

EUCAST DEFINITIVE DOCUMENT E.DEF 9.1: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds

Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST)

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INTRODUCTION

Antifungal susceptibility tests are performed on those fungi causing disease especially if they belong to a species exhibiting resistance to commonly used antifungal agents. Antifungal susceptibility testing is also important in resistance surveillance, epidemiological studies and in comparison of the *in vitro* activity of new and existing agents.

Dilution methods are used to establish the minimum inhibitory concentrations (MICs) of antimicrobial agents: They are the reference methods for antimicrobial susceptibility testing and are mainly used to establish the activity of a new antifungal, to confirm the susceptibility of organisms that give equivocal results in routine tests, and to determine the susceptibility on fungi where routine tests may be unreliable. In dilution tests, fungi are tested for their ability to produce visible growth in microdilution plate wells of broth culture media containing serial dilutions of the antimicrobial agents (broth microdilution). The MIC is defined as the lowest concentration, recorded in mg/L, of an antifungal agent that inhibits the growth of a fungus. The MIC informs about the susceptibility or resistance of the organism to the antifungal agent and can help in treatment decisions.

The increasing number of options for treating invasive mould disease, coupled with documented resistance to antifungal agents among some strains and species, has confirmed the need for having standardised methods for determining the in vitro susceptibilities of both new and established antifungal agents against clinical isolates of filamentous fungi [1-9]. The method described in this document is intended for testing moulds that cause clinically significant fungal infections.

SCOPE

The standard method described here provides a valid method for testing the susceptibility of moulds able to produce conidia to antifungal agents by determination of the MIC. MICs show the activity of a given antifungal drug under the test conditions described, and can be used for patient management when other factors, such as pharmacokinetics, pharmacodynamics and resistance mechanisms are taken into account. The MIC permits moulds to be categorised as “susceptible” (S), “intermediate” (I), or “resistant” (R) to an antifungal drug. In addition, MIC distributions can be used to define wild type or non-wild type fungal populations.

The method described in this document is intended to provide a valid, easy, rapid and economic method for testing the susceptibility to antifungal agents of moulds and to facilitate an acceptable degree of conformity, e.g. agreement within specified ranges, between laboratories. Nevertheless, many factors influence the MIC of filamentous fungi against antifungal agents as shown by Rambali et al. [10]. The MIC of itraconazole against *Aspergillus* was profoundly influenced by shape of the microtitration well, temperature and length of incubation time. Since technical laboratory factors are thus of utmost importance, this standard focuses on testing conditions including inoculum preparation and inoculum size, incubation time and temperature, and medium formulation.

TERMS AND DEFINITIONS

Antifungal agent

A substance of biological, semi-synthetic or synthetic origin that inhibits the growth of a mould or is lethal to it. Disinfectants, antiseptics and preservatives are not included in this definition.

Potency

The antimicrobially active fraction of a test substance, determined in a bioassay against a reference powder of the same substance. The potency is expressed as mass fraction in milligrams per gram (mg/g), or as activity content in International Units (IU) per gram, or as a volume fraction or mass fraction in percent, or as an amount-of-substance concentration (mass fraction) in mole per litre of ingredients in the test substance.

Concentration

The amount of an antimicrobial agent in a defined volume of liquid. The concentration is expressed in SI units as mg/L. Although mg/L \equiv μ g/mL, the use of the latter is not recommended.

Stock solution

The initial solution used for additional dilutions.

Minimum inhibitory concentration (MIC)

The lowest concentration that inhibits the growth of moulds within a defined period of time. The MIC is expressed in mg/l.

Breakpoints (BPs)

Specific values of MICs on the basis of which moulds can be assigned to the clinical categories “susceptible”, “intermediate” and “resistant”. This breakpoint can be altered due to changes in circumstances (e.g. changes in commonly used drug dosages).

Susceptible (S). A mould inhibited *in vitro* by a concentration of an antifungal agent that is associated with a high likelihood of therapeutic success. Fungi are categorized as susceptible by applying the appropriate breakpoints in a defined phenotypic test system.

Intermediate (I). Mould inhibited *in vitro* by a concentration of an antifungal agent that is associated with doubtful therapeutic effect. Fungal strains are categorized as intermediate by applying the appropriate breakpoints in a defined phenotypic test system. Intermediate class implies that an infection caused by the isolate can be properly treated in body sites where the antifungal drug is physiologically concentrated or when a high dosage of drug can be used. This class also indicates a “buffer zone”, to prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

Resistant (R). Mould inhibited *in vitro* by a concentration of an antimicrobial agent that is associated with a high likelihood of therapeutic failure. Fungal strains are categorized as resistant by applying the appropriate breakpoints in a defined phenotypic test system.

Wild type

An isolate without acquired resistance mechanisms to the antifungal agent.

Control strain

A catalogued, characterized fungus with stable, defined antifungal susceptibility phenotypes and/or genotypes. They are obtainable from culture collections and are used for quality control.

Broth dilution susceptibility testing method

A technique in which serial dilutions (usually two-fold) of the antifungal are made in a liquid medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. The objective of this method is the determination of the MIC.

Broth microdilution

The performance of broth dilution in microdilution trays with a nominal capacity of approximately 300 µL per well.

Broth

A fluid medium used for the *in vitro* growth of fungi.

Inoculum

The number of spores (colony-forming units) in a certain volume. The inoculum is expressed as colony-forming units per millilitre (cfu/mL).

TEST PROCEDURE

The test is performed in microdilution trays. The method is based on the preparation of antifungal agent working solutions in 100 µL volumes per well to which 100 µL inoculum is added.

Medium

RPMI 1640 (with glutamine and a pH indicator but without bicarbonate) supplemented with glucose to a final concentration of 2% (RPMI 2% G) is recommended. The RPMI 1640 is at least as satisfactory as other synthetic media [11,12]. The use of a higher concentration of glucose has been shown to result in better growth and facilitated the determination of endpoints [13]. 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L, pH 7.0, is satisfactory for RPMI 1640 media. The composition of RPMI 1640 is outlined in Table 1. The recommended medium, RPMI with 2% of glucose (RPMI 2% G), is prepared as follows:

1. Add components as in Table 2 to 900 mL of distilled water.
2. Stir until components are completely dissolved.
3. With stirring, adjust the pH to 7.0 at 25°C with 1M sodium hydroxide.
4. Add water to a final volume of 1 litre.
5. Filter sterilise with a 0.22µm pore size filter.
6. Store at 4°C.
7. For quality control purposes use one aliquot of the sterilised medium for sterility checks, for retesting the pH (6.9-7.1 is acceptable) and as a growth control with a reference strain.

Antifungal agents

All antifungal drug solutions should be prepared in accordance with Good Manufacturing Practice. Antifungal powders must be obtained directly from the drug manufacturer or from reliable commercial sources. Clinical preparations must not be used, not least because they may contain excipients that interfere with susceptibility testing. Powders must be supplied with generic name of the drug, a lot number, potency, expiry date and recommended storage conditions. Store powders in sealed containers at -20°C or below with a desiccant unless

otherwise recommended by the manufacturers. Ideally, hygroscopic agents should be dispensed into aliquots, one of which is used on each occasion. Allow containers to warm to room temperature before opening them to avoid condensation of water on the powder.

Preparation of stock solutions

Antifungal drug solutions must be prepared taking into account the potency of the lot of antifungal drug powder that is being used. The amount of powder or diluent required to prepare a standard solution may be calculated as follows:

$$\text{Weight (g)} = \frac{\text{Volume (L)} \times \text{Concentration (mg/L)}}{\text{Assay potency (mg/g)}}$$

$$\text{Volume (L)} = \frac{\text{Weight (g)} \times \text{Assay potency (mg/g)}}{\text{Concentration (mg/L)}}$$

Weigh the antifungal drug powder on an analytical balance that has been calibrated to two decimal places when weighing 100 mg. It is recommended that at least 100 mg of powder is weighed.

Prepare antifungal drug stock solutions at concentrations 200 times the highest concentration to be tested. Information on solubility of antifungal compounds should be provided with the drug by the supplier. Solvents other than water are required to dissolve some antifungal drugs (Table 3). Sterilisation of stock solutions is not normally necessary but if required, membrane filtration should be used. Other filter materials should be avoided as they may adsorb significant amounts of drug. If filtration is used, samples obtained before and after filtration must be assayed to ensure that drugs are not adsorbed to the filter.

Unless otherwise indicated by the drug manufacturer, store drug solutions in small volumes in sterile polypropylene or polyethylene vials at -70°C or below. Drugs other than echinocandins may be stored at -70°C for at least six months without significant loss of activity [14]. Echinocandins are unstable and the best option is to prepare stock solutions the same day as the microplates. If this is not possible, stock solutions must be kept at -70°C for no more than 2 months.

Remove vials from -70°C storage and use them the same day that they are defrosted. Discard any drug not used on that day. Significant deterioration of an antifungal drug will be seen in the results of testing the susceptibility of quality control strains. If necessary, the drug can be assayed to determine the potency.

Preparation of working solutions

The range of concentrations tested will depend on the organism and the antifungal drug being tested. The range of concentrations should encompass the breakpoint, when one exists, as well as the expected results for the quality control strains. The drug concentration ranges in Table 3 are recommended. A two-fold dilution series based on 1 mg/L is prepared in double strength RPMI 2% G. The RPMI 2%G medium used in the plates is prepared at double the

final strength to allow for a 50% dilution once the inoculum is added. This approach allows the inoculum to be prepared in distilled water.

Dilutions should be prepared according ISO recommendations [15]. Alternative dilution schemes may be used if they are shown to perform as well as the reference method. For example, an alternative which uses smaller volumes to prepare a dilution series with final concentrations 0.125-64 mg/L is given in Table 4 (also see Table 3 to check the solvents required for each antifungal). A summary of the steps required to prepare working solutions (2 x final concentration) is as follows:

1. Take an antifungal drug stock tube from the -70°C freezer.
2. Dispense the appropriate volumes of solvent (consult Table 3 for solvents and Table 4 for volume of solvents) into nine further tubes.
3. Follow the steps described in Table 4 to produce a dilution series at 200 times final concentration. Similar dilution schemes with a stock concentration of 3,200 mg/L or 1,600 mg/L in step 1 of Table 4 are required for dilution series of 0.03-16 mg/L and 0.015-8 mg/L respectively.
4. Dispense 9.9 mL of double strength RPMI 2% G medium to 10 tubes. Take 100 µL from each of the tubes with 200 x final concentration of antifungal drug in solvent and transfer to the ten tubes with 9.9 mL of culture medium (1:100 dilution). The concentration of solvent in the culture medium tubes is 1% and the concentration of antifungal agents is 2 x final concentration.

Preparation of microdilution plates

Use sterile plastic, disposable, 96 well microdilution plates with flat-bottom wells with a nominal capacity of approximately 300 µL. To wells in each column, from 1 to 10, of the microdilution plate dispense 100 µL from each of the tubes containing the corresponding concentration (2 x final concentration) of antifungal agent. For example, with itraconazole, voriconazole or posaconazole, dispense to column 1 the medium containing 16 mg/L, to column 2 the medium containing 8 mg/L, and so on to column 10 for the medium containing 0.03 mg/L. To each well of column 11 and 12 dispense 100 µL of double strength RPMI 2% G medium. Thus, each well in columns 1 to 10 will contain 100 µL of twice the final antifungal drug concentrations in double-strength RPMI 2% G medium with 1% solvent. Columns 11 and 12 will contain double-strength RPMI 2% G medium.

Storage of microdilution plates

The trays can be sealed in plastic bags or aluminium foil and stored frozen at -70°C or below for up to 6 months or at -20°C for not more than 1 month without loss of drug potency. Once plates are defrosted they must not be refrozen. Echinocandins are unstable, so the prepared trays must be stored at -70°C or below for no more than two months.

Preparation of inoculum

Standardisation of the inoculum is essential for accurate and reproducible antifungal susceptibility tests. The final inoculum must be between 1×10^5 cfu/mL and 2.5×10^5 cfu/mL.

Spore suspension method

Subculture the isolates on potato dextrose agar slants, or other culture media on which the fungus is able to sporulate readily, and incubate at 35°C. Prepare inoculum suspensions from fresh, mature (2-5 day old) cultures. With some isolates an extended incubation is required for adequate sporulation.

Cover colonies with approximately 5 ml of sterile water supplemented with 0.1% Tween 20. Then carefully rub the conidia with a sterile cotton swab and transfer them with a pipette to a sterile tube. Homogenize the suspension for 15 seconds with a gyratory vortex mixer at 2,000 rpm. Make appropriate dilutions in order to attain the right concentration for counting in a haemocytometer chamber. Inoculum preparations should also be examined for the presence of hyphae or clumps. If a significant number of hyphae is detected (> 5% of fungal structures), transfer 5 mL of the suspension to a sterile syringe attached to a sterile filter with a pore diameter of 11 µm, filter and collect in a sterile tube. This step, which is seldom needed for *Aspergillus* spp., removes hyphae and yields a suspension composed of spores. If clumps are detected, the inoculum is shaken again in a vortex mixer for further 15 seconds. Repeat this step as many times as necessary, until clumps are no longer encountered. Adjust the suspension with sterile distilled water to a concentration of $2-5 \times 10^6$ cfu/mL by counting the conidia in a haemocytometer chamber. Then dilute the suspension 1:10 with sterile distilled water to obtain a final working inoculum of $2-5 \times 10^5$ cfu/mL [16-18].

Inoculation of microdilution plates

The microdilution plates should be inoculated within 30 min of standardising the inoculum suspension in order to maintain viable spore concentration. Inoculate each well of a microdilution tray with 100 µL of the $2-5 \times 10^5$ cfu/mL conidial suspension. This will give the required final drug concentration and inoculum density ($1 \times 10^5 - 2.5 \times 10^5$ cfu/mL). Also inoculate the growth control wells (column 11), which contained 100 µL of sterile drug-free medium, with 100 µL of the same inoculum suspension. Fill column 12 of the microdilution plate with 100 µL of sterile distilled water from the lot used to prepare the inoculum as a sterility control for medium and distilled water (drug-free medium only). Test quality control organisms by the same method each time an isolate is tested.

Viable counts should be performed to ensure that test wells contain between 1×10^5 and 2.5×10^5 cfu/mL. This can be done by removing 20 µL from the growth control well immediately after inoculation and diluting it in 2 ml of sterile distilled water supplemented with 0.1% Tween 20. Homogenize the suspension with a gyratory vortex mixer at 2,000 rpm. Then spread 100 µL of this suspension over the surface of a suitable agar plate, which is then incubated for 24-48h, or until colonies can be enumerated. Fifty to 250 colonies would be expected from an acceptable test suspension. Similar results can be obtained by spreading 50 µL or 25 µL of the suspension but the number of

colonies to be counted is proportionally lower and counting is easier. If the correct inoculum is not achieved, the results for this strain can not be used.

Incubation of microdilution plates

Incubate microdilution plates without agitation at $35 \pm 2^\circ\text{C}$ in ambient air for 24-48h. In a few cases, a further 24h incubation period will be required in order to get adequate growth in the control well. Longer incubation is not recommended. Mucorales should be read at 24h. Other moulds should be read at 48h.

Reading results

The endpoint is read visually by recording the degree of growth for each well as seen using a viewing mirror.

MIC endpoint for all drugs except for echinocandins

The concentration of drug yielding no growth is the MIC value. Ignore single colonies on the surface and “skipped-wells”.

Minimum Effective Concentration (MEC) endpoint for echinocandins

The lowest drug concentration that results in macroscopic changes from the filamentous growth similar to that observed in positive control wells to microcolonies or granular growth (which are ignored). Inexperienced personnel can perform microscopic MECs by examining a small volume from the first well(s) that shows the presence of round microcolonies (visual MEC) under the microscope for morphological changes. The MEC is the lowest echinocandin concentration in which abnormal, short, and branched hyphal clusters are observed in contrast to the long, unbranched, hyphal elements that were seen in the growth control well.

INTERPRETATION OF RESULTS

Interpretation of mould MICs is challenging and interpretative breakpoints have yet to be established. The clinical relevance of testing moulds also remains uncertain. Most of the information available comes from *Aspergillus* species causing invasive aspergillosis and can be summarised as follows:

Amphotericin B

No data are available to indicate a clear correlation between MIC and outcome of treatment [10,19,20]. The most useful information is often derived from complete identification of the fungus itself. Experience to date indicates that MICs of amphotericin B for most *Aspergillus* spp. are clustered between 0.5 and 2 mg/L. However, isolates of *A. terreus* may have higher MICs of amphotericin B [5,21] and, in general, *A. terreus* infections are associated with a reduced response to amphotericin B compared to patients with infections caused by

more common species of *Aspergillus* [5]. Therefore, high MICs of amphotericin B should be taken into consideration and alternatives to amphotericin B should be considered when an invasive fungal disease is due to *A. terreus*.

Itraconazole

More is known about the detection of azole resistance than about the relationship between MIC and outcome [3,9]. Two isolates were collected from patients who did not respond to therapy with itraconazole. These isolates were resistant to itraconazole in a murine model of invasive aspergillosis and had elevated itraconazole MICs (MIC \geq 8 mg/L) [3]. In addition, several studies have demonstrated that mutations in *cyp51A* gene are associated with high MICs of itraconazole [4,6-8].

Voriconazole

No data are available to indicate a correlation between MIC and outcome of treatment. However, some isolates with high MICs of itraconazole and mutations in the *cyp51A* gene also had elevated MICs of voriconazole. Therefore, cross resistance cannot be discounted and should be taken into consideration [4,6-8].

Posaconazole

No data are available to indicate a correlation between MIC and outcome of treatment. However, as with voriconazole, isolates with high MICs of itraconazole and mutations in the *cyp51A* gene may also exhibit elevated MICs of posaconazole, so cross resistance should be considered [4,6-8].

Caspofungin

No data are available to indicate a correlation between MIC or MEC and outcome of treatment.

Micafungin

No data are available to indicate a correlation between MIC or MEC and outcome of treatment.

QUALITY CONTROL

Control procedures are the means by which the quality of results is assured and are described in detail by the CLSI [22]. The routine quality of test results is monitored by the use of control strains.

Control strains

MICs for control strains should ideally be close to the middle of the range of the twofold series tested and antifungal drug susceptibility patterns must be stable. The recommended control strains are shown in Table 5 and were selected according to these criteria [22]. Control strains should be obtained from a reliable source such as the American Type Culture Collection (ATCC), National Collection for Pathogenic Fungi (NCPF), Centraal Bureau voor Schimmelcultures (CBS) or commercial suppliers offering similar guarantees of quality.

Storage of control strains

Fungal isolates may be stored lyophilised or frozen at -60°C or below [23,24]. Cultures can be stored short-term on Sabouraud's dextrose agar or potato dextrose agar slopes at 2-8°C, with new cultures prepared from frozen stocks every two weeks.

Routine use of control strains

For routine use of control strains, fresh cultures must be prepared from agar slopes, frozen or lyophilized cultures by inoculation on non-selective nutritive agar medium (eg Sabouraud's dextrose agar or potato dextrose agar)

1. At least two control strains must be included each day the test is performed and the MICs should be within the control ranges given in Table 5. If more than one in 20 tests is out of range the source of error must be investigated.
2. Each test must include a well of medium without antifungal drug to demonstrate growth of the test organisms and provide a turbidity control for reading end points.
3. Subculture inocula on a suitable agar medium to ensure purity and to provide fresh colonies for re-testing if required.
4. Test each new batch of medium, lot of microdilution trays, and lot of RPMI 2% G broth with at least two of the quality control strains listed in Table 5 to ensure that MICs fall within the expected range.

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Table 1. Composition of RPMI 1640 medium

Constituent	g/L
L-arginine (free base)	0.200
L-asparagine (anhydrous)	0.050
L-aspartic acid	0.020
L-cystine 2HCl	0.0652
L-glutamic acid	0.020
L-glutamine	0.300
Glycine	0.010
L-histidine (free base)	0.015
L-hydroxyproline	0.020
L-isoleucine	0.050
L-leucine	0.050
L-lysine HCl	0.040
L-methionine	0.015
L-phenylalanine	0.015
L-proline	0.020
L-serine	0.030
L-threonine	0.020
L-tryptophane	0.005
L-tyrosine 2Na	0.02883
L-valine	0.020
Biotin	0.0002
D-pantothenic acid	0.00025
Choline chloride	0.003
Folic acid	0.001
Myo-inositol	0.035
Niacinamide	0.001
PABA	0.001
Pyridoxine HCl	0.001
Riboflavin	0.0002
Thiamine HCl	0.001
Vitamin B ₁₂	0.000005
Calcium nitrate H ₂ O	0.100
Potassium chloride	0.400
Magnesium sulphate (anhydrous)	0.04884
Sodium chloride	6.000
Sodium phosphate, dibasic (anhydrous)	0.800
D-glucose ^a	2.000
Glutathione, reduced	0.001
Phenol red, Na	0.0053

^aNote that this medium contains 0.2% glucose

Table 2. Components of RPMI 2%G medium

Component	1x concentration	2x concentration
Distilled water	900 mL	900 mL
RPMI 1640 (Table 1)	10.4 g	20.8 g
MOPS	34.53 g	69.06 g
Glucose	18 g	36 g

Table 3. Solvents for preparation of stock solutions, characteristics and appropriate test concentration ranges for antifungal agents

Antifungal agent	Solvent	Characteristics	Test range (mg/L)
Amphotericin B	DMSO	Hydrophobic	0.0312 - 16
Itraconazole	DMSO	Hydrophobic	0.0156 - 8
Voriconazole	DMSO	Hydrophobic	0.0156 - 8
Posaconazole	DMSO	Hydrophobic	0.0156 - 8
Caspofungin	Water	Hydrophilic	0.0312 - 16
Micafungin	Water	Hydrophilic	0.0312 - 16

DMSO, dimethyl sulfoxide

Table 4. Scheme for preparing antifungal dilution series with a final concentration of 0.0312-16 mg/L

Step	Concentration (mg/L)	Source	Volume of antifungal (µL)	Volume of solvent ^a (µL)	Intermediate concentration (mg/L)	Concentration (mg/L) after 1:100 dilution with double strength RPMI 2%G ^b
1	3,200 ^c	Stock	200	0	3,200	32
2	3,200	Stock	100	100	1,600	16
3	3,200	Stock	50	150	800	8
4	3,200	Stock	50	350	400	4
5	400	Step 4	100	100	200	2
6	400	Step 4	50	150	100	1
7	400	Step 4	50	350	50	0.5
8	50	Step 7	100	100	25	0.25
9	50	Step 7	50	150	12.5	0.125
10	50	Step 7	25	175	6.25	0.625

^aConsult table 3 for solvents required to make dilutions of antifungals

^bDilution 1:1 with inoculum suspension gives final concentrations half those indicated

^cFor dilution series with highest final concentrations of 8 mg/L start with a stock concentration of 1600 mg/L

Table 5. Acceptable MIC ranges (mg/L) for quality control strains

Antifungal Agent	<i>Aspergillus fumigatus</i> ATCC 204305	<i>Aspergillus flavus</i> ATCC 204304	<i>Aspergillus fumigatus</i> F 6919	<i>Aspergillus flavus</i> CM 1813
Amphotericin B	0.25-1.0	0.5-2.0	0.25-1.0	1.0-4.0
Itraconazole	0.12-0.5	0.12-0.5	8.0-16.0	0.12-0.5
Voriconazole	0.25-1.0	0.5-2.0	0.5-2.0	0.5-2.0
Posaconazole	0.03-0.25	0.12-0.5	0.5-2.0	0.12-0.5
Caspofungin	NA	NA	NA	NA
Micafungin	NA	NA	NA	NA

NA, not available