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Pulp, paper and board — Microbiological examination —

Part 1: Enumeration of bacteria and bacterial spores based on disintegration

Pâtes, papiers et cartons — Analyse microbienne —

Partie 1: Dénombrement des bactéries et des spores bactériennes basé sur la désintégration

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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

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Contents

Page

Foreword

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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).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 6, *Paper, board and pulps*, Subcommittee SC 2, *Test methods and quality specifications for paper and board*.

This third edition cancels and replaces the second edition (ISO 8784-1:2005), which has been technically revised.

The second edition was applicable to yeast and mould, as well as bacteria. The following main changes have been made with respect to the previous edition:

— This third edition is only applicable to bacteria and bacterial spores, and no longer applicable to yeast and mould;

— incubation temperature changed from (37 °C ± 1 °C) to (32 °C ± 2 °C) (9.4);

— 2 parallel determinations are to be made (Clause 8 and Clause 9);

— the result can be reported "as received" in addition to reporting on a dry-mass basis (8.2, 11.1, and Clause 12).

ISO 8784 consists of the following parts, under the general title *Pulp, paper and board — Microbiological examination*:

— *Part 1: Enumeration of bacteria and bacterial spores based on disintegration*

Introduction

This part of ISO 8784, which deals with the microbiological examination of dry market pulp, paper, and paperboard, is broadly based on ISO 4833[1] although the conditions are not identical. However, it provides specific amplification where necessary. It is intended for the estimation of colony-forming units, CFU, aerobic bacteria, and bacterial spores.

Because of the exacting techniques required in aseptic procedures, reproducible good quality results can only be ensured by skilled microbiological technicians.

Pulp, paper and board — Microbiological examination —

Part 1: Enumeration of bacteria and bacterial spores based on disintegration

1 Scope

This part of ISO 8784 specifies a method for determining the total number of colony-forming units of bacteria and bacterial spores in dry market pulp, paper, and paperboard after disintegration. The enumeration relates to specific media.

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 186:2002, *Paper and board — Sampling to determine average quality*

ISO 7213:1981, *Pulps — Sampling for testing*

ISO 638:2008, *Paper, board and pulps — Determination of dry matter content — Oven-drying method*

3 Terms and definitions

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

3.1
bacteria
microscopic, single-celled organisms that possess a prokaryotic type of cell structure, which reproduce by fission and are able to grow under the test conditions specified in this part of ISO 8784

3.2
bacterial spores
highly resistant, dormant structures

EXAMPLE Endospores from certain genera of bacteria.

3.3
total bacterial count
number of colony-forming units (CFU) of bacteria and bacterial spores formed after incubation in a standard culture medium, under the test conditions specified in this part of ISO 8784

3.4
spore count
number of colony-forming units (CFU) of bacterial spores formed after incubation in a standard culture medium, under the test conditions specified in this part of ISO 8784

4 Principle

This poured plate method involves enumeration of colonies in a standard culture medium. A fibre suspension, prepared from paper, paperboard, or pulp samples, is plated in agar. Two parallel determinations are made. For enumeration of bacterial spores, the fibre suspension is heated for 10 min at 80 °C prior to plating. The plates are incubated at 32 °C for 48 h. The total numbers of bacteria or bacterial spores are enumerated by counting the colonies formed in the agar.

The mean value of 2 parallel determinations is calculated and the results are expressed as the number of CFU per gram of sample.

5 Culture media and diluents

5.1 General

All substrates and diluents shall be appropriately sterilized. When preparing the culture medium, make sure that the ingredients are completely dissolved by mixing while heating prior to dispensing into suitable containers for sterilization. See ISO 11133[2] for quality assurance and guidelines on preparation and production of culture media.

5.2 Water

When water is mentioned in a formula, use distilled water or purified water, see ISO 11133[2].

5.3 Culture media for total bacteria count and spore count

Culture medium shall be prepared as follows, or from commercially available dehydrated culture media according to the manufacturer's instructions. Ready-to-use medium may be used when its composition is comparable to that given in this part of ISO 8784. To test the performance of the medium, see ISO 11133[2].

Plate count agar (PCA) composition per litre:

Tryptone	5,0 g
Yeast Extract	2,5 g
Dextrose	1,0 g
Agar	15,0 g
Water	1 000 ml
Final pH	7,0 ± 0,2

If PCA is not available, Tryptone glucose extract (TGE) agar may be used (see A.3). The use of TGE as an alternative culture medium is acceptable if it gives comparable results as the standard culture medium. The culture medium used shall be stated in the test report (see Clause 12).

5.4 Diluents

Ringer's solution (see A.1) is preferred, although other isotonic solutions may be used. Ringer's tablets are commercially available.

To facilitate the release of cells from the fibres, it is recommended to add 20 µl of Tween 80 (see A.2) per litre to the Ringer's solution prior to sterilization by autoclaving.

The diluent used and if Tween 80 has been added, shall be stated in the test report (see Clause 12).

6 Apparatus and equipment

6.1 General

All laboratory equipment and parts of the equipment in direct contact with the sample and the diluent or the culture medium shall be sterilized.

NOTE For advice on standard microbiological equipment, see ISO 7218[4].

6.2 List of equipment

6.2.1 Use ordinary microbiological laboratory equipment, and the following.

6.2.2 Suitable wrapping material, e.g. aluminium foil (non-coated and inert), ready-to-use envelopes of different sizes or self-closing plastic bags, all of which are commercially available.

6.2.3 Disintegrator, high speed electrical blender with metal (preferably stainless steel) or glass cup that can be sterilized.

NOTE Other homogenizing system with equivalent efficiency may be used.

6.2.4 Incubator, capable of maintaining a constant temperature of 32 °C ± 2 °C.

6.2.5 Petri dishes, having a diameter of 90 mm (standard) or 140 mm to 150 mm (alternative).

6.2.6 Pipettes, of wide-mouth type suitable volume.

The width of the mouth must be large enough so that a 1 % fibre suspension can easily be drawn into the pipette tip.

NOTE A suitable volume is 10 ml or 50 ml.

6.2.7 Water bath, capable of maintaining a temperature of 80 °C ± 2 °C.

6.2.8 Colony-counting equipment or magnifying device, with a magnification between 1,5 × and 2,5 × shall be used.

NOTE The use of an additional lens might be necessary to increase the magnification, up to 10 ×, to facilitate the counting of pin-point bacterial colony-forming units and also to ensure that no other particles except bacterial colonies are counted (see Clause 10).

6.2.9 Balance, with an accuracy of 0,01 g.

6.2.10 Sterilizing unit, an autoclave capable of sterilization at 121 °C.

7 Sampling

Make sure that the sampling procedure is performed using aseptic techniques.

If the sample is to represent a lot of paper or paperboard, the sampling shall be in accordance with ISO 186:2002. From each unit of paper or paperboard to be sampled, cut several top layers and discard them to eliminate surface contamination. Use a sterile knife to cut through several layers of the paper or board sample, producing a stack of sheets. Discard the top sheet.

If the sample is to represent a lot of pulp, the sampling shall be in accordance with ISO 7213:1981. From each unit of dry market pulp to be sampled, discard several top sheets from each bale to eliminate surface contamination.

In other cases, sample a sufficient number of units so that the test material is representative of the paper, the paperboard, or the dry market pulp to be tested. In all sampling and examination procedures, make sure that the test material taken is representative of the sample received.

Ideally, a sample should contain at least four sheets, each of them having a minimum size of 200 mm × 250 mm of dry market pulp, paper, or paperboard (at least 2 sheets for testing and 2 protective sheets).

NOTE For paperboard or thicker material, it might be sufficient to use only 1 sheet for each parallel determination. For thinner paper, more than 2 sheets can be used for each parallel determination.

After sampling, wrap the unexposed test material in suitable wrapping material (6.2.2).

8 Preparation of the test material

8.1 General

Preferably, conduct the procedure under aseptic conditions. A laminar flow hood is recommended for plating. Unwrap the test material under aseptic conditions. Remove the protective sheets on the top and bottom of the sample stack without touching the test sheets in the centre of the sample stack.

The procedure in 8.3 and 8.4 shall be repeated for the 2 parallel determinations.

8.2 Determination of dry-matter content

If the result is to be reported on a dry-mass basis, determine the dry-matter content of the test material, *X*, in accordance with ISO 638:2008.

If the result is to be reported on an "as received"-mass basis (not on a dry-mass basis), omit the determination of dry-matter content and report accordingly [see 11.1 and Clause 12 j)].

8.3 Weighing

Two parallel determinations are made (8.1).

Place a closed Petri dish (6.2.5) on the pan of the balance and determine its tare mass.

With sterile tweezers, hold the sheet or sheets along one edge in one hand, trim and discard the remaining edges with sterile scissors. Cut the sample material into small pieces. Weigh a sufficient amount of the test material (mass approximately 2 g to 3 g), *m*, into the Petri dish, to be able to prepare a fibre suspension having a concentration of 1 %.

NOTE 1 For practical reasons, it can be useful to cut a sufficient amount of small pieces to be able to repeat the test.

NOTE 2 In order to get a short disintegration time it might be useful to cut pieces that are smaller than 5 mm.

Transfer the test material aseptically to the disintegrator jar (6.2.3).

8.4 Disintegration

2 parallel determinations are made (8.1).

Use cooled diluent solution (5.4) to prevent overheating (increase of the suspension temperature above 45 °C) during disintegration. Ensure the sterility of the disintegrator jar (6.2.3) for each test material.

Disintegrate the test material (8.3) in diluent solution (5.4), of a volume, *V*, needed to obtain a 1 % fibre suspension (for 2,0 g use 200 ml, and for 3,0 g use 300 ml). Disintegrate until the suspension is free from fibre clumps.

Other homogenizing systems with equivalent efficiency may be used, and shall be stated in the test report. If it is difficult to obtain a fibre suspension free from fibre clumps by using a disintegrator, some other suitable equipment may be used in addition. If so, the equipment used shall be stated in the test report.

9 Determination of the total bacterial count and spore count

9.1 General

The procedure in 9.2, 9.3, and 9.4 shall be repeated for the 2 parallel determinations.

The procedure shall be carried out in aseptic conditions. The work area shall be cleaned with a suitable disinfectant. If available, a laminar flow hood is recommended for plating.

The procedures for the determination of the total bacterial count and spore count are similar, except that for the determination of bacterial spores the disintegrated test material should be heat-treated as described in 9.3.

After disintegration of the sample, add the fibre suspension to Petri dishes. When using the wide-mouth pipette tip, ensure that no fibre clumps remain in the pipette tip.

NOTE Bacteria and spores might be attached to fibres, and if an inhomogeneous fibre suspension is added to the Petri dishes, the colony counts can be incorrect.

9.2 Plating for total bacterial count

This procedure shall be repeated for the 2 parallel determinations (9.1).

9.2.1 Immediately after disintegration, with a sterile wide mouth pipette (6.2.6), distribute 10 ml, *v*, of the 1 % fibre suspension among 5 sterile standard Petri dishes (6.2.5), i.e. should be as close to 2 ml as possible per 90 mm plate. This will represent 0,1 g of the test material. Within less than 5 min, pour into each plate 15 ml to 20 ml of the selected culture medium (5.3) cooled to approximately 45 °C. Immediately after the addition, rotate the plate with agitation to obtain a uniform distribution of fibre throughout the culture medium. Avoid a swirling motion since the colonies will not be separated this way. It is important that all clumps are broken up, in order that the plates may be examined easily and more accurately. The detection limit is 10 CFU/g.

If the alternative Petri dishes (140 mm to 150 mm) are used, with a wide mouth pipette (6.2.6), distribute 50 ml, *v*, of the 1 % fibre suspension among 5 sterile Petri dishes (6.2.5), i.e. approximately 10 ml per plate. This will represent 0,5 g of test material. In this case, the detection limit is 2 CFU/g.

9.2.2 If a higher dilution is required, add 10 ml of the 1 % fibre suspension to 90 ml of the diluent solution (5.4). Shake the suspension vigorously and plate 10 ml of this suspension as described in 9.2.1. Repeat this procedure (tenfold dilution for each step) until an appropriate dilution is reached.

NOTE If higher counts are expected it might be helpful to distribute 10 ml of the 1 % fibre suspension among five alternative Petri dishes (140 mm to 150 mm) to facilitate counting the number of colony-forming units (CFU) of bacteria.

9.2.3 After plating, allow the agar to solidify at room temperature.

Check the sterility of the culture medium and the diluents by pouring control plates.

9.3 Plating for spore count

This procedure shall be repeated for the 2 parallel determinations (9.1).

9.3.1 For bacterial spores, heat approximately 100 ml of the disintegrated test material (1 % fibre suspension) for 10 min at 80 °C ± 2 °C in a water bath (6.2.7). After heating, cool the disintegrated test material to ≤ 45 °C in cold water and plate the fibre suspension as described in 9.2.1.

9.3.2 If a higher dilution is required, follow 9.2.2.

NOTE To ensure proper heating of the test material, it is advisable to determine the time required to heat the suspension to 80 °C before conducting the test. The precise time used depends on the volume of the suspension and the shape of the container.

9.4 Incubation

This procedure shall be repeated for the 2 parallel determinations (9.1).

After the agar has solidified, invert and store the Petri dishes in an incubator (6.2.4) at 32 °C ± 2 °C for 48 h ± 3 h.

10 Enumeration of the colonies

Before examining the incubated sample Petri dishes, record the absence of colonies on the control plates. If any of the control plates are contaminated, the whole procedure shall be repeated.

Count the number of colony-forming units (CFU) of bacteria using the magnifying device (6.2.8). Record the number of colonies found on each incubated Petri dish.

For statistical reasons, if possible, select Petri dishes with between 15 to 300 colonies for determination of total bacterial count. For undiluted samples, below 15 or fewer colonies are also acceptable.

If more than one third of a plate is covered with swarming bacteria, the plate shall be discarded. If more than two plates are discarded per parallel determination, the test procedure shall be repeated from 8.3.

If, after repeating the test, more than two plates are discarded per parallel determination, the overall result shall be reported as “uncountable due to swarming bacteria “.

NOTE 1 It can be possible to reduce problems with swarming bacteria by distributing the fibre suspension into 10 plates instead of 5 (9.2.1).

NOTE 2 Agglomerations of coating, bundles of fibres, stickies, or yeast colonies can be mistaken for bacterial colonies. Use suitable techniques to confirm that suspect colonies are microbial in nature (i.e. microscopic examination or using additional lens, see also NOTE in 6.2.8).

11 Calculation and report

11.1 Calculation

For total bacterial count and spore count, separately sum up the number of colonies, *n*, from the 5 Petri dishes plated for total bacterial count (9.2), respectively from the 5 Petri dishes plated for spore count (9.3).

Do this separately for each parallel determination.

Calculate the total bacterial count and the spore count, respectively, as N_{rec}, per gram (as received) of the sample, according to Formula (1):

$$N_{rec} = n \cdot V \cdot f / (v \cdot m) \quad (1)$$

where

N_{rec} is the total bacterial count, respectively the spore count, in CFU per gram (as received) of the sample;

n is the total number of colonies on the 5 respective Petri dishes, in CFU;

f is the dilution factor (if no dilution is performed, the factor is 1);

V is the volume of the disintegrated test material (8.4) in millilitres;

v is the volume of the plated fibre suspension (here 10 ml or 50 ml), in millilitres;

m is the mass of the test material (8.3) used to prepare the fibre suspension, in grams.

If reporting on a dry-mass basis, calculate the total bacterial count and the spore count respectively, N_{dry}, per gram (as dry) of the sample, according to Formula (2):

$$N_{dry} = N_{rec} \cdot 100 / X \quad (2)$$

where

N_{dry} is the total bacterial count and the spore count respectively, in CFU per gram (as dry) of the sample;

X is the dry-matter content of the sample as a percentage (determined in 8.2).

Calculate the value for the total bacterial count and the spore count, respectively for each parallel determination.

EXAMPLE 3 g of test material was disintegrated in 300 ml of Ringer's solution and the dry-matter content of the sample was 92 %. The suspension was plated without any further dilution, i.e. $f = 1$.

Colony counts of 5 Petri dishes, each inoculated with 2 ml of the fibre suspension (10 ml in total), gave the following result:

79 86 82 89 67 Sum = 403

N_{rec} = (403 · 300 · 1) / (10 · 3) = 4 030 [according to Formula (1)]

which shall be reported as 4,0 × 10^3 CFU per gram (as received) of the sample.

N_{dry} = 4 030 · 100/92 = 4 380 [according to Formula (2)]

which shall be reported as 4,4 × 10^3 CFU per gram (dry weight) of the sample.

NOTE For estimation of small numbers, see ISO 4833[1].

11.2 Interpretation

When no colonies are observed, the result is reported as follows:

— standard Petri dishes (90 mm) and 10 ml suspension: < 10 CFU/g;

— alternative Petri dishes (140 mm to 150 mm) and 50 ml suspension: < 2 CFU/g.

11.3 Report

Calculate the mean value of the 2 parallel determinations for the total bacterial count and the spore count separately.

Report the results, as the total bacterial count (CFU per gram of sample) and the spore count (CFU per gram of sample) separately.

For CFU values up to 999, express the result as a whole number and round off the result to the nearest multiple of five.

For CFU values from 1 000, express the result preferably as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see EXAMPLE in 11.1).

12 Test report

The test report shall include the following information:

a) a reference to this part of ISO 8784(i.e. ISO 8784-1);

b) identification of the sample, unit, and/or lot tested;

c) date and place of testing;

d) description of the disintegration equipment used, if other than stated in (6.2.3);

e) the culture medium (5.3) used;

f) the diluent used and if Tween 80 has been added to the diluent (5.4);

g) size of Petri dish (6.2.5) used;

h) if determined, the total bacterial count (CFU/g) as stated in 11.3;

i) if determined, the total spore count (CFU/g) as stated in 11.3;

j) if the results are reported on a dry mass basis or “as received” (8.2);

k) if more than two plates are discarded after repeating the test, this shall be reported as “uncountable due to swarming bacteria” (Clause 10);

l) any deviation from the procedure specified in this part of ISO 8784 and any other circumstances which may have affected the results.

Annex A
(informative)

Dilution fluid

A.1 Ringer's solution

Ringer's solution ¼ strength

Composition per litre

Sodium chloride (NaCl)	2,250 g
Potassium chloride (KCl)	0,105 g
Calcium chloride ($CaCl_2$) 6 H_2O	0,120 g
Sodium hydrogen carbonate ($NaHCO_3$)	0,050 g
Water	1 000 ml

Preparation

Dissolve the salts in water and dispense into appropriate containers. Sterilize the solution in the autoclave for 15 min at 121 °C.

NOTE Ringer's tablets are commercially available.

A.2 Non-ionic surfactant

Poly(oxyethylene)-sorbitan monooleate (Tween 80).

A.3 Tryptone glucose extract

Composition per litre

Tryptone (Peptone from Casein, pancr.)	5,0 g
Beef extract	3,0 g
Dextrose	1,0 g
Agar	15,0 g
Water	1 000 ml
Final pH	7,0 ± 0,2

Annex B
(informative)

Precision

B.1 General

Recommendations for reporting and presenting precision data in International Standards developed by ISO/TC 6 and its subcommittees were established in Berlin in 2009.

The recommendations are applicable to test methods for quality control of pulp, paper, and paperboard, where the results are in the form of numerical values that can be statistically treated and both repeatability (standard deviation, s_r, and limit, r) and reproducibility (standard deviation s_R and limit, R) data are to be reported in an informative Annex. The main purpose of the interlaboratory tests and to establish precision data is to get a validation of the test method before it is published as an International Standard.

However, areas were identified where it was considered more difficult to validate the results; the subjective counting in dirt counting and the microbiological examinations. Also the fact that, e.g. micro-organisms are not evenly spread in the product can be the reason for poor precision data.

B.2 Interlaboratory test

In 2013, an interlaboratory test was carried out in order to validate the method. Eight laboratories tested six samples. There was one outlier. The values reported are the mean values of two parallel tests per sample. The data are presented in Tables B.1 to B.4. The one outlier is not included in Tables B.1 to B.4.

Several uncertainty sources might have a role in the reproducibility standard deviations obtained, such as those linked to the sample (spread of bacteria and spores in the material can be uneven), the sub-sampling of the test portion, the initial dilution, and those linked to the reproducibility conditions (operator/time).

NOTE It is also common to report microbiological results in $\log_{10}$ (CFU/g) values, but this is not according to this part of ISO 8784.

Table B.1 — Total bacterial count (CFU/g)

Samples	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Mean	Standard deviation	Coefficient of variation C_V %	Enlarged uncertainty[a]
Carton board, virgin fibre	590	$1{,}9 \cdot 10^3$	$1{,}6 \cdot 10^3$	500	705	$2{,}3 \cdot 10^3$	$1{,}0 \cdot 10^3$	$1{,}2 \cdot 10^3$	705	55	$1{,}4 \cdot 10^3$
Liquid packaging board, virgin fibre	160	245	250	125	110	235	130	180	60	35	125
White kraft top liner, virgin fibre	490	580	350	595	285	825	515	520	175	35	355
White kraft top liner, virgin fibre	$1{,}2 \cdot 10^3$	$2{,}0 \cdot 10^3$	$2{,}5 \cdot 10^3$	$1{,}0 \cdot 10^3$	$2{,}0 \cdot 10^3$	$2{,}9 \cdot 10^3$	$2{,}0 \cdot 10^3$	$2{,}0 \cdot 10^3$	640	35	$1{,}3 \cdot 10^3$
Napkins, virgin fibre	120	145	25	90	95	250	95	115	70	60	140
Hand towel, recycled fibre	70	40	30	35	55	90	25	50	20	45	45

[a] According to ISO/TS 19036.

Table B.2 — Total bacterial count (CFU/g of dry matter)

Samples	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Mean	Standard deviation	Coefficient of variation C_V %	Enlarged uncertainty[a]
Carton board, virgin fibres	630	$2{,}0 \cdot 10^3$	$1{,}7 \cdot 10^3$	530	750	$2{,}4 \cdot 10^3$	$1{,}1 \cdot 10^3$	$1{,}3 \cdot 10^3$	750	55	$1{,}5 \cdot 10^3$
Liquid packaging board, virgin fibre	170	260	265	130	115	255	140	190	65	35	130
White kraft top liner, virgin fibre	515	615	370	630	305	880	550	550	190	35	375
White kraft top liner, virgin fibre	$1{,}3 \cdot 10^3$	$2{,}1 \cdot 10^3$	$2{,}6 \cdot 10^3$	$1{,}1 \cdot 10^3$	$2{,}1 \cdot 10^3$	$3{,}0 \cdot 10^3$	$2{,}1 \cdot 10^3$	$2{,}1 \cdot 10^3$	680	35	$1{,}4 \cdot 10^3$

[a] According to ISO/TS 19036.