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Plastics — Measurement of antibacterial action on plastic surfaces

Plastiques — Mesurage de l'action antibactérienne sur les surfaces en plastique

ICS 83.080.01

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Foreword

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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.

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ISO 22196 was prepared by Technical Committee ISO/TC 61, *Plastics*, Subcommittee SC 6, *Aging, chemical and environmental resistance*.

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Plastics — Measurement of antibacterial action on plastic surfaces

1 Scope

WARNING — Handling and manipulation of microorganisms which are potentially hazardous requires a high degree of technical competence and may be subject to current national legislation and regulations. Only personnel trained in microbiological techniques should carry out such tests. Appropriate practices for disinfection, sterilization and personal hygiene must be strictly observed.

This international standard specifies a test method to evaluate antibacterial activity and efficacy on the surface of antibacterial treated plastic products (including intermediate products).

The secondary effects of antibacterial efficacy, such as bio-deterioration and deodorizing efficacy are NOT included in this standard. This standard should not be used or referenced as a method to document or claim biodegradable activity of plastics. For biodegradation refer to ISO 14855, ISO 14851, ISO 14852 and related standards.

This standard does NOT concern plastic building materials, like PVC or composites for example, unless they act as treated articles.

This standard is NOT intended to evaluate the effects and propagation of bacteria on plastics without antibacterial treatments. ISO 846 describes tests to evaluate the effects and propagation of bacteria on plastics, which are different aspects than those covered by this standard. Those who are interested should refer to ISO 846 Method C.

Any results obtained with this standard should always refer to this standard and the conditions used. Results obtained with this standard indicate antibacterial activity and efficacy under the specified experimental conditions used herein, and do not reflect activity and efficacy under the actual circumstances where a variety of factors, such as temperature, humidity, different bacterial species, nutrient conditions, etc., must be considered.

It is recommended that workers consult ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*.

2 Normative references

The following referenced documents are indispensable for the application 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 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examination*

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3 Terms and definitions

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

3.1
antibacterial
a state where growth of bacteria on the surface of products is suppressed, or the effects of agents which suppress growth of bacteria on surfaces of products

3.2
antibacterial products
products that inhibit the growth of bacteria on their surfaces by use of antibacterial surface treatments or compounded agents

3.3
value of antibacterial activity
a quantitative value that is defined specifically in this standard in terms of the difference in the logarithmic value of viable cell counts between antibacterial products and untreated products after inoculation and incubation of bacteria

3.4
antibacterial efficacy
the ability to inhibit growth of bacteria on the surface of a plastic treated with an antibacterial compound as determined by the value of antibacterial activity

4 Materials

4.1 Bacteria to be used for the tests

Both the following species of bacteria shall be used:

a) *Staphylococcus aureus*

b) *Escherichia coli*

An example of the bacterial strain to be used for the tests is shown in Table 1. If the bacterial strain is contributed by a culture collection other than those shown in Table 1, 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. Stock cultures of these strains should be prepared for use based on manufacturer's directions.

Table 1 — Bacterial strain to be used for the tests

Name	Strain
Staphylococcus aureus	ATCC 6538P CIP 53.156 DSM 346 NBRC 12732 NCIB 8625
Escherichia coli	ATCC 8739 CIP 53.126 DSM 1576 NBRC 3972 NCIB 8545

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4.2 Reagents, culture media and solutions

Any water used shall be distilled or deionized and have a conductivity of < 1 µS/cm.

All reagents shall be of analytical grade and/or of a grade appropriate for microbiological purposes.

4.2.1 onionic surfactant

Polyoxyethylene sorbitan mono-oleate.

4.2.2 Biological materials:

The following biological materials are used.

- Lecithin
- Glucose
- Yeast extract
- Beef extract
- Peptone
- Casein peptone
- Soybean peptone
- Tryptone

4.2.3 Culture medium

The culture medium as shown below shall be used. The medium may be obtained from commercial suppliers and shall be prepared in accordance with the manufacturer’s instructions.

4.2.3.1 1/500 Nutrient broth (1/500 NB)

Prepare nutrient broth by dissolving 3,0 g of beef extract, 10,0 g of peptone, and 5,0 g of sodium chloride in 1000 ml of distilled or deionized water. Dilute the nutrient broth with distilled or deionized water to a 500-fold volume and adjust the pH to a value between 6,8 and 7,2 with sodium hydroxide or hydrochloric acid. Sterilize by autoclaving (see 6.2). If it is not used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use a 1/500 NB that has been kept for one week or longer after preparation.

4.2.3.2 Nutrient agar

Prepare nutrient agar by dissolving 5,0 g of beef extract, 10,0 g of peptone, 5,0 g of sodium chloride, and 15,0 g of agar powder in 1 000 ml of distilled or deionized water. Heat with stirring on a hot plate or in a boiling water bath until agar is dissolved. Adjust the pH to a value between 7,0 and 7,2 (25 °C) using sodium hydroxide or hydrochloric acid. Sterilize by autoclaving (see 6.2). If it is not used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use nutrient agar that has been kept for one month or longer after preparation.

4.2.3.3 Plate count agar

Prepare plate count agar by dissolving 2,5 g of yeast extract, 5,0 g of tryptone, 1,0 g of glucose, and 15,0 g of agar powder in 1 000 ml of distilled or deionized water. Heat while stirring on a hot plate or in a boiling water bath until agar is dissolved. Adjust the pH to a value between 7,0 and 7,2 (25 °C) using sodium hydroxide or

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hydrochloric acid. Sterilize by autoclaving (see 6.2). If it is not used immediately after preparation, preserve it at 5 ºC to 10 ºC. Never use plate count agar that has been kept for one month or longer after preparation.

4.2.3.4 Slant culture medium

Pour 6 ml to 10 ml of nutrient agar, which has been warmed to dissolve, into a screw-capped test tube. Sterilize autoclaving (see 6.2). After sterilization, place the test tube at a slant of about 15 degrees to the horizontal plane and solidify the contents. If it is not used immediately after preparation, preserve it at 5 °C to 10 °C. Never use a slant culture medium that has been kept for one month or longer after preparation.

4.2.3.5 Soybean casein digest broth with lecithin and polyoxyethylene sorbitan mono-oleate (SCDLP broth)

Prepare SCDLP broth by dissolving 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 distilled or deionized water. Mix thoroughly and add 7,0 g of nonionic surfactant. Adjust the pH to a value between 6,8 and 7,2 (25 °C) using sodium hydroxide or hydrochloric acid. Sterilize autoclaving (see 6.2). If it is not used immediately after preparation, preserve it at 5 ºC to 10 °C. Never use a SCDLP broth kept for one month or longer after preparation.

4.2.3.6 Phosphate buffer solution

Prepare phosphate buffer solution by placing 34,0 g of potassium dihydrogen phosphate in a 1 000 ml volumetric flask. Add 500 ml of distilled or deionized water and mix to dissolve. Adjust the pH to a value between 6,8 and 7,2 (25 °C) with sodium hydroxide. Add distilled or deionized water to make 1 000 ml. Sterilize autoclaving (see 6.2). Never use a phosphate buffer solution kept for one month or longer after preparation.

4.2.3.7 Phosphate buffered physiological saline

Dilute the phosphate buffer solution in 4.2.3.6 with physiological saline (0,85 % sodium chloride solution) into an 800-fold volume. Sterilize autoclaving (see 6.2). If it is not used immediately after preparation, preserve it at 5 °C to 10 °C. Never use a phosphate buffered physiological saline kept for one month or longer after preparation.

4.3 Materials

The materials to be used in this standard shall be as follows:

4.3.1 Inoculating loops, 4 mm, sterile

4.3.2 Pipette tips, 1 000 µl, sterile

4.3.3 Stomacher bags, sterile

4.3.4 Film that does not affect bacterial growth or absorb water (polyethylene, polypropylene, or polyester (poly(ethyleneterephthalate)). Films that are 0,05 mm to 0,10 mm in thickness are recommended. Films cut from Stomacher bags are also suitable.

4.3.5 Screw-caped test tubes

4.3.6 Petri dishes, sterile, 90 mm to 100 mm in diameter

4.3.7 Gauze or absorbent cotton

4.3.8 1 000 ml volumetric flask

4.3.9 Stoppered Erlenmeyer flasks or **media bottles**, as required for preparation of media

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5 Apparatus

Unless otherwise specified, the apparatus to be used in this standard shall be as follows.

5.1 Dry-heat sterilizer, capable of maintaining the temperature a value between 160 °C and 180 °C within ± 2 °C of the set point at equilibrium conditions

5.2 Autoclave, capable of maintaining the temperature at (121 ± 2) °C and (103 ± 5) kPa pressure

5.3 Hot plate with stirrer, or hot water bath

5.4 pH meter, capable of measuring to ± 0,2 units

5.5 Balance, capable of weighing to ± 0,01 g

5.6 Pipetters, 1 000 µl

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

6 Method

6.1 Dry-heat sterilization

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

Temperature	Minimum sterilization time
180 °C	30 minutes
170 °C	60 minutes
160 °C	120 minutes

6.2 High pressure steam sterilization

Put the objects to be sterilized in an autoclave and maintain at (121 ± 2) °C for at least 15 min.

6.3 Preparation of glassware

Wash well with alkali or neutral detergent, then rinse well with distilled or deionized water. Sterilize using dry heat or an autoclave prior to use.

6.4 Maintenance of stock cultures

Stock cultures shall be stored at 5 °C to 10 °C on an appropriate medium and transferred monthly. After five transfers or if more than one month has passed between transfers, the stock culture shall be discarded and replaced with a fresh culture, obtained from the institute or culture collection.

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7 Procedures

7.1 Preculture of bacteria

Using a sterile inoculating loop, transfer bacteria from the stock culture to the slant culture medium (see 4.2.3.4) and incubate at (35 ± 1) °C for 16 h to 24 h. From this culture, use a sterile inoculating loop to transfer bacteria onto fresh slant culture medium and incubate at (35 ± 1) °C for 16 h to 20 h.

7.2 Preparation of test specimens

Testing shall be performed on at least three specimens from each treated test material. At least six specimens of the untreated material are required. Half of the untreated test specimens are used to count viable cells immediately after inoculation and half are used to count viable cells after incubation for 24 h.

When testing a series of antibacterial treatments for a single polymer, each antibacterial treatment may be compared to a single set of untreated specimens, if all tests are conducted at one time using the same test inoculum.

NOTE Use of more than three replicate specimens of the treated test material may reduce variability, especially for materials that show smaller antimicrobial effects.

Prepare (50 ± 2) mm flat square specimens of the treated and untreated test materials. Specimens should be no more than 10 mm in thickness. If it is difficult or impossible to cut the product into a square of this size, then test specimens of other sizes and shapes may be used, as long as they can be covered with a film of between 400 and 1 600 square mm surface area. It is desirable to prepare test specimens from the product itself. However, if the shape of the product prevents this, then the test specimens may be prepared in a format suitable for testing using the same raw materials and processing methods normally used for the product. If the test specimen is different from the 50 mm square dimension, the actual dimensions used shall be included in the test report.

When preparing specimens, avoid contamination with microorganisms and extraneous organic debris. Do not allow test specimens to contact each other.

7.3 Cleaning of the test specimens

Wipe the entire surface of the test piece in 7.2 lightly with gauze or absorbent cotton immersed in ethanol two or three times and allow it to dry completely. If changes such as softening of the test specimen, dissolution of the surface coating or elution of components occur after these treatments, and it is considered that these treatments affect the test results, then clean the test specimen with another appropriate method, or use it as it is without cleaning. If an alternate cleaning method or no cleaning is used, it shall be included in the test report.

7.4 Preparation of test inoculum

Evenly disperse one platinum loop of the test bacteria pre-incubated in 7.1 into a small amount of 1/500 NB in 4.2.3.1 and estimate the number of bacteria with direct microscopic observation or another appropriate method. Dilute this suspension with 1/500 NB, as appropriate, to obtain a bacterial concentration of between 2,5 and 10×10^5 cells/ml and use this solution as the test inoculum. If the test inoculum is not used immediately, then chill it on ice (0 °C) and use it within 4 h of preparation.

7.5 Inoculation of test specimen

7.5.1 The surface to be tested is the exposed outer surface of the antibacterial product. Do not test cross sections of the product. Place each test specimen prepared in 7.3 into a separate sterile Petri dish with the test surface up. Pipette 0,4 ml of the test inoculum onto the test surface. Cover the test inoculum with a piece of film (described in 4.3.4) that is 40 mm × 40 mm and gently press down on the film so that the test inoculum spreads to the edges. Make sure that the test inoculum does not leak beyond the edges of the film. After the specimen has been inoculated and the cover film applied, replace the lid of the Petri dish (see Fig. 1).

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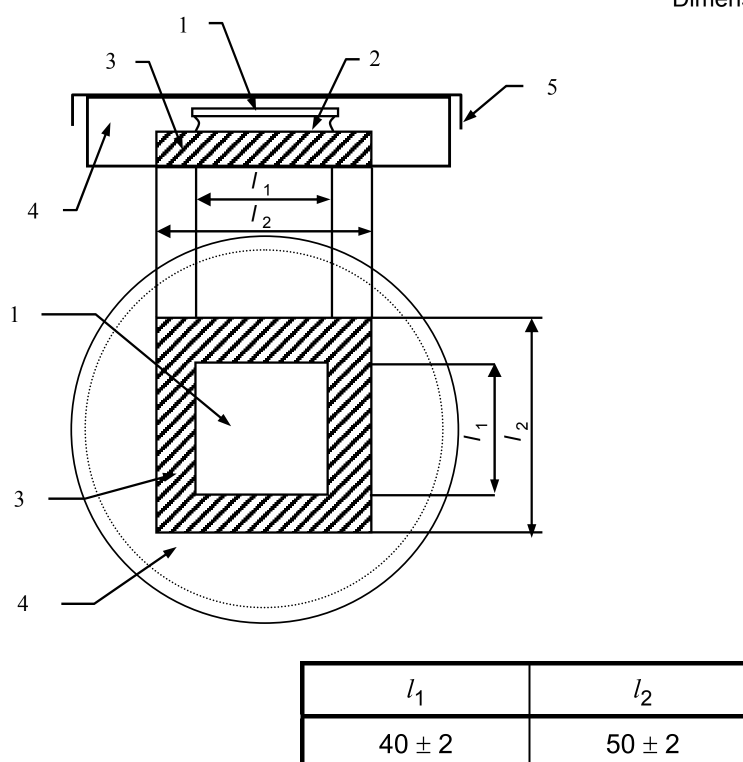
It is essential that the test inoculum does not leak beyond the edges of the cover film. For some surfaces (e.g. those that are very hydrophilic), it could be difficult to prevent leaking beyond the edges of the cover film. When this occurs, use option 1 to prevent leakage. If leakage still occurs with option 1, use option 2. If one of these options is used to ensure that leaking does not occur, it shall be described in the test report.

Option 1 Reduce the volume of the test inoculum applied to the test surface. Do not use less than 0,1 ml of the test inoculum. When the volume of the test inoculum is decreased, the concentration of the bacterial cells shall be increased to provide the same number of bacterial cells as when the normal volume of test inoculum is applied.

Option 2 Increase viscosity of the test inoculum by adding an inert thickener such as an agar or other inert material.

NOTE If the volume is reduced (Option 1) or the viscosity of the test inoculum is increased (Option 2), the measured antimicrobial efficacy may be different compared to tests run with a test inoculum of normal volume or viscosity.

Dimension in millimeters



l_1	l_2
40 ± 2	50 ± 2

Key

1 cover film
2 test inoculum (0,4 ml)
3 test specimen
4 petri dish
5 lid of petri dish

Figure 1 — Inoculation of the test specimen and placement of cover film

7.5.2 Unless otherwise specified, the standard size of the cover film shall be a square of (40 ± 2) mm for the 50 mm × 50 mm test specimen. If the test specimen is not standard size, then the size of the film shall be reduced. Do not, however, reduce the size of film to less than 400 square mm. The edges of the cover film shall be 2,5 mm to 5,0 mm inside the edge of the test specimen on all sides. If the size of the cover film is different from the 40 mm × 40 mm, the actual size used shall be included in the test report.

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7.6 Incubation of the inoculated test specimens

Unless otherwise specified, incubate the Petri dishes containing the inoculated test specimens (including half of the untreated test specimens) at a temperature of (35 ± 1) °C and a relative humidity of not less than 90 % for (24 ± 1) h. The antibacterial efficacy of a product is evaluated based on the value of antibacterial activity obtained from the test at the incubation temperature specified. Other temperatures may be used if agreed upon by all parties. If a temperature other than (35 ± 1) °C is used, it shall be included in the test report.

NOTE If incubation temperatures less than 35 °C are used, the total viable count of the bacteria may be reduced. This may affect the antibacterial activity compared to measurements conducted using a 35 °C incubation temperature.

7.7 Recovery of bacteria from test specimens

7.7.1 Test specimens immediately after inoculation

Immediately after inoculation, process half of the untreated test specimens by adding 10 ml of SCDLP broth (4.2.3.5) to the Petri dish containing the test specimen. This value will be used to confirm that the recovery rate of the bacteria is sufficient. Ensure that the SCDLP broth completely washes the specimens by using a pipette to collect and release the SCDLP broth at least four times.

NOTE Special consideration may be required to achieve a sufficient recovery rate especially if option 2 in 7.5.2 is taken increasing the viscosity of the inoculum. In this case, mechanical agitation may be required, such as stomaching, vortexing or sonicating. If these show a recovery rate equivalent to or superior to the method above, such methods may be used. If an alternate recovery method is used, it shall be described in the test report. If it is difficult to recover the test bacteria with 10 ml of the SCDLP broth due to the size and characteristics of the test specimen, then the volume of solution may be increased. If the volume of the SCDLP broth used is different from 10 ml, the actual volume used shall be included in the test report.

Use of alternative washing procedures may affect the measured antibacterial activity.

7.7.2 Test specimens after incubation

After the incubation in 7.6, process the remaining test specimens according to 7.7.1. Proceed immediately to count the viable bacteria recovered from the test specimen (see 7.8).

7.8 Viable bacteria count by the pour plate culture method

Enumerate the viable bacteria by performing 10-fold serial dilutions of the SCDLP in phosphate buffered physiological saline (4.2.3.7). Place 1 ml of each dilution, as well as 1 ml of the SCDLP recovered from the test specimen, into separate sterile Petri dishes. Pour 15 ml of plate count agar (4.2.3.3) into each Petri dish and swirl gently to disperse the bacteria. All plating shall be performed in duplicate. Invert the Petri dishes, and incubate them at (35 ± 1) °C for 40 h to 48 h.

After incubation, count the number of colonies in the Petri dishes containing 30 to 300 colonies. For each dilution series, record the number of colonies recovered to two significant figures, as well as the dilution factor of the plates used for counting. If the number of colonies in the plates containing the 1 ml aliquots of SCDLP is less than 30, then count and record the number of colonies in these plates. If there are no colonies recovered in any of the agar plates in the dilution series, record the number of colonies as "< 1". If inhibition due to carryover of the antimicrobial agent is observed, it may be necessary to substitute a different neutralizing agent for the SCDLP. If a neutralizer other than SCDLP is used, it shall be documented in the test report.

NOTE Information about selection and evaluation of alternative antibacterial neutralizing agents is found in ASTM E 1054 [8] and EN 1040 [9].

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8 Expression of results

Determine the number of viable bacteria recovered for each test specimen according to the formula (1).

$$N = C \times D \times V \quad (1)$$

where

N number of viable bacteria recovered, per test specimen

C Average plate count for the duplicate plates

D Dilution factor for the plates counted

V Volume (ml) of the SCDLP broth added to the specimen

Calculate the average of the number of viable bacteria recovered for each set of test specimens and express this value to two significant figures. If no colonies were recovered in any of the agar plates for a dilution series, then record the number of colonies counted as "< V" (where V is the volume (ml) of SCDLP broth added to the specimen). For calculating the average when there are no viable bacteria recovered in a dilution series, consider the number of viable bacteria to be "V".

EXAMPLE In the case of V = 10 ml, the number used for calculating the average shall be 10.

8.1 Test results

8.1.1 Conditions for a valid test

When the following three conditions are satisfied, the test is deemed valid. If all conditions are not met, the test is not considered valid and the specimens shall be retested.

8.1.1.1 The following formula is established for the logarithmic value of the number of viable bacteria recovered immediately after inoculation on untreated test specimens;

$$(L_{max} - L_{min})/(L_{mean}) \leq 0,2 \quad (2)$$

where

L_{max} maximum common logarithm* of the number of viable bacteria

* equivalent to base 10 logarithm

L_{min} minimum common logarithm of the number of viable bacteria

L_{mean} average common logarithm of the number of viable bacteria on the untreated test specimens immediately after inoculation

8.1.1.2 The average number of viable bacteria recovered immediately after inoculation from the untreated test specimens shall be within the range of $1,0 \times 10^5$ to $4,0 \times 10^5$ cells

8.1.1.3 The number of viable bacteria recovered from each untreated test specimen after 24 h shall not be less than $1,0 \times 10^3$ cells. When a film is used on the untreated test specimen, the number of viable bacteria recovered from each untreated test specimen after 24 h shall not be less than $1,0 \times 10^4$ cells.

NOTE If incubation temperatures less than 35 °C are used in 7.6, the number of viable bacteria recovered from untreated test specimens would not achieve the above criteria.

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8.1.2 Calculation of the value of antibacterial activity

When the test is deemed valid, calculate the value of antibacterial activity according to the formula (3). Record the value to one decimal place.

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \qquad (3)$$

Where:

R value of antibacterial activity

U_0 average of the common logarithm of the number of viable bacteria recovered from the untreated test specimens immediately after inoculation

U_t average of the common logarithm of the number of viable bacteria recovered from the untreated test specimens after 24 h

A_t average of the common logarithm of the number of viable bacteria recovered from the treated test specimens after 24 h

9 Test Report

The test report shall include the following information:

a) a reference to this international standard

b) the type of plastics, size, shape, and thickness of antibacterial and untreated test specimens

c) the type, size, shape, and thickness of film

d) species of test bacteria and their strain numbers

e) volume of test inoculum used

f) number of viable bacteria in the test inoculum

g) the values of U_0, U_t and A_t used in 8.1.2

h) the value of antibacterial activity

i) any deviation from this international standard, including cleaning methods, use of inert thickeners, volume of SCDLP used, use of alternative recovery methods and neutralizer used

j) identification of test laboratory, the name and signature of the head of the testing laboratory

k) date of commencement of the experiments

l) temperature

10 Repeatability and Reproducibility

Repeatability and reproducibility are discussed quantitatively in Annex A.

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Annex A
(informative)

Repeatability and reproducibility

A.1 Background

The content of this Annex is based on the results of an extensive research conducted to investigate the repeatability and reproducibility of the results from this draft test method. The quoted research was conducted from 2000 to early 2004 by the National Institute of Technology and Evaluation, partly to adopt ISO/IEC 17025:2000 as part of the Japan National Laboratory Accreditation System, and partly to determine the uncertainties for JIS Z 2801:2000, the original method used for this draft.

A.2 Summary

Repeatability and reproducibility of this draft test method were determined by means of a statistical analysis method defined in ISO 5725-2:1994. Antibacterial activity test results obtained in the inter-laboratory testing at four laboratories with two sorts of test samples by replicated experiments were examined.

Repeatability of identical test items in the same laboratory = 0,087

Reproducibility of identical test items in different laboratories = 0,304

These figures give an example for the estimation of repeatability and reproducibility of this draft test method, but should not be rigorously applied to acceptance or rejection of the laboratory test results on different test items.

A.3 Experiment

Materials included in the inter-laboratory test and the test conditions used are summarised in Table A.1.

Five local laboratories in Japan participated in the inter-laboratory testing. Each laboratory carried out replicated experiments with two types of antibacterial film samples. The number of test specimens used in each stage was as described in this standard. Test samples, as well as test bacterial strain, culture media and so on were supplied prior to the experiments.

Table A.1 — Materials and test conditions

Antibacterial sample	PET film, 40 mm square, 0,055 mm thickness Sample I; acrylic coating with 350 µg/g Ag compound Sample II; acrylic coating with 450 µg/g Ag compound
Untreated control sample	PET film, as above, acrylic coating without Ag compound
Cover film	PE film, 50 mm square, 0,09 mm thickness
Test strain	*Staphylococcus aureus* NBRC 12732
Test inoculum volume	0,4 ml

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NOTE 1 For the inter laboratory study, the special standard samples consisted of water soluble acrylic coatings applied on PET films. A required amount of antibacterial agent, a silver compound, was dispersed in the acrylic polymer solution, and it was coated on PET. This achieved uniform amounts of antibacterial agent on specimens for the testing.

NOTE 2 Because the applied coatings were water soluble, inoculums tended to spread over wider than cover film area. In this inter-laboratory testing, a reverse configuration than described in this draft method was necessarily adopted, i.e. put a cover film first, inoculated, and put a sample on it.

A.4 Results and discussion

In the primary analysis, calculated Z score by means of split-plots analysis for one laboratory exceeded 2,0. Thus those results were rejected, and data analyses were conducted only test results reported by the rest of the 4 laboratories. Table A.2 shows mean antibacterial activity and standard deviation for each antibacterial sample. In replicated experiments, here each experiment is expressed as 1st block and 2nd block.

Table A.2 — Mean antibacterial activity and standard deviation

	Mean antibacterial activity (standard deviation)	
	1st block	**2nd block**
Level 1 (sample I)	1,72 (0,42)	1,78 (0,26)
Level 2 (sample II)	2,29 (0,45)	2,42 (0,41)

In this inter-laboratory testing, two replicated experiments were performed by four laboratories for two types of material with two separate measurements of three replicate specimens. It should be noted that repeated measurement was carried out by using three individual samples for each sort of test piece. Sources of variation to consider in the analysis are experimental block, 1st or 2nd, i.e. replication [R], which laboratory performed [L], and contribution of test piece [P]. However, factors [R] and [L] cannot be randomised, and so split-plot based on two blocks are examined. Table A.3 shows ANOVA table and uncertainty.

Table A.3 — ANOVA table and uncertainty

Sources of variation	SS	df	V	F ratio	$F(p = 0{,}05)$	$F(p = 0{,}01)$	F test
Replication [R]	0,120 0	1	0,120 0	0,40	10,13	34,12	
Laboratory [L]	4,656 9	3	1,552 3	5,18	9,28	29,46	
$R \times L$ (1st order error: e_1)	0,899 1	3	0,229 7	13,84	2,90	4,46	**
Test piece [P]	4,440 8	1	4,440 8	205,12	4,15	7,50	**
L x P	0,388 7	3	0,129 6	5,98	2,90	4,46	**
R x P	0,013 3	1	0,013 3	0,62	4,15	7,50	
R x L x P	0,116 0	3	0,038 7	1,79	2,90	4,46	
2nd order error (e_2)	0,692 8	32	0,021 7				
Total	11,327 7	47					

NOTE 1 SS: sum of squares, df: degree of freedom, V: mean square

NOTE 2 ** : significant at 1 % level

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As shown in Table A.3, the main-plot error (e_1: first order error) of $R \times L$ indicates that e_1 is highly significant at 1 % level against the second error e_2 (error for replication of measurement). On the other hand, there is no statistical significance seen for $R \times P$ and $R \times L \times P$ against e_2, and so effects of these two are pooled into sub-plot error (e'_2). Table A.4 shows the result of analysis of variance after pooling the non-significant variations.

Table A.4 – ANOVA table and uncertainty (pooled for non-significant variation into e_2)

Source of variation	SS	df	V	F ratio	$F(p = 0{,}05)$	$F(p = 0{,}01)$	F test
Replication [R]	0,120 0	1	0,120 0	0,40	10,13	34,12	
Laboratory [L]	4,656 9	3	1,552 3	5,18	9,28	29,46	
$R \times L$ (1st order error: e_1)	0,899 1	3	0,299 7	13,84	2,90	4,46	**
Test piece [P]	4,440 8	1	4,440 8	194,46	4,11	7,50	**
$L \times P$	0,388 7	3	0,129 6	5,67	2,87	4,46	**
2nd order error (e'_2)	0,822 1	36	0,022 8				
Total	11,327 7	47					

It is confirmed that [R] of experimental block, i.e. replication, and [L] of laboratory, i.e. where experiments are done, individually are NOT statistically significant. Finally, the following conclusions can be expressed from the results of this research.

Repeatability; the standard uncertainty under repeatability conditions of three repetition of measurement is calculated from the above figures, as follows.

$$\sigma e_2 = (Ve'_2/3)^{1/2} = (0{,}022\ 8/3)^{1/2} = 0{,}087$$

Reproducibility; the standard uncertainty under reproducibility conditions is calculated from the above figures, as follows.

$$\sigma e_1 = [(Ve_1 - Ve'_2)/3]^{1/2} = [(0{,}299\ 7 - 0{,}022\ 8)/3]^{1/2} = 0{,}304$$

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