 10.1111/j.1469-0691.2007.01935.x

EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts

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

Keywords Antifungal agents, EUCAST, fermentative yeasts, MICs, susceptibility testing method

Clin Microbiol Infect 2008; **14:** 398–405

INTRODUCTION

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

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

The method described in this document is intended for testing the susceptibility of yeasts that cause clinically significant infections (primarily *Candida* spp.). The method encompasses only those yeasts that are able to ferment glucose. Thus, the susceptibility of non-fermentative yeasts, e.g., *Cryptococcus neoformans*, cannot be determined by the current procedure, and the method is not suitable for testing the yeast forms of dimorphic fungi.

SCOPE

The standard method described in this document provides a valid method for testing the susceptibility of glucose-fermenting yeasts to antifungal agents by determination of the MIC. MICs indicate the activity of a given antifungal drug under the described test conditions, and can be used in making decisions concerning patient management after taking into account other factors, e.g., pharmacokinetics, pharmacodynamics and resistance mechanisms. The MIC also allows fungi to be categorised as 'susceptible' (S), 'intermediate' (I) or 'resistant' (R) to a drug. In addition, MIC distributions can be used to define wild-type or non-wild-type fungal populations.

This method is intended primarily to facilitate an acceptable degree of conformity, i.e., agreement within specified ranges among laboratories, in measuring the susceptibility of yeasts to antifungal agents. The method is designed to be easy to perform, rapid, economical, and suitable for reading by microdilution plate readers in order to allow direct transfer, storage and manipulation of the data by computer. The method is also intended to yield results that are concordant with those obtained using the procedure

Corresponding author and reprint requests: J. L. Rodriguez-Tudela, Mycology Reference Laboratory, National Center for Microbiology, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km.2, E-28220 Majadahonda, Spain
E-mail: jlrtudela@isciii.es

*J. L. Rodriguez-Tudela (Chairman, Spain), M. C. Arendrup (Denmark), F. Barchiesi (Italy), J. Bille (Switzerland), E. Chryssanthou (Sweden), M. Cuenca-Estrella (Spain), E. Dannaoui (France), D. W. Denning (UK), J. P. Donnelly (The Netherlands), F. Dromer (France), W. Fegeler (Germany), C. Lass-Flörl (Austria), C. Moore (UK), M. Richardson (Finland), P. Sandven (Norway), A. Velegraki (Greece) and P. Verweij (The Netherlands).

© 2008 The Authors
Journal Compilation © 2008 European Society of Clinical Microbiology and Infectious Diseases

recommended by CLSI document M27 for antifungal susceptibility testing of yeasts [1].

TERMS AND DEFINITIONS

Antifungal agent

An antifungal agent is a 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.

Potency

Potency is 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 mg/g, or as activity content in International Units (IU)/g, or as a volume fraction or mass fraction in per cent, or as an amount-of-substance concentration (mass fraction) in mol/L of the ingredients in the test substance.

Concentration

Concentration is 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 is equivalent to μg/mL, the use of the latter is not recommended.

Stock solution

A stock solution is an initial solution used for additional dilutions.

MIC

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

Breakpoint

Breakpoints are specific MIC values that enable fungi to be assigned to the clinical categories 'susceptible', 'intermediate' and 'resistant'. Breakpoints can be altered according to changes in circumstances (e.g., changes in commonly used drug dosages).

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

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

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

Wild-type

The term 'Wild-type' refers to an isolate without acquired resistance mechanisms to the antifungal agent.

Control strain

The term 'Control strain' refers to a catalogued, characterised strain with stable, defined antifungal susceptibility phenotypes and/or genotypes. Such strains are obtainable from culture collections and are used for quality control purposes.

Broth dilution

Broth dilution is a susceptibility testing technique in which serial dilutions (usually two-fold) of an antifungal agent are made in a liquid medium that is inoculated with a standardised number of organisms and incubated for a prescribed period. The objective of this method is the determination of the MIC.

© 2008 The Authors
Journal Compilation © 2008 European Society of Clinical Microbiology and Infectious Diseases,

Broth microdilution

The performance of broth dilution in microdilution plates containing wells with a nominal capacity of *c.* 300 μL/well is referred to as 'broth-microdilution'.

Broth

The term 'broth' refers to the fluid medium used for the in-vitro growth of fungi.

Inoculum

The number of yeasts (CFU) in a defined volume. The inoculum is expressed as CFU/mL.

TEST PROCEDURES

The test is performed in microdilution plates. The method is based on the preparation of working solutions of antifungal agents in 100-μL volumes/well (with the addition of an inoculum also in a volume of 100 μL).

Medium

A completely synthetic medium, RPMI-1640 supplemented with glutamine and a pH indicator, but without bicarbonate, has been recommended [2,3]. However, RPMI-1640 (Table 1) contains only 2 g glucose/L (0.2% w/v), a concentration that is lower than that used commonly for culturing yeasts. Supplementing medium to a final concentration of 20 g glucose/L (2% w/v) has been shown to result in better growth of yeast isolates without altering the MICs of antifungal agents markedly [4]. Zwitterion buffers are preferred to Tris, which antagonises the activity of flucytosine, and also to phosphate buffer, which may give unexpected interactions with antifungal agents. The buffer 3-(*N*-morpholino)-propanesulphonic acid (MOPS), used at a final concentration of 0.165 mol/L, pH 7.0, is satisfactory for RPMI-1640 media. The recommended medium, RPMI containing glucose 2% w/v (RPMI-1640 2% G), is prepared as follows:

1 Add the components listed 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 1 M sodium hydroxide.

Table 1. Composition of RPMI-1640

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-Tryptophan	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
Para-amino benzoic acid	0.001
Pyridoxine.HCl	0.001
Riboflavin	0.0002
Thiamin.HCl	0.001
Vitamin B_{12}	0.000005
Calcium nitrate.H_2O	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 glucose 0.2% w/v and not the recommended concentration of 2% w/v.

Table 2. Components of RPMI-1640 2% G

Component	1× concentration	2× concentration
Distilled water	900 mL	900 mL
RPMI-1640[a]	10.4 g	20.8 g
MOPS	34.53 g	69.06 g
Glucose	18 g	36 g

[a]See Table 1.
MOPs, 3-(*N*-morpholino) propanesulphonic acid.

4 Add water to a final volume of 1 L.
5 Filter sterilise using a 0.22-μm 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.

Medium for testing amphotericin B

No specific medium can currently be recommended for testing susceptibility to amphotericin B.

© 2008 The Authors
Journal Compilation © 2008 European Society of Clinical Microbiology and Infectious Diseases,

Hence, for the time being, it is recommended that the same method used for flucytosine, azole drugs and echinocandins be adopted for amphotericin B. The non-synthetic broth Antibiotic Medium 3 (AM3), supplemented to a final concentration of glucose 2% w/v, has been evaluated for detecting resistance to amphotericin B [5–8]. However, there is batch-to-batch variation in this medium, and also in the performance of the medium from different manufacturers. Preliminary results indicate that an inoculum of $0.5–2.5 \times 10^5$ CFU/mL is too large for testing susceptibility to amphotericin B in AM3 [5].

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 often 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. Powders should be stored in sealed containers at −20°C or below with a desiccant unless otherwise recommended by the manufacturer. Ideally, hygroscopic agents should be dispensed into aliquots, one of which is used on each occasion. Containers should be allowed to warm to room temperature before opening in order to avoid condensation of water on the powder.

Preparation of stock solutions

Antifungal drug solutions must be prepared after taking the potency of the lot of antifungal drug powder into account. 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-fold greater than the highest concentration to be tested. Information on the solubility of antifungal compounds should be provided 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 membrane filtration should be used if required. Other filter materials are best avoided, as they may adsorb significant amounts of drug. When filtration is used, samples must be obtained before and after filtration, and must be assayed to ensure that the drug is 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 6 months without significant loss of activity [9]. Echinocandins are unstable, so stock solutions must not be kept for >2 months at −70°C. Remove vials from −70°C storage and use them on 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. 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

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

Antifungal agent	Solvent	Characteristics	Test range (mg/L)
Amphotericin B	DMSO	Hydrophobic	0.0312–16
Fluconazole	Water	Hydrophilic	0.125–64
Itraconazole	DMSO	Hydrophobic	0.0156–8
Voriconazole	DMSO	Hydrophobic	0.0156–8
Posaconazole	DMSO	Hydrophobic	0.0156–8
Flucytosine	Water	Hydrophilic	0.125–64
Caspofungin	Water	Hydrophilic	0.0312–16
Micafungin	Water	Hydrophilic	0.0312–16
Anidulafungin	DMSO	Hydrophobic	0.0312–16

DMSO, dimethylsulphoxide.

© 2008 The Authors

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-1640 2% G. The RPMI-1640 2% G used in plates is prepared at double the final strength to allow for a 50% dilution following addition of the inoculum. This approach allows the inoculum to be prepared in distilled water.

Dilutions should be prepared according to ISO recommendations [10]. Alternative dilution schemes may be used if they are shown to perform as well as the reference method. For example, an alternative method that uses smaller volumes to prepare a dilution series with final concentrations of 0.125–64 mg/L is shown in Table 4 (see Table 3 to check the solvents required for each antifungal agent). A summary of the steps required to prepare working solutions (2 × final concentration) in this alternative scheme 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-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-1640 2% G into ten tubes.
5. Take 100 μL from each of the tubes with 200 × 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 agent is 2 × final concentration.

Preparation of microdilution plates

Use sterile plastic, disposable, 96-well microdilution plates with flat-bottomed wells that have a nominal capacity of *c.* 300 μL. For the wells in each column, from 1–10, of the microdilution plate, dispense 100 μL from each of the tubes containing the corresponding concentration (2 × 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 in columns 11 and 12, dispense 100 μL of double-strength RPMI-1640 2% G. Thus, each well in columns 1–10 will contain 100 μL of twice the final antifungal drug concentrations in double-strength RPMI-1640 2% G with 1% solvent.

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. 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 2 months.

Table 4. Scheme for preparing dilution series of antifungal agents 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-1640 2% G[b]
1	12 800[c]	Stock	200	0	12 800	128
2	12 800	Stock	100	100	6400	64
3	12 800	Stock	50	150	3200	32
4	12 800	Stock	50	350	1600	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 antifungal agents.
[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.

© 2008 The Authors
Journal Compilation © 2008 European Society of Clinical Microbiology and Infectious Diseases,

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 non-selective nutritive agar medium, in sterile distilled water. The final inoculum must be between 0.5×10^5 and 2.5×10^5 CFU/mL.

Colony suspension method

1 Culture all yeasts in ambient air at 35 ± 2°C on non-selective nutritive agar medium (Sabouraud's dextrose agar or potato dextrose agar) for 18–24 h before testing.

2 Prepare the inoculum by suspending five distinct colonies, ≥1 mm in diameter from 24 h cultures, in 5 mL of sterile distilled water.

3 Evenly suspend the inoculum by vigorous shaking on a vortex mixer for 15 s. Adjust the cell density to the density of a 0.5× McFarland standard (Table 5) by measuring the absorbance in a spectrophotometer at a wavelength of 530 nm and adding sterile distilled water as required. This will give a yeast suspension of $1–5 \times 10^6$ CFU/mL. Prepare a working suspension from a 1 in 10 dilution of the standardised suspension in sterile distilled water to yield $1–5 \times 10^5$ CFU/mL.

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. Inoculate each well of a microdilution plate with 100 μL of the $1–5 \times 10^5$ CFU/mL yeast suspension. This will give the required final drug concentration and inoculum density (final inoculum = $0.5–2.5 \times 10^5$ 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. Viable counts should be performed to ensure that test wells contain between 0.5×10^5 and 2.5×10^5 CFU/mL. This should be done by removing 10 μL from the growth control well immediately after inoculation and diluting it in 2 mL of sterile distilled water. The suspension is homogenised using a gyratory vortex mixer at 2000 rpm, and 100 μL of the resulting suspension is then spread over the surface of a suitable agar plate, which is then incubated for 24–48 h. An acceptable test suspension would yield 5–125 colonies. If this is not achieved, the results for the isolate in question cannot be used.

Table 5. Preparation of 0.5× McFarland turbidity standard

Step	Procedure
1	Add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) to 99.5 mL of 0.18 mol/L (0.18 M) H_2SO_4 1% v/v and mix thoroughly
2	Check the density with a spectrophotometer having a 1-cm light path and matched cuvettes. The absorbance at 530 nm should be 0.12–0.15
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

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 seen most commonly among strains of *Candida parapsilosis* and *Candida guilliermondii*. Such plates should be re-incubated for a further 12–24 h and then read again. Failure to reach an absorbance of 0.2 after 48 h constitutes a failed test.

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 other wavelengths can be used, e.g., 405 or 450 nm. The value of the blank (background) should be subtracted from the readings for the other 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.

© 2008 The Authors

Flucytosine, azole antifungal agents and echinocandins

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

INTERPRETATION OF RESULTS

Interpretative breakpoints have yet to be established for most antifungal agents, and the clinical relevance of testing remains uncertain. EUCAST has recommended breakpoints for fluconazole [11] (http://www.srga.org/eucastwt/MICTAB/index.html) and is developing breakpoints for other agents. CLSI has recommended breakpoints for flucytosine, fluconazole, itraconazole and voriconazole [1,12,13]. There are no available data for echinocandins.

QUALITY CONTROL

Control procedures are the means by which the quality of results is assured; these have been described in detail by CLSI [1]. 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 shown in Table 6 were selected according to these criteria [14,15]. Control strains should be obtained from a reliable source, e.g., the American Type Culture Collection (ATCC), National Collection for Pathogenic Fungi (NCPF), Central Bureau for Schimmelcultures (CBS), or commercial suppliers offering similar guarantees of quality.

Storage of control strains

Yeasts may be stored lyophilised or frozen at −60°C or below [16]. Cultures can be stored in the short-term on Sabouraud's dextrose agar or potato dextrose agar slopes at 2–8°C, with new cultures being prepared from frozen stocks every 2 weeks.

Table 6. Acceptable MIC ranges (mg/L) of antifungal agents for quality control strains

Antifungal agent	*Candida krusei* ATCC 6258	*Candida parapsilosis* ATCC 22019	*Candida albicans* F 8555	*Candida krusei* CL3403
Amphotericin B	0.12–1.0	0.12–1.0	0.06–0.5	0.25–1.0
Flucytosine	1.0–4.0	0.12–0.5	0.06–0.25	2.0–8.0
Fluconazole	16.0–64.0	0.5–2.0	32.0–128.0	16.0–64.0
Itraconazole	0.03–0.12	0.03–0.12	0.25–1.0	0.12–0.5
Voriconazole	0.03–0.25	0.015–0.06	0.5–2.0	0.12–0.5
Posaconazole	0.015–0.06	0.015–0.06	0.12–0.5	0.06–0.25
Caspofungin	NA	NA	NA	NA
Anidulafungin	NA	NA	NA	NA

NA, not available.

Routine use of control strains

For routine use of control strains, fresh cultures on non-selective nutritive agar medium (e.g., Sabouraud's dextrose agar or potato dextrose agar) must be prepared from agar slopes, or frozen or lyophilised cultures.

1. At least two control strains must be included each day that the test is performed, and the MICs should be within the control ranges given in Table 6. 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 endpoints.
3. Subculture inocula on suitable agar medium (preferably a chromogenic medium) to ensure purity and to provide fresh colonies if retesting 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 listed in Table 6 to ensure that MICs fall within the expected range [17].

REFERENCES

1. National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard*, M27-A, 2nd edn. Wayne, PA: NCCLS, 2002.
2. Fromtling RA, Galgiani JN, Pfaller MA *et al.* Multicenter evaluation of a broth macrodilution antifungal susceptibility test for yeasts. *Antimicrob Agents Chemother* 1993; **37**: 39–45.
3. Pfaller MA, Rinaldi MG, Galgiani JN *et al.* Collaborative investigation of variables in susceptibility testing of yeasts. *Antimicrob Agents Chemother* 1990; **34**: 1648–1654.

© 2008 The Authors
Journal Compilation © 2008 European Society of Clinical Microbiology and Infectious Diseases,

4. Rodriguez Tudela JL, Martinez Suarez JV. Improved medium for fluconazole susceptibility testing of *Candida albicans*. *Antimicrob Agents Chemother* 1994; **38**: 45–48.
5. Cuenca-Estrella M, Diaz-Guerra TM, Mellado E, Rodriguez-Tudela JL. Detection of resistance to amphotericin B in *Candida* isolates by using iso-sensitest broth. *Antimicrob Agents Chemother* 2001; **45**: 2070–2074.
6. Lozano-Chiu M, Nelson PW, Lancaster M, Pfaller MA, Rex JH. Lot-to-lot variability of antibiotic medium 3 used for testing susceptibility of *Candida* isolates to amphotericin B. *J Clin Microbiol* 1997; **35**: 270–272.
7. Rex JH, Cooper CR, Merz WG, Galgiani JN, Anaissie EJ. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. *Antimicrob Agents Chemother* 1995; **39**: 906–909.
8. Wanger A, Mills K, Nelson PW, Rex JH. Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant *Candida* isolates. *Antimicrob Agents Chemother* 1995; **39**: 2520–2522.
9. Anhalt JP, Washington JA II. Preparation and storage of antimicrobials. In: Hausler WJ, Herrmann KL, Isenberg HD, Shadomy H, eds, *Manual of clinical microbiology*, 5th edn. Washington, DC: American Society for Microbiology, 1991; 1199–1200.
10. International Standards Organisation. *Clinical laboratory testing and in vitro diagnostic test systems—susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices—part 1: reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases*. Geneva: ISO, 2006.
11. The European Committee on Antimicrobial Susceptibility Testing–Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST). EUCAST technical note on fluconazole. *Clin Microbiol Infect* 2008; **14**; 193–195.
12. Rex JH, Pfaller MA, Galgiani JN *et al*. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of *in vitro–in vivo* correlation data for fluconazole, itraconazole, and *Candida* infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. *Clin Infect Dis* 1997; **24**: 235–247.
13. Pfaller MA, Diekema DJ, Rex JH *et al*. Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints. *J Clin Microbiol* 2006; **44**: 819–826.
14. Pfaller MA, Bale M, Buschelman B *et al*. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. *J Clin Microbiol* 1995; **33**: 1104–1107.
15. Rex JH, Pfaller MA, Lancaster M, Odds FC, Bolmstrom A, Rinaldi MG. Quality control guidelines for National Committee for Clinical Laboratory Standards—recommended broth macrodilution testing of ketoconazole and itraconazole. *J Clin Microbiol* 1996; **34**: 816–817.
16. Kwon-Chung KJ, Bennett JE. *Medical mycology*. Philadelphia: Lea & Feibiger, 1992.
17. Barry AL, Pfaller MA, Brown SD *et al*. Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. *J Clin Microbiol* 2000; **38**: 3457–3459.

© 2008 The Authors
Journal Compilation © 2008 European Society of Clinical Microbiology and Infectious Diseases,