(2) Minimal Growth Inhibitory Concentration Determination Method II (2012 Version)
MIC determination method for photocatalytic antibacterial agents Abolish

1. Scope

This test method shall be applied to photocatalytic antibacterial agents in powder form.

2. Test Strains[1]

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

3. Procurement of Supplies

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

3.1 Instrumentation and equipment

(1) L-shaped test tubes[2] (made of glass, length 130 to 140 mm, height 110 to 120 mm, outside diameter 18 mm)
(2) Mechanical shaker[3], constant-temperature water chamber[4] and shaking incubator (a model that can be operated at an accuracy within ±1°C)
(3) Constant-temperature chamber (a model that can be operated at an accuracy within ±1°C)
(4) Ultraviolet irradiation apparatus[5]
(5) Ultraviolet intensity tester[6] (measuring wavelength band 310 - 400 nm)

3.2 Culture media

(1) Nutrient agar medium (NA medium)

Meat extract	5.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1,000 ml
pH	7.0 to 7.2

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1 These test microorganisms were chosen to represent Gram-positive and -negative bacteria, respectively.

2 The stoppers for the L-shaped test tubes shall be air-permeable silicone stoppers.

3 This mechanical shaker may be of any type: monode shaking, horizontal shaking, or vertical shaking. Commercially available monode shakers include the Unit Shaker Monoshin IIA from Tietech Co., Ltd.

4 As a rule, this constant-temperature water chamber shall be used in combination with the mechanical shaker. Provided that there is sufficient distance between the sample and the light source to avoid a temperature rise in the sample, a shaking incubator may be used.

5 Black light fluorescent lamps generate ultraviolet rays at wavelengths of approximately 310 to 400 nm, longer than those of ultraviolet rays from what are called germicidal lamps. Note that the number of light source units and wattage used are not subject to regulations.

6 The ultraviolet intensity tester shall be calibrated periodically as directed in the instruction manual. In general, ultraviolet intensity testers from different manufacturers have somewhat different measuring wavelength bands, sensitivities and the like; measured values are often inconsistent depending on the ultraviolet intensity tester used. In particular, special attention must be paid to the fact that measured values can differ by about three times between an ultraviolet intensity tester with a dome-shaped light receiving port and one with a flat light receiving port.

(2) Mueller-Hinton broth medium (MHB medium)[1]

Meat extract	300.0 g
Casamino acid	17.5 g
Soluble starch	1.5 g
Purified water	1,000 ml
pH	7.3±0.1

4. Test Procedures

4.1 Incubation of the test microorganisms

Transplant each test microorganism to NA medium and incubate at 35 to 37°C for 24 hours. Inoculate a platinum loopful of this culture to MHB medium and incubate at 35 to 37°C for 16 to 20 hours.

4.2 Preparation of liquid inoculum

Dilute the culture broth using MHB medium[2] to obtain a cell count of 1.0 to 5.0 × 10^4 cells/ml.

4.3 Preparation of test culture media

Dispense 10 ml of sterile MHB medium to a sterile L-shaped test tube[3]. Add the sample[4] to the medium to obtain two series of test culture media (concentrations obtained by multiplying or dividing the basic concentration of 100 μg/ml by a common factor of 2) ("bright conditions test" and "dark conditions test")[5]. Measure the pH of each medium, compare and make any necessary adjustment if the pH is not in the range of ±0.5 compared to the level before sample addition. Separately, provide two series of test culture media not supplemented with the sample ("bright conditions control" and "dark conditions control"). Inoculate 0.1 ml of the inoculum liquid[6] to each test culture medium.

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[1] Mueller-Hinton agar medium is used for the agar plate dilution method of the Japan Society of Chemotherapy. MHB medium is commercially available from Eiken Chemical Co., Ltd. (MHB agar medium only), DIFCO, BBL, Merck and others.

[2] The MHB medium used for dilution has been chosen in accordance with the standard method of the Japan Society of Chemotherapy. The cell count of the liquid inoculum was set at nearly the same level as the standard method.

[3] Before using in the test, the L-shaped test tubes shall be sterilized with dry heat (160°C to 180°C for 120 minutes or more), the MHB medium shall be sterilized with high-pressure steam (at 121°C for 15 minutes), and the silicone stoppers shall be sterilized by an appropriate method (high-pressure steam sterilization followed by drying etc.).

[4] Because the sample is placed directly in the medium, microorganisms possibly derived from the sample can influence the test results. It is desirable, therefore, that the sample be germfree. To this end, it is recommended that the sample be sterilized in advance if possible (in addition to dry heat sterilization, high-pressure steam sterilization, gas sterilization and other methods of sterilization are available).

① Samples allowing high-temperature heating
Heat the sample at 160 to 180°C for 120 minutes or more to achieve sterilization and drying. After drying, allow the sample to cool in a silica gel-containing desiccator.

② Samples not allowing high-temperature heating
After sterilization by an appropriate method, dry the sample within a range of temperature and time that do not deteriorate the sample. Allow the sample to cool in a silica gel-containing desiccator. In this case, the drying and sterilization conditions shall be indicated. Be sure to avoid inadequate heating temperature and time because incorrectly sterilized samples allow the survival of spores of bacteria of the genus *Bacillus*.

[5] Since the specification value is 800 μg/ml, the test may be performed at selected concentrations of 3,200, 1,600, 800, 400, 200, 100, 50, and 25 μg/ml.

[6] To obtain nearly the same cell count as the standard method of the Japan Society of Chemotherapy, 0.1 ml of the inoculum liquid is prepared.

4.4 Cultivation

Perform shaking culture to achieve sample homogenization (shaking rate 100 to 200 rpm; amplitude 40 to 60 mm for horizontal or vertical shaking or 0 to 24 degrees for monodeshaking) at 35 to 37°C for 24 hours, while irradiating ultraviolet rays[1] for the "bright conditions test" culture media and "bright conditions control" test culture media, or under shading for the "dark conditions test" culture media and "dark conditions control" test culture media.

Note that the "bright conditions control" test culture media shall be located at the position of the highest ultraviolet intensity (in the vicinity of center of black light fluorescent lamp) during the cultivation.

4.5 Requirements for test validity

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

(1) Growth of the test microorganism[2] in "dark conditions control" test culture medium shall be observed by macroscopic examination[3] after completion of cultivation.

(2) Growth of the test microorganism in "bright conditions control" test culture medium shall be observed by macroscopic examination after completion of cultivation.

4.6 Judgement[4]

After cultivation, macroscopically examine each test culture medium for growth of the test microorganism, determine the minimum concentrations of the sample in "bright conditions test" culture medium and "dark conditions test" culture medium showing no growth, and use these concentrations as the minimal growth inhibitory concentrations for the "bright conditions" and the "dark conditions", respectively.

[1] Ultraviolet intensity shall be measured above the upper surface of the liquid inoculum with an L-shaped test tube attached to the mechanical shaker. Adjust the position of the black light fluorescent lamp to obtain an ultraviolet intensity of 0.4 to 1.0 mW/cm^2 for an ultraviolet intensity tester with a flat light receiving port, or 1.2 to 3.0 mW/cm^2 for an ultraviolet intensity tester with a dome-shaped light receiving port. Ultraviolet intensity may be changed according to the choice of test microorganism. In this case, however, the ultraviolet intensity used shall be indicated in the MIC value field. Since *Staphylococcus aureus* sometimes does not grow in the "bright conditions control plot" at the upper limit of the above-described range of ultraviolet intensity and hence fails to fulfill the "requirement for test validity in Term 4.5 (2)", it is recommended that the ultraviolet intensity be set at a level close to the lower limit. Prior to determination of ultraviolet intensity, wait at least 30 minutes after turning on the light source.

[2] The judgment on the presence or absence of growth of the test microorganism shall be made by macroscopic examination. If judgment is impossible due to sample turbidity, proceed as described below. Dilute the medium 10,000 fold with phosphate-buffered physiological saline*, streak the dilution on nutrient agar medium using a loop of platinum or Nichrome wire (inside diameter 1 mm), and examine for growth of the test microorganism.
* Phosphate-buffered physiological saline: Dissolve 34 g of KH_2PO_4 in 500 ml of purified water, adjust the solution to pH 7.2 with 1N NaOH, then add purified water to make a total volume of 1,000 ml. Dilute 1.25 ml of this solution with physiological saline (0.85% NaCl) to make a total volume of 1,000 ml, and sterilize with high-pressure steam at 121°C for 15 minutes.

[3] The minimum cell count allowing the macroscopic confirmation of growth of test microorganism is approximately 10^6 cells/ml.

[4] The choice, model, and number of units of the black light fluorescent lamp used, distance to the liquid inoculum upper surface in the L-shaped test tube, ultraviolet intensity above the upper surface of the liquid inoculum (center and ends of the fluorescent lamp), choice of the ultraviolet intensity tester used, and others shall be indicated in the Test Results section.