
**Footwear and footwear
components — Test method to assess
antibacterial activity**

*Chaussure et composants de chaussure — Méthode d'essai pour
évaluer l'activité antibactérienne*

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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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The committee responsible for this document is ISO/TC 216, *Footwear*.

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Footwear and footwear components — Test method to assess antibacterial activity

CAUTION — Test methods specified herein require the use of bacteria. These tests are only to be carried out in facilities with containment techniques for handling microorganisms and by persons with training and experience in the use of microbiological techniques. Appropriate safety precautions are to be observed with due consideration given to country-specific regulations.

1 Scope

This International Standard specifies quantitative test methods to evaluate the antibacterial activity of footwear and components.

This International Standard is applicable to all types of footwear and components employing non-diffusing antibacterial treatments.

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 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 19952, *Footwear — Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 19952 and the following apply.

3.1

antibacterial activity

efficacy of a material or finish used to prevent or mitigate the growth of bacteria, to reduce the number of bacteria or to kill bacteria

3.2

control sample

material identical to the test material but without antibacterial treatment

4 Safety

Handling of microorganisms which are potentially hazardous requires a high degree of technical competence and can be subject to current national legislation and regulations. Only personnel trained in microbiological techniques should carry out such tests. Codes of practice for disinfection, sterilization and personal hygiene shall be strictly observed.

NOTE It is recommended that workers consult IEC 60068-2-10, appendix A “Danger to personnel”, and ISO 7218.

5 Apparatus and materials

5.1 General

Standard laboratory equipment and the following.

5.2 Biological safety cabinet.

5.3 Incubator, capable of maintaining a temperature of (37 ± 2) °C.

5.4 Autoclave.

5.5 Humidity chamber, capable of maintaining a temperature of (37 ± 2) °C and a relative humidity not less than 90 %.

5.6 Ultraviolet lamp.

5.7 Wide mouth jars, with cap, 100 ml, capable of being used with an autoclave (5.4).

5.8 Cover film that does not affect bacterial growth or absorb water, which can be made of either polyethylene, polypropylene or polyester [poly (ethylene terephthalate)]. Film that is 0,05 mm to 0,10 mm thick is recommended. For example, disposal bag suitable for use with an autoclave (5.4).

5.9 Vortex mixer.

5.10 Dimensional shaker, two dimensional or three dimensional, capable of adjusting to 50 rpm.

5.11 Shaking incubator, capable of maintaining a temperature of (37 ± 2) °C and a rotational frequency of (120 ± 10) rpm.

6 Reagents and culture medium

6.1 Principle

The preparation and test shall be freshly prepared in order to ensure the culture quality.

NOTE This can be done according to ISO/TS 11133-1, ISO/TS 11133-2, or according to national standards or regulations.

Reagents used in tests shall be of analytical grade and/or suited for microbiological purposes.

Use only water Grade 3 according to ISO 3696.

6.2 Nutrient broth (NB)

6.2.1 Composition

Beef extract, 3,0 g.

Peptone, 5,0 g.

Sodium chloride (NaCl), 5,0 g.

Water, 1 000 ml.

6.2.2 Preparation

Stir and adjust pH to $(7,2 \pm 0,2)$ (at room temperature). Heat with stirring on a hotplate or in a boiling-water bath until the components are completely dissolved. Sterilize with autoclave (5.4) at (121 ± 2) °C for 15 min.

6.3 Nutrient agar (NA)

6.3.1 Composition

Beef extract, 5,0 g.

Peptone, 10,0 g.

Sodium chloride (NaCl), 5,0 g.

Agar, 15,0 g.

Water, 1 000 ml.

NOTE If solidification is insufficient, 15 g to 18 g of agar can be used.

6.3.2 Preparation

Stir and adjust pH to $(7,2 \pm 0,2)$ (at room temperature). Heat with stirring on a hotplate or in a boiling-water bath until the components are completely dissolved. Sterilize with autoclave (5.4) at $(121 \pm 2) ^\circ\text{C}$ for 15 min. Cool and shake solution well, then pour into the Petri dishes.

6.4 Soybean casein digest broth with lecithin and polyoxyethylene medium (SCDLP)**6.4.1 Composition**

Peptone, digest of casein, 17,0 g.

Peptone, digest of soybean, 3,0 g.

Sodium chloride (NaCl), 5,0 g.

Potassium dihydrogen phosphate, 2,5 g.

Glucose, 2,5 g.

Lecithin, 1,0 g.

Polysorbate 80, 7,0 g.

Water, 1 000 ml.

If the neutralizing power is insufficient, the content of polysorbate 80 or lecithin may be adjusted or another neutralizing agent may be added. The use of any unspecified neutralizer shall be recorded along with the name and concentration.

NOTE Information about selection and evaluation of alternative antibacterial neutralizing agents can be found in ASTM E 1054 and EN 1040.

6.4.2 Preparation

After mixing well, adjust pH to $(7,2 \pm 0,2)$ (at room temperature) and sterilize with autoclave (5.4) at $(121 \pm 2) ^\circ\text{C}$ for 15 min.

6.5 Sodium chloride solution (physiological saline)**6.5.1 Composition**

Sodium chloride (NaCl), 8,5 g.

Water, 1 000 ml.

6.5.2 Preparation

After mixing well, adjust pH to $(6,9 \pm 0,2)$ (at room temperature) and sterilize at (121 ± 2) °C for 15 min.

7 Test microorganisms

7.1 Test strains

The following species shall be used in all antibacterial activity tests.

- a) *Staphylococcus aureus* AS 1.89 or ATCC 6538.
- b) *Klebsiella pneumoniae* AS 1.1736 or ATCC 4352.

NOTE 1 If required, other species or other strains can be used. However, the selected organisms should contain at least one gram-positive and one gram-negative organism as the antibacterial agents may have different activities.

Test strains shall be obtained from agencies of the World Federation of Culture Collection (WFCC).

The bacteria species and their supply sources shall be included in the test report.

NOTE 2 AS refers to the China General Microbiological Culture Collection Centre (CGMCC), ATCC is the American Type Culture Collection.

7.2 Storage of strains

Inoculate the strains to the nutrient agar (NA) (6.3), and incubate at (37 ± 2) °C for 24 h. Store at (5 ± 3) °C (maximum one month) and keep it as stock culture of the strains. Transfer and incubate one time each month.

Strains can be preserved in accordance with the supplier's direction or EN 12353.

8 Preparation of test inoculums

Using a sterile inoculating loop, transfer one colony (7.2) into 20 ml of nutrient broth (NB) (6.2) and incubate in the shaking incubator (5.11) at (37 ± 2) °C for about 16 h (overnight culture). Estimate the number of bacteria with microscopic observation or other methods. Prepare physiological saline (6.5) with 1 % nutrient broth (NB) (6.2). Use this media to prepare a suspension with a bacterial concentration of $(2,5 \text{ approximately } 10) \times 10^5$ CFU/ml as test inoculum.

If necessary, store the test inoculum on ice and use it within 4 h.

9 Preparation of test samples

9.1 General

Test only the components or material which are claimed to be antibacterial. If the whole footwear is claimed as antibacterial, major components, including upper, lining, insole, insock, outsole shall be tested separately.

In the case where only one material of a component is claimed to be antibacterial, it shall be tested separately, if possible. Otherwise, the whole component shall be tested.

Each test sample shall be at least 80 % of the surface area of the component or material. If single material accounts for less than 80 %, take two main materials used in the composition of the component.

The test samples can be obtained directly from the footwear raw materials.

9.2 Test specimen

The area of test specimen should be about 500 mm². For test method A (see [Annex A](#)), the area of test specimen shall have a thickness of less than 2,0 mm. The area and the weight shall be reported in the test report. If a larger test specimen is used then the volume of bacterial suspension should be increased proportionally.

If it is impossible to lower the thickness of the test specimen (for example, components are thicker and cannot be separated or cut without changing critical properties like surface morphology which may affect how the bacteria interact with the surface), the thickness shall be indicated in the test report.

At least six test specimens shall be taken for each material or component and for each test strain.

9.3 Pre-treatment of the test specimen

Pre-treatment of the test specimen is optional and should only be conducted if necessary due to high bioburden (contamination etc.).

If sterilization methods are applied, they shall be reported in detail, and shall not affect the antibacterial properties or the material itself.

NOTE The test and control sample can be sterilized with the autoclave ([5.4](#)) at (121 ± 2) °C and 103 kPa for 15 min, or with ultraviolet rays [ultraviolet lamp ([5.6](#)), 30 W, away from the sample 300 mm, each side for one hour respectively] or other suitable sterilizing methods.

10 Test procedure

[Table 1](#) lists the circumstances under which each test method should be applied.

Test method A (see [Annex A](#)) should be used only for absorbent single material. Test method B (see [Annex B](#)) should be used only for non-absorbent single materials. Test method C (see [Annex C](#)) can be applied to both absorbent and non-absorbent materials or combinations.

NOTE For single material, method A and B are preferred.

Table 1 — List of test methods

No	Type of material	Test method	Remarks
1	Absorbent	Static challenge test in Annex A .	Textile and leather.
2	Non-absorbent	Film contact method in Annex B .	Micropore, i.e. coated or heavy leather, synthetic/artificial materials, EVA foaming material, PU foaming material; and compacted material, i.e. plastic or coated material.
3	Absorbent and non-absorbent	Dynamic challenge test (shake flask test) in Annex C .	Components of different materials; shaped material; materials with fixed antibacterial agent.

11 Expression of results

Antibacterial performance of footwear or footwear components shall be reported separately based on the antibacterial activity ratio.

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Calculate the antibacterial activity ratio (R) in accordance with Formula (1), or R^* in accordance with Formula (2). Record the result in percentage with three significant figures.

$$R = \frac{C_t - T_t}{C_t} \times 100 \% \quad (1)$$

where

C_t is the average number of colonies of three control samples after 24 h or the specified incubation period, expressed as CFU/ml;

T_t is the average number of colonies of three test samples after 24 h or the specified incubation period, expressed as CFU/ml.

In cases where there is no control sample available, calculate R^* by replacing C_t on Formula (1) by T_0 using Formula 2.

$$R^* = \frac{T_0 - T_t}{T_0} \times 100 \% \quad (2)$$

where

T_0 is the average number of colonies of three samples immediately after inoculation, expressed as CFU/ml.

12 Test report

The test report shall include at least the following information:

- a) reference to this standard, i.e. ISO 16187;
- b) the treated test components and their positions, areas and weights;
- c) test methods for different materials;
- d) preparation of the test specimen, including pre-treatment methods, if applied, for different samples (i.e. sterilization method);
- e) species, serial number and number of viable cells of test strains for different materials;
- f) surfactant and its concentration added into the test inoculums;
- g) judgement of test effectiveness;
- h) antibacterial activity ratio of different materials or components;
- i) any deviations from this method.

Annex A (normative)

Static challenge test

A.1 Test procedure

A.1.1 Inoculation

Place each of the six test samples and the six control samples into separate sterilized wide mouth jars (5.7). Pipette $(1,0 \pm 0,1)$ ml of the inoculum prepared in Clause 8 to each test sample and tightly close the screw cap. The number of swatches to be used is dependent on the sample type.

If no control samples are available, inoculate wide mouth jars without samples as control to determine the test effectiveness.

A.1.2 Elution after inoculation (time zero)

Add 20 ml SCDLP medium (6.4) to each of three inoculated test and control samples (if available). Tighten the caps and shake them in an arc of approximately 30 cm for 30 s, or mix for 5 s \times 5 cycles using the vortex mixer (5.9) in order to elute out bacteria into the medium.

A.1.3 Incubating

Culture the three inoculated test and control samples (if available) at (37 ± 2) °C for (24 ± 1) h.

A.1.4 Elution after incubating (time 24 h)

Proceed as in A.1.2.

A.1.5 Determination of the number of viable bacteria — Surface culture

Take 1 ml elution from A.1.2 or elution from A.1.4 with a sterile pipette and add it into a test tube with $(9,0 \pm 0,1)$ ml physiological saline (6.5) and shake it well. Dilute the elution with physiological saline (6.5) and get 10-fold serial dilutions.

Inoculate 100 μ l of each dilution onto nutrient agar (NA) (6.3) two times, turn the agar upside down and incubate it for 24 h to 48 h.

After incubation, count the number of colonies in the Petri dishes containing 30 to 300 colonies. If the minimum number of colonies is less than 30, then count and record the number of colonies in these plates. If there are no colonies recovered in the plate, record the number of colonies as < 1 .

A.2 Expression of results

A.2.1 Calculation of the number of viable bacteria

For each test sample, determine the number of viable bacteria recovered in accordance with Formula (A.1):

$$M = Z \times B \times 20 \quad (\text{A.1})$$

where

- M is the number of viable bacteria of each test sample;
- Z is the average number of viable bacteria in the two Petri dishes;
- B is the dilution ratio;
- 20 is the volume of the elution, in millilitres (ml).

A.2.2 Judgement of test effectiveness

The extreme difference value of the three controls after inoculation and incubation shall be $\Delta (\lg C) \leq 1$. The average number of colonies of the controls immediately after inoculation shall be minimum 1×10^5 CFU. In the plate counting method, calculate the bacteria growth value (F) in accordance with Formula (A.2), and F shall be ≥ 0 .

$$F = \lg C_t - \lg C_0 \quad (\text{A.2})$$

where

- F is the bacteria growth value of the controls;
- C_t is the average number of colonies of the three control samples after incubation, expressed as CFU/ml;
- C_0 is the average number of colonies of the three controls immediately after inoculation, expressed as CFU/ml.

When the above conditions are satisfied, the test is deemed valid. If all are not met, the test is not valid and the samples shall be retested.

A.2.3 Calculation of antibacterial activity ratio

Calculate the antibacterial activity ratio according to [Clause 11](#).

Annex B (normative)

Film contact method

B.1 Preparation of the sample

B.1.1 Test specimen

If available, a larger sample size is recommended instead of the size mentioned in the procedure (9.2). The size of the test specimen shall be indicated in the test report.

NOTE The inoculum can spread over onto the Petri dish when placing the film on top of the small sample depending on the sample composition.

B.1.2 Cover film

Match the size and shape of the cover film to the test or control samples. It is essential that the test inoculum does not leak beyond the edges of the film. The film shall be smaller than the sample. The actual size and shape used shall be stated in the test report.

The film should be made of PE, PP, PET which have no effect on the growing of bacteria. A thickness of approximately 0,05 mm to 0,1 mm is recommended.

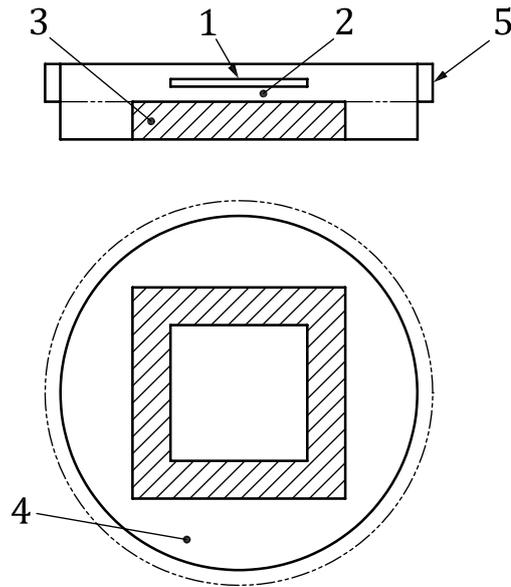
B.1.3 Sterilization of samples (optional)

As an alternative to the procedure given in 9.3, the following procedure can be used. Wipe the surface of the control samples and test samples with 70 % ethanol solution. After 5 min, wash them with sterile distilled water and dry naturally. Samples which cannot be treated with ethanol can be washed directly with sterile distilled water or sterilized by other methods without affecting the antibacterial activity and the experiment results.

B.2 Test procedure

B.2.1 Inoculation

Put six test samples and the six controls into the Petri dishes separately and keep the test surface facing upwards. Accurately pipette 0,1 ml to 0,2 ml of the bacteria suspension and drip it slowly onto the test surface. Grip the cover film (5.8) with a pair of sterile tongs and spread it on the test surface without any bubbles so that the inoculum can contact with the sample evenly. Cover the dish. See Figure B.1.



- Key**
- 1 cover film
 - 2 test inoculum
 - 3 test sample
 - 4 Petri dish
 - 5 cover of the dish

Figure B.1 — Inoculation and the placement of the cover film

If no control samples are available, inoculate the Petri dish without samples as a control to determine the test effectiveness.

B.2.2 Elution after inoculation (time zero)

After the inoculation of the three test samples or controls, add 10 ml of SCDLP medium (6.4) into each Petri dish. Shake at 50 rpm by two or three dimensional shaker (5.10) for 5 min to elute out the bacteria from the test samples and controls.

B.2.3 Incubation

Incubate three test samples and three controls at a temperature of $(37 \pm 2)^\circ\text{C}$ and a relative humidity of not less than 90 % for (24 ± 1) h.

B.2.4 Elution after incubation (time 24 h)

Proceed as in B.2.2.

B.2.5 Determination of the number of viable bacteria

Determine the number of viable bacteria of each sample elution from B.2.2 or B.2.4 according to A.1.5.

B.3 Expression of results

B.3.1 Calculation of the number of bacterial colonies

For each test sample, determine the number of viable bacteria recovered in accordance with Formula (B.1):

$$M = Z \times B \times 10 \times I \quad (\text{B.1})$$

where

- M is the number of viable bacteria of each test sample;
- Z is the average number of viable bacteria in the two Petri dishes;
- B is the dilution ratio;
- 10 is the volume of the elution, in millilitres (ml);
- I is the dilution factor: 10 for inoculum of 0,1 ml or 5 for inoculum of 0,2 ml.

B.3.2 Judgment of test effectiveness

- a) The average number of viable bacteria recovered on the controls immediately after inoculation shall be at least $1,0 \times 10^4$ CFU.
- b) The logarithmic value of the number of viable bacteria recovered on the three controls immediately after inoculation shall satisfy the following requirement (Formula B.2):

$$\frac{L_{\max} - L_{\min}}{L_{\text{mean}}} \leq 0,2 \quad (\text{B.2})$$

where

- L_{\max} is the common logarithm (i.e. base 10 logarithm) of the maximum number of viable bacteria recovered;
- L_{\min} is the common logarithm of the minimum number of viable bacteria recovered;
- L_{mean} is the common logarithm of the mean number of viable bacteria recovered.

The controls shall not possess obvious antibacterial activity. The number of viable bacteria recovered on each control after incubation shall not be less than one tenth of the number of viable bacteria recovered immediately after inoculation. If the above conditions are satisfied, the test is valid. Otherwise it is deemed invalid and a retest is needed.

B.3.3 Calculation of the antibacterial activity ratio

Calculate the antibacterial activity ratio according to [Clause 11](#).

Annex C (normative)

Dynamic challenge test

C.1 Test procedure

C.1.1 Inoculation

Put six test samples and six control samples into sterilized 250 ml conical flasks separately.

Pipette (50,0 ± 0,5) ml of the inoculum prepared in [Clause 8](#) and inoculate each test sample and control.

If no control samples are available, inoculate sterile conical flasks without samples as a control for determining the test effectiveness.

C.1.2 Elution after inoculation (time zero)

Pipette 2 ml from the three inoculated test samples and the three controls into separately prepared sterile flasks each containing 18 ml SCDLP medium ([6.4](#)) in order to neutralize antibacterial activity.

Shake them in an arc of approximately 30 cm for 30 s, or mix for 5 s × 5 cycles using the vortex mixer ([5.9](#)).

C.1.3 Incubating

Culture the three inoculated test samples and the other three inoculated controls at (37 ± 2) °C for (24 ± 1) h in a shaking incubator ([5.11](#)) at 120 rpm.

C.1.4 Elution after incubating (time 24 h)

Proceed as in C.1.2.

C.1.5 Determination of the number of viable bacteria

Determine the number of viable bacteria of each sample elution from C.1.2 and C.1.4 according to A.1.5.

C.2 Expression of results

C.2.1 Calculation of the number of viable bacteria

For each test sample, determine the number of viable bacteria recovered in accordance with Formula (C.1):

$$M = Z \times B \times 10 \quad (\text{C.1})$$

where

M is the number of viable bacteria of each test sample (expressed as CFU/ml);

Z is the average number of viable bacteria in the two Petri dishes;

B is the dilution ratio;

10 is the dilution ratio of neutralization.

C.2.2 Judgement of test effectiveness

The extreme difference value of the three controls after inoculation and incubation shall be $\Delta (\lg C) \leq 1$.

The average number of colonies of the controls immediately after inoculation shall be at least 1×10^5 CFU/ml.

In the plate counting method, calculate the bacteria growth value (F) in accordance with Formula (C.2), and F shall be ≥ 0 .

$$F = \lg C_t - \lg C_o \quad (\text{C.2})$$

where

F is the bacteria growth value of the controls;

C_t is the average number of colonies of the three control samples after incubation, expressed as CFU/ml;

C_o is the average number of colonies of the three controls immediately after inoculation, expressed as CFU/ml.

When the above conditions are satisfied, the test is deemed valid. If all are not met, the test is not valid and the samples shall be retested.

C.2.3 Calculation of the antibacterial activity ratio

Calculate the antibacterial activity ratio according to [Clause 11](#).