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Flexible cellular polymeric materials — Determination of antibacterial effectiveness

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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 (see ~~www.iso.org/directives~~).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see ~~www.iso.org/patents~~).

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

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see ~~www.iso .org/iso/foreword .html~~.

This document was prepared by Technical Committee ISO/TC 45, *Rubber and rubber products*, Subcommittee SC 4, *Products (other than hoses)*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at ~~www.iso .org/members .html~~.

Introduction

Products with a label or marking tag of antibacterial treatment, such as kitchen sponge cleaners, mattresses, pillows and sofas, are available in markets worldwide. However, there is no common standard to evaluate the effectiveness of the antibacterial treatment. The material used for these products is usually a flexible cellular polymeric foam treated with antibacterial agents available in the markets. Because of the porosity of the material, efficient contact between a testing bacterial suspension and the material is critical in an evaluation of the effectiveness of antibacterial treatment. A specific procedure has been developed and adopted for this test method so that the test bacteria can efficiently make contact with the open cell surface of the flexible cellular polymeric test specimens. This document will help consumers to know whether these products have the appropriate quality of antibacterial effectiveness.

Flexible cellular polymeric materials — Determination of antibacterial effectiveness

WARNING — Persons using this document should be familiar with microbiology. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and to determine the applicability of any national regulatory conditions.

1 Scope

This document specifies a method of determining the antibacterial effectiveness of open-cell flexible cellular polymeric antibacterial treated materials, including their intermediate and final products.

This document is suitable for flexible cellular polymeric materials because the test procedure enables the test inoculum to efficiently contact with the surface of open cell in the flexible cellular polymeric materials.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1923, *Cellular plastics and rubbers — Determination of linear dimensions*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at ~~https://www.iso.org/obp~~

— IEC Electropedia: available at ~~http://www.electropedia.org/~~

3.1
surface of flexible cellular polymeric material
surface that is not only the outer peripheral surface but also the true surface of open-cell structure of flexible cellular polymeric material

3.2
antibacterial
condition suppressing the growth of bacteria on the surface of flexible cellular polymeric material

3.3
antibacterial agent
agent that inhibits the growth of bacteria on flexible cellular polymeric materials

3.4
antibacterial treatment
treatment with antibacterial agents

3.5
antibacterial treated material
flexible cellular polymeric material that is treated with antibacterial agents

3.6
antibacterial activity
difference in the logarithm of the viable cell counts found between an antibacterial-treated material and an untreated material after inoculation with bacteria and incubation

3.7
antibacterial effectiveness
ability of an antibacterial agent to inhibit the growth of bacteria on material treated with an antibacterial agent, as determined by the value of the antibacterial activity

4 Judgement criteria of antibacterial effectiveness

When the antibacterial activity value is not less than 2,0, the antibacterial effectiveness of antibacterial treated material is judged to be significant. An antibacterial activity value of more than 2,0 may be agreed between all interested parties.

5 Test methods

5.1 Bacteria to be used for the tests

Both of the following species of bacteria shall be used:

a) *Staphylococcus aureus*;

b) *Escherichia coli*.

The bacterial strains to be used are shown in ~~Table 1~~. If bacterial strains obtained from culture collections other than those shown in ~~Table 1~~ are used, they shall be obtained from a member agency of the World Federation for Culture Collections (WFCC) or the Japan Society for Microbial Resources and Systematics (JSMRS) and shall be the same strains as those shown in ~~Table 1~~. Prepare stock cultures of these species in accordance with the supplier's directions.

Table 1 — Bacterial strains to be used

Name	Strain	Culture collection
Staphylococcus aureus	ATCC 6538P	American Type Culture Collection
	FDA 209P	US Food and Drug Administration
	NBRC 12732	National Institute of Technology and Evaluation Collection des
	CIP 53.156	Bacteries del'Institut Pasteur Deutsche Sammlung von
	DSM 346	Mikroorganismen und Zellkulturen Gmbh National Collection of
	NCIB 8625	Industrial and Marine Bacteria Ltd
Escherichia coli	ATCC 8739	American Type Culture Collection
	NBRC 3972	National Institute of Technology and Evaluation Collection des
	CIP 53.126	Bacteries del'Institut Pasteur Deutsche Sammlung von
	DSM 1576	Mikroorganismen und Zellkulturen Gmbh National Collection of
	NCIB 8545	Industrial and Marine Bacteria Ltd

5.2 Reagents and materials

The following reagents and materials shall be used:

5.2.1 Water, an analytical grade for microbiological media preparation, which is freshly distilled, ion-exchanged, filtered with RO (reverse osmosis) or ultra-filtered, or a combination of these. It shall be free from all toxic or bacteria inhibitory substances.

5.2.2 Meat extract, for microbial test.

5.2.3 Peptone, for microbial test.

5.2.4 Sodium chloride, analytical grade, a grade appropriate for microbiological purposes or both.

5.2.5 Nonionic surfactant, polyoxyethylene sorbitan monooleate.

NOTE The generic name of polyoxyethylene sorbitan monooleate is polysorbate 80 (Tween 80^{R}).

5.2.6 Sodium hydroxide, analytical grade, a grade appropriate for microbiological purposes or both.

5.2.7 Hydrochloric acid, analytical grade, a grade appropriate for microbiological purposes or both.

5.2.8 Agar, analytical grade, a grade appropriate for microbiological purposes or both.

5.2.9 Yeast extract, for microbial test.

5.2.10 Trypton, for microbial test.

5.2.11 Glucose, for microbial test.

5.2.12 Potassium dihydrogen phosphate (KH_2PO_4), analytical grade, a grade appropriate for microbiological purposes or both.

5.3 Equipment and apparatus

The usual laboratory apparatus and, in particular, the following shall be used:

5.3.1 Inoculation loops, 4 mm in ring diameter, made of platinum.

5.3.2 Dry-heat sterilizer, capable of maintaining the temperature at a value between 160 °C and 180 °C.

5.3.3 Stopper, made of cotton, silicone, metal or molleton.

5.3.4 Autoclave, capable of maintaining a temperature of (121 ± 2) °C and a pressure of (103 ± 5) kPa.

5.3.5 Clean bench, for microbial test.

5.3.6 Biological safety cabinet, for antibacterial test.

5.3.7 Balance, capable of weighing to ± 0,01 g.

5.3.8 pH-meter, capable of measuring ± 0,2 units.

5.3.9 Incubator, capable of maintaining the temperature within ± 1 °C of the set point at equilibrium conditions.

5.3.10 Sterilized cup, with an outside diameter of 63 mm to 65 mm, a depth of 31 mm to 35 mm and an internal volume of 50 ml to 60 ml.

NOTE Sterilized cups with dimensions and volumes other than those specified can lead to different results.

5.3.11 Bacteria spreader, for microbial test and with a tip width of 20 mm or more.

5.3.12 Glass rod, with a diameter of approximately 20 mm and a flat tip.

5.3.13 Shaker with thermostatic chamber, capable of shaking at (150 ± 10) rpm with (30 ± 5) mm in amplitude of horizontal direction, and chamber with temperature control accuracy within ± 1 °C.

NOTE A mechanical shaker can be used in a thermostatic chamber.

5.3.14 Pipettes, having the most suitable volume for each use, with a tip made of glass or plastic and a tolerance of 0,5 % or less.

5.3.15 Petri dishes, made of glass, sterilized plastics or both, with an inner diameter of approximately 90 mm.

5.4 Sterilization methods

5.4.1 General

Glass and plastic apparatus are thoroughly washed with alkali or neutral detergent, rinsed thoroughly with water, dried and then sterilized. The method of sterilization is according to 5.4.2 or 5.4.3. In the case of flame sterilization of inoculation loops, follow 5.4.4.

The plastic apparatus shall have heat resistance capable of withstanding the sterilization treatment temperature, or sterile apparatus may be used. When sterile apparatus is used, another sterilization is not necessary.

5.4.2 Dry-heat sterilization

Place the apparatus to be sterilized in a dry-heat sterilizer, using the following minimum times for the given temperature:

Temperature	Minimum sterilization time
170 °C	60 min
160 °C	120 min

If the cotton stopper or wrapping paper of the apparatus to be sterilized gets wet with water after completion of dry-heat sterilization, the apparatus shall not be used.

5.4.3 High-pressure steam sterilization

Pour water into an autoclave and then place the objects to be sterilized in a wire mesh basket on a wire mesh shelf. After locking the lid of the autoclave, increase the temperature and maintain at a temperature of 121 °C and a pressure of 103 kPa for 15 min to 20 min. After sterilization, naturally cool down to 100 °C or lower, before removing the objects from the autoclave. If further cooling is necessary, use a clean bench or a biological safety cabinet. An autoclave should be cleaned with neutral detergent and rinsed with water to prevent contamination by medium and processing chemicals.

5.4.4 Flame sterilization

Flame the whole apparatus with gas or alcohol flames. In the case of an inoculation loop, flame it until it glows. In the case of test tubes, flame them for 2 s to 3 s.

5.5 Medium and buffer

5.5.1 General

As the medium and buffer solution, those having the following composition are used. Commercially available products can be used as long as they have the same composition.

5.5.2 1/500 nutrient broth medium (1/500 NB)

Add 5,0 g of meat extract, 10,0 g of peptone and 5,0 g of sodium chloride to 1 000 ml of water, mix and dissolve, and prepare nutrient broth medium. 800 ml of water is added to 2 ml of nutrient broth medium and 0,5 g of non-ionic surfactant that has been weighed, mixed and dissolved, and water is added to make the total volume 1 000 ml. Adjust the solution with sodium hydroxide solution or hydrochloric acid solution to pH 6,8 to 7,2 (25 °C) using a pH meter, sterilized by autoclaving, 1/500 nutrient broth medium (1/500 NB). If it is not used immediately after preparation, store it at a temperature of 5 °C to 10 °C. 1/500 NB that is one week old or more shall not be used.

5.5.3 Slant culture medium

Add 5,0 g of scaled meat extract, 10,0 g of peptone and 5,0 g of sodium chloride to 1 000 ml of water and mix them. Adjust the solution with sodium hydroxide solution or hydrochloric acid solution to pH 7,0 to 7,2 (25 °C) using a pH meter. Add 15,0 g of agar powder, dissolve it by heating. Pour 6 ml to 10 ml of the mixture into a test tube, close with a cotton stopper and sterilize by autoclaving. After sterilization, place the test tube at an angle of about 15 degrees with respect to the horizontal plane in a clean room and solidify the contents to make the slant culture medium. If it is not used immediately after preparation store it at a temperature of 5 °C to 10 °C. If the condensed water runs out, dissolve it, coagulate again and use it. Slant culture medium that is over one month old shall not be used.

5.5.4 Standard nutrient agar medium (SA medium)

2,5 g of weighed yeast extract, 5,0 g of tryptone and 1,0 g of glucose are added to 1 000 ml of water and mixed, and the mixture is adjusted with sodium hydroxide solution or hydrochloric acid solution to pH 7,0 to 7,2 (25 °C) using a pH meter. 15,0 g of the agar powder is added and dissolved by heating. Sterilize in autoclave with high-pressure steam. Use this as standard nutrient agar medium. If it is not used immediately after preparation, store it at a temperature of 5 °C to 10 °C. SA media that is over one month old shall not be used.

5.5.5 Phosphate-buffered physiological saline

Dissolve 34,0 g of potassium dihydrogen phosphate in 500 ml of water and mix, then adjust the solution with a sodium hydroxide solution to pH 6,8 to 7,2 (25 °C) using a pH meter. Dilute the mixture with water to 1 000 ml. Take 1,25 ml of this solution and dilute with sodium chloride aqueous solution with a mass fraction of 0,85 % (physiological saline) to 1 000 ml. If necessary, aliquot it into a test tube or Erlenmeyer flask, cotton plug it and sterilize by autoclaving to phosphate-buffered physiological saline. If it is not used immediately after preparation, store it at a temperature of 5 °C to 10 °C. Phosphate-buffered physiological saline that is more than one month old shall not be used.

5.6 Preservation of test strain

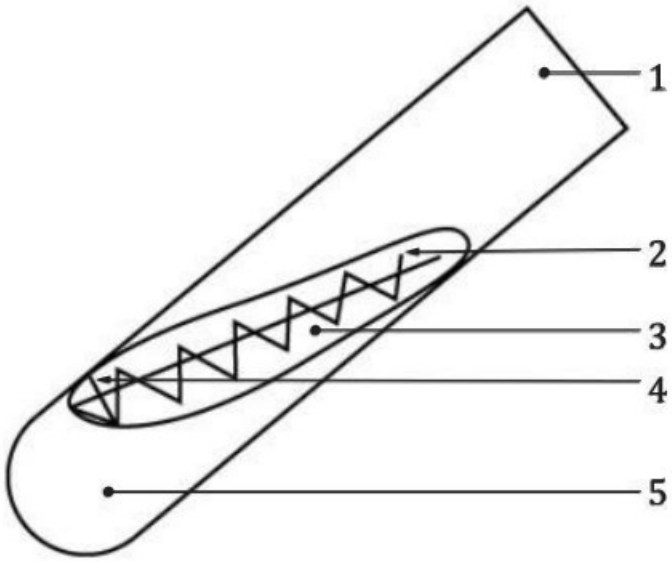
Inoculation of bacteria shall be carried out aseptically. Use a safety cabinet if necessary. Hold the test tube of the slant culture medium prepared in 5.5.3 and the container of original strain in one hand; hold an inoculation loop handle, by which the cotton stopper is pulled out to sterilize the mouth of the test

tube with flame, in the other hand. Sterilize the inoculation loop with flame and cool down the tip of the inoculation loop in a portion of the condensed water on the slant culture medium. Scrape a part of the original strain and smear it on the slant culture medium.

Smearing is done by placing the tip of the inoculation loop in condensed water and drawing a straight line and a zigzag line from the bottom to the upper side of the slope with an inoculation loop to disperse the bacteria (see ~~Figure 1~~).

Carry out flame sterilization of the mouth of the test tube of the inoculated slant culture medium with original strains, then insert a cotton stopper. The inoculation loop used shall be sterilized with flame. The inoculated slant culture medium is incubated at a temperature of (35 ± 1) °C for 24 h to 48 h in an incubator and thereafter stored at a temperature of 5 °C to 10 °C. The next inoculation is carried out in the same way within a month of the first inoculated slant culture medium. Slant culture medium that was inoculated more than one month previously shall not be used for the next inoculation. The number of passages is limited to five from the original strains that were distributed from the strain preservation agency.

This limited total number of passages is also applied to test strains stored for a long period of time in ways such as freeze-drying or freezing before the test. In this case, the number of passages cultured from the original strain shall be counted, and the total number of passages shall not exceed five passages from the original strains.



Key

1 test tube
2 straight and zigzag line in smearing
3 surface of the slant culture medium
4 condensed water pool
5 slant culture medium

Figure 1 — Inoculation on the slant culture medium

5.7 Procedure

5.7.1 General

Handling of the bacteria shall be carried out aseptically and other bacterial contamination from laboratory operators, apparatus and working environment shall be prevented. A safety cabinet may be used if necessary.

5.7.2 Pre-culture of bacteria

Using a sterilized inoculation loop, transfer one loop of the test bacteria from the stock culture (5.6) to the slant culture medium (5.5.3) and incubate at (35 ± 1) °C for 16 h to 24 h.

From this slant culture medium, use a sterilized inoculation loop to transfer the bacteria onto fresh slant culture medium and incubate at (35 ± 1) °C for 16 h to 20 h.

NOTE Culturing twice in pre-culture is necessary to keep the test bacteria in the logarithmic growth phase or stationary phase of bacterial growth.

5.7.3 Preparation of test specimens

When preparing specimens, take care to avoid contamination with microorganisms or extraneous organic debris, or cross-contamination of products.

The determination of the dimensions of the specimens shall be verified in accordance with ISO 1923. 1)

Cut out nine square test specimens from the antibacterial treated material. The size of the specimens shall be (20^{+2}_{-1}) mm long and (3^{+0}_{-1}) mm thick. The total of the outer peripheral surface area of the specimen shall be (32 ± 5) cm^2. Three antibacterial treated test specimens comprise one set and three sets shall be prepared. All of the three sets are used for viable cell count after shaking for 24 h.

When cutting the test piece, do not use any wet agents, lubricants or similar that might affect the contact between the test bacteria suspension and the inside of the test specimens.

2) Cut out nine square test specimens from non-treated material in the same manner described in 5.7.3 1). Three specimens shall be in one set and three sets shall be prepared. All three sets are used for viable cell count after shaking for 24 h. When the non-treated specimens are not available, use the test inoculum suspension prepared in 5.7.6 4) as a control group.

NOTE The non-treated material means flexible cellular polymeric materials which are not treated with antimicrobial agents.

5.7.4 Sterilization of specimens

Sterilization of the specimens prepared in 5.7.3 is usually carried out by high-pressure steam sterilization, as in 5.4.3.

If high-pressure steam sterilization cannot be carried out due to heat resistance of the test specimens, ethylene oxide gas, γ ray or another appropriate method can be used. When applying these sterilization methods, this shall be included in the test report.

5.7.5 Preparation of test inoculum

Using a sterilized inoculation loop, transfer one loop of the pre-incubated bacteria specified in 5.7.2 into a small amount of 1/500 NB prepared in accordance with 5.5.2. Ensure that the test bacteria are uniformly dispersed. Adjust the test bacteria suspension to obtain the number of bacteria between $1{,}0 \times 10^4$ CFU/ml and $5{,}0 \times 10^4$ CFU/ml by diluting it with 1/500 NB adequately, using direct microscopic

observation or another appropriate method. Use this suspension as the test inoculum. If the test inoculum is not used immediately then cool it down on ice (0 °C) and use it within 2 h of preparation.

NOTE 1 CFU stands for colony forming unit.

NOTE 2 In addition to direct observation by microscope, there is a method of measuring the absorbance of the bacterial suspension as turbidity using a spectrophotometer.

5.7.6 Inoculation onto the test specimens

The procedure is as follows:

1) Place the three antibacterial treated test specimens from one set sterilized in ~~5.7.4~~ into a fresh sterilized cup and repeat it for the other two sets. The three sets of the non-treated test specimens shall be placed into fresh sterilized cups in the same manner.

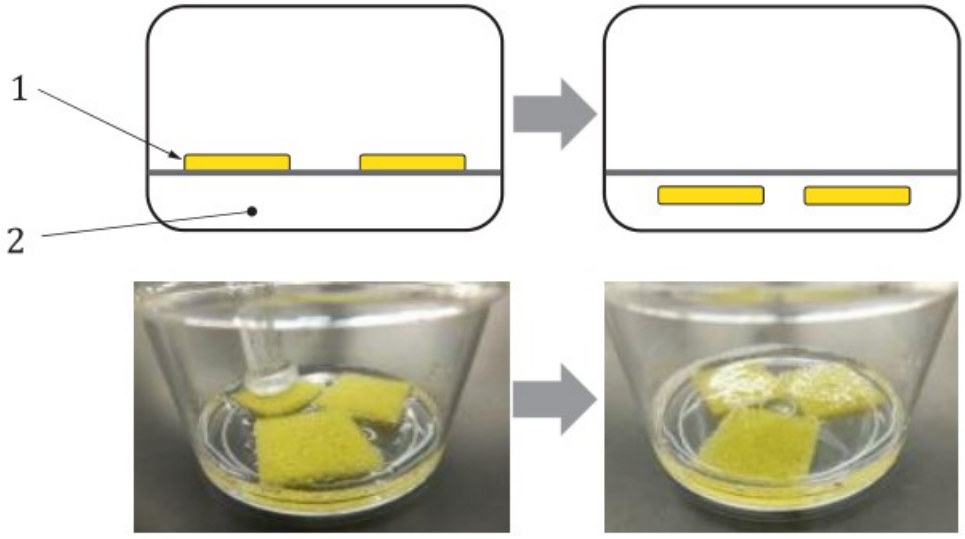
2) Inoculate 10 ml of the test inoculum prepared in ~~5.7.5~~ to each of the antibacterial treated test specimens (three sets). In the same manner, inoculate 10 ml of the test inoculum to each of the non-treated test specimens (three sets).

3) Prepare two sterilized bacteria spreaders (~~5.3.11~~) or a sterilized glass rod (~~5.3.12~~) to remove the air inside the test specimens so that the test inoculum is soaked uniformly throughout the test specimens, as in ~~Figure 2~~. Each operation is as follows:

   a) When using two sterilized bacteria spreaders, press one end of the test specimens with one spreader held in one hand and move it to the other end, while pressing the upper surface of the test specimens with the other spreader in the other hand.

   b) When using a sterilized glass rod, repeatedly press the whole specimen evenly. In either case, the operation shall be kept until the bubbles do not come out from the specimens on visual inspection. Then put a lid on the sterilized cup.

      NOTE Depending on the test specimens, moving the bacteria spreader or pressing with the sterilized glass rod is usually repeated between 50 and 100 times.



Key

1 test specimen
2 suspension of bacteria

Figure 2 — Test specimens in the suspension

4) Prepare six fresh sterilized cups for the control group and inoculate 10 ml of the test inoculum prepared in 5.7.5 to each of them. Three of the six sterilized cups shall be used for measuring the viable bacteria cell count immediately after inoculation. The remaining three cups shall be used for viable bacteria cell count after shaking for 24 h.

5.7.7 Shaking incubation of the test specimens and the control group

Cups inoculated with the test bacteria in 5.7.6 (three sets of antibacterial treated specimens, three sets of non-treated specimens and three cups for the control group) shall be shaken at (35 ± 1) °C in the shaker (5.3.13) for (24 ± 1) h, taking care not to change the position of the cups. Shaking conditions are (30 ± 5) mm in amplitude and (150 ± 10) rpm in the horizontal direction.

5.7.8 Measurement of viable bacteria cell count

5.7.8.1 The just-after-inoculation control plot

Measure the number of viable bacteria for three cups inoculated in 5.7.6 4) immediately, in accordance with the pour plate culture method in Annex A.

The number of viable bacteria per 1 ml of each of three test bacterial suspension according to Annex A is multiplied by 10, and it is regarded as the number of viable bacteria of "the just-after-inoculation control plot".

Each logarithmic value is calculated for the number of viable bacteria of the three "just-after-inoculation control plot", and the arithmetic mean value of these three logarithmic values is regarded as A in 5.8.

5.7.8.2 The after-24-hours'-shaking control plot

Measure the number of viable bacteria for each of the three cups inoculated with shaking for 24 h as the control group in 5.7.7, in accordance with the pour plate culture method in Annex A.

The number of viable bacteria per 1 ml of each of the three test bacterial suspensions in Annex A is multiplied by 10, and this is regarded as the number of viable bacteria of "the after-24-hours-shaking control plot".

Each logarithmic value is calculated for the number of viable bacteria of the three "after-24-hours'-shaking control plots", and the arithmetic mean value of these three logarithmic values is regarded as B in 5.8.

5.7.8.3 The non-treated test plot

After shaking, press each test specimen in each cup with the sterilized bacteria spreader or glass rod about 10 times. Then measure the number of viable bacteria for each of the three cups inoculated with shaking for 24 h as non-treated specimens (three sets) in 5.7.7, in accordance with the pour plate culture method in Annex A.

Each number of viable bacteria per 1 ml of three test bacterial suspensions in Annex A is multiplied by 10, and this is regarded as the number of viable bacteria of "the non-treated test plot".

Each logarithmic value is calculated for the number of viable bacteria of the three "non-treated test plots", and the arithmetic mean value of these three logarithmic values is regarded as C in 5.8.

5.7.8.4 The antibacterial treated test plot

After shaking, press each test specimen in each cup with the sterilized bacteria spreader or glass rod about 10 times. Then measure the number of viable bacteria for each of the three cups inoculated with shaking for 24 h as antibacterial treated specimens (three sets) in 5.7.7, in accordance with the pour plate culture method in Annex A.

Each number of viable bacteria per 1 ml of three test bacterial suspensions in ~~Annex A~~ is multiplied by 10, and this is regarded as the number of viable bacteria of "the antibacterial treated test plot".

Each logarithmic value is calculated for the number of viable bacteria of the three "antibacterial treated test plots", and the arithmetic mean value of these three logarithmic values is regarded as D in ~~5.8~~.

5.7.9 Notation of viable bacteria cell count

The value of viable bacteria count shall be given to two significant figures. The logarithm value of the number of the viable bacteria count is noted by the common logarithm of the second digit after rounding off the third decimal place. The arithmetic average value is the mean value of the three logarithmic values.

When the viable bacteria cell count is less than 10, the notation is "<10" and the logarithmic value is calculated with "10". However, when all measured values are "<10", the average value of the logarithmic value is noted as "<1,00".

5.8 Expression of the results

5.8.1 Requirements for test validity

When the following four conditions for valid tests are satisfied, the test is deemed valid. However, if the non-treated specimens cannot be prepared, it suffices to satisfy the test establishment conditions of items a) to c).

a) Calculate the viable cell count for the three sets in each of the just-after-inoculation control plot and the after-24-hours'-shaking control plot using ~~Formula (1)~~. The calculated value shall not be more than 0,2.

$$\frac{L_{max} - L_{min}}{L_{mean}} \leq 0,2 \qquad (1)$$

where

L_{max} is the common logarithm of the maximum number of viable bacteria found on a specimen;

L_{min} is the common logarithm of the minimum number of viable bacteria found on a specimen;

L_{mean} is the average of the common logarithm of viable bacteria found on the specimens.

b) Calculate the reduction value for B compared to A using ~~Formula (2)~~. The calculation value shall not be more than 1,0.

$$A - B \leq 1,0 \qquad (2)$$

where

A is the average of the common logarithm of viable bacteria of the just-after-inoculation control plot [see 5.7.8.1];

B is the average of the common logarithm of viable bacteria of the after-24-hours'-shaking control plot [see ~~5.7.8.2~~].

c) The average of the viable cell counts from the three sets of the just-after-inoculation control plot shall be between $1,0 \times 10^5$ and $5,0 \times 10^5$ CFU/ml.

d) All of the viable cell counts from the three sets of the non-treated test plot shall not be less than $1,0 \times 10^3$ CFU/ml.

5.8.2 Calculation of the antibacterial activity value

Calculate the antibacterial activity value using Formula (3) and indicate the value to the first decimal place by rounding down the second decimal place.

$$R = (C - A) - (D - A) = C - D \tag{3}$$

where

R is the antibacterial activity value;

A is the average of the common logarithm of viable bacteria of the just-after-inoculation control plot;

C is the average of the common logarithm of viable bacteria of the after-24-hours'-shaking control plot on the non-treated specimens [see 5.7.8.3];

D is the average of the common logarithm of viable bacteria of the after-24-hours'-shaking control plot on the antibacterial treatment specimens [see 5.7.8.4].

However, if the non-treated specimens cannot be prepared, calculate the antibacterial activity value using Formula (4) and indicate the value to the first decimal place by rounding down the second decimal place.

$$R = (B - A) - (D - A) = B - D \tag{4}$$

where

R is the antibacterial activity value;

A is the average of the common logarithm of viable bacteria of the just-after-inoculation control plot;

B is the average of the common logarithm of viable bacteria of the after-24-hours'-shaking control plot;

D is the average of the common logarithm of viable bacteria of the after-24-hours'-shaking control plot on the antibacterial treatment specimens.

6 Test report

The test report shall include the following information:

a) a reference to this document, including its year of publication (i.e. ISO 23641:2021);

b) the date of commencement of the experiments;

c) the type and size of antibacterial treatment specimens and non-treated specimens; if non-treated specimens cannot be prepared then describe it;

d) the species of the test bacteria;

e) the stocked strain number used for the test;

f) the volume of test inoculum used;

g) the number of viable bacteria in the test inoculum;

h) the method of sterilization of specimens;

i) the values of A, B, C and D used in 5.8.2 and the calculated antibacterial activity value;

j) identification of the test laboratory, the name and signature of the head of the laboratory and the date of the examination report;

k) other special matters (include any deviation from this document).

Annex A
(normative)

Pour plate culture method

A.1 General

This annex specifies the test procedure for quantitative measurement by the plate count method.

A.2 Test procedure

A.2.1 Take 1 ml of the suspension for which the viable bacteria is to be measured by using a pipette, add it to a test tube containing (9,0 ± 0,1) ml of phosphate buffered physiological saline (~~5.5.5~~) and shake well.

A.2.2 Take 1 ml from the test tube in ~~A.2.1~~ using a new pipette, add it to another test tube containing (9,0 ± 0,1) ml of phosphate buffered physiological saline (~~5.5.5~~) and shake well. Repeat the procedure successively and prepare a dilution series so that the dilutions are undertaken 10 times in total. Ensure that 1 ml of each dilution of the suspension is pipetted into each of the two Petri dishes.

A.2.3 Add 15 ml to 20 ml standard agar medium (~~5.5.4~~) kept at 46 °C to 48 °C to each of the two Petri dishes in ~~A.2.2~~ and shake well. Cover the dishes and maintain at room temperature. When the medium solidifies, turn the dishes upside down and incubate at (35 ± 1) °C for 40 h to 48 h.

A.2.4 After incubation, count the number of colonies on the Petri dishes of dilution series on which 30 to 300 colonies have appeared. When the number of viable bacteria is fewer than 30 in the Petri dishes with 1 ml of the suspension for which the viable bacteria is to be measured, count the number of colonies. In cases where the formation of colonies is not recognized in any Petri dish, it shall be "<1".

A.2.5 From the measured number of colonies, obtain the number of viable bacteria using ~~Formula (A.1)~~:

$$N = Z \times R \tag{A.1}$$

where

N is the number of viable bacteria for 1 ml of the suspension for which the viable bacteria is to be measured;

Z is the average number of colonies of the two Petri dishes adopted;

R is the dilution rate of the diluted suspension dispensed to the Petri dishes adopted.

The number of viable bacteria for 1 ml indicates the value to the second decimal place by rounding off the third decimal place. When the number of colonies is less than 1, Z is taken as "1" when calculating the number of viable bacteria. However, when Z in each of the two Petri dishes is less than "1", the number of viable bacteria for 1ml shall be "<1".

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