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Textiles—Determination of antibacterial activity and efficacy of textile products

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the original JIS is to be the final authority.

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Foreword

This translation has been made based on the original Japanese Industrial Standard revised by the Minister of Economy, Trade and Industry through deliberations at the Japanese Industrial Standards Committee according to the proposal for revision of Japanese Industrial Standard submitted by Japan Textile Evaluation Technology Council (JTETC)/Japanese Standards Association (JSA) with the draft being attached, based on the provision of Article 12 Clause 1 of the Industrial Standardization Law. Consequently **JIS L 1902**:2008 has been withdrawn and partially replaced with this Standard.

Attention is drawn to the possibility that some parts of this Standard may conflict with patent rights, applications for a patent after opening to the public or utility model rights. The relevant Minister and the Japanese Industrial Standards Committee are not responsible for identifying any of such patent rights, applications for a patent after opening to the public or utility model rights.

Textiles—Determination of antibacterial activity and efficacy of textile products

Introduction

This Japanese Industrial Standard has been prepared based on the second edition of **ISO 20743** published in 2013 with some modifications of the technical contents to correspond to technical trend and actual situation in Japan.

The portions given sidelines or dotted underlines are the matters in which the contents of the corresponding International Standard have been modified. A list of modifications with the explanations is given in Annex JB. Testing examples are given in Annex E.

The matters contained in Annex JA are unique contents of JIS that are not given in the corresponding International Standard.

1 Scope

This Standard specifies quantitative and qualitative test methods to determine the antibacterial activity of all antibacterial textile products including nonwovens and antibacterial efficacy.

This Standard is applicable to all textile products, including cloth, wadding, thread and material for clothing, bedclothes, home furnishings and miscellaneous goods, regardless of the type of antibacterial agent used (organic, inorganic, natural or man-made) or the method of application (built-in, after-treatment or grafting).

Based on the intended application and on the environment in which the textile product is to be used and also on the surface properties of the textile properties, the user can select the most suitable of the following four determination methods on determination of antibacterial activity.

a) **Absorption method** An evaluation method in which test bacterial suspension is inoculated directly onto the specimens.

b) **Transfer method** An evaluation method in which test bacteria are placed on an agar plate and transferred onto the specimens.

c) **Printing method** An evaluation method in which test bacteria are placed on a filter and printed onto specimens.

d) **Halo method** A qualitative method to evaluate by the existence of halo.

The colony plate count method and the ATP (ATP = Adenosine Tri-phosphate) luminescence method are also specified for measuring the enumeration of bacteria.

The evaluation of photocatalytic antibacterial efficacy treated on the photocatalytic finished textile product shall be carried out according to **JIS R 1702**.

NOTE : The International Standard corresponding to this Standard and the symbol of degree of correspondence are as follows.

ISO 20743:2013 *Textiles—Determination of antibacterial activity of textile products* (MOD)

In addition, symbols which denote the degree of correspondence in the contents between the relevant International Standards and **JIS** are IDT (identical), MOD (modified), and NEQ (not equivalent) according to **ISO/IEC Guide 21-1**.

2 Normative references

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

JIS K 0970 *Piston pipettes*

JIS K 3800 *Class II biological safety cabinets*

JIS K 8150 *Sodium chloride (Reagent)*

JIS L 0803 *Standard adjacent fabrics for staining of colour fastness test*

JIS R 1702 *Fine ceramics (advanced ceramics, advanced technical ceramics)—Test method for antibacterial activity of photocatalytic products and efficacy*

JIS R 3505 *Volumetric glassware*

JIS Z 8401 *Guide to the rounding of numbers*

3 Terms and definitions

For the purpose of this Standard, the following term and definition apply.

3.1 control specimen

specimen which is the same textile product as the textile product to be tested, but without antibacterial treatment, used to validate the condition of test effectiveness

If control specimen is not available, a 100 % cotton standard adjacent fabric (cotton No. 3-1) specified in **JIS L 0803** is used as the control specimen, after 10 cycles of washing for 10 min at a temperature of 60 °C without detergents or any brighteners, rinsing twice for 5 min and air-drying.

3.2 antibacterial agent

product designed to prevent or mitigate the growth of bacteria, to reduce the number of bacteria or to kill bacteria

3.3 antibacterial finish

treatment designed to prevent or mitigate the growth of bacteria, to reduce the number of bacteria or to kill bacteria

3.4 antibacterial activity

activity of an antibacterial finish used to prevent or mitigate the growth of bacteria, to reduce the number of bacteria or to kill bacteria

3.5 antibacterial efficacy

efficacy of antibacterial activity shown by antibacterial finish

It is evaluated by the existence of halo and antibacterial activity value.

3.6 plate count method

method in which the number of bacteria present after incubation is calculated by counting the number of colonies according to a ten-time dilution method

The results are expressed in "CFU (Colony Forming Unit)".

3.7 luminescence method

method in which the amount of adenosine triphosphate (hereafter referred to as "ATP") contained in bacterial cells is measured

The results are expressed in "moles of ATP".

3.8 neutralizer

chemical agents used to inactivate, neutralize or quench the antibacterial properties of antibacterial agents

3.9 halo

the part where the growth of test bacteria produced around the sample is suppressed when the antibacterial processed sample is placed and incubated on the culture medium containing the test bacteria

4 Safety precaution

The test methods specified herein require the use of bacteria. These tests should be carried out by person with training and experience in the use of microbiological techniques. Safety precautions shall be checked against and comply with regulations.

5 Apparatus

5.1 Spectrophotometer, capable of measuring at a 620 nm to 660 nm wavelength, or McFarland's nephelometer.

5.2 Incubator, capable of maintaining a constant temperature of 37 °C ± 2 °C.

5.3 Water baths, one capable of maintaining a constant temperature of 46 °C ± 2 °C and another capable of maintaining a temperature of 70 °C to 90 °C.

5.4 Mixer, producing a vortex shaking action.

5.5 Stomacher, capable of speeds of 6 blows per second to 8 blows per second, with the corresponding disposable containers.

5.6 Clean bench or safety cabinet, for microbial test. Safety cabinet is specified in **JIS K 3800**.

5.7 Platinum colony loop, with a loop of approximately 4 mm in diameter.

5.8 Humidity chamber, tropical chamber or other container capable of maintaining a high-humidity more than 70 %RH atmospheric condition.

5.9 Luminescence photometer, capable of measuring ATP of 10^{-13} mol/L to 10^{-7} mol/L at 300 nm to 650 nm with a luminescence-measuring reagent.

5.10 Printing apparatus, capable of applying a 4 N load to a test specimen and rotating the specimen 180° in one direction for a period of 3.0 s.

5.11 Refrigerator, capable of maintaining a temperature of between 2 °C and 8 °C.

5.12 Freezers, one adjustable to a temperature below −70 °C and another to a temperature below −20 °C.

5.13 Balance, which can be read to the nearest 0.01 g.

5.14 Filtering apparatus, consisting of an upper container equipped with a membrane filter and a lower container equipped with a suction opening.

5.15 Pipette, having the most suitable volume for each use, with a tip made of glass or plastic, and with a tolerance to conform to Class A in **JIS K 0970** or **JIS R 3505** or equivalent to it.

5.16 Vials, 30 ml glass bottles, with screw openings, polytetrafluoroethylene or silicone packing and caps made of polypropylene, polycarbonate or another suitable material.

5.17 Petri dishes, that have been sterilized, made of glass or plastic, in diameter sizes of 90 mm to 100 mm. For the specimen of transfer method, in diameter sizes of 55 mm to 60 mm.

5.18 Glass rod, with a diameter of approximately 18 mm.

5.19 Anti-bumping granules (glass beads), with a diameter of 3 mm to 4 mm.

5.20 Erlenmeyer flask with cap, of capacity 100 ml.

5.21 Cutting template, made of a sterilizable material (stainless steel or glass) with a diameter of 38 mm ± 1 mm.

5.22 Disposable plastic bags, sterile bags suitable for containing food products, to be used for one of the shaking methods of the specimens.

5.23 Tweezers, made of a material which can be sterilized.

5.24 Stainless-steel cylinder, with a mass of 200 g ± 10 g and a diameter of 35 mm ± 1 mm.

5.25 Metal wire basket, for autoclaving, to be used for sterilizing test specimens.

5.26 Aluminium foil, capable of wrapping a metal wire basket, etc.

5.27 Reciprocal incubation shaker, capable of adjusting a temperature of 37 °C ± 2 °C, 110 time/min and about 30 mm in amplitude.

5.28 Autoclave, capable of sterilizing at 120 °C ± 2 °C and 103 kPa ± 5 kPa.

6 Reagents and culture media

6.1 Water Water used in tests shall be analytical-grade water for microbiological media preparation, which is freshly distilled and/or ion-exchanged and/or ultra-filtered and/or filtered with RO (reverse osmosis). It shall be free from all toxic or bacteria inhibitory substances.

6.2 Tryptone soya broth (TSB) Mix well the following compositions and adjust pH to 7.2 ± 0.2, then sterilize by autoclave (**5.28**).

— Tryptone, pancreatic digest of casein	17 g
— Soya peptone, papain digest of soya	3 g
— Sodium chloride (NaCl) According to **JIS K 8150**.	5 g
— Glucose	2.5 g
— Dipotassium hydrogen phosphate	2.5 g
— Water (**6.1**)	1 000 ml

6.3 Tryptone soya agar (TSA) Mix well the following compositions and adjust pH to 7.2 ± 0.2, then sterilize by autoclave.

— Tryptone, pancreatic digest of casein	15 g
— Soya peptone, papain digest of soya	5 g
— Sodium chloride (NaCl)	5 g
— Agar	15 g
— Water	1 000 ml

6.4 Agar for transfer Mix well the following compositions and adjust pH to 7.2 ± 0.2, then sterilize by autoclave.

— Tryptone, pancreatic digest of casein	0.75 g
— Soya peptone, papain digest of soya	0.25 g
— Sodium chloride (NaCl)	5 g
— Agar	15 g
— Water	1 000 ml

6.5 Nutrient broth (NB) Mix well the following compositions and adjust pH to 6.9 ± 0.2, then sterilize by autoclave.

— Beef extract	3 g
— Peptone	5 g
— Water	1 000 ml

6.6 Peptone salt solution Mix well the following compositions and adjust pH to 6.9 ± 0.2, then sterilize by autoclave.

— Peptone, pancreatic digest of casein 1 g
— Sodium chloride (NaCl) 8.5 g
— Water 1 000 ml

6.7 Physiological saline Mix well the following compositions and adjust pH to 6.9 ± 0.2, then sterilize by autoclave.

— Sodium chloride (NaCl) 8.5 g
— Water 1 000 ml

6.8 SCDLP medium Mix well the following compositions and adjust pH to 7.2 ± 0.2, then sterilize by autoclave.

— Peptone, digest of casein 17 g
— Peptone, digest of soybean 3 g
— Sodium chloride (NaCl) 5 g
— Dipotassium hydrogen phosphate 2.5 g
— Glucose 2.5 g
— Lecithin 1 g
— Polysorbate 80 7 g
— Water 1 000 ml

6.9 Dilution buffer for shake-out bacterial suspension This buffer solution consists of 0.00 5 mol/L sodium dihydrogen phosphate containing 0.037 % sucrose and adjusting pH to 7.2 ± 0.2.

6.10 Neutralizing solution The composition of the standard neutralizing solution shall be as follows.

— Polysorbate 80 30 g
— Egg-yolk lecithin 3 g
— Histidine hydrochloride 1 g
— Meat or casein peptone 1 g
— Sodium chloride (NaCl) 4.3 g
— Monopotassium phosphate 3.6 g
— Disodium phosphate dihydrate 7.2 g
— Water 1 000 ml

6.11 Enumeration agar (EA) Mix well the following compositions and adjust pH to 7.2 ± 0.2, then sterilize by autoclave.

— Dehydrated yeast extract 2.5 g
— Casein tryptone 5.0 g
— Glucose 1.0 g

— Agar 12 g to 15 g[1)]

— Water 1 000 ml

Note [1)] Necessary mass is depending on the gel strength of the product.

6.12 Agar for printing Mix well the following compositions, then sterilize by autoclave.

— Agar 20 g

— Water 1 000 ml

6.13 Cryoprotective solution for bacterial species For freezing, a cryoprotective solution containing 150 g/L of glycerol or 100 g/L of dimethylsulfoxide shall be used and prepared as follows.

a) Add 1 000 ml of tryptone soya broth (TSB) (**6.2**) or nutrient broth (NB) (**6.5**). Add 150 g of glycerol or 100 g of dimethylsulfoxide, and mix well and sterilize by autoclave.

b) For solutions containing glycerol, sterilize the mixed solution by autoclave. For solutions containing dimethylsulfoxide, sterilize the mixed solution by using 0.22 μm membrane filter.

NOTE : Any commercially available product may be used as long as it is a cryoprotective solution or preserving system that contains glycerol or dimethylsulfoxide and allows preservation of the strains in the same manner as the specified solutions.

6.14 Stock solution of ATP standard reagent Dissolve 60.5 mg of adenosine-disodium 5′-triphosphate trihydrate with water to make 1 000 ml. The concentration of ATP standard reagent is 1×10^{-4} mol/L which is obtained by the following mixing.

After preparation, the solution shall be placed in a tightly sealed container and cryopreserved at a temperature of –20 °C or lower. The solution shall be used no later than six months from the date of preparation.

Do not refreeze frozen solution that has thawed.

NOTE : The suitable amount of adenosine-disodium 5′-triphosphate trihydrate may be calculated from the ATP content of each commercial product.

6.15 Buffer solution for ATP luminescent reagent, prepared from the following compositions to be pH 7.2 ± 0.2.

— N-[Tris(hydroxymethyl)methyl] glycine 1 117 mg

— Ethylenediamine disodium tetraacetatedehydrate 183 mg

— Magnesium acetate tetrahydrate 808 mg

— DL-dithiothreitol 6.7 mg

— Dextrin 25 000 mg

— Sucrose 925 mg

— Water 250 ml

6.16 ATP luminescent reagent, prepared from the following compositions. Once fully dissolved, let sit at room temperature for 15 min before use.

Use within 3 h of preparation. When a different ATP luminescent reagent is used, its composition shall be recorded.

— Luciferase (EC:1.13.12.7) 16.0 mg

— D-luciferin 12.6 mg

— Bovine serum albumin 56 mg

— Buffer solution for ATP luminescent reagent (**6.15**) 30 ml

6.17 ATP extracting reagent, prepared from the following compositions to be pH 7.2 ± 0.2.

When a different ATP extracting reagent is used, its composition shall be recorded.

— N-[Tris(hydroxymethyl)methyl] glycine 45 mg

— 10 % aqueous benzalkonium chloride 0.2 ml

— Water 9.8 ml

6.18 ATP eliminating reagent, prepared from the following compositions to be pH 6.0 ± 0.5. An agent to reduce the ATP in Tryptone soya broth (TSB) (**6.2**) or Nutrient broth (NB) (**6.5**) to less than 10^{-13} mol/L within 15 min.

— Apyrase (EC:3.6.1.5) 46 international units/ml

— Adenosine phosphate deaminase (EC:3.5.4.6 or EC:3.5.4.17) 46 international units/ml

— Sucrose 37 mg

— Bovine serum albumin 20 mg

— 0.05 mol/L buffer solution of 2-morpholinoethanesulfonicacid, monohydrate 10 ml

The reagent shall be used within 8 h of preparation.

When a different ATP eliminating reagent is used, its composition shall be recorded.

6.19 SCDLP or other medium for preparing ATP reference solution, prepared from the following compositions. An ATP reference solution should be prepared if the addition of neutralizing agents causes the ATP content in the shake-out solution to exceed 10^{-11} mol/L.

— SCDLP (**6.8**) or other medium 10 ml

— ATP eliminating agent (**6.18**) 1 ml

After mixing, maintain at 30 °C to 37 °C for 1 h to prevent microbiological contamination. Next, transfer to a hot-water bath (**5.3**) at 70 °C to 90 °C for 1 h and cool down to room temperature. Preserve the solution under refrigeration and use within 24 h.

6.20 Shake-out physiological saline Mix well the following compositions, then sterilize by autoclave (**5.28**).

— Sodium chloride (NaCl) 8.5 g

— Polysorbate 80 2.0 g

— Water 1 000 ml

NOTE 1 Reagents used in tests should be of analytical quality and/or suitable for microbiological purposes.

NOTE 2 Dehydrated products available on the commercial market are recommended for use in preparing the culture media. The manufacturer's instructions for the preparation of these products should be strictly followed.

7 Reference strains

7.1 Strains

The strain to be used shall be selected from Annex A.

7.2 Storage of strains

7.2.1 General

The strains shall be stored in accordance with the supplier's recommendations.

7.2.2 Ceramic bead method

Ceramic bead method shall be as follows.

a) Obtain a sample of the freeze-dried bacterial strain following the recommendations supplied with the culture and resuspend it in 5 ml of Tryptone soya broth (TSB) (**6.2**). Obtain a sample of the suspension and isolate it in a Petri dish (**5.17**) containing Tryptone soya agar (TSA) (**6.3**).

b) Incubate the cultures for 18 h to 24 h at 37 °C ± 2 °C. After incubation, use the culture isolated in the Petri dish to verify the purity of the strain. After verification, prepare the stock cultures.

c) Sample 0.7 ml of the broth culture and spread it over the surface of the Petri dish containing the Tryptone soya agar (TSA). Incubate the culture on plates for 18 h to 24 h under the conditions specified in **b**).

d) Add 10 ml of cryoprotective solution (**6.13**) to the surface of the TSA plate culture and resuspend the cells in the solution using a sterile glass spreader. Sample the suspended cells from the surface of the agar. Dilute them in 100 ml of cryoprotective solution and incubate for 30 min at 20 °C.

e) Using a pipette (**5.15**), sample 1 ml of the suspension and transfer it to a cryogenic vial (**5.16**) containing the beads (**5.19**). Shake the vial in order to spread the suspended cells around the beads. Where a cryoprotective solution containing dimethylsulfoxide is used, do not let it stand longer than 1 min at ambient temperature. Where a cryoprotective solution containing glycerol is used, let it stand for 30 min at 20 °C.

f) Withdraw the excess cryoprotective solution with a sterile pipette. Place the cryogenic vials in a freezer (**5.12**) set at –70 °C or lower.

g) Carry out the quantitative measurement by plate count method specified in Annex C to confirm the number of bacteria. Prepare 10^{-6} and 10^{-7} dilutions of the suspension using the serial dilution method. Take a 1.0 ml sample of each dilution and transfer it to separate Petri dishes. Add 12 ml to 15 ml of nutritive solution, cooled down to 45 °C ± 1 °C. Incubate for 18 h to 24 h under the conditions at 37 °C ± 2 °C.

h) Enumerate the plate cultures and confirm that the suspension contains less than 5×10^8 CFU/ml.

7.2.3 Glycerol suspension method

Glycerol suspension method shall be as follows.

a) Inoculate a 15 ml culture tube containing 5 ml of appropriate medium with a freshly grown isolated colony. Incubate usually for 5 h to overnight at 37 °C until the bacteria culture seems to reach the late logarithm or stationary phase in the growth curve.

b) For each strain to be stored below –70 °C, for the archives, prepare a sterile, labelled cryogenic vial. Place 225 µl of sterile 80 % glycerol in a cryogenic vial.

c) Add 1.0 ml of the bacterial culture (frozen stock shall be 15 % glycerol). Mix well using the vortex mixer (**5.4**) and store in a tube at –70 °C or lower.

d) For each strain to be stored at –20 °C, as liquid glycerol working stock, pipette equal volumes of 80 % glycerol and bacterial culture into a labelled polypropylene tube. Mix the contents well to avoid formation of ice crystals that will decrease the viability of the cells. Place the tube in a freezer at –20 °C. Check the viability of the cells after 1 week if possible.

e) To recover a strain from the glycerol stock stored below –70 °C, use a sterile toothpick to scrape pieces of the solid substance. Then streak the cells onto the appropriate medium. Do not thaw the frozen stock because each freeze-thaw cycle will result in a 50 % loss in cell viability.

f) To use the –20 °C working stock, pipette 50 µl to 100 µl as inoculum for a 5 ml overnight culture.

7.2.4 Slant culture medium method

Slant culture medium method shall be as follows.

a) For the preparation of slant culture medium, pour about 10 ml of Enumeration agar (EA) (**6.11**), dissolved by warming beforehand in a test tube, carry out autoclaved-sterilization by putting cotton stopper, after the sterilization is finished, place the test tube in clean room so as to incline against the horizontal surface by about 15° in angle and coagulate the contents. Dissolve that with the coagulated water lost and coagulate it again and use.

b) The transference of bacteria shall be carried out according to the following procedure. Hold both the stock strain and slant agar to be transferred in one hand, hold the stem of platinum colony loop in the other hand and pull out the cotton stopper from test tube with this hand, and sterilize the mouth of the test tube with flame.

c) Next, sterilize the platinum colony loop with flame, insert the loop into a part on slant agar having condensed water, cool it well and put it into a test tube of stock strain (see Figure 1). Scrape out the bacteria from the breeding surface of bacteria using the platinum colony loop, spread it on a slant agar, again sterilize the mouth of the test tube with flame, and place a cotton stopper as it was. Sterilize the platinum colony loop used with flame.

d) Incubate the bacteria transferred to slant agar at 37 °C ± 2 °C for 24 h to 48 h, and thereafter preserve it at 5 °C to 10 °C.

When spreading one platinum loop on the slant agar, disperse bacteria in condensed water as shown in Figure 1, and draw a straight line slant to get to the upper part. Separate once the point of platinum loop from culture medium, then again insert it into condensed water, and this time draw a zigzag line up to the upper part.

e) Within one month from the transference, carry out the next transference of bacteria as same as previously to make passage culture. The passage culture, shall not be subcultured more than 10 times. When kept for one month or longer from the last transference, it shall not be used for the following transference.

NOTE : In the case of long term preservation, it may be preserved either by freeze drying or by being transferred on high layer culture medium and being layered with sterilized liquid paraffin.

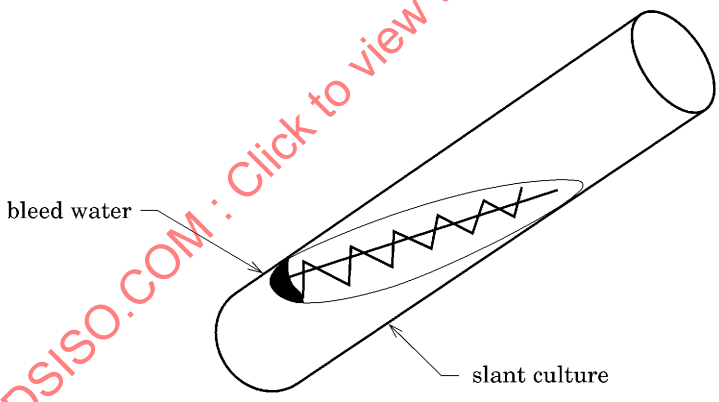


Figure 1 Transference of bacteria on slant culture medium

8 Test procedures

8.1 Absorption method

8.1.1 Incubation

8.1.1.1 Preincubation A

Pick up the preserved stock bacteria from the storage container using a platinum colony loop (**5.7**). Streak onto the plate of Enumeration agar (EA) (**6.11**) so that the colony after incubated is isolated. Incubate for 24 h to 48 h under the conditions at 37 °C ± 2 °C. The plate is kept at 5 °C to 10 °C and used within 1 week after the date of preparation.

The plate with colony taken once shall not be used again.

8.1.1.2 Preincubation B

Pour 20 ml of Nutrient broth (NB) (**6.5**) or Tryptone soya broth (TSB) (**6.2**) into a 100 ml Erlenmeyer flask with cap (**5.20**). Apply a platinum colony loop to pick one colony up from the incubation as specified in **8.1.1.1** and inoculate it in the broth. Incubate under the following conditions.

— Temperature: 37 °C ± 2 °C

— Rate of shaking: 110 time/min and 30 mm width by reciprocal incubation shaker (**5.27**)

— Incubation time: 18 h to 24 h

8.1.1.3 Preincubation C

Pour 20 ml of Nutrient broth (NB) (**6.5**) or Tryptone soya broth (TSB) (**6.2**) into a 100 ml Erlenmeyer flask. Add 0.4 ml of the inoculum from the incubation as specified in **8.1.1.2** that contains 1×10^8 CFU/ml to 3×10^8 CFU/ml in bacteria concentration or a target ATP concentration of 1×10^{-6} mol/L to 3×10^{-6} mol/L to the flask and incubate under the following conditions.

— Temperature: 37 °C ± 2 °C

— Rate of shaking: 110 time/min and 30 mm width by reciprocal incubation shaker

— Incubation time: 3 h ± 1 h

Target CFU or ATP concentration after incubation: 10^7 CFU/ml or 10^{-7} mol/L.

NOTE : The prepared inoculum is preserved by ice-cooling and used within 8 h.

8.1.2 Preparation of test inoculum

Adjust the bacteria after incubation in **8.1.1.3** to a concentration of 1×10^5 CFU/ml to 3×10^5 CFU/ml by a spectrophotometer or McFarland's nephelometer (**5.1**) using Nutrient broth (NB) (**6.5**) or Tryptone soya broth (TSB) (**6.2**) after it has been diluted 20 times with water at room temperature, or measure the ATP concentration of the bacteria after incubation in **8.1.1.3** by the luminescence method and adjust the ATP to a concentration of 1×10^{-9} mol/L to 3×10^{-9} mol/L.

Measure the bacteria concentration and the ATP concentration of prepared test inoculum according to the determination method specified in Annex C and Annex D, and take them as the concentration of test inoculum.

NOTE : The prepared inoculum is preserved by ice-cooling and used within 4 h.

8.1.3 Preparation and setting of test specimens

8.1.3.1 Preparation of test specimens

Obtain six test specimens and six control specimens with a mass of 0.40 g ± 0.05 g and cut to a suitable size for test specimens.

NOTE : Three test specimens and three control specimens are used for zero time, immediately after inoculation. The remaining six specimens are used for the contact time after 18 h to 24 h incubation.

8.1.3.2 Setting of test specimen

Place each of the test specimens in separate vials (**5.16**) by selecting the following method appropriate to the nature of the test sample.

a) If specimens tends to curl easily, or if it contains wadding or down, place a glass rod (**5.18**) onto the specimen in the vial. Alternatively, lace up both ends of the specimen with thread.

b) If the specimen is yarn, arrange the yarn in a bundle and place a glass rod onto the specimen in the vial.

c) If the specimen is from a carpet or a similar construction, cut the carpet itself or pile and place a glass rod onto the specimen in the vial.

Pretreatment may be carried out in accordance with the agreement between the parties concerned, if necessary. The detail of the pretreatment carried out shall be recorded in the test report.

8.1.3.3 Sterilization

Except in the case where it is confirmed that contamination has not been found, sterilize test specimens by autoclave (**5.28**) according to the following procedure.

a) Wrap the opening of vials (**5.16**) containing specimens with aluminium foil (**5.26**).

b) Place the wrapped vials in a metal wire basket (**5.25**) for autoclaving.

c) Wrap the vial caps with aluminium foil and place them in the wire basket.

d) Sterilize the caps and the vials containing the test specimens by autoclave for 15 min to 20 min.

e) After sterilization, remove the aluminium foil and allow the specimens in the vials to dry for 60 min or more by placing them on a clean bench (**5.6**) or any other place where there is no risk of airborne contamination.

f) Cap the vials securely.

NOTE : When autoclaving is not possible, sterilization may be accomplished by ethylene oxide gas, γ-ray or another suitable method. The use of alternative methods shall be recorded in the test report.

8.1.4 Test operation

8.1.4.1 Inoculation of test specimens

Accurately pipette (**5.15**) 0.2 ml of the inoculum prepared in **8.1.2** at several points on each test specimen (**8.1.3.2**) to ensure that no inoculum touches the surface of the vial (**5.16**) and cap the vials.

8.1.4.2 Shake-out after inoculation

Immediately after the inoculation of **8.1.4.1**, add 20 ml of any one of SCDLP medium (**6.8**) or the neutralizing solution (**6.10**) or the shake-out physiological saline (**6.20**) into each of the three vials (**5.16**) in which a test specimen and each of three vials in which a control specimen have been placed, cap the vials and shake out as specified in Annex B.

8.1.4.3 Incubation

Incubate the three vials (**5.16**) for control specimens and the three vials for test specimens which have not been subjected to the shake out in **8.1.4.2** at 37 °C ± 2 °C for 18 h to 24 h.

8.1.4.4 Shake-out after incubation

After the incubation of **8.1.4.3**, add 20 ml of any one of SCDLP medium or of the neutralizing solution or the shake-out physiological saline to each of the six vials (**5.16**), cap the vials and shake out as specified in Annex B.

8.1.4.5 Calculation of number of bacteria or amount of ATP

8.1.4.5.1 General

Obtain the number of bacteria or amount of the ATP as specified in **8.1.4.2** and **8.1.4.4** from the bacteria concentration obtained in Annex C or the ATP concentration obtained in Annex C or Annex D according to the following formulae.

8.1.4.5.2 Number of bacteria

Calculation of the number of bacteria shall be made according to Formula (1).

$$M = c_B \times 20 \quad \cdots\cdots (1)$$

where, M : number of bacteria per test specimen

c_B : bacteria concentration obtained in Annex C

20 : volume of the shake-out solution (ml)

8.1.4.5.3 Amount of ATP

Calculation of the amount of ATP shall be made according to Formula (2).

$$M' = c_{ATP'} \times 20 \quad \cdots\cdots (2)$$

where, M' : amount of ATP per test specimen

$c_{ATP'}$: ATP concentration obtained in Annex D

20 : volume of the shake-out solution (ml)

8.1.5 Test results

8.1.5.1 Judgement of test effectiveness with the control specimen

When the conditions of **a**), **b**) and **c**) or **a**), **b**) and **d**) are satisfied, the test is judged to be effective. When the test is judged to be ineffective, a retest shall be carried out.

a) The test inoculum of **8.1.2** shall be 1×10^5 CFU/ml to 3×10^5 CFU/ml or the ATP concentration shall be 1×10^{-9} mol/L to 3×10^{-9} mol/L.

b) The difference in common logarithm in extremes of the number of bacteria, or the amount of ATP for the three control specimens immediately after inoculation and after incubation, respectively, shall be less than one.

c) The growth value (F) obtained according to the following formula shall be equal or more than 1.0 in the plate count method and round the results to one decimal place according to Rule B of **JIS Z 8401**.

d) The growth value (F) obtained according to the following formula shall be equal or more than 0.5 in the luminescence method and round the results to one decimal place according to Rule B of **JIS Z 8401**. Calculation of the growth value (F) shall be made according to Formula (3).

$$F = \log C_t - \log C_0 \quad \cdots\cdots (3)$$

where, F : growth value on the control specimen

$\log C_t$: common logarithm of arithmetic average of the numbers of bacteria, or amount of ATP, obtained from three control specimens after an 18 h to 24 h incubation

$\log C_0$: common logarithm of arithmetic average of the numbers of bacteria, or the amount of ATP, obtained from three control specimens immediately after inoculation

8.1.5.2 Judgement of test effectiveness with the test specimen and calculation of antibacterial activity value

When the condition in the following is satisfied, the test is judged to be effective. The difference in common logarithm in extreme of the number of bacteria, or the amount of ATP for the three test specimens immediately after inoculation and after incubation, respectively, shall be less than two. When the test is judged to be ineffective, a retest shall be carried out.

When the test has been judged to be effective, obtain the antibacterial activity value according to Formula (4) and round the results to one decimal place according to Rule B of **JIS Z 8401**.

In the case of $\log C_0 > \log T_0$, substitute $\log C_0$ for $\log T_0$.

$$A = (\log C_t - \log C_0) - (\log T_t - \log T_0) = F - G \quad \cdots\cdots (4)$$

where, A : antibacterial activity value

F : growth value on the control specimen ($F = \log C_t - \log C_0$)

G : growth value on the antibacterial test specimen ($G = \log T_t - \log T_0$)

$\log T_t$: common logarithm of arithmetic average of the numbers of bacteria, or the amount of ATP, obtained from three test specimens after an 18 h to 24 h incubation

$\log T_0$: common logarithm of arithmetic average of the numbers of bacteria, or the amount of ATP, obtained from three test specimens immediately after inoculation

8.2 Transfer method

8.2.1 Test bacteria and preparation of test inoculum

8.2.1.1 Incubation of test strain

Pick up the preserved stock bacteria from the storage container using a platinum colony loop. Streak it onto the plate of Tryptone soya agar (TSA) (**6.3**). Incubate at 37 °C ± 2 °C for 18 h to 24 h. After incubation, extract a colony from the plate using a platinum colony loop, streak onto another plate of tryptone soya agar (TSA). Incubate at 37 °C ± 2 °C for 18 h to 24 h.

NOTE : The second transfer constitutes the working culture(s).

When inoculation cannot be completed within a single day, a 48-h culture may be used for the subsequent inoculation, provided that the culture is stored in an incubator (**5.2**) and incubate at 37 °C ± 2 °C for 48 h. In this event, a new 24-h subculture shall be prepared prior to performing the test. A fourth subculture shall not be used.

8.2.1.2 Preparation of test inoculum

Obtain a colony from the second transferred TSA using a platinum colony loop, place it in the Peptone-salt solution (**6.6**). Mix well with the vortex mixer (**5.4**).

Adjust the number of bacteria to a concentration of 1×10^8 CFU/ml to 3×10^8 CFU/ml using the Peptone-salt solution by the spectrophotometer or McFarland's nephelometer (**5.1**). Adjust the number of bacteria to an ATP concentration of 2×10^{-7} mol/L to 6×10^{-7} mol/L using the Peptone-salt solution by luminescence method.

Dilute the inoculum to a concentration of 1×10^6 CFU/ml to 3×10^6 CFU/ml or an ATP concentration of 2×10^{-9} mol/L to 6×10^{-9} mol/L using the Peptone-salt solution.

The final number of bacteria should be checked by the quantitative measurement method specified in Annex C and Annex D.

8.2.2 Preparation of test specimens

Using a template (**5.21**), cut specimens for test that are 38 mm in diameter. Pretreatment may be carried out in accordance with the agreement between the parties concerned, if necessary. The detail of the pretreatment carried out shall be recorded in the test report.

Except in the case where it is confirmed that contamination has not been found, sterilize test specimens by autoclave (**5.28**). When autoclaving is not possible, sterilization may be accomplished by ethylene oxide gas, γ-ray or another suitable method. The use of alternative methods shall be recorded in the test report.

8.2.3 Test operation

8.2.3.1 Inoculation to agar plates

Prepare 12 plates of the agar for transfer (**6.4**), six plates for control specimens and six for test specimens. Inoculate 1 ml of the test inoculum of **8.2.1.2** on the agar, inclining the plate in several directions so as to completely flood the surface of the plate. Suck up as much of the excess liquid as possible. Let stand for 300 s ± 30 s.

8.2.3.2 Transfer to specimens

Prepare six control specimens and six test specimens.

Pretreatment may be carried out in accordance with the agreement between the parties concerned, if necessary. The detail of the pretreatment carried out shall be recorded in the test report.

NOTE : Three of the control specimens and three of the test specimens are used for immediately after transfer. The remaining specimens are used for the contact time, after incubation.

Set each specimen on the agar surface of **8.2.3.1** so that the antibacterial finished surface is the side of agar culture medium in the case of the test sample, and weigh down with a stainless-steel cylinder (**5.24**) for 60 s ± 5 s. Place each specimen in a 55 mm to 60 mm in diameter Petri dish with the transferred surface face up. Incubate in a humidity chamber (**5.8**) for 18 h to 24 h at a temperature of 37 °C ± 2 °C and a humidity of more than 70 %RH.

8.2.3.3 Shake-out after transfer

Immediately after transfer, place each specimen in a sterile bag (**5.22**) or a vial containing 20 ml of the neutralizing solution (**6.10**) and shake out as specified in Annex B.

8.2.3.4 Shake-out after incubation

After incubation, place each specimen in a sterile bag or a vial containing 20 ml of the neutralizing solution (**6.10**) and shake out as specified in Annex B.

8.2.3.5 Calculation of number of bacteria or amount of ATP

8.2.3.5.1 General

Obtain the number of bacteria or amount of the ATP as specified in **8.2.3.3** and **8.2.3.4** from the bacteria concentration obtained in Annex C or the ATP concentration obtained in Annex D according to the formulae specified in **8.2.3.5.2** and **8.2.3.5.3**.

8.2.3.5.2 Number of bacteria

Calculation of the number of bacteria shall be made according to Formula (1) of **8.1.4.5.2**.

8.2.3.5.3 Amount of ATP

Calculation of the amount of ATP shall be made according to Formula (2) of **8.1.4.5.3**.

8.2.4 Test results

8.2.4.1 Judgement of test effectiveness with the control specimen

When the conditions of **a**), **b**) and **c**) or **a**), **b**) and **d**) are satisfied, the test is judged to be effective. When the test is judged to be ineffective, a retest shall be carried out.

a) The test inoculum of **8.2.1.2** shall have a concentration of 1×10^6 CFU/ml to 3×10^6 CFU/ml or the ATP concentration shall be 2×10^{-9} mol/L to 6×10^{-9} mol/L.

b) The difference in common logarithm in extremes of the numbers of bacteria, or the amount of ATP for the three control specimens immediately after inoculation and after incubation, respectively, shall be less than one.

c) The growth value (F) obtained according to the following formula shall be equal or more than 1.0 in the plate count method.

d) The growth value (F) obtained according to the following formula shall be equal or more than 0.5 in the luminescence method.

The growth value (F) shall be obtained by Formula (3) in **8.1.5.1**. Round the results to one decimal place according to Rule B of **JIS Z 8401**.

8.2.4.2 Judgement of test effectiveness with the test specimen and calculation of antibacterial activity value

When the condition in the following is satisfied, the test is judged to be effective. The difference in common logarithm in extreme of the number of bacteria, or the amount of ATP for the three test specimens immediately after inoculation and after incubation, respectively, shall be less than two. When the test is judged to be ineffective, a retest shall be carried out.

When the test has been judged to be effective, obtain the antibacterial activity value according to Formula (4) in **8.1.5.2** and round the results to one decimal place according to Rule B of **JIS Z 8401**.

In the case of $\log C_0 > \log T_0$, substitute $\log C_0$ for $\log T_0$.

8.3 Printing method

8.3.1 Incubation

8.3.1.1 Obtain the strain preserved as stock culture using a platinum colony loop (**5.7**), streak onto the plate of Enumeration agar (EA) (**6.11**) and incubate at 37 °C ± 2 °C for 24 h to 48 h.

The plate is kept at 5 °C to 10 °C and used within one week from the date of preparation.

8.3.1.2 Pour 20 ml of Nutrient broth (NB) (**6.5**) or Tryptone soya broth (TSB) (**6.2**) into a 100 ml Erlenmeyer flask with cap (**5.20**). Apply a platinum colony loop (**5.7**), to pick one colony up from the plate as specified in **8.3.1.1** and inoculate it in the broth.

Incubate under the following conditions.

— Temperature: 37 °C ± 2 °C

— Rate of shaking: 110 time/min and 30 mm width by reciprocal incubation shaker (**5.27**)

— Incubation time: 18 h to 24 h

8.3.1.3 Pour 20 ml of Nutrient broth (NB) (**6.5**) or Tryptone soya broth (TSB) into a 100 ml Erlenmeyer flask. Add 0.4 ml of the inoculum from the incubation as specified in **8.3.1.2** that contains 1×10^8 CFU/ml to 3×10^8 CFU/ml in bacteria concentration or a ATP concentration of 1×10^{-6} mol/L to 3×10^{-6} mol/L to the flask using Nutrient broth (NB) or Tryptone soya broth (TSB).

Incubate under the following conditions.

— Temperature: 37 °C ± 2 °C

— Rate of shaking: 110 time/min and 30 mm width by reciprocal incubation shaker

— Incubation time: 3 h ± 1 h

Target CFU or ATP concentration after incubation: 10^7 CFU/ml or 10^{-7} mol/L.

NOTE : The prepared inoculum is preserved by ice-cooling and used within 8 h.

8.3.2 Preparation of test inoculum

Adjust the bacteria in **8.3.1.3** to a concentration of 1×10^7 CFU/ml to 3×10^7 CFU/ml by a spectrophotometer or McFarland's nephelometer (**5.1**) using Nutrient broth (NB) or Tryptone soya broth (TSB) after it has been diluted 20 times with water at room temperature, or measure the ATP concentration of the bacteria after incubation in **8.3.1.3** by the luminescence method and adjust the ATP to a concentration of 1×10^{-7} mol/L to 3×10^{-7} mol/L using Nutrient broth (NB) or Tryptone soya broth (TSB) after it has been diluted 20 times with water at room temperature.

NOTE : The prepared inoculum is preserved by ice-cooling and used within 4 h.

8.3.3 Pretreatment of test specimens

8.3.3.1 Sampling of test specimens

Prepare six control specimens and six test specimens cut to 60 mm in diameter.

Pretreatment may be carried out in accordance with the agreement between the parties concerned, if necessary. The detail of the pretreatment carried out shall be recorded in the test report.

NOTE : Three control specimens and three test specimens are used for the time zero, immediately after inoculation. The remaining specimens are used for the contact time, after incubation.

8.3.3.2 Sterilization of test specimens

Place the specimens in a Petri dish (**5.17**), cover with aluminium foil and sterilize by autoclave (**5.28**) for 15 min to 20 min.

After sterilizing, remove the foil, place on a clean bench (**5.6**) or any other place where there is no risk of airborne contamination and dry for 60 min or more.

When autoclaving is not possible, sterilization may be accomplished by ethylene oxide gas, γ-ray or another suitable method. The use of alternative methods shall be recorded in the test report.

8.3.3.3 Humidity conditioning of specimens

Pour 10 ml of the agar for printing (**6.12**) into a Petri dish. Place the uncovered dish on a clean bench to cool and solidify. Cool to room temperature avoiding dew formation.

When the agar solidifies, turn the dish upside down with lid. Put the agar containing the agar culture medium aside and place the test specimen on the dish lid. Place the test specimen at the cover bottom of Petri dish and place the agar culture medium at the top surface.

Condition the humidity of the specimen for 18 h to 24 h in humidity chamber (**5.8**) at a temperature of 20 °C ± 2 °C and a humidity of more than 70 %RH.

8.3.4 Test operation

8.3.4.1 Filtering of test bacteria

Set a membrane filter on a filtering apparatus (**5.14**) sterilized by autoclave on a clean bench.

Pour 5 ml of Nutrient broth (NB) (**6.5**) after it has been diluted 20 times on the membrane filter. Add 2 ml of the test inoculum prepared in **8.3.2**. Filtrate under aspiration. Continue the aspiration for approximately 1 min after the liquid on the membrane filter disappears.

NOTE 1 The sterilization for the membrane filter is not carried out because the pore size is changed by sterilization.

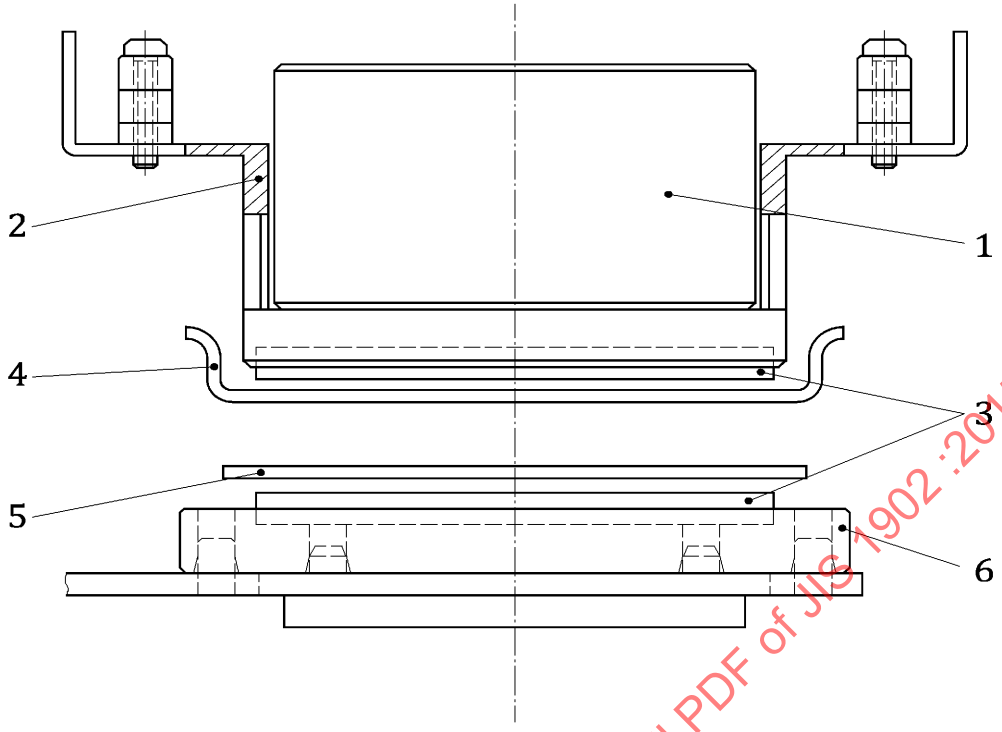
NOTE 2 A sintered glass or polytetrafluoroethylene-coated net should be placed under the membrane filter when using the filtering apparatus. An aspirator, small air pump or other simple apparatus should be used for aspiration.

8.3.4.2 Printing of test bacteria

Printing of test bacteria shall be as follows.

a) Remove the membrane filter collecting the test bacteria from the filtering apparatus using sterilized tweezers (**5.23**). Place on the rotating table of the printing apparatus (**5.10**) with the bacteria facing upwards as shown in Figure 2.

The outer dimension of printing apparatus is 170 mm in height, 160 mm in width, 150 mm in depth.



1 weight (mass 300 g)
2 weighting plate
3 silicone rubber (42 mm)
4 test specimen (60 mm)
5 membrane filter (47 mm)
6 turn plate (54 mm) (anticlockwise rotation)

Figure 2 Example of printing apparatus

b) Remove the specimen of **8.3.3.3** from the Petri dish using sterilized tweezers (**5.23**) and place on the membrane filter facing downwards.

c) Place the weight on the weighting plate, and print the bacteria on the membrane filter by rotating the table by 180° in one direction for 3.0 s.

d) Immediately after printing, transfer the specimen to the lid for the Petri dish containing the agar of **8.3.3.3** with the printed surface facing upwards, place the specimen with the Petri dish in an incubator (**5.2**) at a temperature of 20 °C ± 2 °C and a humidity of more than 70 %RH and incubate in accordance with **8.3.4.3**.

8.3.4.3 Incubation test

Select incubation time of **8.3.4.2 d**) from a period of 1 h ± 0.1 h, 2 h ± 0.1 h, 3 h ± 0.1 h, or 4 h ± 0.1 h according to the defined condition. The incubation time is determined by specific test condition requirements which shall be recorded in the test report.

8.3.4.4 Shake-out after printing

After printing of **8.3.4.2 c**), transfer three control specimens and three test specimens for measurement after printing to each vial containing 20 ml of the SCDLP medium (**6.8**), and shake out the bacteria from each specimen as specified in Annex B.

8.3.4.5 Shake-out after incubation

After incubation of **8.3.4.3**, transfer three control specimens and three test specimens for measurement after incubation to each vial containing 20 ml of the SCDLP medium, and shake out the bacteria from each specimen as specified in Annex B.

8.3.4.6 Calculation of number of bacteria or amount of ATP

8.3.4.6.1 General

Obtain the number of bacteria or amount of the ATP as specified in **8.3.4.4** and **8.3.4.5** from the bacteria concentration obtained in Annex C or the ATP concentration obtained in Annex D according to the formulae specified in **8.3.4.6.2** and **8.3.4.6.3**.

8.3.4.6.2 Number of bacteria

Calculation of the number of bacteria shall be in accordance with Formula (1) of **8.1.4.5.2**.

8.3.4.6.3 Amount of ATP

Calculation of the amount of ATP shall be in accordance with Formula (2) of **8.1.4.5.3**.

8.3.5 Test results

8.3.5.1 Judgement of test effectiveness with the control specimen

When the following **a**) and **b**) are satisfied, the test is judged to be effective. When the test is judged to be ineffective, a retest shall be carried out.

a) The number of bacteria or amount of ATP printed on the control specimen shall not be less than 1.0×10^{6} CFU or 1.0×10^{-11} mol.

b) The amount of increase or decrease on the control specimen (F), obtained according to Formula (3) in **8.1.5.1**, shall be +0.5 to −0.5.

8.3.5.2 Calculation of antibacterial activity value

When the test has been judged to be effective, obtain the antibacterial activity value according to Formula (5) and round the results to one decimal place according to Rule B of **JIS Z 8401**.

$$A = \log C_t - \log T_t \quad \cdots\cdots (5)$$

where, A : antibacterial activity value

$\log C_t$: common logarithm of arithmetic average of the numbers of bacteria, or amount of ATP, obtained from three control specimens after an 1 h to 4 h incubation

$\log T_t$: common logarithm of arithmetic average of the numbers of bacteria, or amount of ATP, obtained from three test specimens after an 1 h to 4 h incubation

8.4 Halo method

The qualitative test by halo method shall be in accordance with Annex JA.

9 Antibacterial efficacy

The antibacterial efficacy shall be as follows.

a) **Quantitative test** Depending on test methods, it shall be as follows.

1) **Absorption method** When tested according to **8.1**, the antibacterial activity value is not less than 2.0.

2) **Transfer method** When tested according to **8.2**, the antibacterial activity value is not less than 2.0.

3) **Printing method** When tested according to **8.3**, the antibacterial activity value is not less than 0.

b) **Qualitative method (halo method)** When tested according to **8.4**, halo (**JA.7.2**) is observed.

10 Test report

The test report shall contain the following information.

a) Reference to this Standard

b) Details of test textile products

c) Name of the test bacteria

d) Strain number and supplier

e) Inoculation method

f) Concentration inoculum

g) Antibacterial activity value

h) Quantitative measurement method applied and test result

i) Sterilization method of test specimen

j) Incubation time after inoculation

k) In the case of $\log C_0 > \log T_0$, substitute $\log C_0$ for $\log T_0$.

l) Any deviation from this Standard

Annex A (normative)
Reference strains

A.1 General

The bacteria to be used in this Standard shall be identical to those listed in Table A.1, which are preserved by the members of the World Federation for Culture Collection (WFCC). For the strains used in the test, the strain numbers shall be stated in the test report together with their supply sources.

A.2 List of bacteria

List of bacteria for testing is shown in Table A.1. Strain number and culture collection organization are shown in Table A.2.

Table A.1 Bacteria for testing [a)]

Bacteria name	WDCM code [b)]
Staphylococcus aureus	00193 http://refs.wdcm.org/getinfo.htm?sid=WDCM_00193
Klebsiella pneumoniae	00192 http://refs.wdcm.org/getinfo.htm?sid=WDCM_00192

Notes [a)] When the tests are confirmed valid, other bacteria can be used after appropriate validation.

[b)] Refer to WDCM and website: http://refs.wdcm.org/search.htm. (Note that WDCM stands for World Data Centre for Microorganisms.)

Table A.2 Strain number and culture collection organization of bacteria for testing (example)

Bacteria type	Strain number	Culture collection organization
Staphylococcus aureus	ATCC 6538 ATCC 6538P	American Type Culture Collection (USA)
	FDA 209P	Food and Drug Administration
	AHU 1142	Hokkaido University, School of Agriculture (Japan)
	CIP 4.83	Institut Pasteur, Collection de l'Institut Pasteur (France)
	DSM 799	German Collection of Microorganism and Cell Culture (Germany)
	GIFU 10391	Gifu University, School of Medicine (Japan)
	IAM 12082	University of Tokyo, Institute of Molecular and Cellular Biosciences (Japan)
	NBRC 12732 NBRC 13276	NITE Biological Resource Center (Japan)
	NRIC 1136	Tokyo University of Agriculture (Japan)
	OUT 8232	Osaka University, School of Engineer (Japan)
	NCIMB 9518	National Collection of Industrial, Food and Marine Bacterial Ltd. (UK)
Klebsiella pneumoniae	ATCC 4352	American Type Culture Collection (USA)
	CIP 104216	Institut Pasteur, Collection de l'Institut Pasteur (France)
	DSM 789	German Collection of Microorganism and Cell Culture (Germany)
	IAM 12015	University of Tokyo, Institute of Molecular and Cellular Biosciences (Japan)
	NBRC 13277	NITE Biological Resource Center (Japan)
	NCIMB 10341	National Collection of Industrial, Food and Marine Bacterial Ltd. (UK)
NOTE : Contact each culture collection organization, when they are acquired in practice.		

Annex B (normative)
Shaking method

B.1 General

There are three methods to shake-out the bacteria from specimen, which are shaking by vortex mixer, shaking by hand and shaking by Stomacher (**5.5**).

B.2 Shaking by vortex mixer

Press the bottom portion of the test tubes or bottles on the plate or rubber holder of the vortex mixer (**5.4**) and mix for 5 s×5 cycles.

B.3 Shaking by hand

Take the vial (**5.16**) by hand and shake in an arc of approximately 300 mm for 30 s, at a rate of 1 round per second.

B.4 Shaking by Stomacher

Place the designated disposable bag (**5.22**) in the Stomacher machine. Run the machine for 1 min on each face of the bag.

Annex C (normative)
Quantitative measurement by plate count method

C.1 General

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

C.2 Test procedure

C.2.1 Take 1 ml of the inoculum which is shake-out bacteria suspension from specimens using a pipette (**5.15**). Add it to a test tube containing 9.0 ml ± 0.1 ml of the Nutrient broth (NB) (**6.5**), the Physiological saline (**6.7**) or the Peptone-salt solution (**6.6**) and shake well.

C.2.2 Take 1 ml of this solution using a new pipette, add it to a separate test tube containing 9.0 ml ± 0.1 ml of the Nutrient broth (NB) (**6.5**), the Physiological saline (**6.7**) or the Peptone-salt solution (**6.6**) and shake well.

Repeat the procedure successively and prepare a dilution series so that the dilutions are undertaken 10 times in total. Ensure that 1 ml of each dilution is pipetted into two Petri dishes.

C.2.3 Add approximately 15 ml of Enumeration agar (EA) (**6.11**) or Tryptone soya agar (TSA) (**6.3**) maintained in a water bath (**5.3**) warmed to a temperature of 45 °C to 46 °C, to the dishes and mix well. Maintain at room temperature and, when the medium solidifies, turn the dishes upside down and incubate at 37 °C ± 2 °C for 24 h to 48 h.

C.2.4 After incubation, count the number of colonies on the Petri dishes of dilution series on which 30 CFU to 300 CFU have appeared. When the number of viable bacteria is less than 30 in the Petri dishes with 1 ml of shake-out bacteria solution, the cell number is used to calculate the average number. When the number of viable bacteria is less than 1 in the Petri dishes with 1 ml of shake-out bacteria suspension, the average number is taken as 1.

C.2.5 Obtain the bacteria concentration in the solution according to the following formula.

$$c_B = Z \times R$$

where, c_B : bacteria concentration, in Colony Forming Units per millilitre (CFU/ml)

Z : average value of two Petri dishes in Colony Forming Units (CFU) per 1 ml of inoculum (CFU/ml)

R : dilution rate

Annex D (normative)
Quantitative measurement by luminescence method

D.1 General

This Annex specifies the test procedure for quantitative measurement by the luminescence method.

D.2 Test procedure

D.2.1 Calibration curve formula

D.2.1.1 Prepare the ATP standard reagent (**6.14**), and dilution medium, such as the physiological saline (**6.7**), the SCDLP medium (**6.8**) or another suitable medium. Dilute the ATP standard reagent with the dilutions corresponding to the procedures (see Table D.1), and prepare three dilution solutions with ATP concentration of 2×10^{-8} mol/L, 2×10^{-9} mol/L and 2×10^{-10} mol/L, respectively.

Table D.1 ATP standard reagent dilution solution and blank

Testing method	Operation	ATP standard reagent dilution solution and blank
Absorption method	Preparation of test inoculum	Water
	Shake-out immediately after inoculation Shake-out after incubation	Shake-out bacteria solution used
Transfer method	Preparation of test inoculum	Water
	Shake-out immediately after printing Shake-out after incubation	Neutralizing solution (**6.10**)
Printing method	Preparation of test inoculum	Water
	Shake-out immediately after printing Shake-out after incubation	SCDLP or other medium for preparing ATP reference solution (**6.19**)

D.2.1.2 Pour 0.1 ml of each solution specified above into three separate test tubes. Add 0.9 ml of the dilution buffer (**6.9**) to each tube and shake amply. Pour 0.1 ml of each solution into two separate test tubes. Designate them as specimens for measuring the diluted standard reagent.

D.2.1.3 Pour 0.1 ml of solution used in **D.2.1.1** (see Table D.1), 0.8 ml of the dilution buffer, and 0.1 ml of ATP eliminating reagent (**6.18**) into a plastic test tube. Shake amply and pour 0.1 ml portions into two separate test tubes. Let them stand for 5 min to 30 min and designate them as specimens for measuring the blank.

D.2.1.4 Add to a test tube containing the specimen for measuring the blank, 0.1 ml of ATP extracting reagent and shake. Add 0.1 ml of ATP luminescent reagent (**6.16**), shake again and immediately apply a luminescence photometer to determine the quantities of luminescence.

D.2.1.5 Add to the specimens for measuring the diluted standard reagent, 0.1 ml portions of ATP extracting reagent (**6.17**) in order, starting from the lowest concentration, and shake. Then add 0.1 ml of ATP luminescent reagent, shake again and immediately apply a luminescence photometer to determine the quantities of luminescence.

D.2.1.6 Divide the ATP concentration by the average of the quantity of luminescence obtained from the measurement of diluted standard reagent (2×10^{-8} mol/L, 2×10^{-9} mol/L and 2×10^{-10} mol/L), and record this value as the average value of the gradient a.

D.2.1.7 Obtain the coefficient b by substituting gradient a, and $c_{ATP}=0$ into the following calibration curve formula.

$$c_{ATP}=a\times X+b$$

where, c_{ATP} : ATP concentration (mol/L)

X : quantity of luminescence (RLU = Relative Light Unit)

a : gradient of linear calibration curve

b : intercept of linear calibration curve

NOTE : The correlation coefficient between c_{ATP} and X is R^2, 0.9 and the confidence level is ≥ 0.95.

D.2.2 ATP concentration of the test inoculum and shake-out bacterial suspension

D.2.2.1 Prepare one test tube for the ATP eliminating treatment and two test tubes for measuring.

D.2.2.2 Pour 0.1 ml of the shake-out bacterial suspension, 0.8 ml of the dilution buffer and 0.1 ml of the ATP eliminating agent (**6.18**) into the test tube designated for the ATP eliminating treatment and shake amply. Pour 0.1 ml portions of the solution into the two test tubes designated for measuring and let them stand for 5 min to 30 min.

D.2.2.3 Add 0.1 ml of the ATP extracting reagent to each measuring test tube and shake amply. Then add 0.1 ml of ATP luminescent reagent, shake again and immediately apply a luminescence photometer to determine the quantities of luminescence.

D.2.2.4 Obtain the ATP concentration according to the formula specified in **D.2.1.7** and obtain the concentration of ATP in the test inoculum and shake-out bacterial suspension according to the following formula. In this case, the lower limit value of the ATP concentration shall be 1×10^{-13} mol/L.

$$c_{ATP'}=\frac{c_{ATP}}{1\,000}$$

where, $c_{ATP'}$: ATP concentration in the test inoculum and shake-out bacterial suspension (mol/ml)

c_{ATP} : ATP concentration (mol/L)

Annex E (informative)
Testing examples

E.1 Absorption method

Example of testing by absorption method measured by plate count method (see Table E.1) and by luminescence method (see Table E.2) are shown.

Table E.1 Example of testing by absorption method measured by plate count method

Test bacteria	Staphylococcus aureus		Klebsiella pneumoniae	
Strain number	ATCC 6538		ATCC 4352	
Concentration of inoculum (CFU/ml)	1.2×10^5		1.1×10^5	
Difference of extremes for three control specimens (log) (condition: less than 1)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.4	0.3	0.5	0.4
Difference of extremes for three test specimens (log) (condition: less than 2)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.6	0.5	0.7	0.6
Growth value of F ($F=\log C_t-\log C_0$)	+2.7 : ($\log C_t$: +7.0, $\log C_0$: +4.3)		+3.2 : ($\log C_t$: +7.5, $\log C_0$: +4.3)	
Growth value of G ($G=\log T_t-\log T_0$)	−1.0 : ($\log T_t$: +3.2, $\log T_0$: +4.2)		+0.7 : ($\log T_t$: +4.9, $\log T_0$: +4.2)	
Antibacterial activity value ($A=F-G$)	3.8		2.6	
Measuring method	Plate count method			
Type of sample material	Socks: Cotton 100 %			
Sterilization method	Autoclave			
Incubation time	20 h			

Table E.2 Example of testing by absorption method measured by luminescence method

Test bacteria	Staphylococcus aureus		Klebsiella pneumoniae	
Strain number	ATCC 6538		ATCC 4352	
Concentration of inoculum (mol/L)	1.2×10^{-9}		1.9×10^{-9}	
Difference of extremes for three control specimens (log) (condition: less than 1)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.5	0.6	0.5	0.4
Difference of extremes for three test specimens (log) (condition: less than 2)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.5	0.7	0.6	0.8

Table E.2 (concluded)

Test bacteria	Staphylococcus aureus	Klebsiella pneumoniae
Growth value of F ($F = \log C_t - \log C_0$)	+1.7 : ($\log C_t$: −11.0, $\log C_0$: −12.7)	+2.1 : ($\log C_t$: −10.4, $\log C_0$: −12.5)
Growth value of G ($G = \log T_t - \log T_0$)	−0.8 : ($\log T_t$: −13.6, $\log T_0$: −12.8)	−0.1 : ($\log T_t$: −12.6, $\log T_0$: −12.5)
Antibacterial activity value ($A = F - G$)	2.6	2.2
Measuring method	Luminescence method	
Type of sample material	Curtain: Polyester 100 %	
Sterilization method	Ethylene oxide gas	
Incubation time	20 h	

E.2 Transfer method

Example of testing by transfer method measured by plate count method (see Table E.3) and by luminescence method (see Table E.4) are shown.

Table E.3 Example of testing by transfer method measured by plate count method

Test bacteria	Staphylococcus aureus		Klebsiella pneumoniae	
Strain number	ATCC 6538		ATCC 4352	
Concentration of inoculum (CFU/ml)	2.2×10^6		1.1×10^6	
Difference of extremes for three control specimens (log) (condition: less than 1)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.3	0.4	0.2	0.3
Difference of extremes for three test specimens (log) (condition: less than 2)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.6	0.5	0.7	0.6
Growth value of F ($F = \log C_t - \log C_0$)	+2.9 : ($\log C_t$: +8.5, $\log C_0$: +5.6)		+2.7 : ($\log C_t$: +8.1, $\log C_0$: +5.4)	
Growth value of G ($G = \log T_t - \log T_0$)	−1.7 : ($\log T_t$: +3.8, $\log T_0$: +5.5)		−2.5 : ($\log T_t$: +2.8, $\log T_0$: +5.3)	
Antibacterial activity value ($A = F - G$)	4.6		5.2	
Measuring method	Plate count method			
Type of sample material	Socks: Cotton 100 %			
Sterilization method	Autoclave			
Incubation time	22 h			

Table E.4 Example of testing by transfer method measured by luminescence method

Test bacteria	Staphylococcus aureus		Klebsiella pneumoniae	
Strain number	ATCC 6538		ATCC 4352	
Concentration of inoculum (mol/L)	3.2×10^{-9}		4.9×10^{-9}	
Difference of extremes for three control specimens (log) (condition: less than 1)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.5	0.6	0.4	0.3
Difference of extremes for three test specimens (log) (condition: less than 2)	Immediately after inoculation	After incubation	Immediately after inoculation	After incubation
	0.4	0.7	0.5	0.6
Growth value of F ($F=\log C_t-\log C_0$)	+3.1 : ($\log C_t$: −9.1, $\log C_0$: −12.2)		+2.6 : ($\log C_t$: −9.4, $\log C_0$: −12.0)	
Growth value of G ($G=\log T_t-\log T_0$)	−2.2 : ($\log T_t$: −14.5, $\log T_0$: −12.3)		−2.2 : ($\log T_t$: −14.1, $\log T_0$: −11.9)	
Antibacterial activity value ($A=F-G$)	5.3		4.8	
Measuring method	Luminescence method			
Type of sample material	Curtain: Polyester 100 %			
Type of neutralizing agent and concentration	Egg-yolk lecithin 0.5 % of the total volume			
Sterilization method	γ-ray			
Incubation time	22 h			

E.3 Printing method

Example of testing by printing method measured by plate count method (see Table E.5) and by luminescence method (see Table E.6) are shown.

Table E.5 Example of testing by printing method measured by plate count method

Test bacteria	Staphylococcus aureus	Klebsiella pneumoniae
Strain number	ATCC 6538	ATCC 4352
Number of bacteria on filter (CFU)	3.0×10^{7}	3.6×10^{7}
Number of bacteria on control specimen immediately after printing (CFU)	4.2×10^{6}	6.4×10^{6}
Antibacterial activity value ($A=\log C_t-\log T_t$)	1.7 : ($\log C_t$: +6.7, $\log T_t$: +5.0)	1.1 : ($\log C_t$: +6.7, $\log T_t$: +5.6)
Quantitative measuring method	Plate count method	
Type of sample material	Curtain: Polyester 100 %	
Sterilization method	Autoclave	
Incubation time	4 h	

Table E.6 Example of testing by printing method measured by luminescence method

Test bacteria	Staphylococcus aureus	Klebsiella pneumoniae
Strain number	ATCC 6538	ATCC 4352
Amount of ATP on filter (mol)	2.9×10^{-10}	1.7×10^{-10}
Amount of ATP on control specimen immediately after printing (mol)	4.0×10^{-11}	3.0×10^{-11}
Antibacterial activity value ($A = \log C_t - \log T_t$)	1.7 : ($\log C_t$: −10.4, $\log T_t$: −12.1)	1.1 : ($\log C_t$: −10.6, $\log T_t$: −11.7)
Quantitative measuring method	Luminescence method	
Type of sample material	Curtain: Polyester 100 %	
Sterilization method	Ethylene oxide gas	
Incubation time	2 h	

E.4 Halo method

Example of testing by halo method (see Table E.7) is shown.

Table E.7 Example of testing by halo method

Bacteria	Staphylococcus aureus	Klebsiella pneumoniae
Strain number	ATCC 6538	ATCC 4352
Concentration of bacteria (CFU/ml)	2.3×10^{6}	3.5×10^{6}
Average value of width of halo (mm)	3.5	2.0
Existence of halo	Exist	Exist
Type and shape of test specimen	Knitted fabric, circle of 28 mm in diameter (stainless steel disc is placed)	

Annex F (informative)
Antibacterial efficacy

From the testing result, the antibacterial efficacy of the test specimen is as shown in Table F.1.

Table F.1 Antibacterial efficacy

Antibacterial value A	Antibacterial efficacy
$2.0 \leq A < 3.0$	Effect
$3.0 \leq A$	Full effect

Annex JA (normative)
Qualitative test (halo method)

JA.1 General

This Annex specifies the test procedure for qualitative measurement by the halo method.

JA.2 Agar

JA.2.1 Nutrient broth, prepared from the following compositions to be pH 7.0 ± 0.2.

— Beef extract	5.0 g
— Peptone	10.0 g
— Sodium chloride (NaCl)	5.0 g
— Water	1 000 ml

JA.2.2 Nutrient agar, prepared from the following compositions to be pH 7.0 ± 0.2. Sterilization in autoclave (**5.28**) is carried out.

— Beef extract	5.0 g
— Peptone	10.0 g
— Sodium chloride (NaCl)	5.0 g
— Agar	15.0 g
— Water	1 000 ml

JA.3 Conditioning of test piece

JA.3.1 Dimension, shape and number of test piece

Dimension, shape and number of test piece is shown in Table JA.1. Prepare test specimens and control specimens. Obtain three test specimens and three control specimens, respectively, for each test bacteria.

Table JA.1 Dimensions and shape of test specimen

Type of test specimen	Dimensions and shape of test specimen
Woven fabric, knitted fabric and non-woven fabric	Circle of 28 mm in diameter [a)]
Yarn	As shown in Figure JA.1, wind yarn in single layer with width 28 mm at the centre part of a slide glass of 38 mm × 26 mm (about 3.3 g) [b)].
Wadding and long pile	Take about 0.2 g, cut it into 3 mm to 5 mm, mix well, put them into the cylinder with 28 mm inside diameter as shown in Figure JA.2, spread uniformly, and press it to make a circular shape of 28 mm [c)] using an inserter with 27 mm outside diameter.
Notes [a)] The test specimen shaped as square measuring 28 mm × 28 mm may be used. [b)] As shown in Figure JA.3, take about 0.2 g with 50 mm long yarn so as to make it parallel, and bind it at about 5 mm apart from both ends with the same yarn (to make a bale shape). This test specimen may be used. [c)] That cut and pressed with about 63.7 MPa pressure for about 10 min may be used.	

Unit: mm

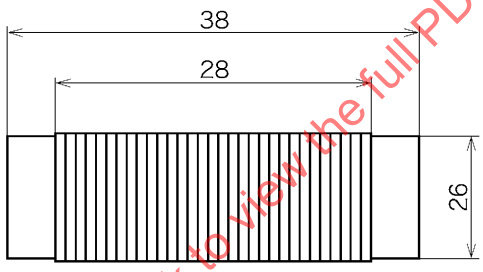


Figure JA.1 Coiled test specimen in spool

Unit: mm

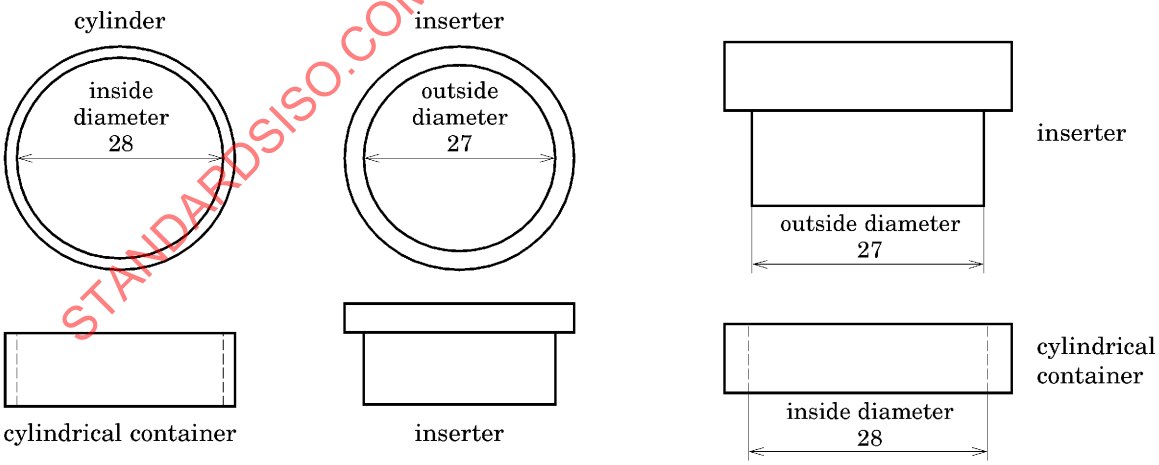


Figure JA.2 Container for test specimen preparation

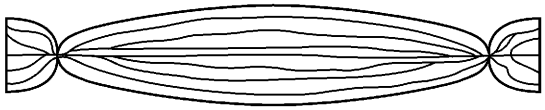


Figure JA.3 Test specimen shaped as bale

JA.3.2 Pretreatment of test specimen

Wrap the test specimen obtained in **JA.3.1** with aluminium foil or put it in a Petri dish made of glass, and sterilized by autoclave. For the test specimens, this process may be omitted except for the yarn coiled on a slide glass.

JA.4 Test operation

JA.4.1 Preparation of inoculum

Inoculate from the stored bacteria to the slant agar in **7.2.4** by one platinum colony loop and incubate at 37 °C ± 2 °C for 24 h to 48 h. After incubated, transplant it to Nutrient broth (**JA.2.1**) and incubate at 37 °C ± 2 °C for 24 h to 48 h. Presume the concentration of bacteria in inoculum according to the absorbance method or the direct observation method by microscope or presume the ATP concentration according to the luminescence method and adjust the concentration of bacteria to 10^6 CFU/ml to 10^7 CFU/ml or adjust the ATP concentration to 10^{-8} mol/L to 10^{-7} mol/L.

JA.4.2 Preparation of pour plate culture medium

In a clean bench, put 1 ml of inoculum of **JA.4.1** in a sterilized Petri dish, add 15 ml of nutrient agar (**JA.2.2**) warmed at 45 °C to 46 °C in the Petri dish and mix them sufficiently, allow it to stand at room temperature and solidify the agar. Turn this upside down, slide its cover and allow to stand as it is for 30 min to 3 h at room temperature to evaporate superfluous water (see Figures JA.4 and JA.5).

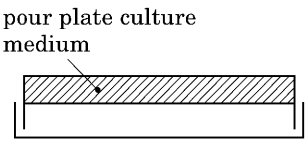


Figure JA.4 Petri dish placed upside down

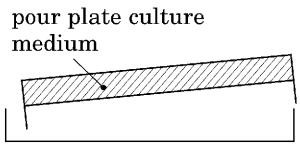


Figure JA.5 Petri dish with its cover slid

JA.4.3 Setting of test specimen

Using tweezers disinfected with gauze sterilized with alcohol, put gently a test specimen of **JA.3.2** on the centre of the pour plate culture medium of **JA.4.2** with care not to give a scratch on the surface of culture medium, and make it adhere closely to the medium. In this case, when treating a test specimen which curls itself easily, or has wadding or long pile, place a sterilized circular stainless steel plate (about 28 mm in diameter, about 20 g of mass, made from stainless steel) on the test specimen. If the circular stainless steel plate is larger than a test specimen, put the plate on several test specimens, which are piled, not to make the circular stainless steel plate touch the surface of culture medium.

JA.5 Incubation test

Place the Petri dish of **JA.3.2** upside down, incubate it at 37 °C ± 2 °C for 24 h to 48 h. When a circular stainless steel plate is put on the test specimen, or when the test specimen is prepared by coiling on a slide glass, do not turn it upside down.