TC 34

International Standard 7251

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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*

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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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

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

0 Introduction

This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for the consideration of 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 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 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.

1 Scope and field of application

This International Standard gives general guidance 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[1] then 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, therefore, be applied and the results interpreted in the light of the information given in 10.4.

2 Reference

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

3 Definition

For the purpose of this International Standard, the following definition applies :

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 double strength liquid enrichment medium [5.3.1 a)][2] in three tubes with a specified quantity of the test sample if the product to be examined is liquid, or with a specified quantity of the initial suspension in the case of other products.

4.2 Inoculation of single strength liquid enrichment medium [5.3.1 b)][2] in three tubes with a specified quantity of test sample if the product to be examined is liquid, or with a specified quantity of the initial suspension in the case of other products.

Then, under the same conditions, inoculation of single strength medium with decimal dilutions of the test sample or of the initial suspension.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

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

4.3 Incubation of the tubes of double and single strength medium at 35 °C or 37 °C[1] for 24 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.

4.5 Incubation at 45 °C for 24 to 48 h and examination of this new series of tubes 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 to 48 h and examination of this new series of tubes for indole production.

4.8 Determination of the most probable number of presumptive *Escherichia coli* by means of an MPN table, 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 Diluent, culture media and reagent

5.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the culture media, dehydrated basic components or complete dehydrated media be used. The manufacturer's instructions shall be rigorously followed.

The chemical products used shall be of recognized analytical quality.

The water used shall be distilled or deionized water, free from substances that might inhibit the growth of micro-organisms under the test conditions.

Measurement of pH shall be made using a pH meter, adjusted to a temperature of 25 °C.

If the diluent, culture media and reagent are not used immediately, they shall, unless otherwise stated, be stored in the dark at approximately 4 °C for no longer than 1 month, in conditions which do not allow any change in their composition.

5.2 Diluent

See ISO 6887, clause 5.

5.3 Culture media

5.3.1 Lauryl sulfate tryptose broth (selective enrichment medium)

Composition

	a) double strength medium	b) single strength medium
tryptose	40 g	20 g
lactose	10 g	5 g
potassium hydrogen-orthophosphate (K_2HPO_4)	5,5 g	2,75 g
potassium dihydrogen-orthophosphate (KH_2PO_4)	5,5 g	2,75 g
sodium chloride	10 g	5 g
sodium lauryl sulfate	0,2 g	0,1 g
water	1 000 ml	1 000 ml

Preparation

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

If necessary, adjust the pH 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 (6.5) in the case of single strength medium, and into test tubes of dimensions 20 mm × 200 mm (6.4) containing Durham tubes (6.5) in the case of the double strength medium.

Sterilize in an autoclave at 121 ± 1 °C for 15 ± 1 min.

The Durham tubes shall not contain air bubbles after sterilization.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

5.3.2 EC Broth (second selective medium)

Composition

tryptose or trypticase	20 g
lactose	5 g
bile salts No. 3[1]	1,5 g
potassium hydrogenorthophosphate (K_2HPO_4)	4 g
potassium dihydrogenorthophosphate (KH_2PO_4)	1,5 g
sodium chloride	5 g
water	1 000 ml

Preparation

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

If necessary, adjust the pH 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 (6.5).

Sterilize in an autoclave at 121 ± 1 °C for 15 ± 1 min.

The Durham tubes shall not contain air bubbles after sterilization.

5.3.3 Tryptone water

Composition

tryptone	10,0 g
sodium chloride	5,0 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

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

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

Sterilize the medium at 121 ± 1 °C for 15 ± 1 min.

5.4 Indole reagent (Kovacs reagent)

Composition

4-dimethylaminobenzaldehyde	5,0 g
2-methyl butanol or pentanol	75,0 ml
hydrochloric acid (ϱ_{20} 1,18 to 1,19 g/ml)	25,0 ml

Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the alcohol by heating gently by means of a water-bath maintained at approximately 50 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 — Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment and in particular :

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

Apparatus that will come into contact with the diluent, the culture media, the sample or dilutions, except for apparatus that is supplied sterile (particularly plastics apparatus), shall be sterilized by one of the following methods:

a) by being kept at 170 to 175 °C for not less than 1 h in an oven;

b) by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

6.2 Incubator, capable of being maintained at 35 ± 1 °C or 37 ± 1 °C, depending on the temperature adopted.[2]

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

6.4 Test-tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm, or **flasks** or **bottles** of suitable capacity.

6.5 Durham tubes, of size suitable for use in the tubes (6.4).

6.6 Total delivery pipettes, of nominal capacities 1 ml and 10 ml.

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

7 Sampling

Carry out sampling in conformity with the specific standard appropriate to the product concerned. If a specific standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

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

2) The temperature should be agreed between the parties concerned and recorded in the test report.

8 Preparation of the test sample

See the specific standard appropriate to the product concerned. If a specific standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and the specific standard appropriate to the product concerned.

Prepare a sufficient number of dilutions in order to ensure that all the tubes for the final dilution will give 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 enrichment medium [5.3.1 a)]. Transfer to each of these tubes, by means of a pipette (6.6), 10 ml of the test sample, if liquid, or 10 ml of the initial suspension.

9.2.1.2 Then take three tubes of single strength enrichment medium [5.3.1 b)]. Transfer to each of these tubes, by means of a pipette (6.6), 1 ml of the test sample, if liquid, or 1 ml of the initial suspension.

9.2.1.3 For each of the subsequent dilutions (from 1/10 or 1/100, according to the test sample), proceed as specified in 9.2.1.2. Use a new pipette for each dilution. Carefully mix the inoculum and the medium.

9.2.2 Incubation

Incubate the tubes of double strength medium (9.2.1.1) and the tubes of single strength medium (9.2.1.2 and 9.2.1.3) in the incubator (6.2) at 35 °C or 37 °C[1] for 24 ± 2 h. If, at this stage, neither gas formation nor opacity preventing the observation of gas formation is observed, incubate for up to 48 ± 2 h.

9.2.3 Inoculation of the second selective medium

From each tube incubated in 9.2.2 and showing gas formation, inoculate, by means of a loop, 10 ml of the selective medium (5.3.2), previously heated to 45 °C.

9.2.4 Incubation

Incubate the tubes inoculated in 9.2.3 in the water-bath (6.3), maintained at 45 ± 0,5 °C, for 24 ± 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 in 9.2.4 and showing gas formation, inoculate, by means of a loop, the tryptone water (5.3.3), previously heated to 45 °C.

9.2.6 Incubation

Incubate the tubes inoculated in 9.2.5 in the water-bath (6.3), maintained at 45 ± 0,5 °C, for 48 h.

9.2.7 Test for indole production

Add 0,5 ml of the indole reagent (5.4) to the tubes containing the inoculated tryptone water, mix well and examine after 1 min.

A red colour in the alcoholic phase indicates the presence of indole (positive tubes).

9.2.8 Interpretation

For each dilution, count the number of positive tubes (9.2.7).

10 Expression of results

10.1 Selection of dilutions[1]

For each sample examined, select three consecutive dilutions as described in the three following cases using 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 10.3, 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 10.3, example 2).

10.1.2 Case 2 — No dilution yields three positive tubes

If no dilution yields three positive tubes (and, hence, the procedure described in 10.1.1 cannot be used), select the three highest dilutions in the series (i.e. those having the lowest sample concentration) (see 10.3, example 3).

See also 10.1.3.

1) For the purpose of selection, the initial suspension and, if necessary, the test sample are considered as dilutions.

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 does 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 10.3, examples 4 and 5), except when positive tubes are found only at the level of the first dilution prepared from the sample. 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 According to the number of samples examined per batch, check, using annex A or B, whether the sequences of numbers of positive tubes corresponding to the dilutions selected in accordance with 10.1 are statistically acceptable. The 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 annex C).

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. On the other hand, 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 acceptable only when the less likely category 3 results are also accepted.

10.2.2 For each sequence found to be acceptable in accordance with 10.2.1, determine the MPN index from annex A or B.

10.3 Calculation of most probable number (MPN)

Determine 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^n, where n is the appropriate power of 10.

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

Example 1 : Solid sample

Initial suspension 1/10	(10 ml)	:	3 positive tubes
Initial suspension 1/10	(1 ml)	:	3 positive tubes
Dilution 1/100	(1 ml)	:	2 positive tubes
Dilution 1/1 000	(1 ml)	:	1 positive tube
Dilution 1/10 000	(1 ml)	:	0 positive tubes

Retain 321.

Reference to annex A or B gives an MPN index of 15 and the calculation gives an MPN of 15 × 10, i.e.

1,5 × 10^2 presumptive *Escherichia coli* per gram

Example 2 : Solid sample

Initial suspension 1/10	(10 ml)	:	3 positive tubes
Initial suspension 1/10	(1 ml)	:	3 positive tubes
Dilution 1/100	(1 ml)	:	3 positive tubes
Dilution 1/1 000	(1 ml)	:	0 positive tubes

Retain 330.

Reference to annex A or B gives an MPN index of 20 and the calculation gives an MPN of 20 × 10, i.e.

2 × 10^2 presumptive *Escherichia coli* per gram

Example 3 : Liquid sample

Test sample (dilution 1/1)	(10 ml)	:	2 positive tubes
Test sample (dilution 1/1)	(1 ml)	:	2 positive tubes
Dilution 1/10	(1 ml)	:	1 positive tube
Dilution 1/100	(1 ml)	:	1 positive tube
Dilution 1/1 000	(1 ml)	:	0 positive tubes

Retain 110.

Reference to annex A or B gives an MPN index of 0,7 and the calculation gives an MPN of 0,7 × 10, i.e.

7 × 10^0 presumptive *Escherichia coli* per millilitre

Example 4 : Solid sample

Initial suspension 1/10	(10 ml)	:	3 positive tubes
Initial suspension 1/10	(1 ml)	:	3 positive tubes
Dilution 1/100	(1 ml)	:	0 positive tubes
Dilution 1/1 000	(1 ml)	:	0 positive tubes

Retain 330.

Reference to annex A or B gives an MPN of 20 and the calculation gives an MPN of $\frac{20}{10} \times 10$, i.e.

2,0 × 10^1 presumptive *Escherichia coli* per gram

Example 5 : Solid sample

Initial suspension 1/10	(10 ml)	:	2 positive tubes
Initial suspension 1/10	(1 ml)	:	2 positive tubes
Dilution 1/100	(1 ml)	:	1 positive tube
Dilution 1/1 000	(1 ml)	:	0 positive tubes
Dilution 1/10 000	(1 ml)	:	0 positive tubes

Retain 210.

Reference to annex A or B gives an MPN index of 1,5 and the calculation gives an MPN of 1,5 × 10, i.e.

1,5 × 10^1 presumptive *Escherichia coli* per gram

10.4 Precision

It is well known 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 annexes A and B.

11 Test report

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

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

Annex A

Determination of MPN index when the examination is carried out on a single sample per batch[1)]

Number of positive tubes at the three successive dilutions selected			MPN index	Category[2)]	Confidence limits[3)]			
1st	2nd	3rd			95 %		99 %	
0	0	0	< 0,30					
0	0	1	0,30	3	0,01	0,95	0,00	1,40
0	1	0	0,30	2	0,01	1,00	0,00	1,60
0	1	1		0				
0	2	0	0,6	3	0,12	1,70	0,05	2,50
0	3	0		0				
1	0	0	0,4	1	0,02	1,70	0,01	2,50
1	0	1	0,7	2	0,12	1,70	0,05	2,50
1	0	2		0				
1	1	0	0,7	1	0,13	2,00	0,06	2,70
1	1	1	1,1	3	0,35	3,50	0,18	4,60
1	2	0	1,1	2	0,36	3,50	0,19	4,60
1	2	1	1,5	3	0,45	3,80	0,24	5,20
1	3	0	1,6	3	0,45	3,80	0,24	5,20
2	0	0	0,9	1	0,15	3,50	0,07	4,60
2	0	1	1,4	2	0,36	3,50	0,19	4,60
2	0	2		0				
2	1	0	1,5	1	0,37	3,80	0,20	5,20
2	1	1	2,0	2	0,45	3,80	0,24	5,20
2	1	2		0				
2	2	0	2,1	1	0,45	4,00	0,24	5,60
2	2	1	2,8	3	0,87	9,40	0,51	14,20
2	2	2		0				
2	3	0	2,9	3	0,87	9,40	0,51	14,20
2	3	1		0				
3	0	0	2,3	1	0,46	9,40	0,25	14,20
3	0	1	3,8	1	0,88	10,40	0,52	15,70
3	0	2	6	3	1,60	18,10	1,00	25,00
3	0	3		0				
3	1	0	4	1	0,91	18,10	0,53	25,00
3	1	1	7	1	1,70	19,90	1,10	27,00
3	1	2	12	3	3,50	36,00	2,10	44,00
3	1	3		0				
3	2	0	9	1	1,80	36,00	1,20	43,00
3	2	1	15	1	3,50	38,00	2,20	52,00
3	2	2	21	2	3,50	40,00	2,50	56,00
3	2	3	29	3	9,00	99,00	4,60	152,00
3	3	0	20	1	3,60	99,00	2,60	152,00
3	3	1	50	1	9,10	198,00	4,70	280,00
3	3	2	110	1	18,20	405,00	11,40	570,00
3	3	3	> 110					

1) From De Man, J.C. *Eur. J. Appl. Microbiol. Biotechnol.* **17** 1983: 301-305.

2) See annex C.

3) The 95 and 99 % confidence limits have been calculated on the basis of statistical considerations only. In reality, the uncertainty in the number of bacteria will be greater than indicated by these limits.