(2) Test Method II (2012 Version)
Shaking method

1. Scope

This test method[1] shall be applied to antibacterial effectiveness tests for antibacterial articles conferred with some antibacterial function (hereinafter referred to as articles)[2]. This test method is suitable for antibacterial power tests of test samples of small products or products of special shape and the like[3]. However, in the case of test pieces to which the antibacterial performance test method for antibacterial products specified in Term 2 of JIS Z28015 is applicable, this test method shall, as a rule, not be used.

2. Test Microorganisms

2.1 Test strains[4]

(1) *Staphylococcus aureus* NBRC 12732 (ATCC 6538P)
(2) *Escherichia coli* NBRC 3972 (ATCC 8739)

1.It is important that the relationship between test piece surface area and liquid inoculum volume/cell count be constant. Otherwise, test results are likely to fluctuate.

2.In case of an article which surface has been partially antibacterial-treated, the test piece shall be prepared in such a way to obtain an antibacterial treated area within the specified range.

3.This test shall not be applied to the following antibacterial articles:

(1) Articles that do not permit the maintenance the specified a ratio between sample surface area and liquid inoculum volume.
(2) Articles that show surface peeling or test piece alteration of the original form during shaking.
(3) Fibrous products of non-negligible thickness, including paper, cloth, and nonwoven fabrics.
(4) Articles to be tested under this protocol having special shape features and have small compatibility with the liquid inoculum such as continuous foam type sponge articles shall be tested under the following conditions:
① A non-ionic surfactant (Tween 80) can be added to the liquid inoculum at 0.05%.
② To make the sample compatible with the liquid inoculum, push the sample with the tip of a pipette several times after inoculation of the liquid inoculum.
③ The total surface area of the test sample shall be calculated on the basis of dimensions assuming a smooth surface with the unlikely occurrence of bubbles. Prepare two to four sample pieces with less than 3 mm in thickness.

- The lid of the shaking container can loosen to cause a leak of the liquid inoculum. To prevent this, it is effective to tape-seal the lid portion.
- Although alcohol spraying is the standard method for sample cleaning, sponge products are difficult to dry because of their porosity. An appropriate method for sample cleaning (autoclaving, dry heat sterilization, ultraviolet irradiation, EOG sterilization and the like) shall be chosen by agreements between the sponsor and the testing organization, and the conditions shall be specified in the Report.
- Sponge products are porous and can require excessive drying time after waterproofing test treatment. Appropriate drying conditions shall be determined by agreements between the sponsor and the testing organization, and the conditions (method, temperature, time, etc.) shall be specified in the Report.

4.*Staphylococcus aureus* and *Escherichia coli* were chosen since are representatives of Gram-positive and -negative bacteria, respectively. Note that *Staphylococcus aureus* and *Klebsiella pneumoniae* are used in SEK.

2.2 Storage of the test microorganisms

Transfer each stock microorganism obtained from the specified stock preservation organization to nutrient agar medium[1], incubate at a temperature of 35±1°C for 48 hours, and then store under refrigeration at 5 to 10°C. The stock microorganisms shall be maintained for 1 month and subcultured at intervals of 1 month or less. The subculture generation number shall be up to 10.

3. Materials and Reagents

The reagents, instruments and other supplies used in this test method shall be in conformity with the Japan Industrial Standards or the Japanese Pharmacopoeia unless otherwise specified.

3.1 Instrumentation, equipment, and materials

(1) Pipettes (milk pipettes, volumetric pipettes allowing dispensing of 10 ml or more)
(2) Constant-temperature chamber (a model that can be operated at an accuracy within ±1°C)
(3) Shaking incubator[2] (a model that can be operated at an accuracy of ±1°C)
(4) Sterile Petri dishes (inside diameter 80 mm to 100 mm, height 15 mm to 25 mm)
(5) Sterile cups[3] (use test cups of 60-ml capacity commercially available for sterile testing (EIKEN KIZAI Co., Ltd. etc.))

3.2 Culture medium

(1) Nutrient broth medium (NB medium)

Meat extract	5.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Purified water	1,000 g
pH	7.0 to 7.2

(2) Nutrient agar medium (NA medium)
NB medium (1) supplemented with 1.5% of agar

(3) Standard Methods agar (SA)

Yeast extract	2.5 g
Tryptone	5.0 g
Glucose	1.0 g
Agar	15.0 g
Purified water	1,000 g
pH	7.1±0.1

(4) Ethanol (purity not less than 99%)

1.Slant medium shall be used for the subculture. Since drug susceptibility of the test microorganisms is influenced by the degree of dryness of the culture medium used, it is recommendable to use the medium within 24 hours of preparation.

2.The shaking incubator can be operated at a horizontal shaking rate of 150±10 rpm, an amplitude of 30±5 mm, and a ±1°C temperature control accuracy.

3.Sterile cups of different shapes lead to variation of test results. It is recommended the use of 60-ml sterile testing cups manufactured by EIKEN KIZAI Co., Ltd. (outside diameter 63 mm, depth 35 mm) or equivalent.

(5) Phosphate-buffered physiological saline
Dissolve 34 g of KH_2PO_4 in 500 ml of purified water, and adjust the solution to pH 7.2 with 1N NaOH, then add purified water to make a total volume of 1,000 ml. Dilute 1.25 ml of this solution 800 fold with physiological saline (0.85% NaCl) to make a total volume of 1,000 ml.

4. Test Samples and Untreated Samples

The test sample subjected to the antibacterial power test shall, as a rule, be an actual supply of the product as is[1]. An untreated sample refers to a product not undergoing antibacterial treatment.

5. Test Procedures

5.1 Incubation of the test microorganisms[2]

(1) Transplant each test microorganism to NA medium and incubate at a temperature of 35 to 37°C for 16 to 24 hours (pre-pre-culture)[3].

(2) Transplant a platinum loopful[4] of the pre-pre-cultured test microorganism obtained in the previous term (1) to NA medium and incubate at a temperature of 35 to 37°C for 16 to 20 hours (pre-culture).

5.2 Preparation of liquid inoculum

Dilute NB medium 500 fold with sterile purified water and adjust the dilution to a pH of 7.0±0.2 [5] to obtain a "1/500 NB medium", uniformly disperse the pre-cultured cells in this 1/500 NB medium to obtain a cell count of 1.0×10^4 to 5.0×10^4 cells/ml, and use this dispersion as the liquid inoculum.

5.3 Preparation of test pieces

(1) Provide more than one test sample[6] having a total surface area[7] of 32±5 cm^2 [8] (cut as required).

1.Since the present method enables testing of small articles or special shape articles, the test sample shall be an actual supply of the article.

2.Since use of broth culture involves risk of contamination with medium components when suspending test microorganism into the 1/500 NB, thus influencing test results, NA slopes should be used. Since drug susceptibility of the test microorganisms is influenced by the degree of dryness of the culture medium used, it is recommendable to use the medium within 24 hours of preparation.

3.For pre-pre-culture and pre-culture operation, use NA slants.

4.The pre-pre culture may be used within 3 days stored at 5 to 10°C to inoculate the pre-culture.

5.Adjust pH to 7.0±0.2 with hydrochloric acid or sodium hydroxide and sterilize. If the pH suffer any variation after sterilization, the medium may be diluted with the phosphate-buffered solution described in (3) 2.6 instead purified water.

6.Water-absorbing test samples shall not be tested with the present protocol.

7.Even in the case of test pieces having complex shape that difficult surface area calculation, the area shall be calculated in the closest possible way keeping constant the liquid inoculum volume-cell count ratio. As mentioned herein, the surface area of a test piece means the area of the original surface of the test sample (intrinsic surface), and does not include the newly formed area resulting from cutting or slicing to obtain the test piece. Because the new surface does not represent the original surface of the test sample and hence involves a possible difference from the intrinsic surface in terms of antibacterial effectiveness, the area of the resulting test piece shall not be more than 10% (3±0.5 cm^2) of the area of the intrinsic surface (32±5 cm^2). If the new surface is an antibacterial surface, however, it shall not be excluded from the calculation.

8.Film-shaped test samples may be shredded into small pieces within the established area range described in the previous term. Resulting pieces may be used as the test piece. Articles showing water absorbing features but with negligible thickness, such as paper, cloth, and nonwoven fabrics, may be handled in the same manner as with films.

As a rule, gently wipe the entire surface[1] two or three times with gauze or absorbent cotton of Japanese Pharmacopoeia grade, previously impregnated[2] with ethanol[3], and dry (pretreatment). Provide three such samples and use these as the antibacterial treatment test pieces.

(2) Provide more than one untreated sample having a total surface area of 32±5 cm^2 (cut as required) and pre-treat in the same manner as with the antibacterial test pieces. Provide three such samples and use these as the no-treatment test pieces.

5.4 Test piece shaking

(1) Place each of the three antibacterial test pieces and three no-treatment test pieces in a sterile cup, inoculate the liquid inoculum at 10 ml (including 1.0 to 5.0×10^5 cells) per 32 cm^2 of surface area, place the lid, then transfer the cups to a constant-temperature mechanical shaker at a temperature of 35±1°C, immobilize the cup on the shaking table, and shake. Shaking conditions shall be of 30 mm amplitude and at a horizontal shaking rate of 150 rpm [4].

(2) Provide three sterile cups for a control plot, inoculate 10 ml of the liquid inoculum (containing 1.0 to 5.0×10^5 cells) to each cup, place the lid, then transfer the cups to a constant-temperature mechanical shaker at a temperature of 35±1°C, and shake under the same conditions as (1).

5.5 Viable cell counting

(1) Provide three sterile cups, inoculate 10 ml of the liquid inoculum to each cup, immediately count the viable cells in 1 m of the liquid by the agar plate culture method[5] using SA medium (cultivation at 35±1°C for 48 hours), calculate the mean[6] of the viable cell counts from the three cups ("just-after-inoculation control plot"), and multiply the cell count by a factor of 10 (value A). Use sterile phosphate-buffered physiological saline as the diluent for viable cell counting.

1.The test piece surface may be contaminated with molding releasing agents, detergents, lubricants and hand contamination. Stable results cannot be obtained unless contaminants are removed to some extent. Therefore, as a rule, the test shall be performed after contaminants removal from the sample surface.

2.The test piece must not be immersed in, or sprayed with ethanol. The main purpose of ethanol treatment (wiping) is to remove oil or organic contaminants from the sample surface to some extent. Complete the treatment in a short time and achieve thorough drying.

3.Since water can fail to remove oily contaminants, ethanol (purity not less than 99%) was chosen as the wiping solution as expected to be more effective in oil removal. Isopropyl alcohol, acetone, toluene and similar solvents must not be used because they can dissolve the test piece surface.

4.Shaking conditions differ between different machines; allowances shall be 30±5 mm for amplitude and 150±10 rpm for horizontal shaking rate. In case of a sample that collapses upon shaking, the amplitude and horizontal shaking rate may be reduced. The amplitude and horizontal shaking rate used shall be specified in the Test Results section.

5.Viable cell counting shall be performed with reference to the "Methods of Counting Bacterial Cells (Viable Cells)" stipulated in the "Specifications and Standards for Foods, Additives etc. (Ministry of Health and Welfare Notification No. 370 dated December 28, 1959)" and the like.

6.To obtain the mean of the viable cell counts from the three sterile cups, calculate the arithmetic mean of the measured values rounded at the third decimal place, and indicate the value to the second decimal place by rounding at the third decimal place. If a viable cell count of less than 10 is obtained, the indication shall be "<10", and use "10" as a basis for calculating the mean viable cell count. However, provided that all measured values are "<10", the mean shall be indicated as "<10".

(2) Count the viable cells in 1 ml of the liquid in each of the three sterile cups for the control plot after 24 hours of storage by the agar plate culture method using SA medium (incubation at 35±1°C for 48 hours), calculate the mean of the viable cell counts from the three cups ("control plot"), and multiply by a factor of 10 (value B).

(3) Count the viable cells in 1 ml of the liquid in each of the three untreated test pieces after 24 hours of storage by the agar plate culture method using SA medium (incubation at 35±1°C for 48 hours), calculate the mean of the viable cell counts from the three test pieces ("no-treatment test plot"), and multiply by a factor of 10 (value C).

(4) Count the viable cells in 1 ml of the liquid in each of the three antibacterial test pieces after 24 hours of storage by the agar plate culture method using SA medium (incubation at 35±1°C for 48 hours), calculate the mean of the viable cell counts from the three test pieces ("antibacterial treatment test plot"), and multiply by a factor of 10 (value D).

6. Requirements for Test Validity

The test shall not be regarded as valid unless all the following four requirements for test validity are fulfilled.

(1) Calculate the viable cell count for the three Petri dishes in each of the "just-after-inoculation control plot" and the "control plot" using the formula shown below: the calculated value[1] shall not be more than 0.2.

(Maximum logarithmic value - minimum logarithmic value) / (logarithmic mean) ≤ 0.2

(2) The reduction rate[2] of value B (mean for "control plot") compared to value A (mean for "just-after-inoculation control plot") shall not be more than 90%.

$\{(A - B)/A\} \times 100 \leq 90$

(3) The mean of the viable cell counts from the three cups of the "just-after-inoculation control plot"

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[1] The calculated values shall be indicated to the first decimal place by rounding up to the second decimal place.

[2] The reduction rate shall be indicated to the second decimal place by rounding up to the third decimal place.

3. If any viable cell count does not comply the requirement of "not less than 1.0×10^3 cells/plate" suggests that the untreated sample per se may have some antibacterial activity; in this situation the test is considered invalid. In this case, make any necessary change in the test procedure or pre-treat the test piece as described below, and proceed to re-test.

① Neutralize the antibacterial activity of the untreated sample by increasing the concentration of the nutrient of the liquid inoculum

In case of an article with relatively weak antibacterial activity, such as ABS resin or vinyl chloride resin, the antibacterial activity of the untreated sample can be neutralized by increasing the concentration of the liquid inoculum, from 1/500 NB to, for example, 1/100 NB, 1/50 NB, 1/10 NB etc. In this case, actual used liquid inoculum concentration shall be indicated in the Test Methods section.

② Volatilize the antibacterial substance of the untreated sample by drying.

The majority of articles containing free formalin, such as melamine resin, FRP resin, amino-series paints and melamine-series paints, exhibit strong antibacterial activity. In this case, their antibacterial activity can be neutralized by drying the sample to volatilize the formalin. Since optimal drying temperature and time vary depending on the sample material, the untreated sample shall be pre-treated at different combinations of drying temperature and time to found the most appropriate pre-treatment condition and then subjected to an antibacterial effectiveness test. Pre-treatment condition shall be indicated in the Test Methods section.

③ Preventing bacterial death due to dehydration in water-absorbing samples by impregnating phosphate-buffered physiological saline

In case of samples which absorb the liquid inoculum, such as floor coatings, the bacteria often die due to dehydration. In this case, it is recommended to perform the test after saturation of the sample with phosphate-buffered physiological saline for about 12 hours in a Petri dish and then wiped to remove excess of buffer from the sample surface. In the case of flooring paints, however, free formalin is contained in the adhesive used in the substrate plywood; testing often fails to produce good results unless the above-described anti-formalin measures are taken.

Pre-treatment condition shall be indicated in the Test Methods section.

shall be between 1.0 and 5.0×10^5 cells/cup.

(4) All of the viable cell counts from the three dishes of the "no-treatment test plot" shall not be less than 1.0×10^3 cells/cup[1].

[1] Any viable cell count that does not fulfill the requirement (not less than 1.0×10^3 cells/dish) suggests that the untreated sample per se may have antibacterial power; the test is invalid. In this case, make any necessary change in the test procedures or pre-treat the test piece as described below, and proceed to performing the test again.

① Canceling the antibacterial power of the untreated sample by increasing the nutrient content of liquid inoculum
In the case of a product of relatively weak antibacterial power, such as ABS resin or vinyl chloride resin, the antibacterial power of the untreated sample can be cancelled by increasing the nutrient content of the liquid inoculum compared to the standard level, 1/500 NB, to, for example, 1/100 NB, 1/50 NB, 1/10 NB etc.
In this case, the nutrient ingredients in the liquid inoculum shall be indicated in the Test Methods section.

② Volatilizing the antibacterial substances contained in the untreated sample by drying
The majority of products containing free formalin, such as melamine resin, FRP resin, amino-series paints and melamine-series paints, exhibit potent antibacterial power in the form of untreated samples. In this case, their antibacterial power can be cancelled by drying the sample to volatilize the formalin. Because optimal drying temperature and time vary depending on sample material and the like, the untreated sample shall be pre-treated at actually varied combinations of drying temperature and time and then subjected to an antibacterial power test. Determine the viable cell count on the basis of these results.
In this case, the pretreatment conditions shall be indicated in the Test Methods section.

③ Preventing bacterial death due to dehydration in water-absorbing samples by impregnating phosphate-buffered physiological saline
In the case of samples likely to absorb the liquid inoculum, such as floor coatings, the bacteria contained often die due to dehydration. In this case, it is recommended that a sterile test piece be tested after being allowed to stand in a Petri dish containing phosphate-buffered physiological saline for about 12 hours, and then wiped to remove water from the sample surface. In the case of flooring paints, however, free formalin is contained in the adhesive used in the substrate plywood; testing often fails to produce good results unless the above-described anti-formalin measures are taken.
In this case, the pretreatment conditions shall be indicated in the Test Methods section.

7. Indication of Test Results

Calculate the "antibacterial activity value"[1] using the equation below, and indicate the value to the first decimal place by rounding down the second decimal place.

{log (C/A) - log (D/A)} = {log (C/D)}

The majority of products containing free formalin, such as melamine resin, FRP resin, amino-series paints and melamine-series paints, exhibit potent antibacterial power in the form of untreated samples. In this case, their antibacterial power can be cancelled by drying the sample to volatilize the formalin. Because optimal drying temperature and time vary depending on sample material and the like, the untreated sample shall be pre-treated at actually varied combinations of drying temperature and time and then subjected to an antibacterial power test. Determine the viable cell count on the basis of these results.
In this case, the pretreatment conditions shall be indicated in the Test Methods section.

[1] If value C (mean value for "no-treatment test plot") or value C (mean value for "antibacterial treatment test plot") is "<10", it shall be regarded as "10" in calculating the antibacterial activity value.