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Milk and milk products — Enumeration of presumptive *Escherichia coli* —

Part 1:
Most probable number technique

*Lait et produits laitiers — Dénombrement d'*Escherichia coli *présumés —*
Partie 1: Technique du nombre le plus probable

Reference number
ISO 11866-1:1997(E)

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.

This part of ISO 11866 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and AOAC INTERNATIONAL, and will also be published by these organizations.

ISO 11866 consists of the following parts, under the general title *Milk and milk products — Enumeration of presumptive* Escherichia coli:

— *Part 1: Most probable number technique*

— *Part 2: Most probable number technique using 4-methylumbelliferyl-β-D-glucuronide (MUG)*

— *Part 3: Colony-count technique at 44 °C using membranes*

The method specified in ISO 11866-1 is preferred for samples in which comparatively low numbers of *Escherichia coli* are suspected.

Annex A forms an integral part of this part of ISO 11866. Annex B is for information only.

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Milk and milk products — Enumeration of presumptive *Escherichia coli* —

Part 1:

Most probable number technique

1 Scope

This part of ISO 11866 specifies a method for the enumeration of presumptive *Escherichia coli* by means of the culture technique involving a liquid medium, and calculation of the number of presumptive *Escherichia coli* per gram or per millilitre by the most probable number (MPN) technique after incubation at 37 °C then incubation at 44 °C.

The method is applicable to

- milk, liquid milk products;
- dried milk, dried sweet whey, dried buttermilk, lactose;
- acid casein, lactic casein and rennet casein;
- caseinate and dried acid whey;
- cheese and processed cheese;
- butter;
- frozen milk products (including edible ices);
- custard, desserts and cream.

The method specified in this part of ISO 11866 is preferred for samples in which comparatively low numbers of presumptive *Escherichia coli* (less than 100 per gram or 10 per millilitre) are suspected.

CAUTION — Some *Escherichia coli* pathogenic species do not grow at 44 °C. The applicability of this part of ISO 11866 is limited by the susceptibility of the method to a large degree of variability. The method should, therefore, be used and the results interpreted in the light of the information given in clause 12.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 11866. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 11866 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 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

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

3 Definition

For the purposes of this part of ISO 11866, the following definition applies.

3.1 presumptive *Escherichia coli:* Bacteria which at 44 °C cause fermentation of lactose with the production of gas, and which at 44 °C produce indole from tryptophan, when the test is carried out in accordance with the method specified in this part of ISO 11866.

4 Principle

4.1 Inoculation of three tubes of double-strength liquid selective enrichment medium [5.3.1.1 a)] 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.

4.2 Inoculation of three tubes of single-strength liquid selective enrichment medium [5.3.1.1 b)] 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 single-strength 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 37 °C for 24 h to 48h. 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 the second selective medium (5.3.2).

4.5 Incubation at 44 °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 liquid selective medium (4.5) which have given rise to gas formation, of a new series of tubes containing tryptone water (5.4).

4.7 Incubation at 44 °C for 24 h to 48 h and examination of this new series of tubes (4.6) for indole production.

4.8 Identification of those tubes inoculated originally in 4.1 and/or 4.2, which show in step 4.5 production of gas from the second selective medium at 44 °C and in step 4.7 formation of indole from tryptone water at 44 °C, as being positive for presumptive *Escherichia coli*.

4.9 Determination of the MPN index from the numbers of positive tubes (4.8) of selected dilutions by means of an MPN table (annex A) and calculation of the most probable number (MPN) of presumptive *Escherichia coli* per gram or per millilitre of the original sample.

5 Dilution fluid, culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218 and ISO 8261.

If the prepared culture media and reagents are not used immediately, they shall, unless otherwise stated, be stored in the dark at a temperature between 0 °C and +5 °C for no longer than 1 month, under conditions which do not produce any change in their composition.

5.2 Dilution fluid

See ISO 8261.

5.3 Culture media

5.3.1 Lauryl sulfate tryptose broth (selective enrichment medium)

5.3.1.1 Composition

	a) Double-strength medium	b) Single-strength medium
Tryptone	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

5.3.1.2 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 6,8 at 25 °C.

Transfer the media in quantities of 10 ml to tubes of dimensions 16 mm x 160 mm (6.2) containing inverted Durham tubes (6.3) in the case of single-strength medium, and to test tubes of dimensions 20 mm x 200 mm (6.2) containing inverted Durham tubes (6.3) in the case of the double-strength medium.

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

The inverted Durham tubes shall not contain air bubbles after sterilization.

5.3.2 EC broth (second selective medium)

5.3.2.1 Components

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 reference [3].

5.3.2.2 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.

Transfer the medium in quantities of 10 ml to test tubes of dimensions 16 mm x 160 mm (6.2) containing inverted Durham tubes (6.3).

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

The inverted Durham tubes shall not contain air bubbles after sterilization.

5.4 Tryptone water

5.4.1 Components

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

5.4.2 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 20 °C.

Dispense the medium in quantities of 5 ml to 10 ml into tubes of dimensions 16 mm x 160 mm (6.2).

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

5.5 Indole reagent (Kovacs reagent)

5.5.1 Composition

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

5.5.2 Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the alcohol by heating gently to between 50 °C and 55 °C by means of the water bath (6.5).

Cool and add the hydrochloric acid.

Protect from light and store at approximately 4 °C.

The colour of the reagent shall be light yellow to light brown.

6 Apparatus and glassware

For general requirements, see ISO 7218 and ISO 8261. Glassware shall be resistant to repeated sterilization.

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

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

For details, see ISO 7218.

6.2 Test tubes, of dimensions approximately 16 mm x 160 mm and 20 mm x 200 mm, or **flasks** or **bottles** of suitable capacity.

6.3 Durham tubes, of size suitable for use in the test tubes (6.2).

6.4 Water bath, capable of operating at 44 °C ± 0,5 °C.

6.5 Water bath, capable of operating at between 50 °C and 55 °C.

6.6 Incubator, capable of maintaining a temperature of 37 °C ± 1 °C at all points within it.

6.7 Loops, made of platinum/iridium or nickel/chromium or plastic, approximately 3 mm in diameter, or **sterile disposable bags.**

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

6.9 Total-delivery pipettes, with nominal capacities of 1 ml and 10 ml.

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 part of ISO 11866. A recommended sampling method is given in ISO 707.

8 Preparation of test sample

Prepare the test sample according to the method given in ISO 8261.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Prepare the test portion, initial suspension (primary dilution) and further decimal dilutions according to the method given in ISO 8261.

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

9.2 Selective enrichment medium

9.2.1 Inoculation

9.2.1.1 Take three tubes of double-strength enrichment medium [5.3.1.1 a)]. Using a sterile pipette (6.9), transfer to each of these tubes 10 ml of the test sample if liquid, or 10 ml of the initial suspension (primary dilution) in the case of other products.

9.2.1.2 Then take three tubes of single-strength enrichment medium [5.3.1.1 b)]. Using a fresh sterile pipette (6.9), transfer to each of these tubes 1 ml of the test sample if liquid, or 1 ml of the initial suspension (primary dilution) in the case of other products.

9.2.1.3 For each of the further decimal dilutions, proceed as specified in 9.2.1.2. Use a fresh sterile pipette for each dilution.

9.2.1.4 Carefully mix the inoculum with the medium by means of a mixer. Avoid the introduction of air into the Durham tubes (6.3).

9.2.2 Incubation

Incubate all inoculated tubes (from steps 9.2.1.1 to 9.2.1.3) in the incubator (6.6) set at 37 °C 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.

9.3 Investigation in second selective medium

9.3.1 Inoculation

From each tube incubated as in 9.2.2 and showing gas formation, and also from each tube of double-strength medium, inoculate with a loop (6.7), the selective medium (5.3.2) previously heated in the water bath (6.4) to 44 °C.

Carefully mix the inoculum with the medium. Avoid the introduction of air into the Durham tubes (6.3).

9.3.2 Incubation

Incubate the tubes inoculated as in 9.3.1 in the water bath (6.4) set at 44 °C for 24 h ± 2 h. If, at this stage, no gas formation is observed, incubate for up to 48 h ± 2h.

9.4 Investigation in tryptone water

9.4.1 Inoculation

From each tube incubated as in 9.3.2 and showing gas formation, inoculate with a loop (6.7) the tryptone water (5.4) previously heated in the water bath (6.4) to 44 °C.

Carefully mix the inoculum with the medium. Avoid the introduction of air into the Durham tubes (6.3).

9.4.2 Incubation

Incubate the tubes inoculated as in 9.4.1 in the water bath (6.4) set at 44 °C for 48 h ± 2 h.

9.4.3 Test for indole production

Add 0,5 ml of the indole reagent (5.5) to the tubes containing the incubated tryptone water. Mix well and examine after 1 min.

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

9.5 Interpretation

Identify those tubes originally inoculated as in 9.2.1.1 to 9.2.1.3, which in 9.3.2 show production of gas and in 9.4.3 the formation of indole, as being positive for the presence of presumptive *Escherichia coli.*

For each dilution, count the number of positive tubes.

10 Selection of dilutions

NOTE — The initial suspension (primary dilution) and the test sample are considered as dilutions.

10.1 For each sample examined, select three consecutive dilutions in accordance with 10.2, 10.3 or 10.4 to obtain the MPN index.

10.2 In the case where only three dilutions were made, use those three dilutions to obtain the MPN index.

10.3 In the case where more than three dilutions were made, the selection of three of these gives combinations with different degrees of probability. This can be expressed in categories as shown in table A.1 (annex A). Explanations of these categories are given in table A.2 (annex A).

10.4 Select the combination of three consecutive dilutions with category 1 to obtain the MPN index; if more than one combination with category 1 is obtained, use the one with the highest number of positive tubes.

If no combination with category 1 is available, use the one with category 2; if more than one combination with category 2 is obtained, use the one with the highest number of positive tubes (see table 1 for examples).

If no combination with category 2 is available, use the one with category 3; if more than one combination with category 3 is obtained, use the one with the highest number of positive tubes (see table 1 for examples).

Table 1 — Examples of the selection of positive results for calculating MPN values

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

11 Determination, calculation and expression of results

11.1 Determination of MPN index

Determine the MPN index of presumptive *Escherichia coli* from the number of positive tubes (9.5) from each dilution selected (clause 10), using table A.1 (annex A).

11.2 Calculation of most probable number (MPN)

Obtain the most probable number (MPN) of presumptive *Escherichia coli* per gram or per millilitre of product by multiplying the MPN index (11.1) by the reciprocal of the lowest dilution selected (i.e. that having the highest sample content).

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.

NOTE — A division of the MPN index by 10 is only necessary with liquid products where 10 ml of the text sample are transferred to the tube with double-strength medium. In the case of other products, 10 ml of the initial suspension (primary dilution), containing 1 g of test sample are transferred (see 9.2.1.1).

11.3 Expression of results

Express the result as the most probable number (MPN) of presumptive *Escherichia coli* per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

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

12 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 table A.1 (annex A).