ml

5.3.1.2 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.

Transfer 10 ml portions to sterile tubes or bottles (6.8).

Do not autoclave the medium.

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

5.3.2 Violet red bile glucose agar (VRBG)

5.3.2.1 Composition

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 in powder or flake form	8 to 18 g [1]
Water	1 000 ml

1) Depending on the gel strength of the agar.

5.3.2.2 Preparation

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

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

Transfer the culture medium to sterile tubes, flasks or bottles (6.8) of capacity not more than 500 ml.

Do not autoclave the medium.

Prepare this medium just before use (see 9.2.2 and 9.3.1).

5.3.2.3 Preparation of agar plates (required only for MPN technique, see 9.2.2)

Dispense immediately approximately 15 ml of the culture medium, cooled to approximately 45 °C, into Petri dishes (6.6) and allow to solidify.

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

5.3.3 Glucose agar

5.3.3.1 Composition

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

1) Depending on the gel strength of the agar.

5.3.3.2 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 (6.8).

Sterilize for 15 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.

5.3.4 Nutrient agar

5.3.4.1 Composition

Beef extract	3,0 g
Peptone	5,0 g
Agar in powder or in flake form	8 to 18 g [1]
Water	1 000 ml

1) Depending on the gel strength of the agar.

5.3.4.2 Preparation

Dissolve the components or 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.

Transfer the culture medium to tubes, bottles or flasks (6.8) of capacity not more than 500 ml.

Sterilize in the autoclave (6.1) set at 121 °C ± 1 °C for 15 min.

5.3.4.3 Preparation of agar plates (see 9.4.1)

Transfer immediately about 15 ml of the culture medium, melted and cooled to approximately 45 °C, to Petri dishes (6.6) and allow to solidify.

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

5.4 Oxidase reagent

5.4.1 Composition

N,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylene-diamine dihydrochloride	1,0 g
Water	100 ml

5.4.2 Preparation

Dissolve the reagent in the cold water.

Prepare the reagent just before use.

6 Apparatus and glassware

NOTE 4 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment 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 at 37 °C ± 1 °C.

6.3 Drying cabinet or **incubator**, capable of operating between 35 °C ± 1 °C and 55 °C ± 1 °C.

6.4 pH-meter, accurate to within ± 0,1 pH unit at 25 °C.

6.5 Water bath, or similar apparatus, capable of operating at 45 °C ± 1 °C.

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

6.7 Loops, of platinum/iridium or nickel/chromium, approximately 3 mm in diameter, and **wires** of the same material, or a **glass rod**.

NOTE 5 A nickel/chromium loop is not suitable for use in the oxidase test (see 9.4.2.1).

6.8 Test tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm, and **flasks** or **bottles**, of capacity between 150 ml and 500 ml.

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.

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. 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, initial suspension and dilutions

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

Prepare a single decimal dilution series from the test sample if the product is liquid, or from the initial suspension in the case of other products.

9.2 MPN technique

9.2.1 Inoculation and incubation

Take three tubes of double-strength medium [5.3.1.1, a)]. Transfer to each of these tubes, using a pipette (6.9), 10 ml of the test sample if the product is liquid, or 10 ml of the initial suspension in the case of other products.

Take three tubes of single-strength medium [5.3.1.1, b)]. Transfer to each of these tubes, using another pipette (6.9), 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take three more tubes of single-strength medium [5.3.1.1, b)]. Transfer to each of these tubes, using another pipette (6.9), 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in the case of other products.

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

9.2.2 Isolation

Streak a loopful (6.7) from each of the nine incubated cultures (see 9.2.1) on the violet red bile glucose agar plates (see 5.3.2.3) and incubate the plates at 35 °C or 37 °C (as agreed) for 24 h.

9.2.3 Selection of colonies for confirmation

From each of the plates incubated as in 9.2.2 on which typical pink to red colonies (with or without precipitation haloes) or colourless, mucoid colonies have developed, select at random five such colonies for biochemical confirmation (see 9.4.2) after subculturing (see 9.4.1).

9.3 Colony-count technique

9.3.1 Inoculation and incubation

9.3.1.1 Take two sterile Petri dishes (6.6). Using a sterile pipette (6.9), transfer to each dish 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take two other sterile Petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in the case of other products.

Repeat the procedure described with the further dilutions, using a fresh sterile pipette for each decimal dilution.

9.3.1.2 Pour into each Petri dish approximately 15 ml of the VRBG medium (5.3.2) which has been prepared then cooled to approximately 45 °C in the water bath (6.5). The time elapsing between the end of the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) and the moment when the medium (5.3.2) is poured into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium by horizontal movements and allow the mixture to solidify with the Petri dishes standing on a cool horizontal surface.

9.3.1.3 After complete solidification of the mixture, add a covering layer of 10 ml to 15 ml of the VRBG medium (5.3.2), prepared then cooled as described in 9.3.1.2, to prevent spreading growth and to obtain semi-anaerobic conditions. Allow to solidify as described above.

9.3.1.4 Invert the prepared dishes and incubate them in the incubator set at 35 °C or 37 °C (as agreed) for 24 h.

9.3.2 Counting and selection of colonies

Select the dishes (9.3.1.4) containing less than 150 typical colonies (see 9.2.3) of diameter 0,5 mm or more; count these suspect colonies. Select at random five such colonies from each dish for biochemical confirmation (see 9.4.2) after subculturing (see 9.4.1).

Consider the determination to be void if half or more than half the surface area of a dish is overgrown. If less than half of the surface area of a dish is overgrown, count the colonies on the clear part and extrapolate so that the number corresponds to the total surface area of the dish.

9.4 Confirmation

9.4.1 Subculturing

Streak on nutrient agar plates (5.3.4) each of the colonies selected for confirmation (see 9.2.3 and 9.3.2).

Incubate these plates at 35 °C or 37 °C (as agreed) for 24 h ± 2 h. Select a well-isolated colony from each of the incubated plates for biochemical confirmation (see 9.4.2).

9.4.2 Biochemical confirmation

9.4.2.1 Oxidase reaction

Using the platinum/iridium loop or wire or glass rod (6.7), take a portion of each well-isolated colony (9.4.1) and streak on a filter paper moistened with the oxidase reagent (5.4) or on 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 in 10 s.

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

9.4.2.2 Fermentation test

Stab, using a wire (6.7), the same colonies selected in 9.4.1 into tubes containing glucose agar (5.3.3). Incubate at 35 °C or 37 °C (as agreed) for 24 h ± 2 h.

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

10 Expression of results

10.1 Calculation of the most probable number (MPN)

10.1.1 Count the number of tubes giving a positive reaction for each dilution.

10.1.2 If one of the selected typical colonies (9.2.3) of a subculture (see 9.4.1) is oxidase-negative and glucose-positive, the tube from which the subculture is derived shall be regarded as being positive.

10.1.3 Using the MPN table (see annex A), determine from the number of positive tubes in the different dilutions, the most probable number (MPN) index

10.1.4 In the case of liquid products, the number of *Enterobacteriaceae* per millilitre is calculated by dividing the MPN index by 10. In the case of other products for which initial suspensions are prepared, the number per gram is equal to the MPN index.