INTERNATIONAL STANDARD

ISO 16649-1

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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* —

Part 1: Colony-count technique at 44 °C using membranes and 5-bromo-4-chloro-3-indolyl β-D-glucuronide

Mircobiologie des aliments — Méthode horizontale pour le dénombrement des Escherichia coli β-glucuronidase positive —

Partie 1: Technique de comptage des colonies à 44 °C au moyen de membranes et de 5-bromo-4-chloro-3-indolyl β-D glucuronate

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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 part of ISO 16649 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 16649-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 16649 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of β-glucuronidase-positive* Escherichia coli:

— *Part 1: Colony-count technique at 44 °C using membranes and 5-bromo-4-chloro-3-indolyl β-D-glucuronide*

— *Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide*

— *Part 3: Most probable number technique*

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this part of ISO 16649 is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this part of ISO 16649 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

This International Standard describes two horizontal methods (ISO 16649-1 and ISO 16649-2) for the enumeration of β-glucuronidase-positive *Escherichia coli*.

The user may choose either ISO 16649-1 or ISO 16649-2. Either part is for general application. However, ISO 16649-1 should be used for foodstuffs which may contain severely stressed cells.

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* —

Part 1:
Colony-count technique at 44 °C using membranes and 5-bromo-4-chloro-3-indolyl β-D-glucuronide

1 Scope

This part of ISO 16649 specifies a horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* in products intended for human consumption or for the feeding of animals. It uses a colony-count technique after resuscitation using membranes and incubation at 44 °C on a solid medium containing a chromogenic ingredient for detection of the enzyme β-glucuronidase.

WARNING — Strains of *Escherichia coli* which do not grow at 44 °C and, in particular, those that are β-glucuronidase negative, such as *Escherichia coli* O157, will not be detected.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 16649. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 16649 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions.*

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

3 Terms and definitions

For the purposes of this part of ISO 16649, the following terms and definitions apply.

3.1
β-glucuronidase-positive *Escherichia coli*
bacteria which at 44 °C form typical blue colonies on tryptone-bile-glucuronide medium (TBX) under the conditions specified in this part of ISO 16649

3.2
enumeration of β-glucuronidase-positive *Escherichia coli*
determination of the number of colony-forming units (CFU) of β-glucuronidase-positive *Escherichia coli*, per millilitre or per gram of sample, when test and calculations are carried out in accordance with this part of ISO 16649

4 Principle

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

Under the same conditions, using decimal dilutions of the test sample or of the initial suspension, two plates per dilution are inoculated.

4.2 For isolation, the membranes from the resuscitation stage on the MMGA are transferred to tryptone-bile-glucuronide agar (TBX), then incubated at 44 °C for 18 h to 24 h.

4.3 The number of colony-forming units (CFU) of β-glucuronidase-positive *Escherichia coli* per gram or per millilitre of sample is calculated from the number of typical blue CFU (see clause 10).

5 Diluent and culture media

For current laboratory practice, see ISO 7218.

5.1 Diluent

See ISO 6887-1 or the specific International Standard dealing with the product to be examined.

5.2 Culture media

5.2.1 Resuscitation medium: Mineral-modified glutamate agar (MMGA)

5.2.1.1 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)	0,900 g
Ammonium chloride	2,5 g
Agar	9 g to 18 g [b]
Water	1 000 ml

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

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

5.2.1.2 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 ± 0,2 at 25 °C.

Transfer aliquots of up to 500 ml to suitable containers (6.10).

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

5.2.1.3 Preparation of agar plates

Pour 12 ml to 15 ml of the medium into sterile Petri dishes (6.11) and allow to solidify.

Dry the plates (see ISO 7218). The plates may be stored at 3 °C ± 2 °C for up to 5 days.

The agar should be dry enough to allow excess moisture to disappear within 15 min of spreading the inoculum (1 ml).

5.2.2 Selective medium: Tryptone-bile-glucuronic medium (TBX)

5.2.2.1 Composition

Enzymatic digest of casein	20,0 g
Bile salts No. 3	1,5 g
5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (BCIG)	144 µmol [a]
Dimethyl sulfoxide (DMSO) [b]	3 ml
Agar	9 g to 18 g [c]
Water	1 000 ml

[a] For example, 0,075 g of cyclohexylammonium salt.

[b] Dimethyl sulfoxide is harmful by inhalation and contact. The use of a fume cupboard when handling is advised. Because of this toxicity, a diluent recommended by the manufacturer may be used.

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

5.2.2.2 Preparation

Dissolve the BCIG in the dimethyl sulfoxide or in the diluent recommended by the manufacturer. Dissolve all components in the water and heat to boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

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

5.2.2.3 Preparation of agar plates

Proceed as described in 5.2.1.3.

6 Apparatus and glassware

Usual microbiological equipment (see ISO 7218) and, in particular, the following.

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

6.2 Incubators, capable of being maintained at 37 °C ± 1 °C and at 44 °C ± 1 °C.

6.3 Drying cabinet or **ventilated oven**, capable of being maintained between 25 °C ± 1 °C and 50 °C ± 1 °C, or a **laminar airflow cabinet**.

6.4 Refrigerator (for storage of prepared media), capable of operating at 3 °C ± 2 °C.

6.5 Sterile and non-inhibitive membranes, made of cellulose acetate or mixed esters of cellulose**,** with 0,45 µm to 1,2 µm pore size and 85 mm diameter.

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

6.7 pH-meter, capable of measuring to an accuracy of ± 0,1 pH unit.

Its minimum measuring threshold shall be 0,01 pH unit. The pH-meter shall be equipped with either manual or automatic temperature equalization.

6.8 Pipettes, total delivery (blow out), having wide openings and having a nominal capacity of 1 ml, graduated in 0,1 ml divisions.

6.9 Measuring cylinders, of appropriate capacity for preparation of the media.

6.10 Test tubes, bottles or **flasks**, of suitable capacity for sterilization and storage of culture media.

6.11 Petri dishes, of approximately 90 mm diameter.

6.12 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 16649. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887-1 and any specific International Standard appropriate to the product.

9.2 Resuscitation

9.2.1 Using sterile forceps (6.6), aseptically place a membrane (6.5) onto the dried surface of each of two plates of the MMGA (5.2.1.3), taking care to avoid trapping air bubbles beneath the membranes. Gently flatten the membranes with a sterile spreader (6.12), if necessary.

Using a sterile pipette (6.8), add 1 ml of the test sample or the initial suspension to the centre of each membrane. Using a sterile spreader, spread the inoculum evenly over the whole membrane surface, avoiding any spillage from the membrane.

9.2.2 Repeat the procedure as specified in 9.2.1 with the further decimal dilutions, if necessary, using another sterile pipette and another sterile spreader for each dilution.

9.2.3 Leave the inoculated plates in a horizontal position at room temperature for approximately 15 min until the inoculum has soaked through the membrane into the agar. Incubate the plates for 4 h ± 1 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 After resuscitation, using sterile forceps (6.6), transfer membranes from MMGA (resuscitation medium) to plates of TBX medium (5.2.2.3).

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, and not more than 45°C, with the membrane/agar surface uppermost. Do not stack dishes more than three high.

9.4 Counting the colony-forming units (CFU)

After the specified period of incubation (9.3.2), count the typical CFU of β-glucuronidase-positive *Escherichia coli* in each dish containing less than 150 typical CFU and less than 300 total (typical and non-typical) CFU.

If they form part of the retained dishes, the dishes containing 0 typical CFU should be taken into consideration in the different calculation methods defined in clause 10.

10 Expression of results

10.1 General

The calculation in 10.2 takes into account those cases most frequently encountered when conducting tests in accordance with good laboratory practice. Some special, fairly improbable, cases can arise (e.g. very different CFU numbers between the two dishes from the same dilution, or very different proportions from that of the dilution factor between the dishes from two successive dilutions). It is then necessary that the counting results be examined, interpreted and possibly rejected by a competent microbiologist.

10.2 Calculation

For a result to be valid, in general it is considered that it is necessary to count the typical CFU on at least one dish containing as a minimum 15 typical CFU.