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Microbiology of the food chain — Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* —

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

Microbiologie de la chaîne alimentaire — Méthode horizontale pour le dénombrement des Escherichia coli *bêta-glucuronidase positive —*

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

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This second edition cancels and replaces the first edition (ISO 16649-1:2001), which has been technically revised with the following main changes:

— samples from the environment and the primary production stage have been added to the Scope;

— the minimum length of incubation (20 h) for tryptone-bile X-glucuronide agar (TBX) during culture on selective medium has been adopted;

— performance testing for the quality assurance of the culture media and the membrane for transfer has been added;

— to improve safety for the user, the solvent dimethyl sulphoxide (DMSO) is no longer recommended to dissolve the chromogenic substrate (BCIG);

— the composition of the minerals-modified glutamate agar (MMGA) has been corrected (aspartic acid 0,024 g and arginine 0,02 g) to the values in the original formulation[12].

A list of all parts in the ISO 16649 series can be found on the ISO website.

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.

The main changes, listed in the Foreword, introduced in this document compared to ISO 16649-1:2001 are considered as minor (see ISO 17468)[5].

When this document 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 document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

There are three horizontal methods (ISO 16649-1, ISO 16649-2 and ISO 16649-3) for the enumeration of β-glucuronidase-positive *Escherichia coli*[3][4].

The user may choose either ISO 16649-1, ISO 16649-2 or ISO 16649-3. All parts are for general application. However, ISO 16649-1 or ISO 16649-3, which both include a resuscitation step, should be used in preference for foodstuffs likely to contain sub-lethally injured cells as a result of properties associated with the food or processing conditions.

Microbiology of the food chain — Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* —

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

1 Scope

This document specifies a horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* by 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[9][10][13][14][17][18][19][20]. It is applicable to

— products intended for human consumption,

— products intended for feeding animals,

— environmental samples in the area of food production and food handling, and

— samples from the primary production stage such as animal faeces, dust, and swabs.

WARNING — Some strains of *Escherichia coli* may grow poorly or not at all in media incubated at 44 °C. This includes strains of *E. coli* O157:H7 and O157:H$^-$. Additionally, some strains of *Escherichia coli*, notably those belonging to serotype O157:H7, are mostly β-glucuronidase negative[11]. Consequently, some strains of *E. coli*, including pathogenic ones, will not be detected by this method. β-glucuronidase activity may also be exhibited at 44 °C by certain other members of the *Enterobacteriaceae*, notably *Shigella*[15] and *Salmonella*[16].

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1
β-glucuronidase-positive *Escherichia coli*
bacteria which at 44 °C forms typical, blue or blue-green colonies on tryptone-bile X-glucuronide agar (TBX) under the conditions specified in this document

3.2
enumeration of β-glucuronidase-positive *Escherichia coli*
determination of the number of colony-forming units (cfu) of β-glucuronidase-positive *Escherichia coli* (3.1), per gram of sample, per millilitre, per square centimetre or per sampling device when the analysis is carried out in accordance with this document

4 Principle

4.1 Test portion, initial suspension, dilutions and resuscitation step

A specified quantity of the test sample, initial suspension or decimal dilutions, is inoculated onto membranes overlaid on minerals-modified glutamate agar (MMGA), then incubated at 37 °C for 4 h[13][14].

4.2 Culture on selective medium

For isolation, the membranes from the resuscitation stage on the MMGA are transferred to tryptone-bile X-glucuronide agar (TBX), then incubated at 44 °C for 20 h to 24 h.

4.3 Calculation

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 or blue-green colonies per plate.

5 Culture media and reagents

For current laboratory practice, see ISO 7218 and ISO 11133.

Composition of culture media and reagents and their preparation are described in Annex A.

For performance testing of culture media and membrane for transfer, see ISO 11133 and Annex A.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

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

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**. As specified in ISO 7218.

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

6.3 Incubator, capable of operating at 37 °C ± 1 °C.

6.4 Incubator, capable of operating at 44 °C ± 1 °C.

6.5 Water bath, capable of operating at 47 °C to 50 °C.

6.6 Refrigerator (for storage of prepared media), capable of operating at 5 °C ± 3 °C.

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

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

6.9 pH-meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ±0,1 pH unit.

6.10 Sterile graduated pipettes or automatic pipettes, of nominal capacity 1 ml.

6.11 Sterile petri dishes with a diameter of approximately 90 mm diameter.

6.12 Sterile spreaders, made of glass or plastic, for example, hockey sticks made from a 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

Sampling is not part of the method specified in this document. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Recommended sampling methods are given in the following documents:

— ISO/TS 17728[7] for food and animal feed;

— ISO 707[1] for milk and milk products;

— ISO 13307[2] for sampling at primary production stage;

— ISO 17604[6] for sampling of carcasses;

— ISO 18593[8] for sampling of surfaces.

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

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: see ISO 6887 (all parts). If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

For general aspects refer to ISO 7218.

9.2 Test portion, initial suspension and dilutions

Use the method specified in the relevant document of ISO 6887 (all parts) and any specific International Standard appropriate to the product concerned.

Prepare a single decimal dilution series from the test sample if the product is liquid, or from the initial suspension (10^{-1} dilution) in the case of other products.

9.3 Resuscitation

9.3.1 Using sterile forceps (6.7), aseptically place a membrane (6.8) onto the dried surface of an appropriate number of plates of MMGA (A.2), 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 (6.10), add 1 ml of the test sample or the initial suspension to the centre of the membrane. If only the initial suspension is used prepare duplicate MMGA/membrane plates, or if more than one dilution is being plated a single plate for each dilution can be used; see ISO 7218. Using a sterile spreader, spread the inoculum evenly over the whole membrane surface, avoiding any spillage from the membrane.

9.3.2 Repeat the procedure as specified in 9.3.1 with the further decimal dilutions, if necessary, using another sterile pipette and another sterile spreader for each dilution.

9.3.3 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 ± 0,25 h in the incubator (6.3) set at 37 °C, with the membrane/agar surface uppermost.

9.4 Transfer to selective medium and incubation

9.4.1 After resuscitation, using sterile forceps (6.7), transfer membranes from MMGA (resuscitation medium) to plates of TBX (A.3).

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

9.4.2 Incubate the plates for 20 h to 24 h in the incubator (6.4) set at 44 °C, and not more than 45 °C, with the membrane/agar surface uppermost. Plates should be stacked no more than three high. Higher stacks may be acceptable in incubators fitted with air circulation systems; in this case, the temperature distribution should be verified.

9.5 Counting the colony-forming units (cfu)

After the specified period of incubation (9.4.2), examine plates of TBX (A.3) for the presence of typical, blue or blue-green colonies indicating the presence of β-glucuronidase-positive *Escherichia coli*.

9.6 Calculation

Calculate the number of β-glucuronidase-positive *Escherichia coli* by counting the typical colonies (9.5) in each plate containing less than 150 typical cfu and less than 300 total (typical and non-typical) cfu.

10 Expression of results

Refer to ISO 7218.

Report the results as the number of β-glucuronidase-positive *Escherichia coli* in cfu per gram of sample, per millilitre, per square centimetre or per sampling device.

11 Performance characteristics of the method

The performance characteristics (specificity, sensitivity and the LOD_{50}) of the method have not been determined in interlaboratory studies.

12 Test report

The test report shall contain at least the following information:

— the test method used, with reference to this document, i.e. ISO 16649-1;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— any deviations from the standardized test method used (e.g. in the media or the incubation conditions used);

— the test result(s) obtained;

— the date of the test.

13 Quality assurance

The laboratory shall have a clearly defined quality control system to ensure that the equipment, reagents and techniques are suitable for the test. The use of positive controls, negative controls and blanks are part of the test. Performance testing of culture media and membrane for transfer is specified in Annex A and described in ISO 11133.

Annex A
(normative)

Composition and preparation of culture media and reagents

A.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been determined in some studies. The user should verify these under their own storage conditions (see ISO 11133).

Performance testing of culture media is described in A.4.

A.2 Resuscitation medium: minerals-modified glutamate agar (MMGA)[12][14]

A.2.1 Composition

Sodium L-glutamate monohydrate	(CAS No. 6106-04-3)	6,35 g
Lactose monohydrate	(CAS No. 10039-26-6)	10,0 g
Sodium formate (sodium methanoate)	(CAS No. 141-53-7)	0,25 g
L-(-)-Cystine	(CAS No. 56-89-3)	0,02 g
L-(-)-Aspartic acid	(CAS No. 56-84-8)	0,024 g
L-(+)-Arginine	(CAS No. 74-79-3)	0,02 g
Thiamine chloride hydrochloride	(CAS No. 67-03-8)	0,001 g
Nicotinic acid	(CAS No. 59-67-6)	0,001 g
Pantothenic acid	(CAS No. 79-83-4)	0,001 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	(CAS No. 10034-99-8)	0,1 g
Ammonium iron(III) citrate[a]	(CAS No. 1185-57-5)	0,01 g
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	(CAS No. 10035-04-8)	0,01 g
Dipotassium hydrogen phosphate (K_2HPO_4)	(CAS No. 7758-11-4)	0,9 g
Ammonium chloride	(CAS No. 12125-02-9)	2,5 g

Agar	9 g to 18 g[b]
Water	1 000 ml

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

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

A.2.2 Preparation

Dissolve the ammonium chloride in the water. Add the other components and dissolve by heating, if necessary.

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, e.g. bottles or flasks.

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

A.2.3 Preparation of the agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.5), mix, and pour 12 ml to 15 ml of the medium into sterile petri dishes (6.11).

Allow to solidify.

Store the poured plates, protected from drying, at 5 °C (6.6) for up to four weeks unless results of the laboratory shelf life validation indicate a longer shelf-life.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.2) set between 25 °C and 50 °C until the surface of the agar is dry, see ISO 11133.

A.3 Selective medium: tryptone-bile X-glucuronide agar (TBX)

A.3.1 Composition

Enzymatic digest of casein		20,0 g
Bile salts No. 3		1,5 g
5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (BCIG), cyclohexylammonium salt monohydrate or sodium salt	(CAS No. 114162-64-0)	144 µmol[a]
Agar		9 g to 18 g[b]
Water		1 000 ml

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

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

A.3.2 Preparation

Dissolve the cyclohexylammonium salt of BCIG in 3 ml of solution consisting of 2,5 ml of a volume fraction of 95 % aqueous ethanol and 0,5 ml of 1M sodium hydroxide solution. Add this solution to the medium. Alternatively the sodium salt of BCIG can be added directly to the medium.

Suspend the ingredients in water and heat until completely dissolved.

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

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

A.3.3 Preparation of the agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.5), mix and pour 18 ml to 20 ml of the molten medium into sterile Petri dishes (6.11). Allow to solidify. For storage and drying of plates before use, see A.2.3.

A.4 Performance testing for the quality assurance of the culture media and the membrane

Performance testing of the agar media described in this annex should be carried out with the membranes in place on the plates. If the results are unsatisfactory the procedure should be repeated on the media with and without the membranes in place in order to identify the cause of the poor performance.

Sufficient testing shall be carried out to demonstrate that a batch of membranes meets the requirements and that the membrane can produce consistent results for the specific analysis used. Verification is achieved by demonstrating the suitability in the whole system: membrane together with culture media in both the resuscitation step and the culture on selective medium step.

Incubate the plates with the membrane/agar surface uppermost.

The definition of selectivity and productivity is specified in ISO 11133. Test the performance of the culture media in accordance with the methods and criteria as described in ISO 11133.

Table A.1 shows the performance criteria of MMGA which shall be tested with membrane for transfer on TBX agar. These criteria shall be used for the performance testing of MMGA as well as for the membrane for transfer. Every batch of membranes shall be tested for its suitability for the test according to A.4, especially since the use of different brands of membrane filters may result in different recovery and colour development after incubation on TBX.

Table A.2 shows the performance criteria of TBX.