EUCAST DEFINITIVE DOCUMENT E.DEF 9.3.1

Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds

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INTRODUCTION

Antifungal susceptibility tests are performed on fungi causing disease especially when infections are 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 in 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 antimicrobial agents, to confirm the susceptibility of organisms that give equivocal results in other test formats (such as commercial susceptibility tests), and to determine the susceptibility of organisms where other test formats may be unreliable or not yet validated (which is still a common scenario for susceptibility testing of moulds). In dilution tests, fungi are tested 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. The MIC informs about the susceptibility or resistance of the organism to the antifungal agent, which 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 [1-10].

The first version of the standard was published as the definitive document in July 2008 [11]. The second version was updated to reflect current knowledge regarding indications for mould AFST, stability of the echinocandins, inoculum preparation specifically for *Aspergillus* (spectrophotometer standardization of inoculum concentration was included), practical tips regarding endpoint reading (picture illustrations of endpoint reading), and interpretation of endpoints (acknowledging the established clinical breakpoints) [12]. This third version has been harmonised with the yeast E.Def 7.3.1 document, some technical details have been included, references have been updated to reflect contemporary knowledge and MIC ranges for quality control strains have been removed. The latter 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 conidia producing moulds to antifungal agents by determination of the MIC. MICs show the *in vitro* activity of a given antifungal drug under the test conditions described, and can be used for patient management in conjunction with other factors, such as pharmacokinetics, pharmacodynamics and resistance mechanisms. The MIC permits moulds to be categorised as "susceptible" (S), "intermediate" (I), or "resistant" (R) to an antifungal drug when appropriate breakpoints are applied [13, 14]. 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 moulds and to facilitate an acceptable degree of conformity, e.g. agreement within specified ranges, between laboratories. Many factors influence the MIC of filamentous fungi against antifungal agents as shown by Rambali et al. [15]. For example the MIC of itraconazole against *Aspergillus* was profoundly influenced by shape of the microdilution well, inoculum concentration, temperature and length of incubation time. Thus, 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 a mould or is lethal to it. 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 moulds 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", "intermediate" 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) **Susceptible (S)**. A mould is defined as susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success.

   b) **Intermediate (I)**. A mould is defined as intermediate by a level of antimicrobial activity associated with a high likelihood of therapeutic success but only when a higher dosage of the agent than normal can be used or when the agent is physiologically concentrated at the site of infection.

   c) **Resistant (R).** A mould is defined as resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure.

6. **Wild type (WT)**. A mould 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 mould 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 mould isolate is categorized as S, I or R by applying breakpoints in a defined phenotypic test system

b) A mould 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, characterized strains 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 standardized 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 plates 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 spores/conidia (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 plates. 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 [16, 17]. 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 [18]. 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L, pH 7.0, is the recommended buffer to be used 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 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 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 1M sodium hydroxide.
4. Add water to a final volume of 1000 mL.
5. Filter sterilise with a 0.22 µm pore size filter.
6. Store at 4°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.

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 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 before freezing, one of which is then 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:

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

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

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°C or below. Drugs may be stored at -70°C for at least six months without significant loss of activity [19]. The echinocandins were previously regarded as unstable at -70°C, however, have been found stable for at least 6 months at this temperature [20].

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 (Table 7). 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 (Table 4). 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 (Table 4) [21]. An example 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. 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 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.
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 [22].

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°C or below for up to 6 months or at -20°C for not more than 1 month without loss of drug potency [23-25]. 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 inoculated.

PREPARATION OF INOCULUM

Standardisation of the inoculum is essential for accurate and reproducible antifungal susceptibility tests.

The final inoculum must be between 1 x 10^5 cfu/mL to 2.5 x 10^5 cfu/mL.

Spore/conidia suspension method

The isolates are subcultured on potato dextrose agar or Sabouraud dextrose agar or other culture media where the fungus is able to sporulate sufficiently and incubated at 35°C. Inoculum suspensions are prepared from fresh, mature (2- to 5-day-old) cultures. In some cases an extended incubation is required for proper sporulation of the isolate.

Cover colonies with approximately 5 ml of sterile water supplemented with 0.1% Tween 20. Then, the conidia are carefully rubbed with a sterile cotton swab and transferred with a pipette to a sterile tube. Alternatively, a damp sterile cotton swab could be used to gently touch the culture, and the spores transferred to a sterile tube containing 5 ml water supplemented with Tween 20. The suspension is vortexed for 15 seconds with a gyratory vortex mixer at approximately 2,000 rpm. In general appropriate dilutions are made in order to attain the right concentration for counting in a haemocytometer chamber (see comment for alternative procedure for *Aspergillus* sp. below). 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 removes hyphae and yields a suspension composed of conidia. 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 2 to 5 x 10^6 conidia/mL by counting the conidia in a haemocytometer chamber. Alternatively, provided the *Aspergillus* conidia suspension is filtered a spectrophotometer can be used to adjust the suspension to a concentration equivalent to McFarland 0.5 (Table 5) [26, 27].

The suspension is then diluted 1:10 with sterile distilled water to obtain a final working inoculum of 2–5 x 10^5 cfu/mL [26-29].

INOCULATION OF MICRODILUTION PLATES

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

Vortex the inoculum suspension and inoculate each well of a microdilution plate with 100 µL of the 2 to 5 x 10^5 cfu/mL conidial suspension, without touching the contents of the well. This will give the required final drug concentration and inoculum density (final inoculum = 1 x 10^5 – 2.5 x 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.

Viability counts should be performed for quality control purposes to ensure that test wells contain between 1 x 10^5 and 2.5 × 10^5 cfu/mL, as follows. 10 µL of the inoculum suspension should be diluted in 2 ml of sterile distilled water supplemented with 0.1% Tween 20. The suspension is then vortexed with a gyratory vortex mixer at 2,000 rpm. Then 100 µL of this suspension is spread over the surface of a suitable agar plate (such as Sabouraud dextrose agar or potato dextrose agar), which is then incubated for 24-48h or until colonies can be enumerated. A hundred to 250 colonies are expected from an acceptable test suspension. A further dilution of 100 µL suspension in 900 µL sterile distilled water supplemented with 0.1% Tween 20, vortexing, and 100 µL plated out 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 dependant on need).

INCUBATION OF MICRODILUTION PLATES

Incubate microdilution plates without agitation at 34 to 37 °C in ambient air. Mucorales should be read at 24h when growth is sufficient, whilst most other moulds should be read at 48h. In few cases, a further 24h incubation period will be required in order to get sufficient growth of the control well (for example *Scedosporium*). A longer incubation than 72h is not recommended.

READING RESULTS

The endpoint is read visually by recording the degree of growth for each well.

- MIC endpoint for all drugs except for echinocandins: the concentration of drug yielding no visible growth by eye is the MIC value. Ignore single colonies on the surface and "skipped-wells" (a single well without growth in between growth positive wells). It is recommended to use a horizontal black and white paper as background for the plate when recorded, as the line between the white and black colour will only appear sharp and distinct when viewed through the elevated plate, when the well is without growth (Fig. 1).
- Minimum Effective Concentration (MEC) endpoint for echinocandins is the lowest echinocandin concentration in which abnormal, short, and branched hyphal clusters are observed in contrast to the long, unbranched, hyphal elements that are seen in the growth control well (Fig. 2). This may on some occasions be recorded by eye as the lowest drug concentration that results in macroscopic changed filamentous growth similar to that observed in positive control wells to microcolonies or granular growth (which are ignored), although this is rarely seen. When this is not the case it is necessary to examine a small volume from the wells under the microscope to observe drug-induced morphological changes, or an inverted microscope can be used to visualise changes within the wells.

INTERPRETATION OF RESULTS

EUCAST has established breakpoints for amphotericin B, isavuconazole, itraconazole, posaconazole, and voriconazole and *Aspergillus* species which together with the relevant background literature is found in

publications and on the EUCAST website [13, 14]. No data are yet available to suggest a correlation between echinocandin MEC and outcome of treatment. Interpretation of MICs for other moulds 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 moulds 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 [30]. 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 (available on the EUCAST website http://www.EUCAST.org) were selected according these criteria [30].

Storage of control strains

Fungal isolates may be stored lyophilised or frozen at -60°C or below [31]. Cultures can be stored short-term (less than 2 weeks) on Sabouraud 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 (e.g. Sabouraud dextrose agar or potato dextrose agar)

1. At least one control strains must be included per test run and the MICs should be within the control ranges (available on the EUCAST website http://www.EUCAST.org). Two or more strains are needed if the MIC for the QC strain falls outside the concentration range tested for one or several compounds. If control strain MIC results are out of range the test should be repeated. 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 if re-testing is required.

4. Test each new batch of medium, lot of microdilution plate, and lot of RPMI 2% G broth 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 double strength RPMI 2%G medium buffered with MOPS

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	0.0312 - 16
Anidulafungin	DMSO	Hydrophobic	0.0312 – 16
Caspofungin	DMSO	Hydrophobic	0.0312 – 16
Isavuconazole	DMSO	Hydrophobic	0.0312 – 16
Itraconazole	DMSO	Hydrophobic	0.0156 – 8
Micafungin	DMSO	Hydrophobic	0.0312 – 16
Posaconazole	DMSO	Hydrophobic	0.0156 – 8
Voriconazole	DMSO	Hydrophobic	0.0312 - 16

DMSO, Dimethyl sulfoxide

Table 4. ISO 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

[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 8 mg/L start with stock concentrations of 1600 mg/L.

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

Figure 1. Illustration of how the use of a black and white paper behind the elevated microdilution plate helps recognising the difference between clear wells (green circle) and wells with weak (orange circle) or prominent growth (red circle).
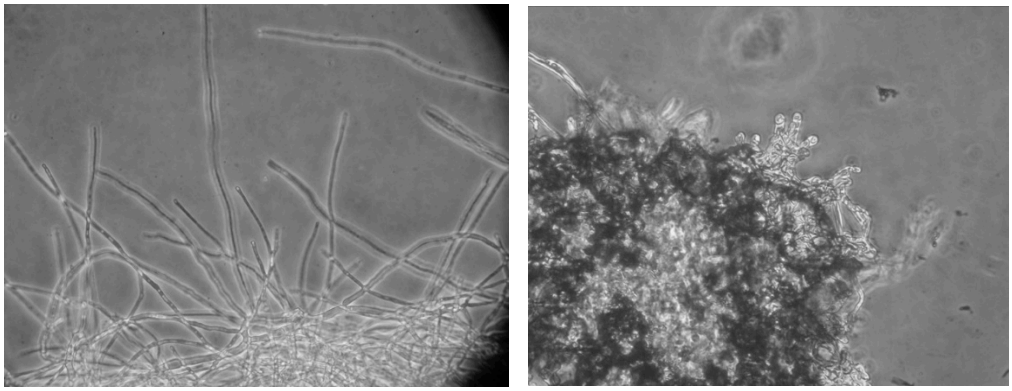
Figure 2. Wet mount illustrating the micromorphological differences between an uninhibited *A. fumigatus* growth control (left) with long and elegant hyphal growth and a caspofungin inhibited *A. fumigatus* isolate (right) with aberrant/amputated hyphal growth.