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

Part 2: Colony-count technique at 44 °C using membranes

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

Partie 2: Technique par comptage des colonies obtenues sur membranes à 44 °C

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11866-2 | IDF 170-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

This edition of ISO 11866-2 | IDF 170-2 cancels and replaces ISO 11866-3:1997, of which it constitutes a minor revision.

ISO 11866-1:1997 has been cancelled and replaced by ISO 7251:2005, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of presumptive Escherichia coli — Most probable number technique.*

ISO 11866 | IDF 170 consists of the following parts, under the general title *Milk and milk products — Enumeration of presumptive* Escherichia coli:

— *Part 1: Most probable number technique using 4-methylumbelliferyl-β-D-glucuronide (MUG)*

— *Part 2: Colony-count technique at 44 °C using membranes*

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 11866-2 | IDF 170-2 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO/IDF/AOAC Group of Experts on *Pathogenic contaminants* (E102), under the aegis of its chairman, Mrs R. Lodi (IT).

This edition of ISO 11866-2 | IDF 170-2 cancels and replaces the former part 3 of IDF 170A:1999, while the former part 1 has been replaced by ISO 7251:2005.

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

Part 2:
Colony-count technique at 44 °C using membranes

1 Scope

This part of ISO 11866|IDF 170 specifies a method for the enumeration of presumptive *Escherichia coli* by means of a colony-count technique 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), and

— custard, desserts and cream.

The method specified in this part of ISO 11866|IDF 170 is the preferred method for samples in which comparatively large numbers of presumptive *Escherichia coli* (more than 100 per gram or 10 per millilitre) are suspected.

CAUTION — Some pathogenic strains of *Escherichia coli* do not grow at 44 °C.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
presumptive *Escherichia coli*
bacteria which at 44 °C form indole-positive (pink) colonies on cellulose acetate membranes overlaid on tryptone-bile agar, under the conditions specified in this part of ISO 11866|IDF 170

4 Principle

4.1 Resuscitation

A specified quantity of the test sample or initial suspension is inoculated onto cellulose acetate membranes overlaid on mineral-modified glutamate agar, then they are incubated at 37 °C for 4 h.

NOTE This procedure enables the presumptive *Escherichia coli* damaged by storage under frozen, dried or chill conditions, or damaged by heat or chemical processes, to be resuscitated. It also permits the diffusion of high concentrations of any fermentable carbohydrate present in the test sample which would otherwise interfere with indole production during the subsequent isolation stage.

4.2 Isolation

The membranes from the resuscitation stage on the mineral-modified glutamate agar are transferred to tryptone-bile agar. They are incubated at 44 °C for 18 h to 24 h.

4.3 Detection

The presence of presumptive *Escherichia coli* on the membrane is demonstrated by the production of indole by each colony.

4.4 Calculation

The number of colony-forming units (CFU) of presumptive *Escherichia coli* per gram or per millilitre of sample is calculated from the number of indole-positive colonies obtained on membranes at dilution levels chosen so as to give a significant result.

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|IDF 122.

5.3 Culture media and reagent

5.3.1 Resuscitation medium: Mineral-modified glutamate agar

5.3.1.1 Composition

Sodium glutamate	6,35 g
Lactose	10,0 g
Sodium formate	0,25 g
L(–)Cystine	0,02 g
L(–)Aspartic acid	0,02 g
L(+)Arginine	0,024 g
Thiamine	0,001 g
Nicotinic acid	0,001 g
Pantothenic acid	0,001 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0,100 g
Ammonium iron(III) citrate [a]	0,010 g
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	0,010 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0,90 g
Ammonium chloride	2,5 g
Agar	12 g to 18 g [b]
Water	1 000 ml

[a] Iron content of at least 15 % (mass fraction).

[b] Depending on the gel strength of the agar.

5.3.1.2 Preparation

Dissolve the ammonium chloride in the water. Add the other components and heat to boiling.

Adjust the pH, if necessary, so that after sterilization it is 6,7 at 25 °C.

Transfer 100 ml volumes of the medium to suitable containers.

Sterilize in the autoclave (6.1) set at 115 °C for 10 min.

5.3.1.3 Preparation of agar plates

Pour into sterile Petri dishes (6.12), 12 ml to 15 ml of the medium cooled to approximately 45 °C, and allow it to solidify. The plates may be stored at 0 °C to +5 °C for up to 4 days.

Immediately before use, dry the plates, preferably with the lids removed and the agar surfaces facing downwards, in the drying cabinet or the oven (6.3) set at 50 °C for 30 min or until the droplets have disappeared from the surface of the medium.

The agar should be dry enough not to allow excess moisture to appear within 15 min of spreading the inoculum (1 ml).

5.3.2 Selective medium: Tryptone-bile agar

5.3.2.1 Composition

Tryptone	20,0 g
Bile salts (refined)	1,5 g
Agar	12 g to 18 g [a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.3.2.2 Preparation

Dissolve the components in the water and heat to boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,2 at 25 °C.

Transfer aliquots of up to 500 ml of the medium to suitable containers.

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

5.3.2.3 Preparation of agar plates

Pour into sterile Petri dishes (6.12), 12 ml to 15 ml of the medium cooled to approximately 45 °C, and allow it to solidify. The plates may be stored at 0 °C to +5 °C for up to 4 days.

Immediately before use, dry the plates, preferably with the lids removed and the agar surfaces facing downwards, in the oven (6.3) set at 50 °C for 30 min or until the droplets have disappeared from the surface of the medium.

5.3.3 Indole detection reagent (Vracko and Sherris reagent)

5.3.3.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
Hydrochloric acid, c(HCl) = 1 mol/l	100 ml

5.3.3.2 Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the hydrochloric acid by heating, if necessary. The reagent may be stored in the dark at 0 °C to +5 °C for a maximum period of 3 months.

6 Apparatus and glassware

For general requirements, see ISO 7218 and ISO 8261|IDF 122. Glassware shall be resistant to repeated sterilization.

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

6.1 Autoclave, capable of operating at 115 °C ± 1 °C and at 121 °C ± 1 °C.

For details, see ISO 7218.

6.2 Incubators, capable of operating at 37 °C ± 1 °C and at 44 °C ± 0,5 °C.

6.3 Drying cabinet or **oven**, ventilated by convection, capable of operating at 50 °C ± 1 °C.

6.4 Refrigerator (for storage of prepared media and reagent), capable of operating at 0 °C to 5 °C.

6.5 Cellulose acetate membranes, 0,45 µm to 1,2 µm pore size and of 85 mm diameter.

6.6 Long-wave ultraviolet (UV) lamp, of wavelength between 360 nm and 366 nm, fitted with a suitable filter to remove UV radiation below 310 nm.

6.7 Blunt-ended forceps, sterile, of approximately 12 cm length.

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

6.9 Pipettes, calibrated for bacteriological use, with 1 ml nominal capacity, graduated in divisions of 0,1 ml and with an outflow opening of 2 mm to 3 mm diameter.

6.10 Measuring cylinders, for preparation of the media and reagent.

6.11 Bottles or **flasks**, for sterilization and storage of culture media.

6.12 Petri dishes, made of glass or plastic, of approximately 90 mm or approximately 100 mm diameter.

6.13 Spreaders, made of glass or plastic, for example hockey sticks made from a glass rod of approximately 3,5 mm diameter and 20 cm length, bent at right angles about 3 cm from one end and with the cut ends made smooth by heating.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 11866|IDF 170. A recommended sampling method is given in ISO 707|IDF 50.

8 Preparation of test sample

Prepare the test sample according to the method given in ISO 8261|IDF 122.

9 Procedure

NOTE If it is required to check whether the repeatability requirement is met (see Clause 11) carry out two single determinations in accordance with 9.1 to 9.5.

9.1 Test portion, initial suspension and further dilutions

Prepare the test portion, initial suspension (primary dilution) and further dilutions according to the method given in ISO 8261|IDF 122.

9.2 Resuscitation

9.2.1 Using sterile forceps (6.7), aseptically place a cellulose acetate membrane (6.5) onto the dried surface of each of two plates of the glutamate agar (5.3.1.3), taking care to avoid trapping air bubbles beneath the membranes. Gently flatten the membranes with a sterile spreader (6.13).

Using a sterile pipette (6.9), add 1 ml of the test sample or the initial suspension to the centre of each membrane. Using a sterile spreader (6.13), spread the inoculum evenly over the whole membrane surface, avoiding any spillage from the membrane.

9.2.2 Using another sterile pipette (6.9), inoculate similar volumes of the further diluted test sample or initial suspension onto other membranes, as specified in 9.2.1.

9.2.3 Leave the inoculated plates in a horizontal position at room temperature for approximately 15 min until the inocula have soaked into the agar. Incubate the plates for 4 h in the incubator (6.2) set at 37 °C with the membrane/agar surface uppermost.

9.3 Transfer to selective medium and incubation

9.3.1 Using sterile forceps (6.7), transfer membranes from the glutamate agar (5.3.1.3) to the tryptone-bile agar plates (5.3.2.3).

WARNING — The moist membrane will adhere to the agar surface. Avoid trapping air bubbles. Do not use a spreader.

9.3.2 Incubate the plates for 18 h to 24 h in the incubator (6.2) set at 44 °C with the membrane/agar surface uppermost. Do not stack dishes more than three high.

9.4 Detection of indole production by colonies on membranes

9.4.1 Label the lid of each dish (9.3.2) for identification.

9.4.2 Pipette 2 ml of the indole reagent (5.3.3) into the upturned lid placed horizontally.

9.4.3 Using sterile forceps (6.7), lift the membrane from the corresponding agar surface and lower it onto the indole reagent. If necessary, tilt the lid so that the whole of the membrane surface is wetted by the indole reagent. After 5 min, remove excess reagent with a pipette.

9.4.4 Indole-positive colonies develop a pink colour within a few minutes. If a permanent record is required, place the membrane under the ultraviolet lamp (6.6) for 30 min.

9.5 Enumeration

Count the indole-positive (pink) colonies on the membranes, which preferably contain between 10 and 150 pink colonies.

For details of the colony-count technique, see ISO 4833.

10 Calculation and expression of results

10.1 Calculation

Calculate N, the number of CFU of presumptive *Escherichia coli* per gram or per millilitre of product using the following equation:

$$N = \frac{\sum a}{(n_1 + 0{,}1 n_2) d}$$

where

$\sum a$ is the sum of the colonies counted on all the dishes retained after two successive dilutions;

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained.

NOTE 1 A dilution factor of 10^{-2} means that 10^{-2} g or 10^{-2} ml of the undiluted test sample (in the diluted state) has been put into the dish.

NOTE 2 The lower dilution is the dilution with the higher content of test sample.

10.2 Expression of results

10.2.1 Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained.

Take as the result the number of CFU 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.

10.2.2 If the two dishes corresponding to the test sample (liquid products) or the initial suspension (other products) contain less than 10 colonies, report the result as follows:

— less than 10 CFU of presumptive *Escherichia coli* per millilitre (liquid products);

— less than $10 \times 1/d$ CFU of presumptive *Escherichia coli* per gram (other products), where d is the dilution factor of the initial suspension.

10.2.3 If there are only dishes containing more than 300 colonies, calculate an estimated count from dishes having a count nearest to 150 colonies and multiply this number by the reciprocal of the value corresponding to the highest dilution.

Report the result as the “estimated number of colony-forming units of presumptive *Escherichia coli* per gram or per millilitre”.

10.3 Example of calculation

A count of the colonies of presumptive *Escherichia coli* at 44 °C gave the following results:

— at the first dilution retained (10^{-2}): 138 and 125 colonies;

— at the second dilution retained (10^{-3}): 20 and 18 colonies.

$$N = \frac{\sum a}{(n_1 + 0{,}1 n_2) d} = \frac{138 + 125 + 20 + 18}{\left[2 + (0{,}1 \times 2)\right] 10^{-2}} = \frac{301}{0{,}022} = 13\,680$$

Rounding the result as specified in 10.2.1 gives 14 000 or $1{,}4 \times 10^4$ CFU of presumptive *Escherichia coli* per gram or per millilitre of product.

11 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not be greater than 50 % of the lower result.

If the repeatability requirements are not met in 5 % or more of cases, an investigation into possible sources of error should be carried out.

NOTE Repeatability definitions are given in ISO 5725-1.

12 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this part of ISO 11866 | IDF 170;

— all operating details not specified in this part of ISO 11866 | IDF 170, or regarded as optional, together with details of any incidents may have influenced the test result(s);

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

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] ISO 4833, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of microorganisms — Colony-count technique at 30 °C*

[3] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[4] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: A basic method for the determination of repeatability and reproducibility of a standard measurement method*

[5] ISO 6391, *Meat and meat products — Enumeration of* Escherichia coli *— Colony-count technique at 44° C using membranes*

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