INTERNATIONAL STANDARD

ISO 11866-3

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

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

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

Partie 3: Technique par comptage des colonies obtenues sur membranes à 44 °C

Reference number
ISO 11866-3:1997(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. It 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 11866-3 is preferred for samples in which comparatively large numbers of presumptive *Escherichia coli* are suspected.

Annex A of this part of ISO 11866 is for information only.

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

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

1 Scope

This part of ISO 11866 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);

— custard, desserts and cream.

The method specified in this part of ISO 11866 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 *Escherichia coli* pathogenic species do not grow at 44 °C.

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 6610:1992, *Milk and milk products — Enumeration of colony-forming units of micro-organisms — Colony-count technique at 30 °C.*

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 form indole-positive (pink) colonies on cellulose acetate membranes overlaid on tryptone-bile agar, under the conditions specified in this part of ISO 11866.

4 Principle

Enumeration of presumptive *Escherichia coli* requires four successive stages.

4.1 Resuscitation

Inoculation of a specified quantity of the test sample or initial suspension onto cellulose acetate membranes overlaid on mineral-modified glutamate agar, then incubation 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

Transfer of membranes from the resuscitation stage on the mineral-modified glutamate agar to tryptone-bile agar. Incubation at 44 °C for 18 h to 24 h.

4.3 Detection

Demonstration of the presence of presumptive *Escherichia coli* on the membrane by the production of indole by each colony.

4.4 Calculation

Calculation of the number of colony-forming units (CFU) of presumptive *Escherichia coli* per gram or per millilitre of sample 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.

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[1)]	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[2)]
Water	1 000 ml

1) Iron content of at least 15 % (*m/m*).
2) 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 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.

NOTE — 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[1)]
Water	1 000 ml

1) 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 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. 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 radiations 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

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

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.

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 6610.

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

Σa 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.

NOTES

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.

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 *Escherichi 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 x 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 *Escherichi coli* per gram or per millilitre".

10.3 Example of calculation

A count of the colonies of presumptive *Escherichi 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.

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

Rounding the result as specified in 10.2.1 gives 14 000 or 1,4 x 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.

NOTES

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

2 Repeatability definitions are given in ISO 5725.1.