INTERNATIONAL STANDARD

ISO 6391

First edition
1988-11-15

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION
ORGANISATION INTERNATIONALE DE NORMALISATION
МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ

Meat and meat products — Enumeration of *Escherichia coli* — Colony count technique at 44 °C using membranes

Viandes et produits à base de viande — Dénombrement des Escherichia coli *— Méthode par comptage des colonies obtenues sur membranes à 44 °C*

Reference number
ISO 6391 : 1988 (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 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.

International Standard ISO 6391 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

Annex A forms an integral part of this International Standard.

Printed in Switzerland

Introduction

Although, for well-accepted statistical reasons, the limit for the lowest number of colonies counted per plate of selective medium is set at 15, for practical purposes it is often desirable to establish an estimated count of lower numbers of *Escherichia coli*. The confidence limits of such determinations (reported as "estimated count") are given in annex A.

Meat and meat products — Enumeration of *Escherichia coli* — Colony count technique at 44 °C using membranes

1 Scope

This International Standard specifies a reference method for the enumeration of viable *Escherichia coli* present in meat and meat products.

The method will detect both typical *E. coli* (biotype 1) and lactose non-fermenting or anaerogenic variants[1].

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards listed below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3100-1 : —[2], *Meat and meat products — Sampling and preparation of test samples — Part 1 : Sampling.*

ISO 3100-2 : 1988, *Meat and meat products — Sampling and preparation of test samples — Part 2 : Preparation of test samples for microbiological examination.*

ISO 6887 : 1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

3 Definition

For the purposes of this International Standard, the following definition applies.

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 International Standard.

4 Principle

In general, the detection of *E. coli* requires three successive stages.

4.1 Resuscitation

Inoculation of a specified quantity of the test sample, if the product is liquid, or of a specified quantity of an initial suspension, in the case of other products, onto cellulose acetate membranes overlaid on mineral modified glutamate agar and incubation at 37 °C for 4 h.

NOTE — This procedure enables *E. 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 20 h.

4.3 Detection

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

4.4 Calculation

Calculation of the number of *E. coli* per millilitre or per gram of sample from the number of indole-positive colonies obtained on membranes at dilution levels chosen to give a significant result.

1) ANDERSON, J.M. and BAIRD-PARKER, A.C. *J. Appl. Bacteriol.* **39**, 1975: 111-117.

2) To be published.

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 culture media, dehydrated basic components or complete dehydrated media should be used. The manufacturer's instructions shall be rigorously followed.

The chemicals used shall be of recognized analytical quality.

The water used shall be distilled or deionized water, and shall be free from substances that might inhibit growth of *E. coli* under the test conditions.

Measure the pH of the medium so that after sterilization it is at the pH required.

If culture media and the prepared reagent are not used immediately, they shall, unless otherwise indicated, be kept in the dark at a temperature between 0 °C and +5 °C, in conditions that prevent any change in their composition. They shall not be kept for longer than 1 month.

5.2 Diluent

See ISO 6887.

5.3 Resuscitation medium — Mineral modified glutamate agar

Composition[1)]

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 ($MgSO_4 \cdot 7H_2O$)	0,100 g
Ammonium iron(III) citrate [at least 15 % Fe (*m*/*m*)]	0,010 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0,010 g
Dipotassium hydrogen phosphate	0,90 g
Ammonium chloride	2,5 g
Agar	12 g to 18 g[2)]
Water	1 000 ml

Preparation

Dissolve the ammonium chloride in the water. Add the other components and dissolve by bringing to the boil. Adjust the pH so that after sterilization it is 6,7 at 25 °C. Transfer 100 ml volumes of the medium to suitable containers (6.6) and sterilize in the autoclave (6.1) for 10 min at 115 °C.

Preparation of agar plates

Add to sterile Petri dishes (6.7) 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 carefully in the cabinet or oven (6.5) at 50 °C, preferably with the lids off and agar surface downwards.

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

5.4 Selective medium — Tryptone-bile agar[3)]

Composition

Tryptone	20,0 g[4)]
Bile salts (refined)	1,5 g[5)]
Agar	12 g to 18 g[2)]
Water	1 000 ml

Preparation

Dissolve the components in the water by bringing to the boil. Adjust the pH so that after sterilization it is 7,2 at 25 °C. Transfer 100 ml volumes of the medium to suitable containers, and sterilize the medium in the autoclave (6.1) for 15 min at 121 °C.

Add 12 ml to 15 ml of the medium, at approximately 45 °C, to sterile Petri dishes and allow to solidify. These plates may be stored at 0 °C to +5 °C for up to 4 days.

Immediately before use, dry the plates carefully in the cabinet or oven (6.5) at 50 °C, with the lids off and agar surface downwards, for 30 min or until the surface of the agar is seen to be dry.

5.5 Indole detection reagent

Composition

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

Preparation

Dissolve the reagent in the hydrochloric acid solution and store between 0 °C and +5 °C.

1) *The bacteriological examination of drinking water supplies*, 1982 Report No. 71. London, Her Majesty's Stationery Office.

2) According to the manufacturer's instructions.

3) DELANEY, J.E., MCCARTHY, J.A. and GRASSO, R.J. *Wat. Sewage Wks.*, 1962: 109; 289.

4) Some commercial brands which favour indole formation are not satisfactory.

5) Oxoid Bile salts No. 3 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptable alternative to glassware if it has appropriate specifications.

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

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

Apparatus that will enter into contact with the culture media, the diluent or the sample, except for apparatus that is supplied sterile (particularly plastics apparatus), shall be sterilized either

— by being kept at 170 °C to 175 °C for not less than 1 h in the oven (6.1), or

— by being kept at 121 °C ± 1 °C for not less than 20 min in the autoclave (6.1).

6.2 Blending equipment.

One of the following shall be used :

a) mechanical meat mincer, laboratory size, capable of being sterilized, fitted with a plate with holes of diameter not exceeding 4 mm;

b) peristaltic-type blender (Stomacher), with sterile plastics bags.

6.3 Incubator, capable of being maintained at 35 °C ± 1 °C or 37 °C ± 1 °C.

6.4 Incubator, capable of being maintained at 44 °C ± 0,5 °C.

6.5 Drying cabinet or oven, ventilated by convection, capable of being maintained at 50 °C ± 1 °C.

6.6 Test tubes, 18 mm × 180 mm, and **flasks** or **bottles,** 125 ml to 300 ml, for sterilization and storage of culture media.

6.7 Petri dishes, made of glass or plastic, of 90 mm to 100 mm diameter.

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

6.9 Pipettes, calibrated for bacteriological use, of 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 Spreaders, made of plastic or glass, for example hockey sticks made from 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.

6.11 Water-bath, or similar apparatus, capable of being maintained at 45 °C ± 0,5 °C.

6.12 Water-bath, or similar apparatus, capable of being maintained at 50 °C ± 0,5 °C.

6.13 Longwave (365 nm) ultra-violet lamp, fitted with a suitable filter to remove UV radiations below 310 nm.

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

7 Sampling

See ISO 3100-1.

8 Preparation of the test sample

Take a representative sample of at least 200 g following the instructions in ISO 3100-2 and using the blending equipment (6.2).

Start the examination of the test sample as soon as possible; it may be stored, if necessary, at a temperature between 0 °C and + 5 °C, but not for longer than 1 h.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887.

9.2 Resuscitation procedure

9.2.1 Using sterile forceps, aseptically place a cellulose acetate membrane (6.8) onto the dried surface of each of two dishes of the mineral modified glutamate agar (5.3), taking care to avoid trapping air bubbles beneath the membranes. Gently flatten the membranes with a sterile spreader (6.10).

Using a sterile pipette (6.9), add 1 ml of the test sample if liquid, or 1 ml of the initial suspension in the case of other products, to the centre of each membrane. Using a sterile spreader (6.10), spread the inoculum evenly over the whole membrane surface, avoiding any spillage from the membrane.

9.2.2 Using another pipette (6.9), inoculate similar volumes of the further diluted test sample or initial suspension onto further 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 membranes. Incubate the Petri dishes at 37 °C or 35 °C[1] in the incubator (6.3) for 4 h with the membrane/agar surface uppermost.

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

9.3 Transfer to selective medium

9.3.1 Using sterile blunt-ended forceps, transfer membranes from the mineral modified glutamate agar to the tryptone-bile agar (5.4).

NOTE — The moist membrane will adhere to the agar surface; avoid trapping air bubbles. **Do not** use a spreader.

9.3.2 Incubate the plates at 44 °C in the incubator (6.4) for 18 h to 24 h 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 plate for identification.

9.4.2 Pipette 2 ml of the indole detection reagent (5.5) into the upturned lid placed horizontally.

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

9.4.4 Place the membrane under the ultra-violet lamp (6.13) for 30 min. Indole-positive colonies develop a pink colour.

9.5 Counting

Count the indole-positive (pink) colonies on membranes preferably containing between 15 and 150 pink colonies.

10 Expression of results

Using the following criteria, determine the number of *E. coli* present.

10.1 Method of calculation

10.1.1 If one or both membranes corresponding to a certain dilution have between 15 and 150 pink colonies, calculate the arithmetic mean of the number of colonies counted on the two membranes.

Retain only two significant figures, proceeding as follows:

— if the number is less than 100, round it to the nearest multiple of 5;

— if the number is greater than 100 and ends in a 5, round it to the nearest multiple of 20;

— if the number is greater than 100 and does not end in a 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the corresponding dilution to obtain the number of *E. coli* per millilitre or per gram of product, according to the circumstances. Express this result as a number between 1,0 and 9,9 multiplied by 10^n, where n is the appropriate power of 10.

10.1.2 If there are membranes containing between 15 and 150 pink colonies at two consecutive dilutions, calculate the number of *E. coli* for each dilution as specified in 10.1.1, and take as the result the arithmetic mean of the two values obtained, except when the ratio of the higher value to the lower value is greater than 2; in this case, take the lower value as the result.

10.1.3 If there are no pink colonies on membranes corresponding to the test sample or to the initial suspension, report the result, according to the circumstances, as

— less than 1 *E. coli* per millilitre (liquid samples), or

— less than $1 \times s$ *E. coli* per gram (solid samples), the dilution of the initial suspension being $1/s$.

10.1.4 If there are fewer than 15 pink colonies on membranes corresponding to the test sample or to the initial suspension, calculate the average of the colony counts and round to the next highest whole number.

Report the result as

— N *E. coli* per millilitre (liquid samples), or

— $N \times s$ *E. coli* per gram (solid samples), the dilution of the initial suspension being $1/s$,

where N is the arithmetic average of the colony counts.

10.2 Precision

10.2.1 Precision of the count of high numbers (between 15 and 150)

For statistical reasons alone, in 95 % of cases the confidence limits of this method[1] vary from ± 16 % to ± 52 %. In practice, even greater variations may be found, especially among results obtained by different analysts.

10.2.2 Precision of the count of low numbers (less than 15)

Confidence intervals for estimated low numbers are given in annex A.

11 Test report

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the results.

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

1) COWELL and MORISETTI. *J. Sci. Food Agric.* **20**, 1969, 573.

Annex A

(normative)

Confidence limits for estimating counts

The confidence levels at 95 % for estimation of low numbers when the average number of colonies counted on double-inoculated dishes starting from the test sample or the initial suspension is less than 15 *E. coli* are given in table A.1.

Table A.1

Number of *E. coli*	95 % confidence level	
	lower	upper
1	< 1	2
2	< 1	4
3	< 1	5
4	1	6
5	2	9
6	2	10
7	2	12
8	3	13
9	4	14
10	4	16
11	5	18
12	6	19
13	7	20
14	7	21
15	8	23