INTERNATIONAL STANDARD

ISO 11866-2

First edition
1997-02-15

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

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

*Lait et produits laitiers — Dénombrement d'*Escherichia coli *présumés —*

Partie 2: Technique du nombre le plus probable avec utilisation de 4-méthylumbelliféryl-β-D-glucuronide (MUG)

Reference number
ISO 11866-2:1997(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.

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 11866-2 is preferred for samples in which comparatively low numbers of presumptive *Escherichia coli* and/or other coliforms are suspected.

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

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Printed in Switzerland

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

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

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1 Scope

This part of ISO 11866 specifies a combined method for the enumeration of presumptive *Escherichia coli* and of presumptive coliforms by means of a culture technique involving a liquid medium with MUG, and calculation of the number of presumptive *Escherichia coli* and/or coliforms per gram or per millilitre by the most probable number (MPN) technique after incubation at 30 °C.

It is a more rapid method than that described in ISO 11866-1 as the incubation time is reduced (no 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* and/or other coliforms (less than 100 per gram or 10 per millilitre) are suspected.

CAUTION — 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 applied and the results interpreted in the light of the information given in clause 12.

NOTE — The methods described in ISO 5541-1 apply for the enumeration of coliforms for reference purposes.

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 Definitions

For the purposes of this part of ISO 11866, the following definitions apply.

3.1 presumptive *Escherichia coli*: Bacteria which at 30 °C cleave 4-methylumbelliferyl-b-D-glucuronide (MUG), with the production of fluorescence, and which produce indole from tryptophan, under the conditions specified in this part of ISO 11866.

3.2 coliforms: Bacteria which at 30 °C cause fermentation of lactose with the production of gas under the conditions 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 with specified quantities of decimal dilutions of the test sample or of the initial suspension.

4.3 Incubation of the tubes of double- and single-strength medium at 30 °C for 24 h to 48 h.

4.4 Identification of those tubes showing fluorescence and formation of indole as positive for presumptive *Escherichia coli*.

4.5 Identification of those tubes showing gas formation as positive for coliforms.

4.6 Determination of the MPN index from the numbers of positive tubes (4.4) 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.

4.7 Determination of the MPN index from the numbers of positive tubes (4.5) of selected dilutions by means of an MPN table (annex A) and calculation of the most probable number (MPN) of coliforms per gram or per millilitre of the original sample.

5 Dilution fluid, culture media and reagents

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.

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5.3 Culture media

5.3.1 Modified 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
4-Methylumbelliferyl-β-D-glucuronide (MUG)	0,2 g	0,1 g
Tryptophan	2,0 g	1,0 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.

5.4 Indole reagent (Kovacs reagent)

5.4.1 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

5.4.2 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.

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5.5 Sodium hydroxide solution, c (NaOH) » 0,5 mol/1.

5.5.1 Composition

Sodium hydroxide	2 g
Water	100 ml

5.5.2 Preparation

Dissolve the sodium hydroxide in the water.

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.

Test tubes should be checked for absence of autofluorescence before being used.

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

6.4 Incubator, capable of maintaining a temperature of 30 °C – 1 °C at all points within it.

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

6.6 Long-wave ultraviolet (UV) lamp, of wavelength between 360 nm and 366 nm, preferably in a UV cabinet or in a dark room, or covered by a box or a carton which provides dark conditions.

NOTE — Short-wave UV (germicidal) lamps are unsatisfactory.

6.7 pH-meter, accurate to within – 0,1 pH units at 25 °C.

6.8 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.

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9 Procedure

9.1 Test portion, initial suspension and further 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 tubes for the final dilution will yield a negative result.

9.2 Inoculation of selective enrichment medium

9.2.1 Take three tubes of double-strength enrichment medium [5.3.1 a)]. Using a sterile pipette (6.8), 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.

9.2.2 Then take three tubes of single-strength enrichment medium [5.3.1.1 b)]. Using a fresh sterile pipette (6.8), 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.

9.2.3 For each of the further dilutions, proceed as specified in 9.2.2. Use a fresh sterile pipette for each dilution.

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

9.3 Incubation

Incubate all inoculated tubes (from 9.2.1 to 9.2.3) in the incubator (6.4) set at 30 °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.

9.4 Confirmatory test for presumptive *Escherichia coli*

Perform the confirmatory test for presumptive *Escherichia coli* on all tubes incubated as in 9.3.

Add to each of the tubes 0,5 ml of the sodium hydroxide solution (5.5). Examine the tubes for fluorescence under a UV lamp (6.6). Add 0,5 ml of the indole reagent (5.4) to the tubes showing fluorescence. Mix well and examine after 1 min.

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

9.5 Interpretation

9.5.1 Test for presumptive *Escherichia coli*

Identify those tubes originally inoculated as in 9.2.1 to 9.2.3 which in 9.4 show fluorescence and formation of indole as positive for the presence of presumptive *Escherichia coli*.

For each dilution, count the number of positive tubes.

9.5.2 Test for coliforms

Identify those tubes inoculated as in 9.2.1 to 9.2.3 which in 9.3 show production of gas as being positive for the presence of coliforms.

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.

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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 / **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 products** ml^{-1}	**Other products** g^{-1}
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).

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11 Determination, calculation and expression of results

11.1 Determination of MPN index

11.1.1 Determine the MPN index of presumptive *Escherichia coli* from the number of positive tubes (9.5.1) from each dilution selected (clause 10), using table A.1 (annex A).

11.1.2 Determine the MPN index of coliforms from the number of positive tubes (9.5.2) 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 of presumptive *Escherichia coli* and/or coliforms 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 — Dividing the MPN index by 10 should be done only with liquid products where 10 ml of the test sample are transferred to the tube with double-strength medium. In the case of other products, 10 ml of the initial suspension containing 1 g of test sample are transferred to the tube with double-strength medium.

11.3 Expression of results

Express the result as the most probable number (MPN) of presumptive *Escherichia coli* or coliforms 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* or coliforms per millilitre or per gram, and if the appropriate procedure for a low number of presumptive *Escherichia coli* or coliforms was used, express the result in the following way: "No presumptive *Escherichia coli* or coliforms 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).

13 Test report

The text report shall specify:

— the method in accordance with which sampling was carried out, if known;

— the method used;

— the test result(s) obtained, indicating clearly the method of expression used.

It shall also mention all operating details not specified in this part of ISO 11866 or regarded as optional, together with details of any incidents may have influenced the test result(s).

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

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Annex A
(normative)

Determination of most probable number

Table A.1 — MPN indexes and confidence limits

Number of positive results			MPN index[1]	Category[2]	Confidence limits at 95 % level[1) 3)]	
					Lower	Upper
0	0	0	< 0,30		0,00	0,94
0	0	1	0,30	3	0,01	0,95
0	1	0	0,30	2	0,01	1,00
0	1	1	0,61	0	0,12	1,70
0	2	0	0,62	3	0,12	1,70
0	3	0	0,94	0	0,35	3,50
1	0	0	0,36	1	0,02	1,70
1	0	1	0,72	2	0,12	1,70
1	0	2	1,1	0	0,4	3,5
1	1	0	0,74	1	0,13	2,00
1	1	1	1,1	3	0,4	3,5
1	2	0	1,1	2	0,4	3,5
1	2	1	1,5	3	0,5	3,8
1	3	0	1,6	3	0,5	3,8
2	0	0	0,92	1	0,15	3,50
2	0	1	1,4	2	0,4	3,5
2	0	2	2,0	0	0,5	3,8
2	1	0	1,5	1	0,4	3,8
2	1	1	2,0	2	0,5	3,8
2	1	2	2,7	0	0,9	9,4
2	2	0	2,1	1	0,5	4,0
2	2	1	2,8	3	0,9	9,4
2	2	2	3,5	0	0,9	9,4
2	3	0	2,9	3	0,9	9,4
2	3	1	3,6	0	0,9	9,4
3	0	0	2,3	1	0,5	9,4
3	0	1	3,8	1	0,9	10,4
3	0	2	6,4	3	1,6	18,1
3	1	0	4,3	1	0,9	18,1
3	1	1	7,5	1	1,7	19,9
3	1	2	12	3	3	36
3	1	3	16	0	3	38
3	2	0	9,3	1	1,8	36,0
3	2	1	15	1	3	38
3	2	2	21	2	3	40
3	2	3	29	3	9	99
3	3	0	24	1	4	99
	3	1	46	1	9	198
3	3	2	110	1	20	400
3	3	3	> 110			

1) From reference [4].

2) See table A.2.

3) The confidence limits given in this table are meant only to provide some idea of the influence of statistical variations on results. There will always also be other sources of variation, which may sometimes be even more important.

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Table A.2 — Explanation of categories for results

Category[1)]	Definition
1	When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have the greatest chance of being obtained. There is only at most 5 % chance of obtaining a result that is less likely than the least likely one in this category.
2	When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 1, but there is only at most 1 % chance of obtaining a result that is less likely than the least likely one in this category.
3	When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 2, but there is only at most 0,1 % chance of obtaining a result that is less likely than the least likely one in this category.
0	When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 3. There is only a chance of 0,1 % of obtaining a result in this category, without anything being wrong.

1) Before starting testing it should be decided which category will be acceptable, i.e. only 1, 1 and or even 1, 2 and 3. When the decision to be taken on the basis of the results is of great importance, only the results of category 1 or at most those of categories 1 and 2 should be accepted. Category 0 results should be considered with the greatest caution.

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Annex B
(informative)

Bibliography

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[5] DE MAN, J.C. *Eur. J. Appl. Biotechnol.*, **17,** 1983, pp. 301-305.

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1) To be published. (Revision of ISO 707:1985)