INTERNATIONAL STANDARD

ISO 16649-2

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

Part 2: Colony-count technique at 44 °C 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 2: Technique de comptage des colonies à 44 °C au moyen 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 16649-2 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 2:
Colony-count technique at 44 °C using 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 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 colony 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 Duplicate plates of tryptone-bile-glucuronic medium (TBX) are inoculated with the specified quantity of the test sample or the initial suspension.

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

The dishes are incubated for 18 h to 24 h at 44 °C ± 1 °C then examined to detect the presence of colonies which, from their characteristics, are considered to be β-glucuronidase-positive *Escherichia coli*.

4.2 The number of colony-forming units (CFU) of β-glucuronidase-positive *Escherichia coli* per gram or per millilitre of sample is calculated (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 medium: Tryptone-bile-glucuronic medium (TBX)

5.2.1 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] e.g. 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 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 set at 121 °C for 15 min. Immediately cool the medium in the water bath (6.3) at 44 °C to 47 °C.

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 operating at 44 °C ± 1 °C.

6.3 Water-bath, capable of being maintained at 44°C to 47 °C.

6.4 Test tubes, flasks or **bottles** of suitable capacity.

6.5 Pipettes or **micropipettes, total delivery (blow out)**, having wide openings and having a nominal capacity of 1 ml and 10 ml, graduated respectively in 0,1 ml and 0,5 ml divisions.

6.6 Petri dishes, of approximately 90 mm diameter.

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.

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 Inoculation and incubation

9.2.1 Using a sterile pipette or a micropipette (6.5), transfer to a sterile Petri dish (6.6) 1 ml of the test sample (if liquid), or 1 ml of the initial dilution (10^{-1}) in the case of other products.

Inoculate two plates per dilution.

Repeat the procedure with the further decimal dilutions, if necessary, using a new sterile pipette for each dilution.

9.2.2 Pour into each Petri dish approximately 15 ml of the TBX medium (5.2), previously cooled at 44°C to 47°C in the water bath (6.3).

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

The time which elapses between the distribution of the inoculum in a dish and pouring of the medium shall not exceed 15 min.

9.2.3 Invert the inoculated dishes (9.2.2) so that the bottom is uppermost and place them in an incubator (6.2) set at 44 °C for 18 h to 24 h. The total incubation time shall not be longer than 24 h.

WARNING — If the presence of stressed cells is suspected, incubate for an initial period of 4 h at 37 °C, and then raise the incubation temperature to 44 °C for 18 h to 24 h. The incubation temperature shall not exceed 45 °C.

9.3 Counting the colony-forming units

After the specified period of incubation (9.2.3) 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 CFU on at least one dish containing as a minimum 15 blue CFU.

Calculate N, the number of CFU of β-glucuronidase-positive *Escherichia coli* present in the test sample per millilitre or per gram, as the weighted mean from two successive dilutions using the following equation:

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

where

$\sum a$ is the sum of the CFU counted on all the dishes retained from two successive dilutions, at least one of which contains a minimum 15 blue CFU;

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

V is the volume of inoculum, in millilitres, applied to each dish;

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

d is the dilution factor corresponding to the first dilution retained [d = 1 in the case (liquid products) where the directly inoculated test sample is retained].

Round off the results to two significant figures (see ISO 7218).

Take as the result the number of β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products) expressed as a whole number to two significant figures (if below 100) or as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

10.3 Estimation of low numbers

10.3.1 If the two dishes [at the level of the test sample (liquid products) or of the initial suspension (other products) or of the first inoculated or retained dilution] contain less than 15 blue CFU, calculate N_E, the number of CFU of β-glucuronidase-positive *Escherichia coli* present in the test sample, as the arithmetical mean from two parallel plates using the following equation:

$$N_E = \frac{\sum c}{V \cdot n \cdot d} \quad (2)$$

where

$\sum c$ is the sum of the blue CFU counted on the two dishes;

V is the volume of the inoculum, in millilitres, applied to each dish;

n is the number of dishes retained (n = 2 in this case);

d is the dilution factor to the initial suspension or the first inoculated or retained dilution [d = 1 in the case (liquid products) where the directly inoculated test sample is retained].

Round off the results to two significant figures (see ISO 7218).

Express the result as follows:

— estimated number of β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products): $N_E = Y$.

10.3.2 If the two dishes at the level of the test sample (liquid products) or the initial suspension (other products) of the first inoculated or retained dilution do not contain any blue CFU, express the result as follows:

— less than 1/d of β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products),

where d is the dilution factor of the initial suspension or the first inoculated or retained dilution [d = 1 in the case (liquid products) where the directly inoculated test sample is retained].

10.3.3 If for the two dishes from the first dilution d_1 the total number of blue and non-typical CFU is higher than 300 with visible blue CFU, and if for the two dishes from the subsequent dilution d_2 containing less than 300 colonies, no blue CFU can be counted, express the result as follows:

— less than 1/d_2 and more than 1/d_1 β-glucuronidase positive *Escherichia coli* per millilitre (liquid products) or per gram (other products),

where d_1 and d_2 are the dilution factors corresponding to dilutions d_1 and d_2.

10.3.4 If for the two dishes from the first dilution d_1 the total number of typical CFU and non-typical CFU is higher than 300 without visible blue CFU, and if for the two dishes from the subsequent dilution d_2 containing less than 300 colonies, no blue CFU can be counted, express the result as follows:

— less than $1/d_2$ CFU of β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products),

where d_2 is the dilution factor corresponding to dilution d_2.

10.4 Method of calculation: Special cases

10.4.1 In the case where the number of blue CFU is higher than 150 for the two dishes from the first dilution d_1, with a number of blue CFU below 15 for the two dishes from the subsequent dilution d_2:

— if the number of blue CFU on each of the two dishes from dilution d_1 is within the range 167 to 150 (upper part of the confidence interval of a weighted mean equal to 150), use the calculation method for the general case (10.2);

— if the number of blue CFU on each of the two dishes from dilution d_1 is higher than 167 (upper limit of the confidence interval of a weighted mean equal to 150 CFU), only take into account the result of the counts of dilution d_2 and carry out a low number count (10.3).

10.4.2 In the case where counting the blue CFU on each of the dishes from all the inoculated dilutions gives a number higher than 150, express the result as follows:

— more than $150/d$ β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products),

where d is the dilution factor of the last inoculated dilution.

10.4.3 In the case where only the two dishes from the lowest dilution (highest concentration) contain less than 150 typical CFU, calculate the number N' of β-glucuronidase-positive *Escherichia coli* present in the test sample as the arithmetical mean of the colonies counted on the two dishes, using the following equation:

$$N' = \frac{\sum c}{V \cdot n \cdot d} \qquad (3)$$

where

$\sum c$ is the sum of the blue CFU counted on the two dishes, of which at least one contains at the minimum 15 typical CFU;

V is the volume of the inoculum, in millilitres, applied to each dish;

n is the number of dishes retained (n = 2 in this case);

d is the dilution factor corresponding to the dilution retained.

Round off the results to two significant figures (see ISO 7218).

10.5 Confidence limits

See ISO 7218.

11 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this part of ISO 16649;

— all operating details not specified in this part of ISO 16649, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the result(s) obtained, indicating clearly the method of expression used; and

— if the repeatability has been checked, the final result obtained.

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