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Textiles — Determination of antibacterial activity of textile products

Textiles — Détermination de l'activité antibactérienne des produits textiles

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2. www.iso.org/directives

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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

The committee responsible for this document is ISO/TC 38, *Textiles*.

This second edition cancels and replaces the first edition (ISO 20743:2007), which has been technically revised.

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Introduction

Speciality products of antibacterial-treated textiles have been introduced in the market and are expanding year by year in various applications. Those textiles certainly meet the consumer's requirement to seek prevention and protection from the negative effects caused by bacteria and to secure the quality of life.

In this situation, the test methods to determine the antibacterial activity for antibacterial textile products were expected to be established in order to address the substantial need for an International Standard.

The test method for antibacterial activity was developed as ISO 20645 which was a qualitative test method. There are no testing standards for the quantitative method which gives more objective information for the antibacterial activity of the textile products.

There are several practical test methods to determine the quantitative antibacterial activity specified in this International Standard. The test methods are composed of 2 major steps, such as inoculation of bacteria and quantitative measurement of bacteria.

The methods for the inoculation of bacteria specified in this International Standard are the absorption method, transfer method and printing method.

The methods of the quantitative measurement of bacteria specified in this International Standard are colony plate count method and ATP luminescence methods.

Although there are 6 ways for the combination of inoculation methods and quantitative measurements to execute this test, the choice of the ways depends on the user's availability and consensus between the concerned parties.

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Textiles — Determination of antibacterial activity of textile products

1 Scope

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

This International 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 three inoculation methods on determination of antibacterial activity:

a) absorption method (an evaluation method in which the test bacterial suspension is inoculated directly onto specimens);

b) transfer method (an evaluation method in which test bacteria are placed on an agar plate and transferred onto specimens);

c) printing method (an evaluation method in which test bacteria are placed on a filter and printed onto specimens).

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

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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 6330, *Textiles — Domestic washing and drying procedures for textile testing*

3 Terms and definitions

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

3.1
control fabric
fabric used to validate the growth condition of test bacteria and validate the test

Note 1 to entry: The same fabric as the fabric to be tested but without antibacterial treatment or a 100 % cotton fabric without fluorescent brighteners or other finish can be used.

3.2
antibacterial agent
product designed to prevent or mitigate the growth of bacteria, to reduce the number of bacteria or to kill bacteria

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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
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

Note 1 to entry: The results are expressed in "CFU (Colony Forming Unit)".

3.6
luminescence method
method in which the amount of ATP contained in bacterial cells is measured

Note 1 to entry: The results are expressed in "moles of ATP".

3.7
neutralizer
chemical agents used to inactivate, neutralize or quench the antibacterial properties of antibacterial agents

4 Safety precaution

The test methods specified in this International Standard require the use of bacteria.

These tests should be carried out by persons with training and experience in the use of microbiological techniques.

Appropriate safety precautions should be observed with due consideration given to country-specific regulations.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

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, for microbial test.

5.7 Washing machine, in accordance with the specifications of ISO 6330.

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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^{-12} 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 of 0,5 % or less.

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 or 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, of capacity 100 ml.

5.21 Cutting template, made of a sterilizable material (stainless steel or glass) with a diameter of 3,8 cm ± 0,1 cm.

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 3,5 cm ± 0,1 cm.

5.25 Metal wire basket, for autoclaving.

5.26 Aluminium foil.

5.27 Reciprocal incubation shaker.

5.28 Autoclave, capable of sterilizing at 121 °C ± 2 °C and 103 kPa ± 5 kPa.

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6 Reagents and culture media

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

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.

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)

Tryptone, pancreatic digest of casein	17 g
Soya peptone, papain digest of soya	3 g
Sodium chloride (NaCl)	5 g
Glucose	2,5 g
Dipotassium hydrogen phosphate	2,5 g
Water	1 000 ml
Mix well and adjust pH,	7,2 ± 0,2
then sterilize by autoclave (5.28).	

6.3 Tryptone soya agar (TSA)

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
Mix well and adjust pH,	7,2 ± 0,2
then sterilize by autoclave (5.28).	

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6.4 Agar for transfer

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
Mix well and adjust pH,	7,2 ± 0,2
then sterilize by autoclave (5.28).	

6.5 Nutrient broth (NB)

Beef extract	3 g
Peptone	5 g
Water	1 000 ml
Mix well and adjust pH, then sterilize by autoclave (5.28).	
pH	6,9 ± 0,2

6.6 Peptone salt solution

Peptone, pancreatic digest of casein	1 g
Sodium chloride (NaCl)	8,5 g
Water	1 000 ml
Mix well and adjust pH,	6,9 ± 0,2
then sterilize by autoclave (5.28).	

6.7 Physiological saline

Sodium chloride (NaCl)	8,5 g
Water	1 000 ml
Mix well, then sterilize by autoclave (5.28).	

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6.8 SCDLP medium

Peptone, digest of casein	17 g
Peptone, digest of soybean	3 g
Sodium chloride (NaCl)	5 g
Dipotassium hydrogenphosphate	2,5 g
Glucose	2,5 g
Lecithin	1 g
Polysorbate 80	7 g
Water	1 000 ml
Mix well and adjust pH,	7,2 ± 0,2
then sterilize by autoclave (5.28).	

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

6.9 Dilution buffer for shake-out bacterial suspension

This buffer solution consists of 0,005 mol/l sodium dihydrogenphosphate containing 0,037 % sucrose.

pH	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
Mix well and sterilize by autoclave (5.28).	

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

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6.11 Enumeration agar (EA)

Dehydrated yeast extract	2,5 g
Casein tryptone	5,0 g
Glucose	1,0 g
Agar	12 g to 18 g (depending on the gel strength of the product)
Water	1 000 ml
Mix well and adjust pH,	7,2 ± 0,2

then sterilize by autoclave (5.28).

6.12 Agar for printing

Agar	20 g
Water	1 000 ml

Mix well and sterilize by autoclave (5.28).

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,

TSB (6.2) or NB (6.5): 1 000 ml

Add,

Glycerol: 150 g

or

dimethylsulfoxide: 100 g

Mix well and sterilize by autoclave (5.28).

For solutions containing glycerol, sterilize the mixed solution by autoclave (5.28). 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

The concentration of ATP standard reagent is 1 X 10^{-4} mol/l which is obtained by the following mixing.

Adenosine-disodium 5'-triphosphate trihydrate	60,5 mg
Water	1 000 ml (final volume)

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 6 months from the date of preparation.

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

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6.15 Buffer solution for ATP luminescent reagent

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 (final volume)
pH	7,5 ± 0,2

6.16 ATP luminescent reagent

Luciferase (EC: 1.13.12.7)	16,0 mg
D-luciferin	12,6 mg
Bovine serum albumin	56 mg
Buffer solution (6.15)	30 ml

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.

6.17 ATP extracting reagent

N-[Tris (hydroxymethyl) methyl] glycine	45 mg
10 % aqueous benzalkonium chloride	0,2 ml
Water	9,8 ml
pH	12,0 ± 0,5

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

6.18 ATP eliminating reagent

An agent to reduce the ATP in NB (6.5) to less than 10^{-13} mol/l within 15 min.

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Use within 8 h of preparation.

Apyrase (EC: 3.6.1.5)	4,6 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
pH	6,0 ± 0,5

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

NOTE Commercially available reagent.

6.19 SCDLP or other medium for preparing ATP reference solution

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 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.

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.

6.20 Shake-out physiological saline

Sodium chloride (NaCl): 8,5 g

Polysorbate 80: 2,0 g

Water: 1 000 ml

Mix well and sterilize by autoclave (5.28).

7 Reference strains

7.1 Strains

The following strains shall be used in all antibacterial activity tests as the details are described in Annex A.

— *Staphylococcus aureus*

— *Klebsiella pneumoniae*

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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

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

Sample 0,7 ml of the broth culture and spread it over the surface of the Petri dish containing the TSA. Incubate the culture on plates for 18 h to 24 h under the conditions specified for the strain in the standard.

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.

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.

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

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

Store the cryogenic vials in a freezer at a temperature below –70 °C.

7.2.3 Glycerol suspension method

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.

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. 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.

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.

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.

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

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8 Test procedures

8.1 Absorption method (see Annex E)

8.1.1 Incubation

8.1.1.1 Pick up the preserved stock bacteria from the storage container using an inoculating loop. Streak onto the plate of EA (6.11) and incubate at 37 °C ± 2 °C for 24 h to 48 h.

NOTE The plate is kept at 5 °C to 10 °C and used within 1 week after the date of preparation.

8.1.1.2 Pour 20 ml of NB (6.5) or TSB (6.2) into a 100 ml Erlenmeyer flask. Apply an inoculating 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 min^{-1} and 3 cm width by reciprocal incubation shaker (5.27)

Incubation time: 18 h to 24 h

8.1.1.3 Pour 20 ml of NB (6.5) or 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 an 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 min^{-1} and 3 cm width by reciprocal incubation shaker (5.27)

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 to a concentration of 1×10^5 CFU/ml to 3×10^5 CFU/ml by a spectrophotometer or McFarland's nephelometer (5.1).

or

adjust the ATP to a concentration of 1×10^{-9} mol/l to 3×10^{-9} mol/l by the luminescence method using NB (6.5) or TSB (6.2) 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.

8.1.3 Preparation of test specimens

8.1.3.1 Mass and shape of test specimens

8.1.3.1.1 Obtain test specimens with a mass of 0,40 g ± 0,05 g and cut to a suitable size for test specimens.

8.1.3.1.2 Obtain six test specimens of the antibacterial testing sample and six test specimens of the control fabric or untreated fabric if available.

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NOTE Three of the control specimens and three of the antibacterial testing 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 the test specimen

Place each of the test specimens in separate vials 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 pile and place a glass rod onto the specimen in the vial.

When necessary, test samples may be washed in accordance with ISO 6330 or another suitable method, and after the final washing, the samples are rinsed with water to eliminate the washing detergent. The use of an unspecified method shall be recorded.

8.1.3.3 Sterilization

When contamination is suspected or was found, sterilize test specimens by autoclave (5.28) according to the following procedure.

8.1.3.3.1 Wrap the opening of vials containing specimens with aluminium foil (5.26).

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

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

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

8.1.3.3.5 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.

8.1.3.3.6 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.

8.1.4 Test operation

8.1.4.1 Inoculation of test specimens

Accurately pipette 0,2 ml of the inoculum prepared in 8.1.2 at several points on each test specimen prepared in 8.1.3.2 to ensure that no inoculum touches the surface of the vial 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 SCDLP medium (6.8) or the neutralizing solution (6.10) or the shake-out physiological saline (6.20) into each of the six vials in which a control specimen and an antibacterial testing specimen have been placed, cap the vials and shake out as specified in Annex B by hand or mixer (5.4).

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8.1.4.3 Incubation

Incubate the six vials (three control specimens, three testing specimens) 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 SCDLP medium (6.8) or of the neutralizing solution (6.10) or the shake-out physiological saline (6.20) to each of the six vials, cap the vials and shake out as specified in Annex B by hand or mixer (5.4).

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 or the ATP concentration obtained by the quantitative measurement methods in Annex C or Annex D according to the following formulae:

8.1.4.5.2 Number of bacteria

$$M = c_B \times 20$$

where

M is the number of bacteria per specimen;

c_B is the bacteria concentration obtained in Annex C;

20 is the volume of the shake-out solution, in millilitres (ml).

8.1.4.5.3 Amount of ATP

$$M' = c_{ATP'} \times 20$$

where

M' is the amount of ATP per specimen;

$cATP'$ is the ATP concentration obtained in Annex D;

20 is the volume of the shake-out solution, in millilitres (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 .

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c) The growth value obtained according to the following formula shall be equal or more than 1,0 in the plate count method.

d) The growth value obtained according to the following formula shall be equal or more than 0,5 in the luminescence method.

$$F = \lg C_t - \lg C_0$$

where

F is the growth value on the control specimen;

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

$\lg C_0$ is the 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 Calculation of antibacterial activity value

When the condition in the next paragraph is satisfied, the test is judged to be effective. When the test is judged to be ineffective, a retest shall be carried out.

The difference in common logarithm in extreme of the number of bacteria, or the amount of ATP for the three antibacterial testing specimens immediately after inoculation and after incubation, respectively, shall be less than two.

To validate the test, it is necessary that the difference in logarithm of extremes for the specimens of the treated sample is less than 2 after inoculation and incubation. To repeat the testing because the neutralizer is not effective, it is necessary to know from the manufacturer how to neutralize the antibacterial agent.

When the test has been judged to be effective, obtain the antibacterial activity value according to the following formula, in case of $C_0 > T_0$, substitute C_0 for T_0.

$$A = (\lg C_t - \lg C_0) - (\lg T_t - \lg T_0) = F - G$$

where

A is the antibacterial activity value;

F is the growth value on the control specimen $(F = \lg C_t - \lg C_0)$;

G is the growth value on the antibacterial testing specimen $(G = \lg T_t - \lg T_0)$;

$\lg T_t$ is the common logarithm of arithmetic average of the numbers of bacteria, or the amount of ATP, obtained from three antibacterial testing specimens after an 18 h to 24 h incubation;

$\lg T_0$ is the common logarithm of arithmetic average of the numbers of bacteria, or the amount of ATP, obtained from three antibacterial testing specimens immediately after inoculation.

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8.2 Transfer method (see Annex E)

8.2.1 Preparation of test inoculum

8.2.1.1 Incubation of test strain

Obtain the strain preserved as stock culture using an inoculating loop, streak onto the plate of TSA (6.3) and incubate at 37 °C ± 2 °C for 18 h to 24 h. After incubation, extract a colony from the plate, streak onto another plate of TSA and 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) 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 an inoculating loop, place it in the Peptone-salt solution (6.6) and 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 or an ATP concentration of 2×10^{-7} mol/l to 6×10^{-7} mol/l using the Peptone-salt solution (6.6) by the spectrophotometer or McFarland's nephelometer (5.1) or 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 (6.6). 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 specimens

Using a template (5.21), cut specimens for test that are 3,8 cm in diameter.

The specimens for test should not contain any seams, selvages, embroidery, fasteners, etc.

A sufficient number of specimens should be prepared to allow for repeat tests, with a minimum of 0,5 m^2 in size from the same batch and without from selvages or stemming.

When necessary, test samples may be washed in accordance with ISO 6330 or another suitable method, and after the final washing, the specimens are rinsed with water to eliminate the washing detergent. The use of an unspecified method shall be recorded.

When contamination is suspected or was found, the specimens for test shall be sterilized by autoclave (5.28). The ethylene oxide gas, γ-ray or any other suitable method could be used with recording 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). 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 three control specimens and three antibacterial testing specimens for using immediately after transfer (six specimens) and after incubation (six specimens), respectively. Set each specimen on the agar surface of 8.2.3.1 and weigh down with a 200 g stainless-steel cylinder (5.24) for 60 s ± 5 s. Place each specimen in a 55 mm to 60 mm in diameter Petri dish (5.17) with the transferred surface face up. Incubate in a humidity chamber (5.8) for 18 h to 24 h at 37 °C ± 2 °C.

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8.2.3.3 Shake-out after transfer

Immediately after transfer, 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 by hand, mixer (5.4), or stomacher (5.5).

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, by hand, mixer (5.4) or stomacher (5.5).

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 the amount of ATP from 8.2.3.3 and 8.2.3.4 from the bacteria concentration or ATP concentration obtained by the quantitative measurement methods in Annex C and Annex D according to the following formulae.

8.2.3.5.2 Number of bacteria

$$M = c_B \times 20$$

where

M is the number of bacteria per specimen;

c_B is the bacteria concentration obtained in Annex C;

20 is the volume of the shake-out solution, in millilitres (ml).

8.2.3.5.3 Amount of ATP

$$M' = c_{ATP'} \times 20$$

where

M' is the amount of ATP per specimen;

$c_{ATP'}$ is the ATP concentration obtained in Annex D;

20 is the volume of the shake-out solution, in millilitres (ml).

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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 logarithm in extremes of the numbers of bacteria, or the amount of ATP for the three control fabrics immediately after transfer and after incubation, respectively, shall be less than one.

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c) The growth value obtained according to the following formula shall be equal or more than 1,0 in the plate count method.

d) The growth value obtained according to the following formula shall be equal or more than 0,5 in the luminescence method.

$$F = \lg C_t - \lg C_0$$

where

F is the growth value on the control fabric;

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

$\lg C_0$ is the common logarithm of the arithmetic average of the numbers of bacteria or for the amount of ATP, obtained from three control specimens immediately after transfer to the control fabric.

8.2.4.2 Calculation of antibacterial activity value

When the condition in the next paragraph is satisfied, the test is judged to be effective. When the test is judged to be ineffective, a retest shall be carried out.

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

To validate the test, it is necessary that the difference in logarithm of extremes for the specimens of the treated sample is less than 2 after inoculation and incubation. To repeat the testing because the neutralizer is not effective, it is necessary to know from the manufacturer how to neutralize the antibacterial agent.

When the test has been judged to be effective, obtain the antibacterial activity value according to the following formula:

$$A = (\lg C_t - \lg C_0) - (\lg T_t - \lg T_0) = F - G$$

where

A is the antibacterial activity value;

F is the growth value on the control specimen ($F = \lg C_t - \lg C_0$);

G is the growth value on the antibacterial testing specimen ($G = \lg T_t - \lg T_0$);

$\lg T_t$ is the common logarithm of the arithmetic average of the numbers of bacteria or for the amount of ATP, obtained from three antibacterial testing specimens after an 18 h to 24 h incubation;

$\lg T_0$ is the common logarithm of the arithmetic average of the numbers of bacteria or for the amount of ATP, obtained from three antibacterial testing specimens immediately after transfer.

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8.3 Printing method (see Annex E)

8.3.1 Incubation

8.3.1.1 Obtain the strain preserved as stock culture using an inoculating loop, streak onto the plate of 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 should be used within 1 week from the date of preparation.

8.3.1.2 Pour 20 ml of NB (6.5) into a 100 ml Erlenmeyer flask. Apply an inoculating loop to pick one colony up from the incubation A 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 min^{-1} and 3 cm width by reciprocal incubation shaker (5.27)

Incubation time: 18 h to 24 h

8.3.1.3 Pour 20 ml of NB (6.5) 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 an 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 min^{-1} and 3 cm width by reciprocal incubation shaker (5.27)

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 inoculum to a concentration of 1 × 10^7 CFU/ml to 3 × 10^7 CFU/ml by the spectrophotometer or McFarland's nephelometer (5.1) method or an ATP concentration of 1 × 10^{-7} mol/l to 3 × 10^{-7} mol/l by the luminescence method using a 20 times dilution with NB (6.5) at room temperature.

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

8.3.3 Pretreatment of specimen

8.3.3.1 Sampling

Obtain six control specimens and six antibacterial testing specimens cut to 60 mm in diameter.

When necessary, specimens may be washed in accordance with ISO 6330 or another suitable method, and after the final washing, the specimens are rinsed with water to eliminate the washing detergent. The use of an unspecified method shall be recorded.

NOTE Three of the control specimens and three of the antibacterial testing specimens are used to measure the number of bacteria immediately after the printing of test bacteria. The remaining specimens are used to measure the number of bacteria after incubation (total of six).

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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.

8.3.3.3 Humidity conditioning of specimens

Pour 10 ml of the agar for printing (6.12) into a Petri dish (5.17). Place the uncovered dish on a clean bench (5.6) to cool and solidify. Cool to room temperature avoiding dew formation. When the agar solidifies, turn the dish upside down. Place the specimen on the inside of the dish lid and 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 sterilized by autoclave on a clean bench (5.6).

Pour 5 ml of 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, and 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 is placed under the membrane filter when using the filtering apparatus. An aspirator, small air pump or other simple apparatus is used for aspiration.

8.3.4.2 Printing of test bacteria

8.3.4.2.1 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 1.

8.3.4.2.2 Remove the specimen of 8.3.3.3 from the Petri dish (5.17) using sterilized tweezers (5.23). Place on the membrane filter facing downwards.

8.3.4.2.3 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.

8.3.4.2.4 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 and let it stand for 1 h to 4 h in an incubator (5.2).

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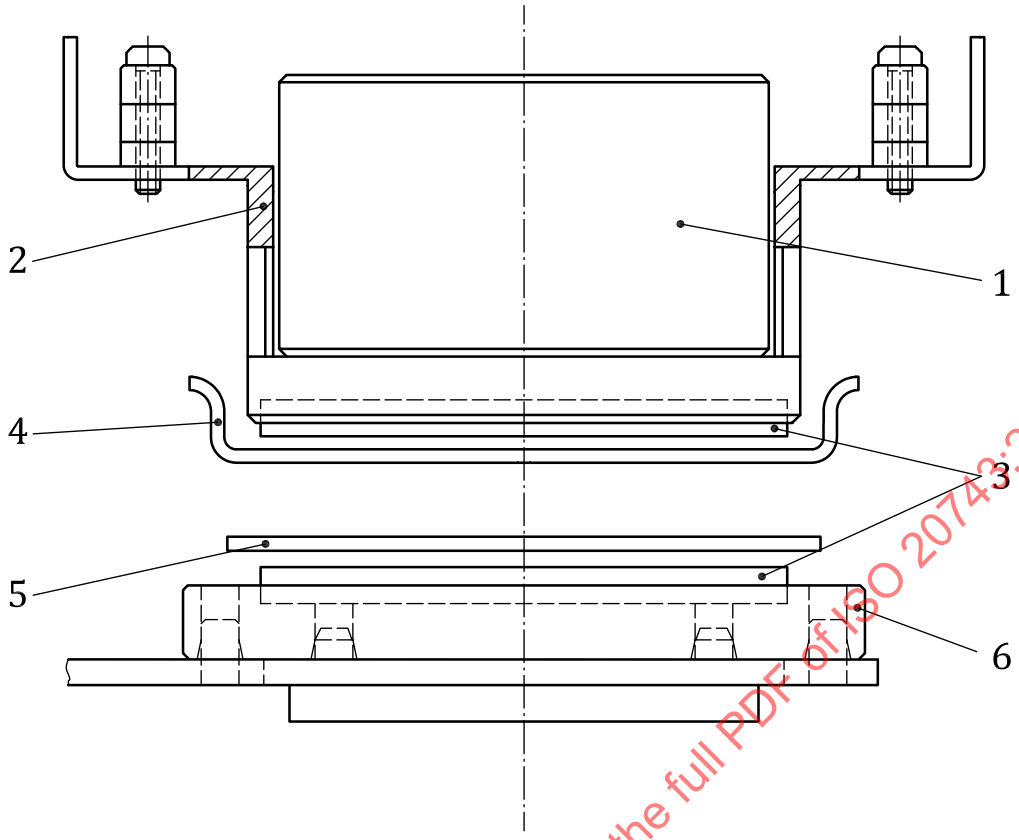

Key

1	weight (17 kg)	4	specimen (Ø 60 mm)
2	weighting plate	5	membrane filter (Ø 47 mm)
3	silicone rubber (Ø 42 mm)	6	turn plate (Ø 54 mm) (anticlockwise rotation)

NOTE This figure illustrates the side of the printing apparatus (5.10). External dimensions (height 170 mm, width 160 mm, depth 150 mm).

Figure 1 — Printing apparatus[1)]

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8.3.4.3 Incubation test

Incubate the printed specimen in the Petri dish of 8.3.4.2.4 in an incubator (5.2) conditioned at 20 °C ± 2 °C and 70 % RH or more for a period of 1,0 h ± 0,1 h, 2,0 h ± 0,1 h, 3,0 h ± 0,1 h, or 4,0 h ± 0,1 h. The incubation time is determined by specific test condition requirements which shall be recorded.

8.3.4.4 Shake-out after printing

Immediately after printing, transfer each specimen of the control fabric of 8.3.4.2 to a vial containing 20 ml of the SCDLP medium (6.8), and shake out the bacteria from each specimen as specified in Annex B by hand or mixer (5.4).

8.3.4.5 Shake-out after incubation

After incubation, transfer each specimen of 8.3.4.3 to a vial containing 20 ml of the SCDLP medium (6.8), and shake out the bacteria from each specimen as specified in Annex B by hand or mixer (5.4).

1) This apparatus is available from e.g. Aloka Co., Ltd., 6-22-1, Mure, Mitaka-shi, Tokyo 181-8622, Japan. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

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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 ATP from 8.3.4.4 and 8.3.4.5 from the bacteria concentration or ATP concentration obtained by the quantitative measurement methods in Annex C and Annex D according to the following formulae.

8.3.4.6.2 Number of bacteria

$$M = c_B \times 20$$

where

M is the number of bacteria per specimen;

c_B is the bacteria concentration obtained in Annex C;

20 is the volume of the shake-out solution, in millilitres (ml).

8.3.4.6.3 Amount of ATP

$$M' = c_{ATP'} \times 20$$

where

M' is the amount of ATP per specimen;

$c_{ATP'}$ is the ATP concentration obtained in Annex D;

20 is the volume of the shake-out solution, in millilitres (ml).

8.3.5 Test results

8.3.5.1 Judgement of test effectiveness

When the following two items 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 test bacteria or amount of ATP printed on the control fabric shall not be less than $1{,}0 \times 10^{6}$ CFU or $1{,}0 \times 10^{-11}$ mol.

b) The amount of increase or decrease on the control specimen, F, obtained according to the following formula, shall be +0,5 to −0,5.

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$$F = \lg C_t - \lg C_0$$

where

F is the value of increase or decrease on the control specimen;

$\lg C_t$ is the common logarithm of the arithmetic average of the numbers of bacteria, or the average amount of ATP, obtained from three control specimens after a 1 h to 4 h incubation;;

$\lg C_0$ is the common logarithm of the arithmetic average of the numbers of bacteria, or the average amount of ATP, obtained from three control specimens immediately after printing.

8.3.5.2 Calculation of antibacterial activity value

When the test is determined to be effective, obtain the antibacterial activity value according to the following formula:

$$A = \lg C_t - \lg T_t$$

where

A is the antibacterial activity value;

$\lg C_t$ is the common logarithm of the arithmetic average of the numbers of bacteria or for the average amount of ATP, obtained from three control specimens after a 1 h to 4 h incubation;

$\lg T_t$ is the common logarithm of the arithmetic average of the numbers bacteria or for the average amount of ATP, obtained from three antibacterial testing specimens after a 1 h to 4 h incubation.

9 Test report

The test report shall contain the following information:

a) a reference to this International Standard, ISO 20743;

b) details of the testing specimens;

c) name of the test bacteria;

d) strain number;

e) inoculation method;

f) concentration inoculum;

g) antibacterial activity value;

h) quantitative measurement method;

i) any deviation from this International Standard.

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Annex A
(normative)

Strain numbers

A.1 General

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

A.2 List of bacteria

Table A.1 — Bacteria for testing

Bacteria type	WDCM code
Staphylococcus aureus	00193
	http://refs.wdcm.org/getinfo.htm?sid=WDCM_00193
Klebsiella pneumoniae	00192
	http://refs.wdcm.org/getinfo.htm?sid=WDCM_00192

NOTE 1 Other bacteria can be used after appropriate validation.

NOTE 2 Refer to WDCM and website: http://refs.wdcm.org/search.htm. (Note that WDCM stands for World Data Centre for Microorganisms.)

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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 test tube or bottle by hand and shake in an arc of approximately 30 cm for 30 s.

B.4 Shaking by Stomacher

Place the designated disposable bag (5.22) in the Stomacher machine (5.5) and run the machine for 1 min on each face of the bag.

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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 NB (6.5) 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 medium 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 Warm approximately 15 ml of EA (6.11) or TSA (6.3) to a temperature of 45 °C to 46 °C using a water bath (5.3), add 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 1ml 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 1ml 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 is the bacteria concentration, in Colony Forming Units per millilitre (CFU/ml);

Z is the average value of two Petri dishes in Colony Forming Units (CFU) per 1 ml of inoculum;

R is the dilution rate.

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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, 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.

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 three separate test tubes and designate them as specimens for measuring the diluted standard reagent.

D.2.1.3 Pour 0,1 ml of physiological saline (6.7), SCDLP (6.8) or another suitable medium, 0,8 ml of the dilution buffer (6.9), and 0,1 ml of ATP eliminating reagent (6.18) into a plastic test tube. Shake amply and pour 0,1 ml portions into three 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 (6.17) and shake. Add 0,1 ml of ATP luminescent reagent (6.16), shake again and immediately apply a luminescence photometer (5.9) 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 (6.16), shake again and immediately apply a luminescence photometer (5.9) 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:

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