4. Antifungal Potency Evaluation Test Method for Antifungal Agents (2012 Version)

Minimal Growth Inhibitory Concentration Determination Method
MIC determination for molds by the agar plate dilution method

1. Scope
This test method shall be applied to antifungal agents.

2. Test Strains[1]
(1) *Aspergillus niger* NBRC 105649
(2) *Penicillium pinophilum* NBRC 6345

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) Glass test tubes (length 170 to 200 mm, outside diameter 18 mm)
(2) Sterile Petri dishes (inside diameter 80 to 100 mm, height 15 to 25 mm)
(3) Constant-temperature chamber (a model that can be operated at an accuracy within ±1°C)
(4) Pipettes (milk pipettes, volumetric pipettes allowing dispensing of 1 ml and 10 ml or more or automated pippetter)
(5) Balance (chemical balance)
(6) Autoclave
(7) Microscope (light microscope)
(8) pH meter
(9) Platinum loop

[1] With reference to JIS Z 2911 etc., *Penicillium pinophilum*, often used for fungal resistance testing, and *Aspergillus niger*, reportedly spontaneously resistant thereto, were chosen as the test microorganisms. As both are intermediately hygrophilous fungi, it is preferable to use them in combination with a xerophilous fungus such as *Eurotium tonophilum* or *Aspergillus penicilloides*, a hygrophilous fungus such as *Aureobasidium pullulans*, a cellulose-decomposing fungus such as *Chaetomium globosum* or *Trichoderma virens*, and the like, depending on the intended use.

3.2 Culture Media

(1) Potato dextrose agar medium (PDA medium)

Potato extract	200 g
Glucose	20 g
Agar	15 g
Purified water	1000 ml
pH	5.6±0.2

(2) Glucose peptone (GP medium)

Glucose	20.0 g
Yeast extract	2.0 g
Magnesium sulfate	0.5 g
Peptone	5.0 g
Potassium dihydrogen phosphate	1.0 g
Purified water	1,000 ml
pH	5.7±0.1

(3) Dioctyl sodium sulfosuccinate solution[1]

Dioctyl sodium sulfosuccinate solution	0.05g
Purified water	1,000 ml

4. Test Procedures

4.1 Incubation of the test microorganisms

Transplant each test microorganism to PDA medium and incubate at 25±1°C for 7 to 14 days.

4.2 Preparation of liquid inoculum

Suspend the conidium of the cultured test microorganism in 0.005% dioctyl sodium sulfosuccinate solution, remove fruiting bodies and mycelia, then dilute the suspension with GP medium to obtain a density of 1.0×10^5 to 1.0×10^6 conidia/ml [2].

[1] With reference to JIS Z 2911, 0.005% dioctyl sodium sulfosuccinate solution was chosen as the suspending agent.

[2] In the agar plate dilution method of the Japan Society of Chemotherapy, bacteria are used as the test microorganisms at a cell count of 10^6 cells/ml. In the present test method, however, the test microorganisms are fungi, which produce conidia larger than bacterial cells. Accordingly, the cell count was set at lower-by-one-digit levels between 1.0×10^5 cells and 1.0×10^6 cells/ml.

4.3. Preparation of test liquids

Dissolve, emulsify, or suspend or dilute the sample in a solvent such as purified water, ethanol or DMSO to prepare a series of dilutions (concentrations obtained by multiplying or dividing the basic concentration of 1000 μg/ml by a common factor of 2) and use these dilutions as the test liquids.

4.4. Preparation of susceptibility determination plates

Add each test liquid, in an amount of 1/9 of the volume of the culture medium, to "GP medium supplemented with 1.5% of agar", previously sterilized and cooled to 50 to 60°C, mix thoroughly, then dispense the mixture to a Petri dish and allow to solidify, and use this as the susceptibility determination plate[1].

4.5. Cultivation

Apply the liquid inoculum in a streak about 1 cm in length over the susceptibility determination plate using a Nichrome wire loop (inside diameter 1 mm) [2] and culture at 25°C±1°C for 7 days.

4.6 Judgement

After the cultivation, macroscopically examine the plate for growth of the test microorganism and determine the minimum sample concentration showing no microbial growth to obtain the minimal growth inhibitory concentration.

If two or more continuous colonies[3] are observed, even though they are micro-colonies, the test microorganism shall be judged to have "grown".

[1] It is desirable that a control test be performed with a separately prepared plate not supplemented with any test liquid.

[2] In the agar plate dilution method of the Japan Society of Chemotherapy, the test microorganism is applied in a streak about 2 cm in length. In the present test method, however, the test microorganisms are molds. In the case of fungi (molds, yeast), it is common practice to apply the test microorganism in a streak about 1 cm in length. Hence, the latter was adopted.

[3] In the agar plate dilution method of the Japan Society of Chemotherapy, the test microorganism is judged to have "grown" if five or more colonies are observed. In the present test method, however, the test microorganisms are fungi. In the case of molds, it is common practice to judge the test microorganism to have "grown" if two or more continuous colonies are observed. Hence, the latter was adopted.