Antimicrobial products
Test for antimicrobial activity and efficacy

Introduction

This Japanese Industrial Standard has been prepared for standardizing the method for evaluating antimicrobial efficacy in antimicrobial products shown in the report of the Meeting on Life-Related Processed Products with New Functions (Antimicrobial Products): December 1998 (so-called "Guidelines for Antimicrobial Products"). This standard specifies the antimicrobial efficacy, a part of the performance considered important for antimicrobial products, and the testing methods. Other information considered being important for antimicrobial products including safety, duration of antimicrobial efficacy, and marking on the product shall be referred to in the "Guidelines for Antimicrobial Products".

1 Scope

This Standard specifies the testing methods to evaluate antimicrobial activity and antimicrobial efficacy on bacteria on the surface of antimicrobial products (including intermediate products).

Remarks: The secondary effects of antimicrobial efficacy, such as being antifungal and deodorizing efficacy shall not be included in this Standard.

2 Normative references

The following standards contain provisions, which, through reference in this Standard constitute provisions of this Standard. The most recent editions of the standards indicated below (including amendments) shall be applied.

- JIS K 0950 *Sterilized plastic petri dishes*
- JIS K 0970 *Piston operated micro-volumetric apparatus*
- JIS K 3800 *Class II biological safety cabinets*
- JIS K 8101 *Ethanol (99.5)*
- JIS K 8150 *Sodium chloride*
- JIS K 8180 *Hydrochloric acid*
- JIS K 8576 *Sodium hydroxide*
- JIS K 9007 *Potassium dihydrogenphosphate*
- JIS K 9017 *Dipotasslum hydrogen phosphate*
- JIS L 1902 *Testing method for antibacterial of textiles*
- JIS R 3505 *Volumetric glassware*

3 Definitions

The definitions of the main terms, used in this Standard are as follows:

a) Antimicrobial: The condition inhibiting the growth of bacteria on the surface of products.

b) Antimicrobial finish: A finishing treatment for antimicrobial efficacy.

c) Antimicrobial products: Products treated with antimicrobial finish.

d) Value of antimicrobial activity: This value shows the difference in the logarithmic value of viable cells counts between antimicrobial products and untreated products after inoculation and incubation of

bacteria.

Informative reference: The value of bacteriostatic activity in **JIS L1902** expresses that of the antimicrobial activity in this Standard.

e) Antimicrobial: The efficacy of antimicrobial products judged from the value of antimicrobial activity.

4 Antimicrobial efficacies

The value of antimicrobial activity obtained by the testing methods of this Standard shall not be less than 2.0 for the antimicrobial efficacy of antimicrobial products. Values of over 2.0 may be applicable subject to the agreement between parties concerned with delivery.

5 Testing methods

5.1 Testing method for textile products.

The testing method for textile products shall be in accordance with **8** of **JIS L 1902**.

5.2 Testing method for plastic products, etc.

This testing method is applicable to products other than textile products, such as plastic products, metal products, and ceramic products. Testing method **5.1** maybe used, however, for products judged to be suitable for using the testing method for textile products from the usage and form of the product.

5.2.1 Bacteria to be used for the tests

The species of bacteria to be used for the test shall be as follows, and each bacterium shall be used for the test:

a) ***Staphylococcus aureus***

b) ***Escherichia coli***

An example of the bacterial strain to be used for the tests is shown in Table 1. The bacterial strain is contributed by other culture collection shown in Table 1, and then it shall be obtained from member agencies of the World Federation of Culture Collection (WFCC) or the Japan Society of Culture Collection (JSCC) and the same strain as that shown in Table 1.

Table 1 Bacterial strain to be used for the tests

Bacteria	Strain number	Institution of culture collection
Staphylococcus aureus	ATCC 6538P	American Type Culture Collection
	FDA 209P	Food and Drug Administration
	IFO 12732	Institute For Fermentation
Escherichia coli	ATCC 8739	American Type Culture Collection
	IFO 3972	Institute For Fermentation

5.2.2 Chemicals, materials, and apparatus

The chemicals, materials land apparatus to be used in this Standard shall be given as follows, unless otherwise designated.

Ethanol (C_2H_5OH)	Class 1 as specified in **JIS K 8102** or superior.
Beef extract	For microbial tests.
Peptone	For microbial tests.
Sodium chloride (NaCl)	Guaranteed reagent specified in **JIS K8150**.
Purified water	Conforms to the reference of the 13th revised Japanese Pharmacopoeia.
Agar	Guaranteed or superior reagent specified in **JIS K 8263**.
Yeast extract	For microbial tests.
Tryptone	For microbial tests.
Glucose	For microbial tests.
Casein peptone	For microbial tests.
Soybean peptone	For microbial tests.
Lecithin	For microbial tests.
Nonionic surfactant	Polyoxyethylene sorbitan monooleate [Polysorbate 80 (Tween 80)]
Potassium dihydrogenphosphate (KH_2PO_4)	Guaranteed reagent specified in **JIS K 9007**.
Dipotassium hydrogenphosphate (K_2HPO_4)	Guaranteed reagent specified in **JIS K 9017**
Sodium hydroxide (NaOH)	Guaranteed reagent specified in **JIS K 8576**.
Hydrochloric-acid (HCI)	Guaranteed reagent specified in **JIS K 8180**.
Cotton stopper, etc.	OME cotton, otherwise silicone, metal, or mouton stopper.
Platinum loop	With a loop of about 4 mm at the point.
Dry-heat sterilizer	Capable of keeping the temperature from 160℃ to 180℃.
Autoclave	Capable of keeping at 121℃ temperature and 103 kPa of pressure.
Safety cabinet	A performance conforming to or equivalent to **JIS K 3800**.
Pipette	Precision conforming .to or equivalent to **JIS K 0970** or the class A specified in **JIS R 3505**.
Incubator	Capable of keeping the temperature ±1℃
Petri dish	Made of glass with about 90 mm of inside diameter, or conforming to No.90A or 90B as specified in **JIS K 0950**.
Stomacher	For microbial tests
Film	A material not affecting microbial growth and that does not absorb water. The thickness is not specified, but one with good adherence shall be used.

5.2.3 Sterilization method

a) Dry-heat sterilization

Put the object to be sterilized in a dry-heat sterilizer heated from 160℃ to l80℃, and heat it for 30 min to 60 min(1)

Note (1): After dry-heat sterilization is finished, when the cotton stopper or packaging paper is wet with water, do not use the apparatus referred to.

b) High pressure steam sterilization

Place water-in an autoclave, and put the objects to be sterilized, which have been kept in a metal net basket, on a metal net shelf in the autoclave. Tighten the lid on the autoclave, heat it, and keep it at 121°C temperature and 103 kPa pressure for 15 min to 20 min. Stop heating it, naturally cool it to lower than 100 °C, and open an exhaust valve to draw off the steam. Then take out the sterilized objects after opening the lid, and if necessary, cool them on a clean bench or in a safety cabinet. In order to keep the autoclave clean from contamination, by incubation or processing chemicals, wash it with neutral detergent if needed, and rinse it sufficiently with water.

c) Flame sterilization

Put he objects to be sterilized into a gas or alcohol flame. For a platinum loop, red heat it sufficiently, and for a test tube, let it touch the flame for 2 s or 3 s.

d) Preparation of apparatus

Wash well glassware such as test tubes or flasks with alkali or neutral detergent, rinse them sufficiently with water, dry them, and use them after either dry-heat sterilization or high pressure steam sterilization.

5.2.4 Culture medium, etc.

The culture medium as shown below shall be used. A commercially available one may be used if it is of the same composition.

a) Nutrient broth

Take 3.0 g of beef extract, 10.0 g of peptone, and 5.0 g of sodium chloride in 1000 ml of purified water or don-exchanged water. Put them in a flask, mix them, dissolve them completely, and adjust the pH to 7.0 to 7.2 (25°C) using a sodium hydroxide solution or a hydrochloric acid solution. When the medium is used, take a part into a test tube, put in a cotton stopper, and sterilize it with high-pressure steam. - If it is not used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use a nutrient broth that has been kept for one month or longer after preparation.

b) Nutrient agar

Take 5.0 g of beef extract, 10.0g of peptone, 5.0 g of sodium chloride, and 15.0 g of agar powder in 1000 ml of purified water or ion-exchanged water. Put them in a flask mix them, arid heat in a boiling water bath to dissolve them sufficiently. Adjust the pH to 7.0 to 7.2 (25 °C) using a sodium hydroxide solution or a hydrochloric acid solution, put in a cotton stopper, and sterilize it with high-pressure steam. If it is not used immediately after preparation, then preserve it at 5°C to 10°C. Never use a nutrient agar kept for one month or longer after preparation.

c) Plate count agar

Take 2.5 g of yeast extract, 5.0 g of tryptone, 1.0 g of glucose, and 15.0 g of agar, powder in 1000 ml of purified water or ion-exchanged water. Put them in a flask, mix them, and heat in a boiling water bath to dissolve them sufficiently. Adjust the pH to 7.0 to 7.2 (25°C) using a sodium hydroxide solution or a hydrochloric acid solution, put in cotton stopper, and sterilize with high-pressure steam. If it is not used immediately after preparation, then preserve it at 5°C to 10°C. Never use a plate count agar kept for one month or longer after preparation.

d) Slant culture medium

Pour 6 ml to 10 ml of nutrient agar **b**) which has been warmed to dissolve into a test tube. Put in a cotton

stopper and sterilize with high-pressure steam. After sterilization, place the test tube in a clean room at a slant of about 15" to the horizontal plane, and solidify the contents. If it is not used immediately after preparation, then preserve it at 5°C to 10°C. If there is no condensed water, dissolve it, and employ it after solidifying it again. Never use a slant culture medium kept for one month or longer after preparation.

e) SCDLP broth

Take 17.0 g of casein peptone, 3.0 g of soybean peptone, 5.0 g of sodium chloride, 2.5 g of disodium hydrogen phosphate, 2.5 g of glucose, and 1.0 g of lecithin in 1 000 ml of purified water Orion-exchanged water. Put them in a flask, mix them, and add 7.0 g of nonionic surfactant to dissolve them. Adjust the pH to 6.8 to 7.2 (25°C) using a sodium hydroxide solution or a hydrochloric acid solution. When the medium is used, dispense it into test tubes or Erlenmeyer flasks, put in cotton stoppers, and sterilize with high-pressure steam. If it is not used immediately after preparation, then preserve it at 5°C to 10°C. Never use an SCDLP broth kept for one month or longer after preparation.

f) Phosphate buffer solution

Take 34.0 g of potassium dibydrogen phosphate in a volumetric flask, then add and mix .500 ml of purified water or ion-exchanged water to dissolve the content sufficiently. Adjust the pH to 6.8 to 7.2 (25°C) with a sodium hydroxide solution. Further, add purified water or ion-exchanged water to make 1000 ml. When the solution is used, dispense the solution into test tubes or Erlenmeyer flasks, put in cotton stoppers, and sterilize with high-pressure steam. Never use a phosphate buffer solution kept for one month or longer after preparation.

g) Phosphate buffered physiological saline

Dilute the phosphate buffer solution into with physiological saline. (0.85 % sodium chloride solution) into a 800-fold volume. When the solution is used, dispense it into test tube Erlenmeyer flasks, put in cotton stoppers, and sterilize with high-pressure steam. If it is not used immediately after preparation, then preserve it at 5°C to 10°C. Never use a phosphate buffered physiological saline kept for one month or longer after preparation.

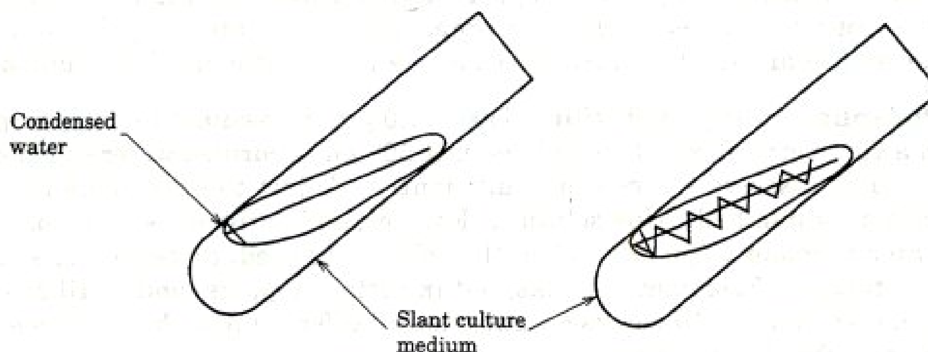
5.2.5 Preservation of bacteria

Transplant the test bacteria aseptically. Use a safety cabinet if necessary. Hold both the stock strain and slant culture medium in **5.2.4 d**) (nutrient agar) in one hand ready to be transferred, hold the stem of the platinum loop in the other hand, and pull out the cotton test tube stopper with this hand. Then sterilize the mouth of the test tube with flame. Sterilize the platinum loop with flame, apply the tip of the platinum loop to a part with condensed water on the new slant culture medium, and cool it. Scrape out the bacteria from the breeding surface of the bacteria using the platinum loop and spread it in streaks on a fresh slant culture medium (2). Sterilize the mouth of the test tube again with flame, and place a cotton stopper as it was before. Sterilize the platinum loop with flame. Incubate the transferred slant culture medium at 35°C±1°C for 24h to 48h and then store it at 5°C to 10°C. Within one month of the transfer, transfer it on a fresh slant culture medium to make the passage transfer. The passage transfer shall be, however, not more than ten passages counting from the original strain contributed by the institution of culture collection. Furthermore, when kept for one month or more from the last transfer it shall not be used for the following transfer.

Note (2): As shown in Fig. I, insert the tip of the platinum loop into condensed-water to disperse the bacteria, and draw a straight line aslant to get to the upper part with the platinum loop, of insert the tip of the platinum loop into condensed water again, and draw zigzag line up to the upper

part.

Remarks: For bacterial strains contributed by the institution's' of culture collection which were preserved by methods such as lyophilization and freezing for long time preservation, the number of passages cultured from the original strain to prepare the bacterial strain for preservation shall be considered as the number of passages of the stock strain.
If this stock strain is used for the test, the passage transfer shall be not more than the number obtained by subtracting the number of passages of the stock strain from l0.



5.2.6 Test operation

Bacteria shall be handled aseptically, and attention shall be paid to the contamination of testing personnel, apparatus, and working environment with bacteria. A safety cabinet shall be used if necessary.

a) Preincubation of bacteria

Using a platinum loop, transfer one platinum loop of bacteria from the stock culture in **5.2.5** to the slant culture medium in **5.2.4 d**), and incubate at 35℃±1℃ for 16 h to 24 h. Further, from this inoculum, transfer one platinum loop of bacteria into a fresh slant culture medium and incubate.

b) Preparation of test piece

Cut the flat part of the product into a square of 50 mm±2 mm (within 10 mm in thickness) (3), and use it as the standard size test piece. Prepare 6 pieces (5) for the untreated test pieces (4) and 3 pieces for the antimicrobial test pieces. If untreated test pieces cannot be prepared, then the film in 5.2.2 may be used. Pay close attention to contamination with microorganisms and mutual contamination between products and filth for preparing test pieces. It is desirable to collect the test pieces from the product itself, but it is difficult to prepare the test pieces because of the shape of the product, then the test pieces may be prepared from the product separately processed to a plate with the same raw material and processing method.

Notes (3) If it is difficult or impossible to cut the product into a square of 50 mm±2 mm (within 10 mm in thickness), then a test piece of shape and size other than specified may be used if it can be covered with a film of the surface area of 400 mm^2 to 1600 mm^2

(4) Test piece cut from untreated product or film.

(5) Among 6 untreated test pieces, us~ 3 test pieces to count viable cells immediately after

inoculation and 3 test pieces to count viable cells afterjncubationfor24h.

c) Cleaning of the test piece

Wipe the whole surface of the test piece in **b**) lightly with gauze or absorbent cotton immersed in ethanol 2 or 3 times and dry it completely.

If changes such as softening of the test piece, dissolution of the surface coating and elution of components occur after these treatments, and it is considered that these treatments affect the test results, then clean the test piece with another appropriate method, or use it as it is without cleaning.

d) Preparation of test inoculum

Dilute the nutrient broth in **5.2.4 a**) with purified water to a 500-fold-volume, adjust the pH to 6.8 to 7.2 with a sodium hydroxide solution or a hydrochloric acid solution, sterilize with high pressure steam, and use it as 1/500 NB. Disperse one platinum loop of the test bacteria preincubated in **a**) into a small amount of 1/500 NB evenly, and estimate the number of bacteria with direct microscopic observation or another appropriate method. Dilute this inoculum with 1/500 NB as appropriate so' that the number of bacteria is 2.5 to l0 $\times 10^5$ cells/ml, and use this solution as the test inoculum. If the test inoculum is not used immediately, then cool it on ice (0℃) and use it within 4 h of storage.

e) Inoculation of test inoculum

Place each test piece in **c**) in a sterilized petri dish making the test surface (6) up. Take exactly 0.4 ml of the test inoculum in **d**) with a pipette (7) and instill it onto each test piece in the petri dish. Cover the instilled test inoculum with a film (8), press the film so that the test inoculum spreads over the film while paying attention so that the inoculum does not spill over from the edge of film, and place the lid of the petri dish on (see Fig. 2).

Notes (6) The test surface shall be the surface of the antimicrobial product. Even when the product uses antimicrobial processing to a depth, never use the cross section as the test surface.

(7) The volume of the inoculated of the test piece to be inoculated of a size other than the standard on 6 shall be divided proportionally by the ratio of the area of coated film. Even if the test pieces of standard size, when the inoculum is inoculated on the volume based on provisions, the film of the test piece of very good wet ability, such as ceramics, tile, enamel, and glass, may move at a small slant, and the inoculum may escape from the edge of film. In this case, the volume of inoculum may be reduced up to 1/4 of the specified volume. If the volume of inoculum is reduced, however, the number of bacterial test pieces shall be 1.0 to 4.0 $\times 10^5$ per test piece similar to a standard size test piece. In this case, the number of cells in the test inoculum shall not be in accordance with the provisions in **d**) but shall be calculated from the volume of the inoculum to be inoculated.

(8) The standard size of film shall be the size of a square of 40 mm 2 mm. if the test piece is not standard size, then adjust the size so that the film may be' located more than 2.5 mm inside of the test piece. However, do not reduce the size of film to less than 400 mm^2. Further, If it is difficult to adhere the film closely since the shape of test piece is not flat, and if the inoculum' spreads over the test piece without covering the film since the test piece is hydrophilic or water absorbent, then the process of covering the film can be omitted.

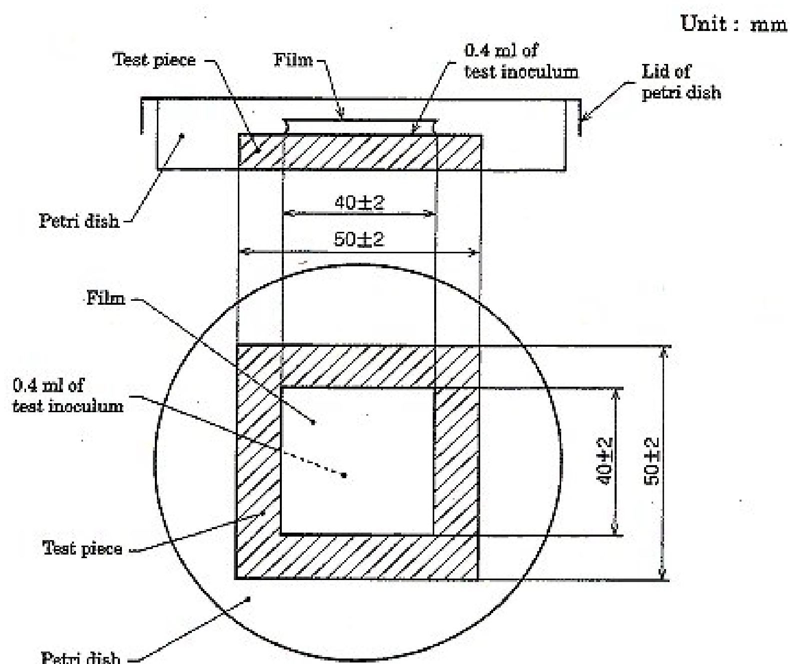


Fig.2 Instillation of inoculum onto the test piece and covering with film

f) Incubation of the test piece' inoculated with the test inoculum

Incubate the petri dish containing the test piece inoculated with the test inoculum (3 untreated test pieces and 3 antimicrobial test pieces) at a temperature of 35°C±1°C and a relative humidity of not less than 90 % for 24 h±1 h.

Informative reference: The antimicrobial efficacy of a product is evaluated from the value of antimicrobial activity obtained from the test at the incubation temperature specified here, but the temperature established considering the actual use of the antimicrobial product (such as room temperature) may be examined together.

g) Washing out the test bacteria inoculated

1) Test piece immediately after inoculation of test inoculum (9): For the 3 untreated test pieces immediately after the inoculation of the test inoculum, place the covering film and the test piece in a sterilized stomacher pouch using sterilized tweezers with caution so that the test inoculum does not spill. 'Add 10 ml of the SCDLP broth in **5.2.4 e**) with a pipette and massage the test piece and the covering film sufficiently with hands or an extractor (such as stomacher) for the microbial test to wash out the test bacteria. Proceed immediately to count viable cells of bacteria in the washings.

2) Test piece after incubation (9): For the test piece after the incubation in **f**), wash out the test bacteria in a similar manner to **1**). Proceed immediately to count viable cells of bacteria in the washings.

Note (9) For washing out the test bacteria, if other methods show a recovery rate equivalent to or superior to the method above, such methods .may be used. If it is difficult to wash out the test bacteria with l0 ml of the SCDLP broth because of the size and characteristics of the test piece, then the volume of solution may be increased.

h) Viable cell Count of bacteria by the agar plate culture method

Take exactly I ml of the washings in **g)** with a pipette and place it in attest tube containing 9.0 ml of phosphate buffered physiological saline in **6.2.4 g)**, and mix it well. Then take 1 ml from this test tube with a new pipette and place it in another test tube containing 9.0 ml of phosphate buffered physiological saline and mix it well. Repeat these procedures to prepare 10-fold serial dilutions. Dispense 1 ml each of the washings and each dilution into 2 sterilized petri dishes. To each petri dish, add 15 ml to 20 ml of the plate~cb66ttit agar in **5.2.4 c)** warmed at 46 ℃ to 48 ℃ and mix it Well. Place the lids oh the petri dishes, and allow them to stand at room temperature. After solidifying the culture medium, invert the petri dishes, and incubate them at 35℃±1℃ for 40 h to 48 h. After incubation, count the number of colonies in a serially diluted petri dish in which 30 to 300 colonies appear. If the number of colonies is less than 30 in the agar plate dispensed with 1 ml of the washings, then count the number of colonies of this plate. If there are not any colony formations in any agar plate, then record it as "< 1". Further, if the number of colonies is not inversely proportional to the dilution ratio, since it is considered that the formation of colonies is inhibited by the effects of the antimicrobial agent. Then determine the number of viable cells of bacteria using a method, which forms colonies without being affected by the antimicrobial agent with the use of an inactivating agent or dilution.

Informative reference:	For the methods of adoption of the number of colonies other than specified above, refer to 1.2 Microorganism tests, 3) Viable cell count of bacteria, (1) *Pour plating method of Standard Method of Analysis for Hygienic Chemists* (2000) edited by the Pharmaceutical Society of Japan, or 2. *Spoilage indicator bacteria*, 1. *Total bacterial count of Standard Methods of Analysis in Food Safety Regulation* supervised by the Environmental Health Bureau of the ministry of Health and Welfare, Japan.

5.2.7 Calculation of the number of viable cells of bacteria

Determine the number of viable cells of bacteria by the counts of colonies according to the formula:

$$N = C \times D \times V$$

Where, N: number of viable cells of bacteria (per test piece)
C: number of colonies (average of the number of colonies in two petri dishes adopted)
D: dilution ratio (dilution ratio of the diluted solution dispensed into the petri dish adopted)
V: volume (ml) of the SCDLP broth used for washing out

Record the number of viable cells of bacteria with two significant figures after rounding the third significant figure off. The number of viable cells of bacteria in the number of colonies of "< 1" shall be recorder as "< 10". (In the case of the V of 10 ml). When an average of the number of viable cells, of bacteria is determined, average the number of viable cells of bacteria in each of the three test pieces arithmetically and express it with two significant figures after rounding the third significant figure off. When the number of viable cells of bacteria is "< 10", calculate the average considering the number of viable cells of bacteria as 10.

5.2.8 Test results

a) Judgment of the conditions of test effectiveness

When the following three conditions of test effectiveness are all satisfied, and then judge the test effective. Unless all the conditions are satisfied, judge the test as not effective, and carry out a retest.

1) The following formula is established for the logarithmic value of the number of viable cells of bacteria immediately after inoculation on untreated test pieces:

$$(L\max - L\min)/(L\text{mean}) \leqq 0.2$$

Where, Lmax: maximum logarithm of the number of viable cells of bacteria
Lmin: minimum logarithm of the number of viable cells of bacteria
Lmean: average of the logarithm of the number of viable cells of bacteria in three test pieces

2) The average of the number of viable cells of bacteria immediately after inoculation on an untreated test piece shall be within the range of 1.0 to 4.0×10^5 cells.

3) The number of viable cells of bacteria on an untreated test piece after 24 h shall not be less than 1.0×10^3 cells on all 3 test pieces. When a film is used on the untreated test piece, however, the number of viable cells of bacteria after 24 h shall not be less than 1.0×10^4 cells on all 3 test pieces.

b) Calculation of the value of antimicrobial activity

When the test has been effective, calculate the value of antimicrobial activity according to the formula (2) Record the value to the first decimal place after rounding the second decimal place down.

$$R = [\log (B/A) - \log (C/A)] = [\log (B/C)]$$

Where, R: value of antimicrobial activity
A: average of the number of viable cells of bacteria immediately after inoculation on the untreated test piece
B: average of the number of viable cells of bacteria on the untreated test piece after 24 h
C: average of the number of viable cells of bacteria on the antimicrobial test piece after 24 h

6 Record of test results

6.1 Textile products

The species of test bacteria, strain number of bacteria, concentration of inoculum (number of viable cells of bacteria in the test inoculum), value of antimicrobial activity (bacteriostatic activity), and the type of sample (type of test piece) shall be recorded. Further, if nonionic surfactant is added to the test inoculum, then the name and concentration shall be mentioned.

6.2 Plastic products

Following items shall be recorded:

- The type, size, shape, and thickness of antimicrobial and untreated test pieces
- The type, size, shape, and thickness of film
- Species of test bacteria

- Strain number of bacteria
- Volume of test inoculum inoculated
- Number of viable cells of bacteria in the test inoculum
- The values of A, B and C in **5.2.8 b**)
- The value of antimicrobial activity