TC 34

International Standard ISO 7937

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Microbiology — General guidance for enumeration of *Clostridium perfringens* — Colony count technique

Microbiologie — Directives générales pour le dénombrement de Clostridium perfringens *— Méthode par comptage des colonies*

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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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

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Microbiology — General guidance for enumeration of *Clostridium perfringens* — Colony count technique

0 Introduction

0.1 This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for the reference of bodies preparing microbiological methods of test for application to food products or to animal feeding stuffs. In view of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, 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 these guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard 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 which necessitated deviation 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 that do not comply with the guidelines may already exist. In cases where International Standards already exist for the product to be tested, they should be followed, but it is hoped that, when they are reviewed, they will be aligned with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well established technical reasons.

0.2 For practical reasons, the definition of *Clostridium perfringens* given in clause 3 and used as the basis for the procedure does not exclusively describe strains of *Clostridium perfringens.* In particular, the confirmatory tests are inadequate to distinguish between *Clostridium perfringens* and other closely related but less commonly encountered *Clostridium* species such as *C. paraperfringens* and *C. absonum.*

0.3 For statistical reasons, the lowest limit for the number of colonies counted per dish has been set at 15, but, for practical purposes, a count of lower numbers of *Clostridium perfringens* is often required. The confidence limits of such determinations (estimated counts) are given in the annex.

1 Scope and field of application

This International Standard gives general guidance for the enumeration of viable *Clostridium perfringens* in products intended for human consumption or feeding of animals.

2 Reference

ISO 6887, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

3 Definitions

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

3.1 *Clostridium perfringens:* Bacteria that form black colonies in the specified selective medium and which give positive confirmatory reactions when the test is carried out by the method specified in this International Standard.

3.2 enumeration of *C. perfringens:* Determination of the number of viable and confirmed *C. perfringens* bacteria per millilitre or per gram of sample, when the test is carried out by the method specified in this International Standard.

4 Principle

4.1 Inoculation of Petri dishes with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of the initial suspension in the case of other products.

Inoculation, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

Mixing with a selective medium (poured plate technique) and adding an overlayer of the same medium.

4.2 Anaerobic incubation of the plates at 35 °C or 37 °C[1] for 20 h.

4.3 Calculation, from the number of black colonies appearing on the plates, of the number of characteristic colonies.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

4.4 Subjection of characteristic colonies to confirmatory procedures and calculation of the number of *C. perfringens* per millilitre or per gram of sample.

5 Diluent, culture media and reagents

5.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent and culture media, dehydrated basic components, or complete dehydrated media, be used. Similarly, commercially prepared reagents may also be used. The manufacturer's instructions shall be rigorously followed.

Chemical products shall be of recognized analytical quality.

The water used shall be distilled or deionized water, free from substances that might inhibit the growth of *Clostridium perfringens* under the test conditions.

Measurements of pH shall be made using a pH meter (6.5), measurements being referred to a temperature of 25 °C. If the diluent and culture media are not used immediately, they shall, unless otherwise stated, be stored in the dark at approximately 4 °C, in conditions which do not produce any change in their composition.

5.2 Diluent

See ISO 6887 and the specific standard dealing with the product to be examined.

5.3 Egg-yolk-free tryptose-sulfite-cycloserine agar (SC)[1]

5.3.1 Base

Composition

Tryptose[2]	15,0 g
Soytone[2]	5,0 g
Yeast extract	5,0 g
Disodium disulfite ($Na_2S_2O_5$), anhydrous	1,0 g
Ammonium iron(III) citrate[3]	1,0 g
Agar	12,0 to 18,0 g[4]
Water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that it will be 7,6 after sterilization.

Transfer the base to tubes or flasks or bottles of capacity not more than 500 ml.

Sterilize for 10 min at 121 °C.

Store in a refrigerator at 4 ± 2 °C.

Discard unused medium 2 weeks after preparation.

Preparation of agar plates for confirmation

Transfer portions of about 15 ml of the base, melted and cooled to approximately 45 °C, to Petri dishes and allow to solidify.

Immediately before use, dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet (6.2) at 50 °C for 30 min.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or 1 day at 4 °C.

5.3.2 D-Cycloserine solution

Composition

D-Cycloserine (use white crystalline powder only)	4,0 g
Water	100 ml

Preparation

Dissolve the D-cycloserine in the water and sterilize the solution by filtration.

Store in a refrigerator at 4 ± 2 °C.

Discard unused solution 4 weeks after preparation.

5.3.3 Complete medium

Before plating (see 9.2), add 1 ml of the sterilized D-cycloserine solution (5.3.2) to each 100 ml of sterile melted base (5.3.1) at 50 °C.

5.4 Fluid thioglycollate medium

Composition

Pancreatic casein digest	15,0 g
L-Cystine	0,5 g
Dextrose (D-glucose)	5,0 g
Yeast extract	5,0 g
Sodium chloride	2,5 g
Sodium thioglycollate (mercaptoacetate)	0,5 g
Agar	0,5 to 0,8 g[4]
Resazurin	0,001 g
Water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that it will be 7,1 after sterilization.

Dispense 10 ml portions into test-tubes and sterilize at 121 °C for 15 min.

1) This was originally designated EY-free TSC (HAUSCHILD and HILSHEIMER. *Appl. Microbiol.* **27** 1974: 78-82).

2) The names tryptose and soytone are used at present only by certain producers of media. Any other pancreatic casein or soybean digest giving comparable results may be used.

3) This reagent should contain at least 15 % (*m*/*m*) of iron.

4) According to the manufacturer's instructions.

5.5 Motility-nitrate medium

Composition

Peptone	5,0 g
Beef extract	3,0 g
Galactose	5,0 g
Glycerol	5,0 g
Potassium nitrate (KNO_3)	1,0 g
Disodium hydrogenorthophosphate (Na_2HPO_4)	2,5 g
Agar	1,0 to 5,0 g[1]
Water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that it will be 7,3 after sterilization.

Transfer the medium to culture tubes in 10 ml quantities and sterilize at 121 °C for 15 min.

If not used the same day, store in a refrigerator at 4 ± 2°C; just prior to use, heat in boiling water or flowing steam for 15 min and then cool rapidly to the incubation temperature.

Discard unused medium 4 weeks after preparation.

5.6 Lactose-gelatine medium

Composition

Tryptose[2]	15,0 g
Yeast extract	10,0 g
Lactose	10,0 g
Gelatine	120,0 g
Phenol red	0,05 g
Water	1 000 ml

Preparation

Dissolve the components, except the lactose and phenol red, in the water.

Adjust the pH so that it will be 7,5 after sterilization.

Add the lactose and phenol red, dispense 10 ml portions into test-tubes and sterilize at 121 °C for 15 min.

If not used the same day, store in a refrigerator at 4 ± 2 °C.

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

Discard unused medium 3 weeks after preparation.

5.7 Nitrite-detection reagent

5.7.1 5-amino-2-naphthalenesulfonic acid (5-2 ANSA) solution

Dissolve 0,1 g of 5-2 ANSA in 100 ml of 15 % (*V*/*V*) acetic acid solution. Filter through a filter paper.

Store in a well-stoppered brown bottle (preferably with a bulb-type dropper) at 4 °C.

5.7.2 Sulfanilic acid solution

Dissolve 0,4 g of sulfanilic acid in 100 ml of 15 % (*V*/*V*) acetic acid solution. Filter through a filter paper.

Store in a well-stoppered brown bottle (preferably with a bulb-type dropper) at 4 °C.

5.7.3 Preparation of complete reagent

Mix equal volumes of the two solutions (5.7.1 and 5.7.2) just before use.

Discard unused reagent immediately.

5.8 Zinc dust

6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptable alternative to glassware if it has suitable specifications.

Usual microbiological laboratory equipment and in particular

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave) (autoclave operating either separately or as part of an apparatus for preparing and distributing media).

Apparatus that will come into contact with the diluent, culture media, or the sample, except for apparatus that is supplied sterile (in particular plastics apparatus), shall be sterilized by one of the following methods:

a) by being kept at 170 to 175 °C for not less than 1 h in an oven;

b) by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

6.2 Drying cabinet or **oven,** ventilated by convection, (for drying the surface of agar plates), capable of being maintained at 50 ± 1 °C.

6.3 Incubator, capable of being maintained at 35 ± 1 °C or 37 ± 1 °C, depending on the temperature adopted[3] (for main-

1) According to be manufacturer's instructions.

2) The name tryptose is used at present only by certain producers of media. Any other pancreatic casein digest giving comparable results may be used.

3) The temperature should be agreed between the parties concerned and recorded in the test report.

taining the inoculated media, plates and tubes within one of these temperature ranges).

6.4 Anaerobic jars or any other apparatus appropriate for anaerobic culture.

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

6.6 Loops, of platinum-iridium or nickel-chromium, of diameter approximately 3 mm, and stab-inoculation needle of the same material.

6.7 Filtration apparatus, for sterilization of solutions.

6.8 Test-tubes, of diameter 18 mm and length 180 mm and **culture bottles** or **flasks,**[1] for sterilization and storage of culture media and incubation of liquid media.

6.9 Measuring cylinders, for preparation of complete media.

6.10 Graduated pipettes, calibrated for bacteriological use only, of nominal capacities 1 ml and 10 ml, graduated in 0,1 ml and 0,5 ml divisions, respectively, and with an outflow opening of diameter 2 to 3 mm.

6.11 Rubber bulbs, for use with the graduated pipettes for distributing the components of the nitrite-detection reagent.

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

7 Sampling

Carry out sampling in accordance with the specific standard appropriate to the product concerned. If no such specific standard exists, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of the test sample

See the specific standard appropriate to the product concerned. If no such specific standard exists, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and the specific standard appropriate to the product concerned.

9.2 Inoculation and incubation (poured plate technique)

Transfer, by means of a sterile pipette, 1 ml of each dilution of the initial suspension, or of the test sample if the initial product is liquid, in duplicate, to the centres of empty Petri dishes, pour 15 to 20 ml of the SC agar (5.3.3) into the dish and mix well with the inoculum by gently rotating each dish. When the medium has solidified, add an overlayer of 10 ml of the same SC agar. Allow to solidify and place the plates with the lid uppermost in anaerobic jars or other suitable containers (6.4) and incubate at 35 °C or 37 °C[2] for 20 h. Longer incubation may result in excess blackening along the bottom rim of the plates.

9.3 Counting of colonies

9.3.1 After the specified period of incubation (see 9.2), count and record the number of characteristic colonies on the plates in accordance with 9.3.2, 9.3.3, 9.3.4 and 9.3.5. Colonies of *C. perfringens* are black.

9.3.2 If both plates corresponding to a particular dilution contain between 15 and 150 characteristic colonies, count the characteristic colonies on each plate and record the arithmetic mean of the counts from the two plates; otherwise record the count on one plate.

9.3.3 If there are plates containing between 15 and 150 characteristic colonies at two consecutive dilutions, count the characteristic colonies on each plate from each respective dilution and determine the arithmetic mean of the counts from the two plates from each of the two dilutions as specified in 9.3.2. Record the arithmetic mean of the two values obtained except when the ratio of the higher value to the lower value is greater than 2; in this case, record the lower value of the two.

9.3.4 If parts of the plates (see 9.3.2) are completely blackened, or if it is difficult to count well isolated characteristic colonies, count colonies on plates at the next higher dilution, even though their number may be less than 15, and proceed as in 9.3.5.

9.3.5 If there are fewer than 15 characteristic colonies on plates from the initial suspension or the test sample (if the initial product is liquid), count the actual number of characteristic colonies on each plate and record the arithmetic mean of the counts from the two plates.

9.3.6 If there are no characteristic colonies on plates from the initial suspension or the test sample (if the initial product is liquid), record the statement "No colonies observed".

1) Bottles or flasks with metal screw-caps may be used.

2) The temperature should be agreed between the parties concerned and recorded in the test report.

9.4 Confirmation

9.4.1 Selection and purification of colonies for confirmation

Select a total of 10 characteristic colonies from the plates counted in accordance with either 9.3.2, 9.3.3 or 9.3.4. If less than 10 colonies are available on the plates counted, select all the characteristic colonies present. Confirm these colonies as described in 9.4.2.

If the surface area of the plates is overgrown and it is not possible to select well-isolated characteristic colonies, inoculate 10 characteristic colonies into fluid thioglycollate medium (5.4). Incubate under anaerobic conditions at 35 °C or 37 °C[1] for 18 to 24 h. Streak the colonies on SC base agar plates (see 5.3.1), and add an overlayer of 10 ml of the SC base agar.

Allow to solidify and incubate anaerobically at 35 °C or 37 °C[1] for 18 to 24 h.

Select from each plate at least one characteristic and well separated colony.

Confirm this colony as described in 9.4.2.

If necessary, repeat the streaking and inoculation on SC base agar plates until well isolated, characteristic black colonies are obtained.

9.4.2 Biochemical confirmation

9.4.2.1 Confirmation using motility-nitrate medium

Stab-inoculate the selected colonies (see 9.4.1) into motility-nitrate medium (5.5). Incubate under anaerobic conditions at 35 °C or 37 °C[1] for 24 h.

Examine the tubes of motility-nitrate medium for the type of growth along the stab line. Motility is evident from diffuse growth out into the medium away from the stab line.

Test for the presence of nitrite by adding 0,2 to 0,5 ml of the nitrite-detection reagent (5.7) to each tube of motility-nitrate medium.[2] The formation of a red colour confirms the reduction of nitrate to nitrite.

If no red colour is formed within 15 min, add a small amount of zinc dust (5.8) and allow to stand for 10 min. If a red colour is formed, after the addition of zinc dust, no reduction of nitrate has taken place.

9.4.2.2 Confirmation using lactose-gelatine medium

Inoculate the selected colonies (see 9.4.1) into lactose-gelatine medium (5.6). Incubate under anaerobic conditions at 35 °C or 37 °C[1] for 24 h.

Examine the tubes of lactose-gelatine medium for the presence of gas and of a yellow colour (due to acid) indicating fermentation of lactose. Chill the tubes for 1 h at 5 °C and check for gelatine liquefaction. If the medium has solidified, reincubate for an additional 24 h and again check for gelatine liquefaction.

9.4.3 Interpretation

Bacteria which produce black colonies in SC medium, are non-motile, reduce nitrate to nitrite, produce acid and gas from lactose, and liquefy gelatine in 48 h are considered to be *C. perfringens.*

Cultures that show a faint reaction for nitrite (i.e. a pink colour) should be eliminated, since *C. perfringens* consistently gives an intense and immediate reaction.

10 Expression of results

10.1 Calculation of colony count

10.1.1 If 80 % or more of the selected characteristic colonies (see 9.4.1) are confirmed as *C. perfringens,* the number of these micro-organisms present shall be taken to be the same as the number of *C. perfringens* given by the count made in 9.3.2, 9.3.3 and 9.3.5.

10.1.2 In all other cases, the number shall be calculated on the basis of the percentage of the count of characteristic colonies (9.3.2, 9.3.3 or 9.3.5) that were confirmed (in 9.4.1 and 9.4.2) as *C. perfringens.*

Example:

If the mean colony count was recorded as 75 in accordance with 9.3 and six of the 10 colonies (60 %) tested were confirmed as *C. perfringens,* the number of *C. perfringens* colonies should be reported as 75 × 0,60 = 45.

10.1.3 Retain only two significant figures for the expression of results, proceeding as follows:

a) if the number is less than 100, round it to the nearest multiple of 5;

b) if the number is greater than 100 and ends in 5, round it to the nearest multiple of 20;

c) if the number is greater than 100 and does not end in 5, round it to the nearest multiple of 10.

For estimating low numbers (see 9.3.4 and 9.3.5), take the average of the counts of confirmed characteristic colonies and round to the next highest whole number.

Multiply this value by the reciprocal of the corresponding dilution of the test sample to obtain the number of *Clostridium perfringens* per millilitre or per gram of product, as appropriate.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

2) For health reasons, it may be desirable to carry out this test under a fume hood.

10.2 Reporting of results

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

If there were no characteristic colonies on plates from the initial suspension (if the initial product is solid), the number of *Clostridium perfringens* per gram of product should be reported as fewer than 10.

If there were no characteristic colonies on plates from the test sample (if the initial product is liquid), the number of *C. perfringens* per millilitre of product should be reported as fewer than 1.

10.3 Precision of the count

10.3.1 Precision of the count of high numbers (between 15 and 150)

For statistical reasons, the confidence intervals of this method vary, in 95 % of cases, from ± 16 % to ± 52 %.[1)] In practice, even greater variation may be found, especially between results obtained by different workers.

10.3.2 Precision of the count of low numbers (less than 15)

Confidence intervals for estimated counts of low numbers (10.3) are given in the annex.

11 Test report

The test report shall show the method used, the temperature of incubation, and the results 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.

1) See COWELL and MORISETTI. *J. Sci. Fd. Agric.* **20**, 1969 : 573.