INTERNATIONAL STANDARD

ISO 4833-2

First edition
2013-09-01

Microbiology of the food chain — Horizontal method for the enumeration of microorganisms —

Part 2: Colony count at 30 °C by the surface plating technique

Microbiologie de la chaîne alimentaire — Méthode horizontale pour le dénombrement des micro-organismes —

Partie 2: Comptage des colonies à 30 °C par la technique d'ensemencement en surface

Reference number
ISO 4833-2:2013(E)

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Published in Switzerland

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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, www.iso.org/directives.

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

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This first edition, together with ISO 4833-1, cancels and replaces ISO 4833:2003.

ISO 4833 consists of the following parts, under the general title *Microbiology of the food chain — Horizontal method for the enumeration of microorganisms*:

— *Part 1: Colony count at 30 °C by the pour plate technique*

— *Part 2: Colony count at 30 °C by the surface plating technique*

Microbiology of the food chain — Horizontal method for the enumeration of microorganisms —

Part 2:
Colony count at 30 °C by the surface plating technique

1 Scope

This part of ISO 4833 specifies a horizontal method for enumeration of microorganisms that are able to grow and form colonies on the surface of a solid medium after aerobic incubation at 30 °C. The method is applicable to:

a) products intended for human consumption or for animal feed;

b) environmental samples in the area of food and feed production and food handling.

This part of ISO 4833 is applicable to:

1) products containing heat-sensitive organisms that are likely to form a significant proportion of the total flora (e.g. psychrotrophic organisms in chilled and frozen foods, dried foods, other foods that may contain heat-sensitive organisms);

2) products containing obligately aerobic bacteria that are likely to form a significant proportion of the total flora (e.g. *Pseudomonas* spp.);

3) products that contain small particles that can prove difficult to distinguish from colonies in a pour plate;

4) products whose intense colour prevents the recognition of colonies in a pour plate;

5) products for which distinction between different types of colony is required as part of the assessment of food quality.

In addition to the manual spread plating technique, this part of ISO 4833 also specifies the use of a spiral plater, a rapid method of performing surface colony counts (Annex A).

The applicability of this part of ISO 4833 to the examination of certain fermented food and animal feeds is limited and other media or incubation conditions can be more appropriate. However, this method can be applied to such products even though it is possible that the predominant microorganisms in these products are not detected effectively.

For some matrices, the method described in this part of ISO 4833 can give different results to those obtained using the method described in ISO 4833-1.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 food and animal feeding stuffs — 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.

3.1
microorganism
entity of microscopic size, encompassing bacteria, fungi, protozoa and viruses

[SOURCE: ISO/TS 11139:2006,[3] 2.26]

Note 1 to entry: For the purposes of this part of ISO 4833, microorganisms are bacteria, yeasts and moulds that are able to produce colonies under the conditions specified in this part of ISO 4833.

4 Principle

A specified quantity of the test sample, or a specified quantity of an initial suspension in the case of other products, is surface plated on a solid agar culture medium contained in Petri dishes.

Other plates are prepared under the same conditions using decimal dilutions of the test sample or of the initial suspension.

The plates are incubated under aerobic conditions at 30 °C for 72 h.

The number of microorganisms per gram of sample or the number of microorganisms per millilitre of sample is calculated from the number of colonies obtained on the plates containing fewer than 300 colonies.

5 Culture media and diluents

5.1 General

Follow ISO 11133 for the preparation, production and performance testing of culture media.

5.2 Diluents

Use the diluent(s) specified in ISO 6887 for the product concerned or the specific International Standard dealing with the product under examination.

5.3 Agar medium: plate count agar (PCA)

5.3.1 Composition

Enzymatic digest of animal tissues	5,0 g
Yeast extract	2,5 g
Glucose, anhydrous ($C_6H_{12}O_6$)	1,0 g
Agar[a]	9 g to 18 g
Water	1 000 ml

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

When dairy products are examined, add skimmed milk powder at 1,0 g/l of the culture medium. The skimmed milk powder shall be free from inhibitory substances.

5.3.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Mix thoroughly and leave to stand for several minutes.

Adjust the pH (6.5), if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium into flasks or bottles (6.9) of suitable capacity. Sterilize in an autoclave (6.1) at 121 °C for 15 min.

If the medium is to be used immediately, cool it in a water bath (6.4) maintained at 47 °C to 50 °C before use. If not, allow the medium to solidify in the flask or bottle. Before use, melt the medium completely in a boiling water bath, then cool it in the water bath (6.4) maintained at 47 °C to 50 °C.

5.3.3 Preparation of agar plates

Pour 15 ml to 20 ml of the medium into sterile Petri dishes (6.6) and allow to solidify.

The plates may be stored at (5 ± 3) °C for up to 4 weeks.

Immediately before use, the agar plates should be dried in accordance with ISO 11133.

5.3.4 Performance testing of the culture medium

5.3.4.1 General

Plate count agar is a non-selective medium, used in this part of ISO 4833 as a pre-poured plate for surface inoculation. Productivity shall be tested according to ISO 11133.

5.3.4.2 Productivity

Incubation	(30 ± 1) °C for (72 ± 3) h
Control strains	*Escherichia coli* WDCM 00013 or WDCM 00012[a] [World Data Centre for Microorganisms (WDCM)]
	Bacillus subtilis subsp. *spizizenii* WDCM 00003[a]
	Staphylococcus aureus WDCM 00032 or WDCM 00034
Reference medium	Tryptone soya agar
Control method	Quantitative
Criterion	Productivity ratio (PR) ≥0,7

[a] The strains to be used by the user laboratory (minimum). See Reference [15] for information on culture collection strain numbers and contact details.

6 Apparatus

Disposable apparatus is an acceptable alternative to re-usable glassware and plastic if it has suitable specifications.

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

6.1 **Oven** for dry sterilization or **autoclave** for wet sterilization, used in accordance with ISO 7218.

6.2 **Drying cabinet** or **incubator**, ventilated by convection, for drying plates, capable of being maintained between 37 °C and 55 °C, or **laminar flow cabinet**.

6.3 **Incubator,** capable of being maintained at (30 ± 1) °C.

6.4 **Water baths**, capable of being maintained at 47 °C to 50 °C, and another capable of maintaining water at boiling point.

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

6.6 **Petri dishes**, made of glass or plastic, of diameter 90 mm to 100 mm, or140 mm.

6.7 **Total delivery graduated pipettes**, sterile, of nominal capacities 0,1 ml and 1 ml, ISO 835[1] class A, or automatic pipettes, ISO 8655-2,[2] with use of sterile tips.

6.8 **Colony-counting equipment** (optional), consisting of an illuminated base and, optionally, a mechanical or electronic digital counter.

6.9 **Bottles** or **flasks**, of appropriate capacity, for preparation, sterilization and, if necessary, storage of culture media.

6.10 **Spreaders,** made of glass, plastic or steel, sterile, for spreading the inoculum on the surface of the culture medium.

7 Sampling

Sampling is not part of the method specified in this part of ISO 4833. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Follow the specifications of ISO 6887 or the specific International Standard appropriate to the product concerned.

9.2 Inoculation and incubation

9.2.1 Transfer, using a sterile pipette (6.7), 0,1 ml of the test sample, if the product is liquid, or of the initial suspension in the case of other products, to the centre of each of two agar plates (5.3). If plates from more than one dilution are prepared, this may be reduced to one agar plate (see ISO 7218).

If, for certain products, it is desirable to count low numbers of organisms, the limits of detection can be raised by a factor of 10 by inoculating 1,0 ml of the test sample, if liquid, or 1.0 ml of the initial suspension for other products, either on the surface of one large agar plate (140 mm) or on the surface of three small agar plates (90 mm). In both cases, prepare duplicates by using two large plates or six small ones.

9.2.2 Take one other agar plate (5.3). Use another sterile pipette (6.7) to dispense 0.1 ml of the 10^{-1} dilution (liquid products) or 0,1 ml of the 10^{-2} dilution (other products).

9.2.3 If necessary, repeat the procedure using further decimal dilutions, using a new sterile pipette for each dilution.

9.2.4 Carefully spread the inoculum uniformly and as quickly as possible over the surface of the agar plate, without touching the sides of the dish with the spreader (6.10). It is possible to use the same spreader for all dilutions from one sample beginning with the highest dilution and progressing in order to the dilution having the greatest amount of test material.

Leave the plates with the lids on for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.

9.2.5 Invert the prepared plates and place them in the incubator (6.3) set at 30 °C, in accordance with ISO 7218. Incubate for (72 ± 3) h.

NOTE For inoculation using a spiral plater, see Annex A.

9.3 Counting of colonies

9.3.1 After the specified incubation period (see 9.2.3), select the agar plates with, if possible, fewer than 300 colonies. Count all colonies using the colony-counting equipment (6.8) if necessary. It is important that pinpoint colonies be included in the count; however, it is essential that the operator avoid mistaking particles of food for pinpoint colonies.

9.3.2 Spreading colonies shall be considered as single colonies. If spreading colonies are expected, examine the plates after 24 h or 48 h and mark the colonies visible. If less than one-quarter of the plate is overgrown by spreading colonies, count the colonies on the unaffected part of the plate and calculate the corresponding number for the entire plate. If more than one-quarter of the plate is overgrown by spreading colonies, discard the count for that plate. If all plates are affected by spreading colonies, count the most suitable plates and note in the test report that the result may be affected by spreading colonies.

10 Expression of results

Follow the procedure specified in ISO 7218.

11 Test report

The test report shall contain at least the following information:

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 part of ISO 4833 (ISO 4833-2:2013);

d) all operating details not specified in this part of ISO 4833, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained.

Annex A
(normative)

Surface colony count using a spiral plater

A.1 General

This annex specifies a method for the enumeration of microorganisms present in foods, animal feeds, and environmental samples using a spiral plater and counting the colonies growing on a solid medium after aerobic incubation (for a definition of microorganism, see 3.1).

A.2 Principle

The liquid sample or a suspension in the case of other products is deposited continuously on the surface of a rotating agar plate in the form of an Archimedes spiral.

The volume dispensed is decreased while the dispensing system (stylus or disposable sterile micro-syringe) moves from the centre to the edge of the plate, so that an exponential relationship exists between the volume deposited and the radius of the spiral.

On incubation, colonies develop along the lines where the liquid was deposited on the agar. A counting grid is calibrated for the sample volume deposited on different areas of agar.

The number of colonies in a known area is counted and calculated to give the count per millilitre or the count per gram. Alternatively, the counting can be done using an automated system.

A.3 Culture media and diluent

See Clause 5.

The following solutions are used for cleaning and decontamination of the stylus. They are not required for spiral platers using disposable micro-syringes.

A.3.1 Sterile water. If the food or feed is likely to contain fatty material, 1 % by volume polysorbate 80 can be included.

A.3.2 Sodium hypochlorite solution, 5 % available chlorine.

A.4 Apparatus

Usual microbiological apparatus — see Clause 6.

A.4.1 Spiral plater, adjusted to deliver the desired total sample volume of, for example, 0,05 ml, 0,1 ml, 0,2 ml or 0,4 ml per plate and usually including a vacuum trap to control loading and dispensing of samples, disposal of residual sample, and disinfection and rinsing of system. The residual pressure required is 24 kPa to 35 kPa (160 mmHg to 260 mmHg).

A.4.2 Colony-counting equipment, with calibrated grid, relating the deposited sample volume to specific areas of agar. Alternatively, several automated counting systems are available.

A.4.3 Disposable sterile 5 ml sample beakers. Some newer models use different sized containers, particularly for disinfection and rinsing.

A.4.4 Disposable sterile microsyringes (optional).

A.4.5 Prepared agar plates, prepared as in 5.3. It is particularly important that the plates contain a sufficient and constant depth of agar, and that the agar surface is level. Label the plates on the side of the dish.

A.5 Sampling

See Clause 7.

A.6 Preparation of test sample

See Clause 8.

A.7 Procedure

A.7.1 Test portion and dilutions

See 9.1.

In general, fewer or no dilutions are needed when using the spiral plate technique. Transfer, using a sterile pipette, 3 ml to 5 ml of the sample homogenate to a sterile disposable 5 ml beaker (A.4.3).

If necessary, allow the sample homogenate to settle for a few minutes before removing the portion of the supernatant liquid for spiral plating, as the presence of particles may block the tubing. The use of sterile plastic bags with built-in filters is recommended for preparation of the initial suspension of non-liquid samples if blockages are frequent.

A.7.2 Setting up

See ISO 7218.

Set up, and if necessary adjust, the instrument in accordance with the manufacturer's instructions, in particular checking that:

a) for mechanically operated machines, the cam follower arm rests on the cam at the correct height, so that the correct volume is dispensed;

b) the labelled plate is centred on the turntable;

c) the stylus tip or micro-syringe is at the correct angle to the agar surface as defined by the manufacturer;

d) the stylus starts and picks up at the correct points — for electronic apparatus, only the start point needs to be checked.

Repeat these checks if, during instrument operation, the stylus becomes damaged or misaligned (indicated by uneven deposition or misalignment of the stylus tip with the start point mark on the instrument turntable).

A.7.3 Inoculation

A.7.3.1 General

The following applies to manually operated models — newer models are semi-automated, and should be operated according to the manufacturer's instructions.

Fill one disposable beaker (A.4.3) with sodium hypochlorite solution (A.3.2), a second with sterile water and a third with sample. Clean the stylus tip before use, and disinfect the stylus between plating each sample, by rinsing for 1 s with the sodium hypochlorite and then for 1 s with sterile water.

After rinsing, lower the stylus into the sample and open the vacuum filling valve. Draw the sample through the stylus tip until a continuous column of liquid is present in the tube above the vacuum filling valve. With the stylus tip still below the level of the liquid, close the vacuum valve. Raise the stylus tip and rotate the sample holder out of the way. Place the base of a pre-poured agar plate, labelled on its side, on the turntable and lower the stylus until the tip rests freely on the agar surface. Start the motor and allow the turntable to rotate until the stylus is lifted and the apparatus stops automatically. Replace the lid and remove the plate from the turntable.

After each sample has been tested, rinse the apparatus with the hypochlorite solution and water as described previously. When not in use, leave filled with water.

If more than one dilution of the sample is to be plated, start with the highest dilution.

Leave the plates with the lids on for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.

A.7.3.2 Sterility check

Check the sterility of the spiral plater by plating sterile water before and after each series of samples examined.

A.7.4 Incubation

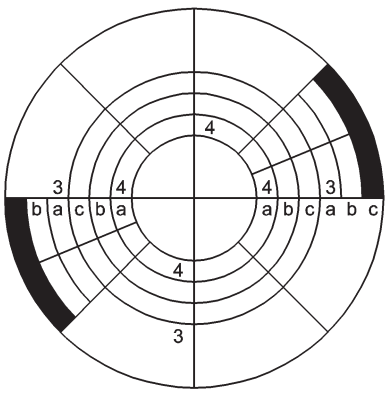
See 9.2.3.

A.7.5 Counting of colonies

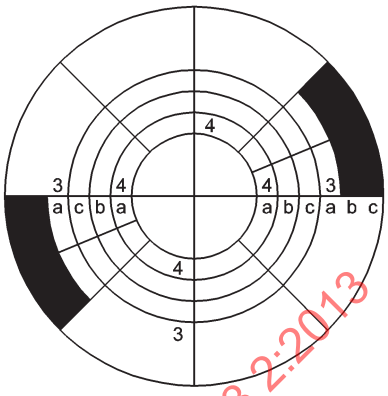
A.7.5.1 Counting grid

Two counting grids are available according to the size of the dish used. Alternatively, the counting grid is a transparent disc 150 mm in diameter but for counting 90 mm plates only the inner part of the circle with diameter 90 mm is used. Use counting grids provided with the apparatus and according to

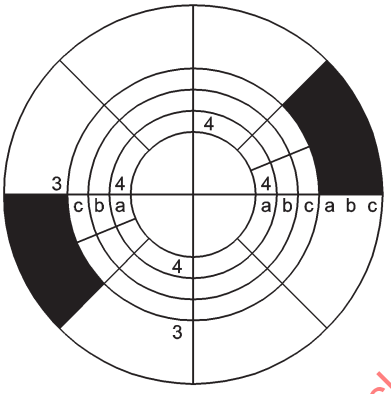
manufacturer's instructions. The grid is used to relate the colonies counted in an area of a spiral plate to the volume of inoculum spread on this area. See examples in Figure A.1.



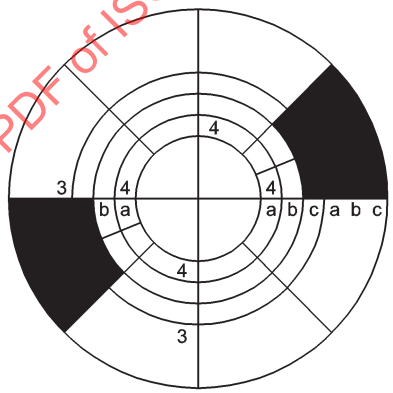
Area 3c — 0,000 5 ml



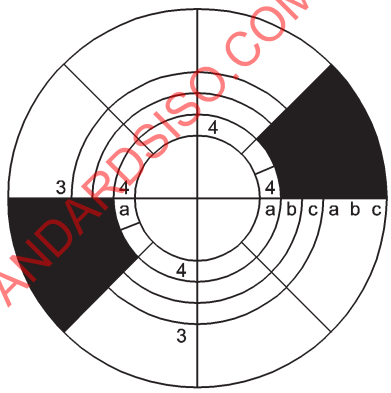
Area 3b — 0,001 5 ml



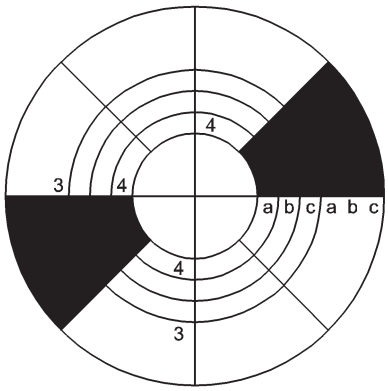
Area 3a — 0,002 5 ml



Area 4c — 0,003 8 ml



Area 4b — 0,006 0 ml



Area 4a — 0,009 0 ml

Figure A.1 — Counting areas

A.7.5.2 Calibration and verification

The volumes deposited on various segments of the grid are given in the operator's manual accompanying the spiral plater. For precise calibration, the grid area volumes-shall be checked by an expert.

To verify the volumes deposited in each segment, prepare 11 bacterial concentrations in the range 10^6 cells/ml to 10^3 cells/ml by making 1 + 1 dilutions of a non-spreading bacterial suspension. Plate all dilutions in duplicate as specified in 9.2 and by the spiral plater, using the same medium and incubator. After incubation, count the colonies. Calculate the volume deposited in the counted grid area, given by:

$$V = \frac{C_A}{C_{\text{ml}}}$$

where

V is the volume for a grid area (in ml);

C_A is the spiral colony count in that area;

C_{ml} is the standard colony count per millilitre.

Check the total volume dispensed by the spiral plater by weighing the amount dispensed on an analytical balance with a precision of ±2 mg.

A.7.5.3 Examination and reporting of spiral plate counts (manual method)

Centre the incubated plate over the grid. Choose any segment and count the colonies from the outward edge into the centre until 20 colonies have been counted.

Continue to count the remaining colonies contained in the area (i.e. segment or subdivision of segment) in which the 20th colony was observed. Record this count, together with the number of the area which included the 20th colony (e.g. 3c, 3b, 3a, 4c, 4b, 4a in Figure A.1). Count in the same area on the opposite side of the plate, and divide the total count of the two areas by the volume known to be deposited on the counted areas. This gives the count per millilitre.

If the total number of colonies counted exceeds 75 and the count in the area containing the 20th colony is completed, the count is generally low because of coincidence error associated with crowding of colonies. It is recommended that the count be made by counting adjacent annular segments round the circumference until a total of at least 50 colonies is counted. Calculate the count by dividing the counted colonies by the volume of the counted areas.

If less than 20 colonies are counted on the total plate, the confidence interval of the count obtained is wide. If the colony count exceeds 75 in the first area of a wedge, i.e. area 3c, record results as estimated at >300 000 colonies/ml.

A.7.5.4 Examination and reporting of spiral plate counts (using electronic colony counter)

Follow the manufacturer's instructions, but check manually (A.7.5.3), at least when first using the equipment or when examining a new food or feed.

A.8 Calculation and expression of results

Compute the spiral plate colony count. Report counts as the spiral plate count per millilitre or count per per gram as applicable.

A.9 Test report

The test report shall contain at least the following information:

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

b) the test method used, with reference to this annex (ISO 4833-2:2013, Annex A);

