EUCAST DEFINITIVE DOCUMENT E.DEF 7.3.2

Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts

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INTRODUCTION

Antifungal susceptibility tests are performed on fungi causing disease especially if the infection is invasive, relapsing or failing therapy, when inherent or acquired resistance is a possibility or when susceptibility cannot reliably be predicted from the species identification alone. Antifungal susceptibility testing (AFST) is also important for resistance surveillance, epidemiological studies and for 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 new antifungal agents, to confirm the susceptibility of organisms that give equivocal results in other test formats (such as commercial tests), and to determine the susceptibility of organisms where other test formats may be unreliable or not yet validated. In dilution tests, fungi are evaluated for their ability to produce sufficient growth in microdilution plate wells of broth culture media containing serial dilutions of the antimicrobial agents (broth microdilution).

The antifungal MIC is defined as the lowest concentration, recorded in mg/L, of an agent that inhibits the growth of a fungus to a predefined degree (e.g. 50%, 90%, or complete growth inhibition). The MIC informs about the susceptibility or resistance of the organism to the antifungal agent, which can help in treatment decisions.

This 7.3.1 method update is intended for testing the susceptibility of medically important yeasts (primarily *Candida* and *Cryptococcus* spp). The first version of the standard was published as a discussion document (7.1) in 2003 [1] and the definitive document in 2008 [2]. The second version contained additional information concerning solvent for caspofungin, micafungin and fluconazole, shelf-life of plates containing the echinocandins, testing of *Cryptococcus* and reference MIC ranges for quality control strains and anidulafungin [3]. This third version has been harmonised in wording and formatting to align with the mould 9.3.2 definitive document, the revised definition of the I category has been introduced and MIC ranges for quality control strains have been removed (this has been done acknowledging the new separate document summarising all antifungal MIC ranges for quality control strains (available on the EUCAST website http://www.EUCAST.org and in order to avoid a need for method document updating whenever a new QC range is established). Furthermore, the section concerning the preparation and calibration of the spectrophotometer has been modified in the current document.

SCOPE

This EUCAST standard describes a suitable method for testing the susceptibility of yeasts to antifungal agents by determination of the MIC. MICs show the *in vitro* activity of a given antifungal drug under the described test conditions, and can be used for patient management in conjunction with other factors, such as pharmacokinetics, pharmacodynamics and resistance mechanisms. The MIC also allows fungi to be categorised as "susceptible" (S), "susceptible, increased exposure" (I), or "resistant" (R) to an antifungal drug when appropriate breakpoints have been established. In addition, MIC distributions can be used to define wild-type or non-wild-type fungal populations when species-specific epidemiological cut-off values (ECOFFs) are applied.

The method described herein is intended to provide a suitable, easy, rapid and economic method for testing the susceptibility to antifungal agents of yeasts and to facilitate a degree of conformity, e.g. agreement within specified ranges, between laboratories. Since technical laboratory factors are of outmost importance, this standard focuses on testing conditions including inoculum preparation and inoculum size, incubation time and temperature, and medium formulation.

TERMS AND DEFINITIONS

1. **Antifungal agent:** substance of biological, semi-synthetic or synthetic origin that inhibits the growth of fungi or is lethal to them. Disinfectants, antiseptics and preservatives are not included in this definition.
2. **Properties of antifungal agents**
    a. **Potency.** Antimicrobially active fraction of a test 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.
    b. **Concentration.** Amount of an antimicrobial agent in a defined volume of liquid. The concentration is expressed in SI units as mg/L.
3. **Stock solution.** Initial solution used for additional dilutions.
4. **Minimum inhibitory concentration (MIC).** Lowest concentration that inhibits the growth of yeasts within a defined period of time. The MIC is expressed in mg/L.

5. **Breakpoint (BP).** Specific values of MICs on the basis of which fungi can be assigned to the clinical categories “susceptible”, “Susceptible, increased exposure” and “resistant”. The breakpoints can be altered due to changes in circumstances (e.g. changes in commonly used drug dosages) or when additional data/knowledge emerges.

    a) S - Susceptible, standard dosing regimen: A microorganism is categorised as "Susceptible, standard dosing regimen", when there is a high likelihood of therapeutic success using a standard dosing regimen of the agent.

    b) I – Susceptible, increased exposure*: A microorganism is categorised as "Susceptible, Increased exposure*" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.

    c) R - Resistant: A microorganism is categorised as "Resistant" when there is a high likelihood of therapeutic failure even when there is increased exposure.

    *Exposure is a function of how the mode of administration, dose, dosing interval, infusion time, as well as distribution and excretion of the antimicrobial agent will influence the infecting organism at the site of infection.

6. **Wild type (WT).** A yeast isolate is defined as WT for a species by the absence of phenotypically detectable acquired and mutational resistance mechanisms to the agent in question.

7. **Non-wild type (NWT)**. A yeast isolate is defined as NWT for a species by the presence of phenotypically detectable acquired or mutational resistance mechanisms to the agent in question.

Notes

a) A yeast isolate is categorized as S, I or R by applying breakpoints in a defined phenotypic test system.

b) A yeast isolate is categorized as WT or NWT by applying the appropriate cut-off value (ECOFF) in a defined phenotypic test system.

c) NWT micro-organisms harbour one or more resistance mechanisms but, depending on the values of the clinical breakpoints, WT and NWT micro-organisms may or may not respond clinically to treatment with the agent.

d) The wild type is presented as WT ≤z mg/L and non-wild type as NWT >z mg/L (where z is the ECOFF). The ECOFF is the highest MIC value for isolates devoid of phenotypically detectable resistance mechanisms.

e) The ECOFF will not be altered unless accumulated additional MIC distributions indicate the need for adjustment.

8. Reference strain for quality control. Catalogued, characterised strain with stable, defined antifungal susceptibility phenotypes and/or genotypes. They are obtainable from culture collections and used for quality control.

9. Susceptibility testing method

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

b) **Microdilution.** Performance of broth dilution in microdilution plate with a nominal capacity of approximately 300 µL per well.

10. Broth. Liquid medium used for the in-vitro growth of fungi.

11. Inoculum. Number of yeasts (colony forming units) suspended in a certain volume. The inoculum is expressed as colony forming units per millilitre (cfu/mL).

TEST PROCEDURES

General

The test is performed in flat-bottom well microdilution plate. Low evaporation lids should not be used, as this affects oxygen concentrations.

Preliminary data suggests that tissue-treated versus non-tissue-treated microdilution plates produce different MIC values (unpublished data). Furthermore, different plastics are likely to impact on drug binding, which may affect MIC values. Future studies are required to clarify these issues. For the most part, MIC distributions created by the EUCAST committee for ECOFF and breakpoint setting have been generated using tissue-treated microdilution plates, and are therefore more likely to yield similar MIC values. 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 L-glutamine and a pH indicator but without bicarbonate) supplemented with glucose to a final concentration of 2% (RPMI 2% G) is recommended [4]. The use of 2% rather than the standard 0.2% concentration of glucose has been shown to result in better growth and facilitate the determination of endpoints [5]. 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L, pH 7.0 is recommended buffer to be used for RPMI 1640 medium. The composition of RPMI 1640 is outlined in Table 1. The recommended medium, RPMI with 2% of glucose (RPMI 2% G), is prepared to double strength (to allow for a 50% [1:1] dilution, once the fungal inoculum is added; see "Preparation of working solutions and onwards) as follows:

1. Add the 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 ^{o}C with 1 M sodium hydroxide.
4. Add water to a final volume of 1000 mL.
5. Filter sterilise using a 0.22-µm pore size filter.
6. Store at 4 ^{o}C or lower for up to 6 months.
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.

Medium for testing amphotericin B

For the time being it is recommended that the RPMI 2% G is used also for amphotericin B. The non-synthetic broth Antibiotic Medium 3 (AM3), supplemented to a final concentration of 2% glucose, has been evaluated for detecting resistance to amphotericin B [6-9]. However, there is batch to batch variation in this medium, and also in the performance of the medium from different manufacturers. Preliminary results also indicate that an inoculum of 0.5-2.5 x 10^5 cfu/mL is too high for testing amphotericin B in AM3 [6].

ANTIFUNGAL AGENTS

General

All antifungal drug solutions should be prepared in accordance with Good Manufacturing Practice. Antifungal pure powders must be obtained directly from the drug manufacturer or from reliable commercial sources. Clinical preparations must not be used because they may contain excipients that may interfere with

susceptibility testing. Powders must be supplied with the generic name of the drug, a lot number, potency, expiry date and recommended storage conditions. Store powders in sealed containers at -20 ^{o}C or below with a desiccant unless otherwise recommended by the manufacturers. Ideally, hygroscopic agents should be dispensed into aliquots before freezing, one of which is used on each occasion. Containers should be allowed 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:

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

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

The antifungal powder should be weighed on an analytic balance that has been calibrated by approved reference weights from a certified metrology organisation. The portion of antifungal powder weighed should exceed the precision of the balance by at least 10-100 fold. Prepare antifungal drug stock solutions at concentrations at least 200 times higher than the highest concentration to be tested in the microdilution plate. Information on solubility of antifungal compounds should be provided with the drug by the supplier. Solvents other than water are required to dissolve most antifungal drugs (Table 3). It is essential to ensure the drug is fully dissolved before freezing. Several antifungals can be difficult to dissolve resulting in artificially elevated MICs. Placing the stock tube on a rocking table for an hour or more before continuing overcomes this problem. Sterilisation of stock solutions is not normally necessary. However, if required the sterilisation procedure shall be validated by appropriate means (e.g. samples obtained before and after filtration must be assayed) to ensure that drugs are not adsorbed (for example to a sterile filter) or degraded during the process.

Unless otherwise indicated by the drug manufacturer, store drug solutions in small volumes in sterile polypropylene or polyethylene vials at -70 ^{o}C or below. Drugs may be stored at -70 ^{o}C for at least six months without significant loss of activity [10, 11]. The echinocandins were previously regarded as unstable at -70 °C, however, they have been found stable for at least 6 months at this temperature [11].

Remove vials from -70 °C 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 reflected in the results of testing the susceptibility of quality control strains (available on the EUCAST website http://www.EUCAST.org). 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, if 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 to ISO recommendations [12]. For example, an alternative which uses smaller volumes to prepare a dilution series with final concentrations 0.125-64 mg/L is shown 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. Several antifungals can be difficult to dissolve resulting in artificially elevated MICs. Placing the stock tube on a rocking table for an hour or more before continuing overcomes this problem.
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-fold the final concentration. Similar dilution schemes with a stock concentration of 3200 mg/L or 1600 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 ten tubes.
5. 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.

Alternative dilution schemes may be used if they are shown to perform as well as the reference method [13].

Preparation of microdilution plates

Use sterile plastic (avoiding high binding plastic), disposable, 96 well microdilution plates with flat-bottom wells, not low evaporation lids, with a nominal capacity of approximately 300 µL.

Into wells 1 to 10 of each column 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, 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 plates can be sealed in plastic bags or aluminium foil and stored frozen at -70 ^{o}C or below for up to 6 months or at -20 ^{o}C for not more than 1 month without loss of drug potency [11]. Echinocandins are less stable, so the prepared plates must be stored at -70°C (and not -20°C) (unpublished data, M Cuenca-Estrella).

Once plates are defrosted they must not be refrozen. The plates should be used immediately when they have thawed as particularly anidulafungin MICs may increase if plates are left at room temperature after they have thawed and before inoculation.

PREPARATION OF INOCULUM

Standardisation of the inoculum is essential for accurate and reproducible antifungal susceptibility tests. The inoculum should be prepared by suspending five representative colonies, obtained from an 18-24 h culture on nutritive agar medium, in sterile distilled water. The final inoculum must be between 0.5 x 10^5 and 2.5 x 10^5 CFU/mL.

Colony suspension method

1. Culture all yeasts in ambient air at 34 to 37ºC on non-selective nutritive agar medium (Sabouraud's dextrose agar or potato dextrose agar) for 18-48 h before testing.
2. Prepare the inoculum by suspending five distinct colonies, ≥1 mm diameter from 24 h cultures, in at least 3 mL of sterile distilled water.

3. The suspension is homogenized for 15 seconds with a gyratory vortex mixer at approximately 2,000 rpm. Adjust the cell density to the density of a 0.5 McFarland standard (Table 5) by measuring absorbance in a spectrophotometer at a wavelength of 530 nm and adding sterile distilled water as required. This will give a yeast suspension of 1-5 x 10^6 CFU/mL. Prepare a working suspension by a 1 in 10 dilution of the standardised suspension in sterile distilled water to yield 1-5 x 10^5 CFU/mL. The cell density may also be adjusted in validated autocalibrating turbidimeters assuring a 0.5 McFarland inoculum.

***Cryptococcus* spp.**

Cryptococcus spp. are non-fermentative yeasts. The lack of fermentation compromises growth in microdilution plates when done according to the protocols suggested by both the CLSI and EUCAST. A comprehensive study recently explored variations of the EUCAST susceptibility testing procedure in comparison with the standard procedure for *Candida* [14]. Modifications included 1) growth medium (RPMI medium versus yeast nitrogen base [YNB]), 2) glucose concentration (0.2% versus 2%), 3) nitrogen source (ammonium sulphate), 4) temperature (30°C versus 35°C), 5) shaking, and 6) inoculum size (10^3, 10^4, and 10^5 cells). Growth rates and MIC were analyzed and compared. Although the use of YNB medium, reduction of the incubation temperature to 30°C and shaking of the plates during incubation increased the growth rate, there were no significant differences between the MICs obtained with the different methods. MIC values differed by no more than two 2-fold dilutions. It is therefore recommended for the time being that the EUCAST methodology is adopted for the testing of *Cryptococcus* species. Hence it is recommended to use RPMI 2% G as growth medium, a final inoculum of 0.5 x 10^5 and 2.5 x 10^5 CFU/mL, incubation without shaking and to read the plates when the OD value exceeds the background level by 0.2. In cases with insufficient growth it is suggested that the test is repeated but with incubation of the plates at 30°C.

INOCULATION OF MICRODILUTION PLATES

The microdilution plates should be inoculated within 30 min of preparing the inoculum suspension, in order to maintain the viable cell concentration.

Vortex the inoculum suspension and inoculate each well of a microdilution plate with 100 µL of the 1-5 x 10^5 CFU/mL yeast suspension, without touching the contents of the well. This will give the required final drug concentration and inoculum density (final inoculum = 0.5-2.5 x 10^5 CFU/mL). Also, inoculate the growth control wells (column 11), containing 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.

Viability counts should be performed for quality control purposes to ensure that test wells contain between 1-5 x 10^5 CFU/mL as follows. The suspension is homogenized with a gyratory vortex mixer at 2,000 rpm. Then 10 µL loop of this suspension is streaked over the surface of a suitable agar plate (such as Sabouraud dextrose agar or a chromogenic agar), which is then incubated for 24-48 h or until colonies can be checked for purity. A further dilution of 50 µL suspension in 4.95 mL sterile distilled water, homogenising, and 10 µL spread out over the surface of an agar plate would provide an optional/additional count – ten to fifty colonies would be expected. It is recommended that this is completed for every isolate when the laboratory is setting up this test/conduct the test rarely, when unexplained results are suspected, or periodically (to be locally defined dependent on need).

INCUBATION OF MICRODILUTION PLATES

Incubate microdilution plates without agitation at 35 ± 2 °C in ambient air for 24 ± 2 h. An absorbance of ≤0.2 indicates poor growth and is most commonly seen amongst strains of *Candida parapsilosis* and *Candida guilliermondii*. Such plates should be re-incubated for a further 12-24 h and then re-read. Failure to reach an absorbance of 0.2 after 48 h constitutes a failed test. As described above, an absorbance of ≤0.2 after 48 h for *Cryptococcus* spp. should prompt a repeat test with incubation at 30°C [14].

READING RESULTS

The microdilution plates must be read with a microdilution plate reader. The recommended wavelength for measuring the absorbance of the plate is 530 nm, although others can be used e.g. 405 nm or 450 nm. The value of the blank (background) should be subtracted from readings for the rest of the wells.

Amphotericin B

The MIC of amphotericin B is the lowest concentration giving rise to an inhibition of growth of ≥90% of that of the drug-free control.

Flucytosine, azole antifungal agents and echinocandins

The MIC of flucytosine (5-flucytosine), azole antifungal drugs and echinocandins is the lowest drug concentration giving inhibition of growth of ≥50% of that of the drug-free control.

INTERPRETATION OF RESULTS

EUCAST has established breakpoints for most yeast active compounds and *Candida* species (which together with the relevant background literature are found in publications and on the EUCAST website [15-17] http://www.EUCAST.org). Interpretation of MICs for other drug:organism combinations in the absence of breakpoints is challenging and should be done very carefully taking any available data including clinical experience, drug exposure during therapy etc. into account. However, the MIC may still provide some information regarding susceptibility, and importantly generation of MICs for other yeasts is a vital prerequisite for future ECOFF and breakpoint selection.

QUALITY CONTROL

Control procedures are the means by which the quality of results is assured and are described in detail by the CLSI [18]. 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 two-fold series tested and antifungal drug susceptibility patterns must be stable. The recommended control strains (available on the EUCAST website http://www.EUCAST.org) were selected according to these criteria [19, 20]. A recent study has indicated that the two most commonly used control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, are not sufficiently sensitive in detecting variation in caspofungin potency and that *C. albicans* ATCC 64548 or *C. albicans* 64550 are superior for this purpose [11]. Control strains should be obtained from a reliable source such as the American Type Culture Collection (ATCC), National Collection for Pathogenic Fungi (NCPF), Central Bureau voor Schimmelcultures (CBS) or commercial suppliers offering similar guarantees of quality.

Storage of control strains

Yeast isolates may be stored lyophilised or frozen at -60 ^{o}C or below [21]. Cultures can be stored in the short-term (less than 2 weeks) on Sabouraud dextrose agar or potato dextrose agar slopes at 2-8 ^{o}C, with new cultures being 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, or frozen or lyophilised cultures by inoculation on non-selective nutritive agar medium (e.g. Sabouraud dextrose agar or potato

dextrose agar).

1. At least one control strain must be included per test run and the MICs should be within the control ranges (available on the EUCAST website http://www.EUCAST.org). 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 (preferably a chromogenic medium) to ensure purity and to provide fresh colonies if re-testing is required.

4. Test each new batch of medium, lot of microdilution plates and lot of RPMI 1640 2% G medium with at least two of the quality control strains (available on the EUCAST website http://www.EUCAST.org) 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-aspargine (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_20	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

[a]Note that this medium contains 0.2% glucose

Table 2. Components of RPMI 2% G medium

Component	**Double strength concentration**
Distilled water	900 mL [a]
RPMI 1640 (Table 1)	20.8 g
MOPS	69.06 g
Glucose	36 g

[a] Dissolve the powders in 900 mL distilled water. When dissolved and while stirring, adjust the pH to 7.0 at 25 °C using 1 M sodium hydroxide. Add additional water to a final volume of 1 L. Filter sterilize before use.

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	0.008 - 4
Anidulafungin	DMSO	Hydrophobic	0.008 - 4
Caspofungin	DMSO	Hydrophobic	0.008 - 4
Fluconazole	DMSO/Water[a]	Hydrophobic/hydrophilic	0.125 - 64
Flucytosine	Water	Hydrophilic	0.125 - 64
Isavuconazole	DMSO	Hydrophobic	0.008 - 4
Itraconazole	DMSO	Hydrophobic	0.008 - 4
Micafungin	DMSO	Hydrophobic	0.008 - 4
Posaconazole	DMSO	Hydrophobic	0.008 - 4
Voriconazole	DMSO	Hydrophobic	0.008 - 4

DMSO, Dimethyl sulfoxide.

[a] According to the manufacturer's instructions. The original pure substance from Pfizer was readily soluble in water. The powder from Sigma-Aldrich is highly hydrophobic and poorly soluble in water, and should therefore be dissolved in DMSO, as recommended by the supplier (http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=F8929|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC).

Table 4. ISO Scheme for preparing antifungal dilution series with a final concentration of 0.125-64 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	12 800[c]	Stock	200	0	12,800	128
2	12 800	Stock	100	100	6,400	64
3	12 800	Stock	50	150	3,200	32
4	12 800	Stock	50	350	1,600	16
5	1600	Step 4	100	100	800	8
6	1600	Step 4	50	150	400	4
7	1600	Step 4	50	350	200	2
8	200	Step 7	100	100	100	1
9	200	Step 7	50	150	50	0.5
10	200	Step 7	25	175	25	0.25

[a] Consult Table 3 for solvents required to make dilutions of antifungals.

[b] Dilution 1:1 with inoculum suspension gives final concentrations half those indicated.

[c] For dilution series with highest final concentrations of 16 mg/L or 8 mg/L start with stock concentrations of 3200 mg/L and 1600 mg/L respectively.

Table 5. Preparation of McFarland 0.5 turbidity standard

Step	Procedure
1	Add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$ x $2H_20$) to 99.5 mL of 0.18 mol/L (0.36 N) H_2S0_4 (1% v/v) and mix thoroughly
2	Check the density with a spectrophotometer having a 1 cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13
3	Distribute in screw-cap tubes of the same size as those used for test inoculum adjustment
4	Store sealed standards in the dark at room temperature
5	Mix the standard thoroughly on a vortex mixer immediately before use
6	Renew standards or check their absorbance after storage for 3 months