INTERNATIONAL STANDARD

ISO 7251

Second edition
1993-12-15

Microbiology — General guidance for enumeration of presumptive *Escherichia coli* — Most probable number technique

*Microbiologie — Directives générales pour le dénombrement d'*Escherichia coli *présumés — Technique du nombre le plus probable*

Reference number
ISO 7251:1993(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 7251 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 7251:1984), which has been technically revised.

Annex A forms 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 provided guidelines 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 deviations 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.

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Microbiology — General guidance for enumeration of presumptive *Escherichia coli* — Most probable number technique

1 Scope

This International Standard gives general guidelines for the enumeration of presumptive *Escherichia coli* in products intended for human consumption or feeding of animals, by means of the liquid-medium culture technique and calculation of the most probable number (MPN) after incubation at 35 °C or 37 °C (this temperature forming the subject of agreement between the parties concerned), then incubation at 45 °C.

CAUTION — Some *Escherichia coli* pathogenic species do not grow at 45 °C.

A limitation of the applicability of this International Standard is imposed by the susceptibility of the method to a large degree of variability. The method should be applied and the results interpreted in the light of the information given in 10.4.

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 Definition

For the purposes of this International Standard, the following definition applies.

3.1 presumptive *Escherichia coli*: Bacteria which, at 45 °C, cause fermentation of lactose with the production of gas, and which, at 45 °C, produce indole from tryptophan, when the test is carried out in accordance with the method specified in this International Standard.

4 Principle

4.1 Inoculation of three tubes of double-strength liquid selective enrichment medium [5.3.1 a)][1] 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.

4.2 Inoculation of three tubes of single-strength liquid enrichment medium [5.3.1 b)][1] with a specified quantity of test sample if the initial 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 the medium [5.3.1 b)] with decimal dilutions of the test sample or of the initial suspension.

4.3 Incubation of the tubes of double- and single-strength medium at 35 °C or 37 °C (as agreed) for 24 h to 48 h. Examination of the tubes for gas formation.

4.4 Inoculation, from the tubes of double- and single-strength medium which have given rise to gas formation, of a new series of tubes containing a liquid selective medium.

1) If necessary, another liquid enrichment medium may be used prior to inoculation of the selective medium.

4.5 Incubation at 45 °C for 24 h to 48 h, and examination of the new series of tubes (4.4) for gas formation.

4.6 Inoculation, from the tubes of selective medium which have given rise to gas formation, of a new series of tubes containing tryptone water.

4.7 Incubation at 45 °C for 24 h to 48 h, and examination of the new series of tubes (4.6) for indole production.

4.8 Determination of the most probable number of presumptive *Escherichia coli* by means of an MPN table (see annex A), according to the number of incubated tubes which gave rise to gas formation in the selective medium and in which indole was produced in the tryptone water.

5 Dilution fluid, culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Dilution fluid

See ISO 6887:1983, clause 5, and the specific International Standard dealing with the product under examination.

5.3 Lauryl sulfate tryptose broth (selective enrichment medium).

5.3.1 Composition

	a) Double-strength medium	b) Single-strength medium
Tryptose	40,0 g	20,0 g
Lactose	10,0 g	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5,5 g	2,75 g
Potassium dihydrogen phosphate (KH_2PO_4)	5,5 g	2,75 g
Sodium chloride	10,0 g	5,0 g
Sodium lauryl sulfate [$CH_3(CH_2)_{11}OSO_3Na$]	0,2 g	0,1 g
Water	1 000 ml	1 000 ml

5.3.2 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 6,8 at 25 °C.

Dispense the media in quantities of 10 ml into tubes of dimensions 16 mm × 160 mm (6.4) containing Durham tubes (6.6) in the case of single-strength medium, and into test tubes of dimensions 20 mm × 200 mm (6.4) containing Durham tubes (6.6) in the case of the double-strength medium.

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

The Durham tubes shall not contain air bubbles after sterilization.

5.4 EC Broth (second selective medium)

5.4.1 Composition

Tryptose or trypticase	20,0 g
Lactose	5,0 g
Bile salts No. 3[1)]	1,5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Sodium chloride	5,0 g
Water	1 000 ml

1) See *ICMSF Microorganisms in Foods 1*, 2nd edition, 32, p. 280, University of Toronto Press, Canada.

5.4.2 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 6,8 at 25 °C.

Dispense the medium in quantities of 10 ml into tubes of dimensions 16 mm × 160 mm (6.4) containing Durham tubes (6.6).

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

The Durham tubes shall not contain air bubbles after sterilization.

5.5 Tryptone water

5.5.1 Composition

Tryptone	10,0 g
Sodium chloride	5,0 g
Water	1 000 ml

5.5.2 Preparation

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

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

Dispense the medium in quantities of 5 ml to 10 ml into tubes of dimensions 16 mm × 160 mm (6.4).

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

5.6 Indole reagent (Kovacs reagent)

5.6.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
2-Methylbutan-1-ol or pentan-1-ol	75,0 ml
Hydrochloric acid (ρ_{20} 1,18 to 1,19 g/ml)	25,0 ml

5.6.2 Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the alcohol by heating gently by means of a water bath maintained at approximately 50 °C to 55 °C.

Cool and add the acid.

Protect from light and store at approximately 4 °C.

The reagent shall be light yellow to light brown.

6 Apparatus and glassware

NOTE 1 Disposable apparatus is an acceptable alternative to reusable glassware if it has similar 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 37 °C ± 1 °C, depending on the temperature adopted.

6.3 Water bath, capable of being maintained at 45 °C ± 0,5 °C (to allow the inoculated tubes to be maintained at this temperature).

6.4 Test tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm.

6.5 Loop, made of platinum/iridium or nickel/chromium, approximately 3 mm in diameter, or **sterile disposable bags**.

6.6 Durham tubes, of a size suitable for use in the test tubes (6.4).

6.7 Total-delivery pipettes, having nominal capacities of 1 ml and 10 ml.

6.8 pH-meter, accurate to within ± 0,1 pH units at 25 °C.

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 the specific International Standard appropriate to the product concerned.

Prepare a sufficient number of dilutions to ensure that all the tubes for the final dilution will yield a negative result.

9.2 MPN technique

9.2.1 Inoculation of the selective enrichment medium

9.2.1.1 Take three tubes of double-strength selective enrichment medium [5.3.1 a)]. Using a sterile pipette (6.7), transfer to each of these tubes 10 ml of the test sample, if liquid, or 10 ml of the initial suspension, in the case of other products.

9.2.1.2 Then take three tubes of single-strength selective enrichment medium [5.3.1 b)]. Using a fresh sterile pipette (6.7), transfer to each of these tubes 1 ml of the test sample, if liquid, or 1 ml of the initial suspension, in the case of other products.

9.2.1.3 For each of the further dilutions (from 10^{-1} or 10^{-2}, according to the test sample), proceed as in 9.2.1.2. Use a fresh sterile pipette for each dilution. Carefully mix the inoculum and the medium.

9.2.2 Incubation

Incubate the tubes of double-strength selective medium (9.2.1.1) and the tubes of single-strength selective medium (9.2.1.2 and 9.2.1.3) in the incubator (6.2) set at 35 °C or 37 °C (as agreed) for 24 h ± 2 h. If, at this stage, neither gas formation nor opacity preventing the observation of gas formation is observed, incubate for up to 48 h ± 2 h.

9.2.3 Inoculation of the second selective medium

From each tube incubated as in 9.2.2 and showing gas formation, inoculate with a loop (6.5) 10 ml of the second selective medium (5.4), previously heated to 45 °C.

9.2.4 Second incubation

Incubate the tubes inoculated as in 9.2.3 in the water bath (6.3) set at 45 °C for 24 h ± 2 h. If, at this stage, no gas formation is observed, incubate for 48 h.

9.2.5 Inoculation of tryptone water

From each tube incubated as in 9.2.4 and showing gas formation, inoculate with a loop (6.5), the tryptone water (5.5), previously heated to 45 °C.

9.2.6 Incubation

Incubate the tubes inoculated as in 9.2.5 in the water bath (6.3) set at 45 °C for 48 h.

9.2.7 Test for indole production

Add 0,5 ml of the indole reagent (5.6) to the tubes containing the inoculated tryptone water (9.2.5), mix well and examine after 1 min.

9.2.8 Interpretation

For each dilution, count the number of tubes with a red colour in the alcoholic phase, indicating the presence of indole (positive tubes).

10 Expression of results

10.1 Selection of dilutions[2)]

For each sample examined, select three consecutive dilutions in accordance with 10.1.1, 10.1.2 or 10.1.3, whichever is appropriate.

10.1.1 Case 1 — At least one dilution yields three positive tubes

Select the highest dilution (i.e. that having the lowest sample concentration) yielding three positive tubes, together with the next two higher dilutions (i.e. those having sample concentrations of 1/10 and 1/100 of that of the first dilution selected) (see table 1, example 1).

See also 10.1.3.

If insufficient further dilutions were made beyond the highest dilution yielding three positive tubes, select instead the three highest dilutions in the series (i.e. those having the lowest sample concentration) (see table 1, example 2).

10.1.2 Case 2 — No dilution yields three positive tubes

Case 1 cannot be applied. Select the three highest dilutions in the series (i.e. those having the lowest sample concentration) amongst which at least one positive result was obtained (see table 1, example 3).

See also 10.1.3.

10.1.3 Special cases

In all cases where more than one of the three dilutions selected in accordance with 10.1.1 and 10.1.2 do not yield positive tubes, select from these dilutions the lowest one not yielding positive tubes (i.e. that having the highest sample concentration) and the two next lower dilutions in the series (i.e. those having sample concentrations of 10 times and 100 times that of the first dilution selected) (see table 1, examples 4 and 5), except when positive tubes are found only at the level of the first dilution prepared from the sam-

2) In this subclause, the initial suspension and, if necessary, the test sample are considered as dilutions.

ple. In this last case, it is necessary to select the first three dilutions for calculation of the MPN even though this series includes two dilutions yielding no positive tubes.

10.2 Determination of MPN index

10.2.1 Check according to the number of samples examined per batch, using table A.1, whether the sequences of numbers of positive tubes corresponding to the dilutions selected in accordance with 10.1 are statistically acceptable. Acceptability depends both on the number of samples examined and on the decision as to whether or not to accept category 2 or 3 results (see table A.2).

Thus, for example, if only category 1 results are accepted, the sequence 221 is acceptable only when 10 samples (of the batch concerned) have been examined. However, if the less likely category 2 results are also accepted, the sequence 221 is also acceptable when only 2, 3 or 5 samples have been examined. However, when the sequence 221 is the result of a single examination, it is never acceptable.

10.2.2 For each sequence found to be acceptable in accordance with 10.2.1, obtain the MPN index using table A.1.

10.3 Calculation of most probable number (MPN)

Obtain the number of presumptive *Escherichia coli* per millilitre or per gram of product by multiplying the MPN index (see 10.2) by the reciprocal of the lowest dilution selected (i.e. that having the highest sample concentration).

When the lowest dilution selected corresponds to the tubes prepared with double-strength medium (inoculation with 10 ml), first divide the MPN index by 10.

Express the result as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

If the MPN is lower than 0,3 microorganisms per millilitre or per gram, and if the appropriate procedure for a low number of presumptive *Escherichia coli* was used (see 9.2.1), express the result in the following way: "No presumptive *Escherichia coli* in 1 ml or 1 g of the product".

10.4 Precision

It is recognized that wide variations in results may occur with the MPN technique. Results obtained with this method should therefore be used with caution.

Confidence limits are given in annex A.

EXAMPLE

For a solid sample, in 95 % of the cases, the confidence limits vary from 13 to 200 *Escherichia coli* per gram for an MPN of $7,4 \times 10^1$ *Escherichia coli* per gram, and from 4 to 99 *Escherichia coli* per gram for an MPN of $2,4 \times 10^1$ *Escherichia coli* per gram.

11 Test report

The test report shall specify the method used, the temperature of incubation and the test 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 likely to have influenced the test results.

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

Table 1 — Examples of the selection of positive results for calculation of the MPN

Example	Number of positive tubes obtained from three incubated tubes for the following amounts of sample inoculated per tube[1]						MPN[2]	
	Liquid product **Other products**	10 ml 1 g	1 ml 10^{-1} g	10^{-1} ml 10^{-2} g	10^{-2} ml 10^{-3} g	10^{-3} ml 10^{-4} g	**Liquid product** ml^{-1}	**Other products** g^{-1}
1		3	**3**	**2**	**1**	0	$1,5 \times 10^1$	$1,5 \times 10^2$
2		3	**3**	**3**	**0**		$2,4 \times 10^1$	$2,4 \times 10^2$
3		2	2	**1**	**1**	**0**	7,4	$7,4 \times 10^1$
4		**3**	**3**	**0**	0	0	2,4	$2,4 \times 10^1$
5		**2**	**2**	**0**	1	0	$2,1 \times 10^{-1}$	2,1

1) **Bold:** combination selected.

2) Calculated using the MPN index for three tubes (table A.1).