TECHNICAL SPECIFICATION

ISO/TS 16649-3

First edition
2005-09-15

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

Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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

Partie 3: Technique du nombre le plus probable utilisant le bromo-5-chloro-4-indolyl-3 β-D-glucuronate

Reference number
ISO/TS 16649-3:2005(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published 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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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.

ISO/TS 16649-3 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 using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide* (Technical Specification)

Introduction

This Technical Specification is intended to provide general guidance for the examination of products not dealt with by existing International Standards and to be taken into account by organizations preparing microbiological methods of test for application to foods or to animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate in every detail for certain products, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply the provided guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this Technical Specification is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviations from them 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 these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed, but it is hoped that when such standards are reviewed they will be changed to comply with this Technical Specification so that eventually the only remaining departures from these guidelines will be those necessary for well-established technical reasons.

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

Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

1 Scope

This Technical Specification specifies a horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli*, by means of the liquid-medium culture technique and calculation of the most probable number (MPN) after incubation at 37 °C, then at 44 °C.

This Technical Specification is applicable to

— products intended for human consumption and the feeding of animals, and

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

The method is suitable for the enumeration of cells of *Escherichia coli* that may have been subjected to stress arising from dehydration, freezing, exposure to a saline (such as marine) environment or damage by disinfectants such as chlorine-containing products.

A limitation of the applicability of this Technical Specification is imposed by the susceptibility of the method to a large degree of variability. The method is to be applied and the results interpreted in the light of the information given in Clause 11.

WARNING — Strains of *Escherichia coli* that do not grow at 44 °C and, in particular, those that are β-glucuronidase negative, such as *Escherichia coli* O157 and some other strains of pathogenic *E. coli*, will not be detected by the method described in this Technical Specification.

2 Normative references

The following referenced documents are indispensable for the application 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 6887-1, *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 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

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

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

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

3.1
β-glucuronidase-positive *Escherichia coli*
bacteria which, at 44 °C, form typical blue or blue-green colonies on tryptone bile glucuronide medium under the conditions specified in this Technical Specification

3.2
enumeration of β-glucuronidase-positive *Escherichia coli*
determination of the most probable number of β-glucuronidase-positive *Escherichia coli* per millilitre or per gram of sample, when the test is carried out in accordance with this Technical Specification

4 Principle

4.1 Three tubes[1)] of double-strength liquid selective enrichment medium are inoculated 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.

4.2 Three tubes[1)] of single-strength liquid enrichment medium are inoculated with a specified quantity of test sample if the initial product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Then, under the same conditions, the single-strength liquid enrichment medium is inoculated with decimal dilutions of the test sample or of the initial suspension.

4.3 The tubes of double- and single-strength medium are inoculated at 37 °C for 24 h. The tubes are examined for acid production, indicating lactose fermentation.

4.4 Each tube of selective enrichment medium showing acid production is subcultured to tryptone bile glucuronide agar.

1) Five tubes can be used, see 9.2.1.

4.5 The tryptone bile glucuronide agar is incubated at 44 °C for 20 h to 24 h. The tryptone bile glucuronide agar is examined for the presence of blue or blue-green colonies, indicating the presence of β-glucuronidase-positive *Escherichia coli*.

4.6 The most probable number of β-glucuronidase-positive *Escherichia coli* (see ISO 7218) are determined according to the number of tubes of selective enrichment medium whose subcultures have produced blue or blue-green colonies on tryptone bile glucuronide agar.

5 Dilution fluids and culture media

For current laboratory practice, see ISO 7218.

5.1 Dilution fluids

See the appropriate part of ISO 6887 or ISO 8261.

5.2 Culture media

5.2.1 **Mineral-modified glutamate medium** (selective enrichment medium)

5.2.1.1 Composition

	a) Double-strength medium	b) Single-strength medium
Sodium glutamate	12,7 g	6,35 g
Lactose	20,0 g	10,0 g
Sodium formate	0,5 g	0,25 g
L-Cystine	0,04 g	0,02 g
L(–)-Aspartic acid	0,048 g	0,024 g
L(+)-Arginine	0,04 g	0,02 g
Thiamine	0,002 g	0,001 g
Nicotinic acid	0,002 g	0,001 g
Pantothenic acid	0,002 g	0,001 g
Magnesium sulfate septahydrate	0,2 g	0,1 g
Ammonium iron(III) citrate	0,02 g	0,01 g
Calcium chloride dihydrate	0,02 g	0,01 g
Dipotassium hydrogen phosphate	1,8 g	0,9 g
Bromocresol purple	0,02 g	0,01 g
Ammonium chloride	5,0 g	2,5 g
Water	1 000 ml	1 000 ml

5.2.1.2 Preparation

Dissolve the ammonium chloride in the water. Add the remaining components, or the dehydrated complete medium, and dissolve by heating if necessary.

To improve the storage stability of the dehydrated medium, the sodium glutamate may be supplied separately.

Adjust the pH, if necessary, so that after sterilization it is 6,7 ± 0,1 at 25 °C.

Dispense the media in volumes of 10 ml into tubes of dimensions 16 mm × 160 mm (6.6) in the case of single-strength medium, and into test tubes of dimensions 18 mm × 180 mm or 20 mm × 200 mm (6.6) in the case of the double-strength medium.

Sterilize for 10 min in an autoclave (6.1) set at 116 °C. Alternatively, heat to 100 °C for 30 min on three successive days.

5.2.2 Tryptone bile glucuronide agar (second selective medium)

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 and appropriate personal protective equipment when handling is advised.

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

5.2.2.2 Preparation

Dissolve the BCIG in the dimethyl sulfoxide. 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

Pour 12 ml to 15 ml of the molten medium into sterile Petri dishes (6.9) and allow to solidify.

Dry the plates (6.3) (see ISO 7218). The plates may be stored at 5 °C ± 3 °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.

5.2.3 Performance testing for the quality assurance of the culture media

For the definition of productivity and selectivity, refer to ISO/TS 11133-1 and ISO/TS 11133-2. Tables 1 and 2 give the performance tests for mineral-modified glutamate medium and for tryptone bile glucuronide agar.

Table 1 — Performance testing of mineral-modified glutamate medium

Function	Incubation	Control strains	Method of control	Criteria	Characteristic reactions
Productivity	37 °C/24 h	*E. coli* ATCC 25922 or 8739	Semi-quantitative	Acid production	Colour change to yellow
Selectivity	37 °C/24 h	*E. faecalis* ATCC 29212 or 19433	Qualitative	No growth	—

Table 2 — Performance testing of tryptone bile glucuronide agar

Function	Incubation	Control strains	Method of control	Criteria	Characteristic reactions
Productivity	44 °C/20 h to 24 h	*E. coli* ATCC 25922 or 8739	Qualitative	Good growth (2)	Blue to blue-green colonies
		E. coli NCTC 13216 (weakly β-glucuronidase-positive)	Qualitative	Good growth (2)	Blue to blue-green colonies
Selectivity	37 °C/24 h	*E. faecalis* ATCC 29212 or 19433	Qualitative	No growth	—

6 Apparatus and glassware

Disposable apparatus is an acceptable alternative to reusable glassware if it has similar specifications.

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 Incubators, capable of operating at 37 °C ± 1 °C and 44 °C ± 1 °C.

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

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

6.5 pH-meter, having a resolution of 0,01 pH units and accurate to within ± 0,1 pH units at 25 °C.

The pH meter shall be equipped with either manual or automatic temperature equalization.

6.6 Test tubes, of dimensions approximately 16 mm × 160 mm and 18 mm × 180 mm or 20 mm × 200 mm.

6.7 Total-delivery pipettes, having nominal capacities of 1 ml and 10 ml, graduated in 0,1 ml divisions.

6.8 Sampling loops, made of platinum/iridium or nickel/chromium, approximately 3 mm in diameter, or 10 µl sterile disposable sampling loops.

6.9 Petri dishes, of approximately 90 mm diameter.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this Technical Specification. If there is no specific International Standard dealing with sampling of the product concerned, 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, 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 the appropriate part of ISO 6887 and the specific International Standard appropriate to the product concerned.

Prepare a sufficient number of dilutions to ensure that all the tubes for the final dilution will yield a negative result.

9.2 Inoculation of the selective enrichment

9.2.1 As a general case, the following procedure specifies series of three tubes for each dilution. For live shellfish, or other special products, and/or whenever a greater accuracy in the results is needed, it is necessary to inoculate a series of five tubes per dilution.

9.2.2 Take three tubes of double-strength selective enrichment medium [5.2.1.1 a)]. Using a sterile pipette (6.7), transfer to each of these tubes 10 ml of the test sample if liquid, or 10 ml of the initial suspension in the case of other products.

9.2.3 Then take three tubes of single-strength selective enrichment medium [5.2.1.1 b)]. Using a fresh sterile pipette (6.7), transfer to each of these tubes 1 ml of the test sample if liquid, or 1 ml of the initial suspension in the case of other products.

9.2.4 For each of the further dilutions (from 10^{-1} or 10^{-2}, according to the test sample), proceed as in 9.2.3. Use a fresh sterile pipette for each dilution. Carefully mix the inoculum and the medium.

9.3 Incubation

Incubate the tubes of double-strength selective medium inoculated in 9.2.2 and the tubes of single-strength selective medium inoculated in 9.2.3 and 9.2.4 in the incubator (6.2) set at 37 °C for 24 h ± 2 h.

9.4 Subculturing

From each tube incubated according to 9.3 showing the presence of acid, indicated by the presence of any yellow coloration, subculture with a loop (6.8) to a plate of tryptone bile glucuronide agar (5.2.2) and streak to obtain isolated colonies.

9.5 Second incubation

Incubate the plates inoculated as in 9.4 for 20 h to 24 h in an incubator (6.2) set at 44 °C. Do not stack dishes more than three high.

9.6 Examination of the plates

After the specified period of incubation (9.5), examine the plates for the presence of colonies showing any shade of dark or light blue or blue-green, indicating the presence of β-glucuronidase-positive *Escherichia coli*.

9.7 Interpretation

Consider as positive each tube of double-strength or single-strength selective enrichment medium incubated according to 9.3 that has given rise, after subculturing (9.4) and incubation (9.5), to the presence of blue or blue-green colonies on the plate of selective medium.

For each dilution, count the number of positive tubes of medium.

10 Expression of results

Calculate the most probable number from the number of positive tubes at each dilution.

See ISO 7218.

11 Precision

It is well known that wide variations in results can occur with the MPN technique using a series of three tubes per dilution. Results obtained with this method should therefore be used with caution. When using series of five tubes, it has been reported that the precision obtained would be comparable to that of colony-count methods. Confidence limits are given in ISO 7218.

EXAMPLE For a solid sample, in 95 % of the cases, the confidence limits vary from 13 to 200 β-glucuronidase-positive *Escherichia coli* per gram for an MPN of $7{,}4 \times 10^1$ β-glucuronidase-positive *Escherichia coli* per gram, and from 4 to 99 β-glucuronidase-positive *Escherichia coli* per gram for an MPN of $2{,}4 \times 10^1$ β-glucuronidase-positive *Escherichia coli* per gram.

12 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this Technical Specification;

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

e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Bibliography

[1] BLAZKO N. Evaluation of the β-glucuronidase substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in a 24 hour direct plating method for *Escherichia coli*. *J.Food Protection*, **51**, 1,988, p. 402

[2] DAMARE J.M., CAMPBELL D.F. and JOHNSON R.W.. Simplified direct plating method for enhanced recovery of *Escherichia coli* in food. *J Food Sci.*, **50**, 1985, pp. 1736-1737, 1746

[3] DELISLE G.L. and LEY A. Rapid detection of *Escherichia coli* in urine samples by a new chromogenic β-glucuronidase assay. *J.Clin.Microbiol*., **27**, 1989, pp. 778-779

[4] DONOVAN T.J., GALLACHER S., ANDREWS N.J., GREENWOOD M.H., GRAHAM J., RUSSELL J.E., ROBERTS D. and LEE R. Modification of the standard method used in the United Kingdom for counting *Escherichia coli* in live bivalve molluscs. *Comm Dis & Public Health*, **1**, 1998, pp. 188-196

[5] KILIAN M. and BULOW P. Rapid identification of Enterobacteriaceae. Use of a β-glucuronidase detecting agar (PGUA) for the identification of *Escherichia coli* in primary cultures of urine samples. *Acta Pathol Microbiol Scand., Sect. B*, **87**, 1979, pp. 271-276

[6] KILIAN M. and BULOW P. Rapid identification of Enterobacteriaceae. Detection of bacterial glycosidases. *Acta pathol Microbiol Scand., Sect. B*, **84**, 1976, pp. 245-251

[7] LEY A.N., BOWERS R.J. and WOLFE S. Indoxyl-β-D-glucuronide, a novel chromogenic reagent for the specific detection and enumeration of *Escherichia coli* in environmental samples. *Canadian J Microbiol*., **34**, 1988, pp. 690-693

[8] MANAFI M. and KNEIFEL W. A combined chromogenic-fluorogenic medium for the simultaneous detection of total coliforms and *E.coli* in water. *Zentralbl. Hyg.*, **189**, 1989, pp. 225-234

[9] OGDEN I.D. and WATT A.J. An evaluation of fluorogenic and chromogenic assays for the direct enumeration of *Escherichia coli*. *Letters in Applied Microbiology*, **13**, 1991, pp. 212-215

[10] RESTAINO L., FRAMPTON E.W. and LYON R.H. Use of chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC) for enumeration of *Escherichia coli* in 24 hours from ground beef. *J.Food Protection*, **53** (6) 1990, pp. 508-510

[11] WATKINS W.D., RIPPEY S.C., CLAVET C.R., KELLY-REITZ D.J. and BURKHART W. Novel compound for identifying *Escherichia coli*. *Appl. Envir. Microbiol.*, **54**, 1988, pp. 1874-1875

ICS 07.100.30

Price based on 8 pages