INTERNATIONAL STANDARD

ISO 5552

Second edition
1997-12-01

Meat and meat products — Detection and enumeration of *Enterobacteriaceae* without resuscitation — MPN technique and colony-count technique

Viande et produits à base de viande — Recherche et dénombrement des Enterobacteriaceae *sans ressuscitation — Technique de NPP et technique de comptage de colonies*

Reference number
ISO 5552:1997(E)

Contents

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet central@iso.ch
X.400 c=ch; a=400net; p=iso; o=isocs; s=central

Printed in Switzerland

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 second edition cancels and replaces the first edition (ISO 5552:1979), which has been technically revised.

International Standard ISO 5552 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 6, *Meat and meat products*.

Annexes A and B form an integral part of this International Standard. Annex C is for information only.

Introduction

During a meeting in March 1996 of Subcommittees SC 5, *Milk and milk products*, SC 6, *Meat and meat products*, and SC 9, *Microbiology*, it was recommended to put forward one horizontal standard for the detection and enumeration of *Enterobacteriaceae*. This needs a revision of the two horizontal standards ISO 7402 and ISO 8523 into one standard, divided into three parts.

ISO 5552 will be withdrawn as soon as the combined horizontal method is published.

Meat and meat products — Detection and enumeration of *Enterobacteriaceae* without resuscitation — MPN technique and colony-count technique

1 Scope

This International Standard specifies a method for the detection and enumeration of *Enterobacteriaceae* present in all kinds of meat and meat products, including poultry. Enumeration is carried out as follows:

– by calculation of the most probable number (MPN) after incubation at 35 °C or 37 °C in liquid medium; or

– by counting colonies in a solid medium after incubation at 35 °C or 37 °C.

The temperature used is to be the subject of agreement between the parties concerned, and is to be stated in the test report.

NOTE In the case of frozen foods, an incubation temperature of 30 °C is preferred when the aim of the enumeration is technological.

For low numbers, the MPN method is preferable, otherwise the colony-count method is preferred.

This International Standard does not include resuscitation procedures and the results should not, therefore, be related to criteria or specifications based on the assumption that resuscitation has been carried out.

A limitation on the applicability of this International Standard is imposed by susceptibility of the methods to a large degree of variability. The methods should be used and the results interpreted in the light of the information given in 10.3.

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 indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

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 of food and animal feeding stuffs — General guidance for the preparation of dilutions for microbiological examination.*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

ISO 7402:1993, *Microbiology — General guidance for the enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony-count technique.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 *Enterobacteriaceae*
Microorganisms which ferment glucose and show a negative oxidase reaction when the test is carried out according to the method specified.

3.2 detection of *Enterobacteriaceae*
Determination of the presence or absence of these bacteria, in a particular mass of product, when tests are carried out in accordance with this International Standard.

3.3 count of *Enterobacteriaceae*
Number of *Enterobacteriaceae* found per millilitre or per gram of the test sample when the test is carried out according to the method specified.

4 Principle

4.1 Preparation of dilutions

Preparation of decimal dilutions from the test sample.

4.2 Detection of *Enterobacteriaceae* in a specified quantity of sample

Introduction of 1 ml of the test sample if the product is liquid or of the initial suspension in the case of other products (or of decimal dilutions of these), into a tube containing a selective enrichment broth.

Incubation of the tubes at 35 °C or 37 °C for 24 h, followed by streaking of the cultures onto violet red bile glucose agar. After incubation of the streaked agar plates at 35 °C or 37 °C for 24 h, subjection of suspected colonies to biochemical confirmation tests.

4.3 Enumeration of *Enterobacteriaceae*

4.3.1 Most probable number (MPN) technique

NOTE This technique is recommended when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of the test sample.

Inoculation of three tubes of double-strength medium with a specified quantity of test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation of three tubes of single-strength medium with a specified quantity of the test sample if the 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 three tubes of single-strength medium with the first decimal dilution of the test sample or of the initial suspension.

Incubation of the tubes at 35 °C or 37 °C (as agreed) for 24 h.

From the number of confirmed positive tubes, calculation of the most probable number of *Enterobacteriaceae* per millilitre or per gram of the test sample using the MPN table (see annex A).

4.3.2 Colony-count technique

NOTE This technique is recommended when the number sought is expected to be greater than 100 per millilitre or per gram of the test sample.

Inoculation of violet red bile glucose agar contained in two Petri dishes (poured-plate technique) with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of the initial suspension in the case of other products. Covering with an overlayer of the same medium.

Preparation of other pairs of plates under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

Incubation of the dishes at 35 °C or 37 °C (as agreed) for 24 h ± 2 h.

Calculation of the number of *Enterobacteriaceae* per millilitre or per gram of the test sample from the number of confirmed typical colonies per dish.

5 Diluent, culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Diluent

See ISO 6887.

5.3 Culture media

5.3.1 Buffered brilliant green bile glucose broth (EE broth)

5.3.1.1 Composition

	a) Double-strength medium	b) Single-strength medium
Peptone	20,0 g	10,0 g
Glucose	10,0 g	5,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	12,90 g	6,45 g
Potassium dihydrogen phosphate (KH_2PO_4)	4,0 g	2,0 g
Ox bile, dehydrated	40,0 g	20,0 g
Brilliant green	0,027 g	0,0135 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 boiling. Adjust the pH, if necessary, so that after boiling it is 7,2 at 25 °C. Do not heat the medium for longer than 30 min. Cool the medium rapidly.

Aseptically transfer 10 ml portions to sterile tubes or bottles (6.8).

Do not autoclave the medium.

The medium may be stored for up to 1 week at between 0 °C and 5 °C.

5.3.2 Violet red bile glucose agar (VRBG)

5.3.2.1 Composition

Peptone	7,0 g
Yeast extract	3,0 g
Bile salts	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Neutral red	0,03 g
Crystal violet	0,002 g
Agar in powder or flake form	8 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 or the dehydrated complete medium in the water by boiling. The medium must not be boiled for more than 2 min.

Adjust the pH, if necessary, so that after boiling it is 7,4 at 25 °C.

Transfer the culture medium to sterile tubes, flasks or bottles (6.8) of capacity not more than 500 ml.

Do not autoclave the medium.

Prepare this medium just before use (see 9.3.2 and 9.4.1).

5.3.2.3 Preparation of agar plates (required for detection and MPN technique, see 9.3.2)

Dispense immediately approximately 15 ml of the culture medium, cooled to approximately 47 °C in the water bath (6.5), into Petri dishes (6.6) and allow to solidify.

Immediately before use, dry the plates, preferably with the lids off and the agar surface downwards, in the oven (6.3) until the agar surface is dry.

If prepared in advance, the undried plates shall not be kept for longer than 4 days at between 0 °C and 5 °C.

5.3.3 Glucose agar

5.3.3.1 Composition

Tryptone	10,0 g
Yeast extract	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Bromocresol purple	0,015 g
Agar in powder or flake form	8 g to 18 g[1)]
Water	1 000 ml
1) Depending on the gel strength of the agar.	

5.3.3.2 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 7,0 at 25 °C.

Dispense the culture medium in quantities of 15 ml into tubes or flasks (6.8).

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

Leave the tubes or flasks in a vertical position.

The medium may be stored for up to 1 week at between 0 °C and 5 °C.

Just before use, heat in boiling water or flowing steam for 15 min, then cool rapidly to the incubation temperature.

5.3.4 Nutrient agar

5.3.4.1 Composition

Beef extract	3,0 g
Peptone	5,0 g
Agar in powder or flake form	8 g to 18 g[1)]
Water	1 000 ml
1) Depending on the gel strength of the agar.	

5.3.4.2 Preparation

Dissolve the components or dehydrated complete medium in the water by heating, if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0 at 25 °C.

Transfer the culture medium to tubes, bottles or flasks (6.8) of capacity not more than 500 ml.

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

5.3.4.3 Preparation of agar plates (see 9.4.1)

Transfer immediately about 15 ml of the culture medium, melted and cooled to approximately 47 °C, to Petri dishes (6.6) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in the oven (6.3), until the agar surface is dry.

If prepared in advance, the undried plates may be stored for up to 2 weeks at between 0 °C and 5 °C.

5.4 Oxidase reagent

5.4.1 Composition

N,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine dihydrochloride	1,0 g
Water	100 ml

5.4.2 Preparation

Dissolve the reagent in the cold water. The reagent shall be prepared immediately prior to use.

6 Apparatus and glassware

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

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

See ISO 7218.

6.2 Incubator, capable of operating at 35 °C ± 1 °C or at 37 °C ± 1 °C.

6.3 Drying cabinet or **incubator**, capable of operating between 35 °C ± 1 °C and 55 °C ± 1 °C.

6.4 pH-meter, accurate to within ± 0,1 pH unit at 25 °C.

6.5 Water bath, or similar apparatus, capable of operating at 47 °C ± 2 °C.

6.6 Petri dishes, made of glass or plastics, of diameter 90 mm to 100 mm.

6.7 Loops, of platinum/iridium, nickel/chromium, or disposable plastic, approximately 3 mm in diameter, and wires of the same material, or a **glass rod**.

NOTE A nickel/chromium loop is not suitable for use in the oxidase test (see 9.5.2.1).

6.8 Test tubes, of dimensions approximately 16 mm x 160 mm and 20 mm x 200 mm, and **flasks** or **bottles**, of capacity between 150 ml and 500 ml.

6.9 Total-delivery graduated pipettes, of nominal capacities 1 ml and 10 ml, graduated respectively in 0,1 ml and 0,5 ml divisions.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1 [1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

Take, in accordance with the method described in ISO 3100-2, a representative test sample. Start examination of the pretreated sample as soon as possible. It may be stored, if necessary, at a temperature between 0 °C and +2 °C but for no longer than 24 h.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887.

Prepare a single decimal dilution series from the test sample if the product is liquid, or from the initial suspension in the case of other products.

9.2 Detection

NOTE This presence/absence test is often carried out in triplicate.

9.2.1 Inoculation and incubation

Transfer, with a sterile pipette (6.9), a 1 ml portion of the test sample if the product is liquid or of the initial suspension in the case of other products (9.1), or a 1 ml portion of one of the two dilutions 10^{-2} or 10^{-3} of both dilution series, to a tube containing buffered brilliant green bile glucose broth (5.3.1). Incubate the tube for 24 h ± 4 h at 35 °C or at 37 °C (as agreed).

9.2.2 Isolation

Streak a loopful (6.7) from the incubated culture (see 9.2.1) on the violet red bile glucose agar plate (see 5.3.2.3) and incubate the plate at 35 °C or 37 °C (as agreed) for 24 h ± 4 h.

9.2.3 Selection of colonies for confirmation

From the plate incubated as in 9.2.2 on which typical pink to red colonies (with or without precipitation haloes) or colourless mucoid colonies have developed, select at random five such colonies for biochemical confirmation (see 9.5.2) after subculturing (see 9.5.1).

9.3 MPN technique

9.3.1 Inoculation and incubation

Take three tubes of double-strength EE broth [5.3.1.1 a)]. Transfer to each of these tubes, using a pipette (6.9), 10 ml of the test sample if the product is liquid, or 10 ml of the initial suspension in the case of other products.

Take three tubes of single-strength EE broth [5.3.1.1 b)]. Transfer to each of these tubes, using another pipette (6.9), 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take three more tubes of single-strength EE broth [5.3.1.1 b)]. Transfer to each of these tubes using another pipette (6.9), 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in the case of other products.

Incubate these nine tubes at 35 °C or 37 °C (as agreed) for 24 h ± 4 h.

9.3.2 Isolation

Streak a loopful (6.7) from each of the nine incubated cultures (see 9.3.1) on the violet red bile glucose agar plates (see 5.3.2.3) and incubate the plates at 35 °C or 37 °C (as agreed) for 24 h ± 4 h.

9.3.3 Selection of colonies for confirmation

From each of the plates incubated as in 9.3.2 on which typical pink to red colonies (with or without precipitation haloes) or colourless mucoid colonies have developed, select at random five such colonies for biochemical confirmation (see 9.5.2) after subculturing (see 9.5.1).

9.4 Colony-count technique

9.4.1 Inoculation and incubation

9.4.1.1 Take two sterile Petri dishes (6.6). Using a sterile pipette (6.9), transfer to each dish 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take two other sterile Petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in the case of other products.

Repeat the procedure described with the further dilutions, using a fresh sterile pipette for each decimal dilution.

9.4.1.2 Pour into each Petri dish approximately 15 ml of the VRBG medium (5.3.2) which has been prepared then cooled to approximately 47 °C in the water bath (6.5). The time elapsing between the end of the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) and the moment when the medium (5.3.2) is poured into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium by horizontal movements and allow the mixture to solidify, with the Petri dishes standing on a cool horizontal surface.

9.4.1.3 After complete solidification of the mixture, add a covering layer of 6 ml to 10 ml of the VRBG medium (5.3.2), prepared then cooled as described in 9.4.1.2, to prevent spreading growth and to obtain semi-anaerobic conditions. Allow to solidify as described above.

9.4.1.4 Invert the prepared dishes and incubate them in the incubator set at 35 °C or 37 °C (as agreed) for 24 h ± 4 h.

9.4.2 Counting and selection of colonies

Select the dishes (9.4.1.4) containing less than 150 typical colonies (see 9.3.3) of diameter 0,5 mm or more; count these suspect colonies. Select at random five such colonies from each dish for biochemical confirmation (see 9.5.2) after subculturing (see 9.5.1).

Consider the determination to be void if half or more than half of the surface area of a dish is overgrown. If less than half of the surface area of a dish is overgrown, count the colonies on the clear part and extrapolate so that the number corresponds to the total surface area of the dish.

9.5 Confirmation

9.5.1 Subculturing

Streak on nutrient agar plates (5.3.4) each of the colonies selected for confirmation (see 9.2.3, 9.3.3 and 9.4.2).

Incubate these plates at 35 °C or 37 °C (as agreed) for 24 h ± 2 h. Select a well-isolated colony from each of the incubated plates for biochemical confirmation (see 9.5.2).

9.5.2 Biochemical confirmation

9.5.2.1 Oxidase reaction

Using the platinum/iridium loop or wire or glass rod (6.7), take a portion of each well-isolated colony (9.5.1) and streak on a filter paper moistened with the oxidase reagent (5.4) or on a commercially available disc. A nickel/chromium loop or wire shall not be used.

Consider the test to be negative when the colour of the filter paper has not turned dark within 10 s.

Consult the manufacturer's instruction for ready-to-use discs.

9.5.2.2 Fermentation test

Stab, using a wire (6.7), the same colonies selected in 9.5.1 into tubes containing glucose agar (5.3.3). Incubate at 35 °C or 37 °C (as agreed) for 24 h ± 2 h.

If a yellow colour develops throughout the contents of the tube, the reaction is regarded as positive. Most strains produce gas.

10 Expression of results

10.1 Detection of *Enterobacteriaceae* in a specified quantity of sample.

10.1.1 If the tubes yield colonies confirmed as *Enterobacteriaceae* (see 10.2.2), report the result as:

"Enterobacteriaceae were detected in the specified quantity".

10.1.2 If the tubes do not (9.2) yield colonies confirmed as *Enterobacteriaceae* (see 10.2.2), report the result as:

"Enterobacteriaceae were not detected in the specified quantity".

10.2 Calculation of the most probable number (MPN)

10.2.1 Count the number of tubes giving a positive reaction of each dilution.

10.2.2 If one of the selected typical colonies (9.3.3) of a subculture (see 9.5.1) is oxidase-negative and glucose-positive, the tube from which the subculture is derived shall be regarded as being positive.

10.2.3 Using the MPN table (see annex A), determine from the number of positive tubes in the different dilutions, the most probable number (MPN) index.

10.2.4 In the case of liquid products, the number of *Enterobacteriaceae* per millilitre is calculated by dividing the MPN index by 10. In the case of other products for which initial suspensions are prepared, the number per gram is equal to the MPN index.

10.3 Calculation of colony count

10.3.1 General

If at least 80 % of the selected typical colonies (see 9.4.2) are oxidase-negative and glucose-positive and thus confirmed as *Enterobacteriaceae*, the number of microorganisms present will be the same as that given by the count made in 9.4.2.

In all other cases, the number shall be calculated from the percentage of oxidase-negative and glucose-positive colonies in relation to the total number of selected colonies (see 9.4.2).

Round the result to a whole number of colonies.

10.3.2 General case

Calculate the number, *N,* of *Enterobacteriaceae* per millilitre or per gram of product, using the following equation:

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

where

$\sum a$ is the sum of colonies counted after identification on all the dishes retained;

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

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

d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significant figures.

Take as the result the number of microorganisms per millilitre or per gram of product, expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

A direct count of microorganisms gave the following results:

– at the first dilution retained (10^{-3}): 66 and 80 colonies;

– at the second dilution (10^{-4}): 4 and 7 colonies.

The following numbers were stabbed:

for 66 colonies: 5 colonies, 4 of which agreed with the criteria, giving a = 53;

for 80 colonies: 5 colonies, 3 of which agreed with the criteria, giving a = 48;

for 7 colonies: 5 colonies, 4 of which agreed with the criteria, giving a = 6;

for 4 colonies: all 4 were found to be the microorganism sought.

Therefore

$$N = \frac{\sum a}{(n_1 + 0{,}1 n_2)\mathrm{d}} = \frac{53 + 48 + 6 + 4}{(2 + 0{,}2) \times 10^{-3}} = \frac{111}{2{,}2 \times 10^{-3}} = 50\,455$$

Rounding the result as specified above gives 50 000 or $5{,}0 \times 10^4$ *Enterobacteriaceae* per millilitre or per gram of product.

10.3.3 Estimation of small numbers

If the two dishes, at the level of the test sample (liquid products) or of the initial suspension (other products), contain less than 15 colonies, calculate the arithmetic mean, y, of the colonies counted on both dishes.

Express the result as follows:

– for liquid products: estimated number of *Enterobacteriaceae* per millilitre $N_E = y$

– for the other products: estimated number of *Enterobacteriaceae* per gram $N_E = y/d$

where *d* is the dilution factor of the initial suspension.

10.3.4 No characteristic colonies

If the two dishes, at the level of the test sample (liquid products) or of the initial suspension (other products) do not contain characteristic colonies, express the result as follows:

- less than 1 microorganism per millilitre (liquid products);

- less than 1/*d* microorganism per gram (other products),

where *d* is the dilution factor of the initial suspension.

10.4 Precision

10.4.1 MPN Technique

It is known that wide variations in results may occur with the MPN technique. Results obtained from this method should therefore be used with caution. Confidence limits are given in annex A.

10.4.2 Colony-count technique

For statistical reasons alone, in 95 % of cases the confidence limits of the colony-count technique vary from ± 16 % to ± 52 % (see reference [2]) for colony counts of less than 15 per plate. The confidence limits are given in annex B. In practice, even greater variation may be found, expecially among results obtained by different workers.

11 Test report

The test report shall specify the method used, the temperature of incubation and the result obtained. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

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