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Paper and board — Determination of microbiological properties —

Part 1:
Total bacterial count

Papier et carton — Détermination des propriétés microbiologiques —

Partie 1: Dénombrement bactériologique total

Reference number
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Foreword

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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.

International Standard ISO 8784-1 was prepared by Technical Committee ISO/TC 6, *Paper, board and pulps*.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

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Paper and board — Determination of microbiological properties —

Part 1: Total bacterial count

0 Introduction

This part of ISO 8784 for the bacterial examination of paper and board is broadly based on ISO 4833 and provides specific amplification where necessary. It is intended to deal with the estimation of the total bacterial content without any attempt to isolate species of particular public health significance.

Because of the exacting techniques required in bacteriological procedures, reproducible results can be secured only by a well-trained technician. In addition, health risks may arise from employment of inadequately trained staff.

1 Scope and field of application

This part of ISO 8784 specifies a method of determining the total bacterial population in paper and board (i.e. all bacteria both within and on the surface of the sheet). It is applicable to most kinds of paper and board, especially food grades.

The method is not suitable for materials such as vegetable parchment or wet-strength papers which cannot be readily disintegrated.

2 References

ISO 186, *Paper and board — Sampling to determine average quality.*

ISO 4833, *Microbiology — General guidance for enumeration of micro-organisms — Colony count technique at* 30 °C.

3 Definition

For the purpose of this International Standard, the following definition applies.

total bacterial count: The total number of bacterial colonies formed in a standard culture medium after incubation under the conditions specified.

4 Principle

Preparation of culture plates from specified dilutions of a paper or board dispersion on a specified culture medium. Aerobic incubation of the culture plates for 72 h at 30 °C and calculation of the number of bacteria per gram of sample from the number of colonies counted on selected plates and from the dilution factor.

5 Materials

5.1 Alcohol: ethanol, methanol or iso-propanol, for sterilizing instruments.

5.2 Plugs, of non-absorbent cotton, or disposable plugs.

5.3 Nutrient substrate: tryptone glucose extract agar shall be used for bacterial counts. This culture medium is available in dehydrated form. The formula is given in the annex.

If tryptone glucose extract agar is not available, plate count agar or other suitable culture medium may be substituted. The use of an alternative nutrient agar shall be stated in the test report.

5.4 Dilution fluid: Ringer's solution is preferred; however, other isotonic solutions may be used.

The selection of a dilution fluid other than Ringer's solution shall be stated in the test report. Different dilution fluids shall not be used in the same test. The formula for Ringer's solution is given in the annex.

6 Apparatus and glassware

Usual microbiological laboratory equipment, and

6.1 Balance, accurate to 0,1 g, with a pan large enough to hold a Petri dish.

6.2 Colony-counting equipment, consisting of an illuminated base with a dark background, fitted with a lens having a magnifying power of at least 1,5 times. The use of an additional hand lens in conjunction with the lens on the colony counter may be necessary to increase the magnification to 8 to 10 times to facilitate the counting of pin-point bacterial colony-forming units.

6.3 Disintegrators, with metal or glass jars of about 500 ml capacity, fitted with a high-speed impeller near the bottom and fitted with a cap or lid; or other suitable disintegrator which ensures disintegration of the paper or board. Place a paper or aluminium foil hood over the cap of each disintegrator jar prior to sterilization.

6.4 Incubator, capable of being controlled at a temperature of 30 ± 1 °C.

6.5 pH meter.

6.6 Autoclave for steam sterilization, capable of operation at 120 °C and 100 kPa*.

6.7 Hot-air oven, with thermometer, capable of maintaining 165 ± 2 °C for 3 h.

6.8 Flaming equipment (alcohol lamp or Bunsen burner).

6.9 Knife, preferably a surgical knife with a disposable blade, to cut the paper or board.

6.10 Bottles for dilution: 250 ml, narrow-mouthed, glass or plastic bottles fitted with screw-caps or with Escher rubber stoppers.

6.11 Containers for dry samples, of a type that can be readily sterilized.

NOTE — Plastics or glass containers are suitable; however, paper envelopes are most convenient. Use two envelopes, one placed inside the other. The larger one may be as large as 230 mm × 300 mm and the smaller one may be as large as 160 mm × 240 mm. The envelopes should be made from heavy kraft paper capable of withstanding sterilization in a hot-air oven without undue embrittlement or the generation of deleterious by-products. After sterilization, seal the flap on the outer envelope with an adhesive or pressure-sensitive tape.

6.12 Flasks: Erlenmeyer flasks or screw-cap bottles for holding sterile media.

6.13 Petri dishes: dimensions of 100 mm × 15 mm are recommended.

6.14 Pipettes, pipette bulb (aspirator) and **containers.** Graduated 10 ml Mohr pipettes. The 10 ml Mohr pipette, with the tip cut off to give a 3 mm opening at the delivery end, is best suited for measuring fibre suspensions. 10 ml serological pipettes are also satisfactory. Special Mohr pipettes calibrated from the large end are available and preferable to the usual Mohr pipette. Enclose them in metal boxes or wrap them in a heavy kraft wrapper for sterilization.

Suitable sized syringes with single-use pipettes are also suitable.

All pipettes should be plugged with cotton at the mouth end prior to sterilization.

6.15 Scissors, preferably with a 100 mm to 160 mm cutting edge.

6.16 Tongs, suitable for handling samples of paper or board.

* 100 kPa = 1 bar

7 Sterilization of equipment and media

Depending upon the nature of the equipment to be sterilized, use one of the following three methods.

7.1 Steam heat (autoclave)

Sterilize the following for 20 min at 120 °C and 100 kPa:

a) disintegrator jar assemblies (6.3);

b) culture media (5.3);

c) sample bottles;

d) dilution fluid (5.4);

e) tongs (6.16);

f) scissors (6.15).

7.2 Dry heat

Sterilize the following by heating for 3 h at a temperature of 165 ± 2 °C:

a) kraft envelopes (6.11);

b) pipettes (6.14);

c) knives (6.9).

NOTE — Pipettes should be completely dry before heating. Scorching the paper envelopes or wrappers must be avoided.

7.3 Flaming

Immerse scissors, tongs, knives and similar instruments in alcohol (5.1). When needed for cutting or handling samples, remove from the alcohol, allow to drain for a few moments, then burn off excess alcohol (see 6.8).

8 Sampling

8.1 Sample a sufficient number of units so that the specimens are representative of the paper or board being tested. If applicable, follow the sampling frequency given in ISO 186.

NOTE — A specimen should contain more than one sheet of paper or board.

8.2 Use either a single sterile envelope or two sterile envelopes, one within the other, for each specimen (see note to 6.11). From each unit cut away several top layers of the paper or board to be sampled and discard them to eliminate surface contamination. Use a sterile knife (6.9) and cut exposed areas parallel to the edge of the roll or bale and through several thicknesses, then make a second parallel cut. Then cut at right angles to the bottom of the first two parallel cuts. Cut off and discard the top sheet. Carefully open the sterilized envelope and slip the end of the exposed paper or board specimens into the envelope. Make a horizontal cut across the top of the flap.

about 200 mm from the bottom cut, and allow the specimens to slip into the inner envelope. Seal the outer envelope with an adhesive or pressure-sensitive tape. The specimens should be about 100 mm × 200 mm.

9 Preparation of the test pieces

Place a closed Petri dish (6.13) on the pan of the balance (6.1) and determine its tare mass. Cut the envelope holding the specimens (8.2) along the top flap with a sterile knife (6.9) or scissors (6.15). Open the envelope by pressing the sides, without touching the inner surface; remove the specimen with sterile tongs (6.16). Hold the edge of the specimens with the tongs in one hand, trim and discard the edges with sterile scissors. Make a series of cuts 10 to 20 mm apart, parallel to the side of the specimens. Partially remove the cover of the tared Petri dish, but retain the cover on the pan of the balance. Cut squares of paper or board directly into the Petri dish by making a series of cuts perpendicular to those made previously. Cut enough paper or board to give about 2,0 g of test pieces. Replace the cover of the Petri dish. Weigh the Petri dish plus test piece and from the tare difference calculate the test piece mass.

10 Procedure

10.1 Disintegration

Empty the 2,0 g of test pieces of paper or board into a sterile disintegrator jar (6.3) with 200 ml of sterile Ringer's solution (5.4) to give a fibre concentration of 1,0 %, and disintegrate until the suspension is free from fibre clumps. Use a separate sterile disintegrator jar for each test. When adding the paper, board or dilution fluid to the disintegrator jar, do not touch the metal cap with the hands, but lift it by grasping the paper hood placed over the cap before sterilization. Lift both the hood and the metal cap only enough to permit access to the jar. When many tests are run on the same disintegrator, cool the sterile Ringer's solution to prevent the temperature from exceeding 45 °C in the disintegrator jar. Allow the paper hood to remain on the cap at all times to prevent possible contamination through any opening in the cap or around the top of the jar.

10.2 Plating out and incubation

The room in which the test pieces are weighed and the plating out done shall be free of air currents and dust. About 30 min before plating, sponge the surface of the work table with a suitable disinfectant.

Plate out the suspension, immediately after disintegration (10.1). (See note 1.)

Distribute 10 ml of the suspension, corresponding to 0,1 g of paper or board, with a sterile 10 ml wide-mouth pipette (6.14), in approximately equal portions among five sterile Petri dishes (6.13). Prepare and plate additional higher dilutions of the 1 % suspension if it is suspected that the specimen has a high bacterial population. The following dilutions are recommended for plating: 10^{-1}, 10^{-2} and 10^{-3} (see notes 2 and 3).

Within less than 5 min, add to each inoculated plate 15 to 20 ml of the selected culture medium (5.3) cooled to about 45 °C.

Immediately after the addition of the culture medium to the suspension in the Petri dish, agitate the plates individually to disintegrate clumps of fibres and to obtain an equal distribution of fibre throughout the culture medium. It is important that all lumps be broken up in order that the plates may be examined easily and more accurately. Pour one control plate from each container of culture medium to check on its sterility and air contamination.

Agitate the inoculated plates and place them on a level surface to harden, then invert and place in an incubator (6.4). Allow the plates to incubate at 30 ± 1 °C for 72 h.

NOTES

1 In some cases considerable foaming may occur, in which case it is necessary to allow the foam to clear from the suspension prior to plating. When such cases occur, the cap and hood must remain on the jar at all times to maintain sterility.

2 When higher dilutions of the disintegrated test pieces are required, for example a 10^{-1} dilution, add 10 ml of the 1 % suspension to 90 ml of sterile dilution fluid and distribute 10 ml of this suspension equally among five Petri dishes, which together will then contain 0,01 g of the paper or board. Higher dilutions can also be made from this suspension.

3 Adjust the dilution of the paper and board test pieces to give a bacterial count of 30 to 300 colonies per plate. It may not be possible to obtain the lower range for those paper or board samples that have a low bacterial content.

4 The amount of fibrous material distributed over a given area of plate surface has been found to be an important factor in the number of bacterial colonies observed in a given specimen of paper or board. It is therefore important to follow carefully directions regarding the fibre concentration, the volume used for plating and the number and size of plates used.

10.3 Calculation of results

Examine the incubated plates for the presence of bacterial colonies. Count the number of colonies with a suitable colony counter (6.2) and record the number and the dilution. Discard plates with more than 300 colonies.

11 Expression of results

Express the results as the number of bacterial colony-forming units per gram of paper or board. To obtain this value, multiply the number of colonies at each dilution by the dilution factor. For example, if the 1,0 % suspension is used without dilution, the amount of the original test pieces in five dishes is 0,1 g. If the total number of colonies counted is 22, the bacterial count is 22 × 10 = 220 colonies/g.

If the same number of colonies had been obtained with the 10^{-1} dilution, the result would be 2 200 colonies/g.

12 Precision

Microbiologists agree that there is an intrinsic error of about 10 % in normal plate counts. Since these methods employ plate counts this error is understood among microbiologists. A sample plated out at the same time by several competent microbiologists showed about 5 % deviation from each other.

13 Test report

The test report shall include the following particulars:

a) reference to this International Standard;

b) identification of the test pieces, unit and lot tested;

c) date and place of testing;

d) results expressed as number of bacterial colonies per gram;

e) the number of bacterial colonies in the control plate;

f) any deviation from the procedure specified in this part of ISO 8784.

Annex

Culture media and dilution fluid

(This annex forms an integral part of the Standard.)

A.1 Culture media

Tryptone glucose extract agar

Composition

Beef extract	3,0 g
Tryptone	5,0 g
Dextrose (*d*-glucose)	1,0 g
Agar	15,0 g
Distilled water	1 000 ml
pH	7,0

Peptone glucose agar

Composition

Peptone	5,0 g
Yeast extract	2,5 g
Dextrose (*d*-glucose)	1,0 g
Agar	14,0 g
Distilled water	1 000 ml
pH	7,0

Preparation

When the culture medium is prepared in the laboratory according to the above formula, make certain that the ingredients are completely dissolved prior to dispensing into suitable containers and sterilization.

NOTE — The tryptone glucose extract agar is available commercially in dehydrated form. When using the dehydrated medium, follow the instructions printed on the container.

A.2 Dilution fluid

Ringer's solution

Composition

Sodium chloride (NaCl)	2,500 g
Potassium chloride (KCl)	0,105 g
Calcium chloride ($CaCl_2$)	0,120 g
Sodium hydrogencarbonate ($NaHCO_3$)	0,050 g
Distilled or deionized water	1 000 ml

Preparation

Dissolve the salts in the water and dispense into appropriate containers. Sterilize the solution in the autoclave for 15 min at 120 °C and 100 kPa or carry out sterile filtration using appropriate membrane filters (pore size less than 0,5 µm).

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