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Clinical laboratory testing and in vitro diagnostic test systems — Reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

Essais de laboratoire clinique et systèmes de diagnostic in vitro — Méthode de référence pour soumettre à essai l'activité in vitro des agents antimicrobiens par rapport aux levures impliquées dans les maladies infectieuses

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 16256 was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

Introduction

In vitro susceptibility tests are performed on microorganisms suspected of causing disease, particularly if the organism is thought to belong to a species that may exhibit acquired resistance to frequently used antimicrobial agents. The tests are also important in resistance surveillance, epidemiological studies of susceptibility and in comparisons of new and existing agents.

Dilution procedures are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and represent the reference method for antifungal susceptibility testing. MIC methods are used in resistance surveillance, comparative testing of new agents for research or registration purposes, to establish the susceptibility of organisms that give equivocal results in routine tests, for tests with organisms where routine tests may be unreliable and when a quantitative result is needed for clinical management. In dilution tests, microorganisms are tested for their ability to produce discernible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing serial dilutions of the antimicrobial agent.

The lowest concentration of an antimicrobial agent (in mg/l) that, under defined *in vitro* test conditions, reduces visible or optically measurable growth of a microorganism within a defined period of time is known as the MIC. The MIC is a guide for the clinician to the susceptibility of the organism to the antimicrobial agent and aids treatment decisions. Careful control and standardization is required for intra- and inter-laboratory reproducibility, as results may be influenced by the method used. It is generally accepted that broth MIC tests are reproducible to within one doubling dilution of the true end point (i.e. ±1 well or tube in a doubling dilution series).

Broth dilution is a technique in which containers holding identical volumes of broth with antimicrobial agent solutions in incrementally (usually twofold) increasing concentrations are inoculated with a known number of microorganisms.

Broth microdilution denotes the performance of the broth dilution test in microdilution trays.

The reference methods described in this International Standard are intended for the testing of pure cultures of yeast fungi. The broth microdilution methods described in this part of this International Standard are essentially the same as those described by the Clinical and Laboratory Standards Institute (CLSI)[1] and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[2]. These methods have been shown to provide MICs of fluconazole that are essentially the same, if not identical up to 2 mg/l[3]. Studies with various other antifungal agents are planned or under way. The laboratory that wishes to use this International Standard for conducting studies of newer antifungal agents, or as a reference method for comparison to MICs generated by a diagnostic device, should select which of the procedure options to use based upon the choice of MIC reading determined by visual inspection (CLSI method) or by use of a spectrophotometer (EUCAST method). In either case, the procedural details for that option are to be followed explicitly.

Clinical laboratory testing and in vitro diagnostic test systems — Reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

WARNING — The use of this International Standard may involve hazardous materials, operations and equipment. This International Standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard describes a method for testing the susceptibility to antifungal agents of yeasts, including *Candida* spp. and *Cryptococcus neoformans,* that cause infections. The reference method described here has not been used in studies of the yeast forms of dimorphic fungi, such as *B. dermatitidis* and/or *H. capsulatum* variety *capsulatum*. Moreover, testing filamentous fungi (moulds) introduces several additional problems in standardization not addressed by the current procedure. Reference methods for broth dilution antifungal susceptibility testing of filamentous fungi have been developed and are now available as CLSI document M38 and EUCAST document E.DEF 9.1[4][5][6][7][8].

This International Standard describes the broth microdilution reference method which can be implemented by either of two pathways. One pathway involves visual determination of MICs (CLSI method) [1]; the second pathway involves spectrophotometric determination of MICs (EUCAST method)[2]. The MIC reflects the activity of the drug under the described test conditions and can be interpreted for clinical management purposes by taking into account other factors, such as drug pharmacology or antifungal resistance mechanisms. MICs can be categorized as "susceptible" (S), "susceptible dose-dependent" (S-DD), "intermediate" (I), "non-susceptible" (NS) or "resistant" (R). In addition, MIC distributions can be used to define wild type or non-wild type fungal populations. Clinical interpretation of the MIC value is beyond the scope of this International Standard; interpretive category breakpoints specific to the CLSI- and EUCAST-derived methods can be found by consulting the latest interpretive tables provided by the organizations[2][9]. It is advisable to compare routine susceptibility testing methods or diagnostic test devices with this reference method in order to ensure comparable and reliable results for validation or registration purposes.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
antifungal agent
substance of biological, semi-synthetic or synthetic origin that inhibits the growth of or kills fungi, and is thus of potential use in the treatment of infections

NOTE Disinfectants, antiseptics and preservatives are not included in this definition.

2.2
antifungal agents — properties
2.2.1
potency
active fraction of a test substance, determined in a bioassay against a reference powder of the same substance

NOTE 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.

2.2.2
concentration
amount of an antifungal agent in a defined volume of liquid

NOTE 1 The concentration is expressed as mg/l.

NOTE 2 mg/l = µg/ml but use of the unit µg/ml is not recommended.

2.3
stock solution
initial solution used for further dilutions

2.4
minimum inhibitory concentration
MIC
lowest concentration that, under defined *in vitro* test conditions, reduces growth by an agreed amount within a defined period of time

NOTE The MIC is expressed in mg/l.

2.5
breakpoint
BP
specific MIC values that can be used to assign fungi to the clinical categories “susceptible”, “susceptible dose-dependent,” “intermediate,” “nonsusceptible” and “resistant”

NOTE For current interpretive breakpoints, reference can be made to the latest publications of organizations employing the reference method (e.g. CLSI and EUCAST)[1][2][9].

2.5.1
visual reading pathway
2.5.1.1
susceptible
S
fungal strain inhibited *in vitro* by a concentration of an antifungal agent that is associated with a high likelihood of therapeutic success

NOTE 1 Fungal strains are categorized as susceptible by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This breakpoint can be altered in certain circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.1.2
susceptible dose-dependent
S-DD
fungal strain inhibited *in vitro* by a concentration of an antifungal agent that may be achieved *in vivo* by using higher than normal doses of the agent when such dosage schedules can be safely employed

NOTE 1 Fungal strains are categorized as susceptible dose-dependent by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This class of susceptibility implies that an infection due to the isolate can be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used.

NOTE 3 This breakpoint can be altered in certain circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.1.3
intermediate
I
micro-organism having a level of antimicrobial agent activity associated with uncertain therapeutic effect

NOTE This implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

2.5.1.4
nonsusceptible
NS
category used for yeast fungi that currently have only a susceptible interpretive category, but not susceptible dose-dependent, intermediate or resistant interpretive categories (i.e. susceptible-only interpretive category)

NOTE This category is often given to new antifungal agents for which no resistant isolates have yet been encountered.

2.5.1.5
resistant
R
fungal strain inhibited *in vitro* by a concentration of an antifungal agent that is associated with a high likelihood of therapeutic failure

NOTE 1 Fungal strains are categorized as resistant by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This breakpoint can be altered in certain circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.2
spectrophotometric reading pathway
2.5.2.1
susceptible
S
micro-organism having a level of antimicrobial activity associated with a high likelihood of therapeutic success

2.5.2.2
intermediate
I
micro-organism having a level of antimicrobial agent activity associated with uncertain therapeutic effect

NOTE This implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

2.5.2.3
resistant
R
micro-organism having a level of antimicrobial activity associated with a high likelihood of therapeutic failure

2.6
wild type
absence of acquired resistance mechanisms to the antifungal agent in a given fungal strain

2.7
reference strain
catalogued, well-characterized fungal strain with stable, defined antifungal susceptibility phenotypes and/or genotypes

NOTE Reference strains are kept as stock cultures, from which working cultures are derived. They are obtainable from culture collections and used for quality control.

2.8
susceptibility testing method
2.8.1
broth dilution
technique in which containers are filled with appropriate volumes of an antifungal solution, employing incrementally (usually two-fold) increasing concentrations of the antifungal agent and appropriate volumes of broth with a defined inoculum

NOTE The aim of this method is the determination of the MIC.

2.8.2
microdilution
performance of broth dilution in microdilution trays with a capacity of ≤ 300 µl per well

2.9
broth
fluid medium used for the *in vitro* growth of yeast fungi

2.10
inoculum
number of yeast in a suspension, calculated with respect to the final volume

NOTE The inoculum is expressed as colony-forming units per millilitre (CFU/ml).

2.11
inoculum effect
change in MIC related to change in inoculum

3 Test procedures

3.1 General

The tests are performed in plastic disposable microdilution trays. The method is based on the preparation of double strength antifungal agent working solutions in 100 µl volumes per well with the addition of an inoculum also in a volume of 100 µl.

3.2 Medium

3.2.1 General

RPMI-1640 broth shall be used (see Appendix A for details for preparation of the two versions of RPMI-1640 glucose broth).

3.2.2 Visual reading pathway

The RPMI-1640 medium should contain 0,2 % glucose. The RPMI-1640 broth is prepared and dispensed at single strength with double strength antifungal agent dilutions and the inoculum is delivered in equal volumes of RPMI-1640 broth containing the adjusted yeast inoculum suspension.

3.2.3 Spectrophotometric reading pathway

The RPMI-1640 medium should contain 2 % glucose. The RPMI-1640 broth and antifungal agents are both prepared at double strength with the inoculum subsequently added in an equal volume of sterile distilled water.

3.3 Antifungal agents

3.3.1 General

Antifungal agents shall be obtained directly from the manufacturer or from reliable commercial sources; pharmaceutical preparations for clinical use are not acceptable. The antifungal agents shall be supplied with a lot number, potency, an expiry date and details of recommended storage conditions. Substances shall be stored in tightly closed containers in the dark, at –20 °C, with a desiccant unless otherwise recommended by the manufacturer. Hygroscopic agents should be dispensed into aliquots, one of which is used on each test occasion.

Allow containers to warm to room temperature before opening them in order to avoid condensation and loss of potency.

3.3.2 Preparation of stock solutions

The use of a calibrated analytical balance is required for weighing antifungal agents. Allowance for the potency of the powder shall be made by use of the following formula to obtain the amount of antifungal agent substance or the volume of diluent needed for a standard solution:

$$m = \frac{V \times \rho}{P} \tag{1}$$

$$V = \frac{m \times P}{\rho} \tag{2}$$

where

ρ is the concentration of the stock solution, in mg/l;

m is mass of the antifungal agent (powder), in g;

P is the potency of the antifungal agent (powder), in mg/g;

V is the volume of diluent, in l.

Concentrations of stock solutions should be 1 000 mg/l or greater, although the solubility of some agents is a limiting factor. The actual concentrations of stock solutions depend on the method of preparing working solutions (serial dilutions). Some agents require alternative solvents (see Table 1). Sterilization of solutions is not usually necessary. If required, sterilization should be done by membrane filtration and samples before and after sterilization should be compared by assay to ensure that adsorption has not occurred.

Unless information is available on stability of stock solutions under specified storage conditions, they should be prepared fresh for each test batch.

Table 1 — Solvents and diluents for preparation of stock solutions of antifungal agents

Antifungal agent	Solvent (Full strength and intermediate solutions)	Diluent
Amphotericin B	DMSO [a]	Medium
Anidulafungin	DMSO [a]	Medium
Caspofungin	DMSO [a]	Medium
Flucytosine	Water	Medium
Fluconazole	Water or DMSO according to manufacturer's instructions	Medium
Itraconazole	DMSO [a]	Medium
Ketoconazole	DMSO [a]	Medium
Micafungin	DMSO [a]	Medium
Posaconazole	DMSO [a]	Medium
Ravuconazole	DMSO [a]	Medium
Voriconazole	DMSO [a]	Medium

[a] DMSO (dimethyl sulfoxide) is potentially toxic.

3.3.3 Preparation of working solutions

The interval of concentrations selected for testing depends on the organisms and antifungal agent. The chosen range shall allow full end point MIC determination for appropriate reference strains. A twofold dilution series based on 1 mg/l is prepared in RPMI-1640 glucose broth. The procedure outlined in Tables 2 and 3 are known to reliably produce a satisfactory dilution series and should be followed unless an alternative method is carefully validated. For example, the work of one group has reported that serial dilutions of the more hydrophilic compounds can produce acceptable results[10]. Working solutions shall be used the same day unless information is available from the manufacturer on stability of the solutions under specified storage conditions.

Table 2 — Scheme for preparing dilutions of water-soluble antifungal agents to be used in broth dilution susceptibility tests

Antifungal solution										
Step	Concentration	Source	Volume	+	Medium	=	Intermediate Concentration	=	Final Concentration at 1:10	Log_2
	mg/l		ml		ml		mg/l		mg/l	
1	5120	Stock	1,0		3,0		1280		128	7
2	5120	Stock	1,0		7,0		640		64	6
3	640	Step 2	1,0		1,0		320		32	5
4	640	Step 2	1,0		3,0		160		16	4
5	160	Step 4	1,0		1,0		80		8	3
6	160	Step 4	0,5		1,5		40		4	2
7	160	Step 4	0,5		3,5		20		2	1
8	20	Step 7	1,0		1,0		10		1	0
9	20	Step 7	0,5		1,5		5		0,5	-1
10	20	Step 7	0,5		3,5		2,5		0,25	-2
11	2.5	Step 10	1,0		1,0		1,25		0,125	-3
12	2.5	Step 10	0,5		1,5		0,625		0,0625	-4
13	2.5	Step 10	0,5		3,5		0,3125		0,03125	-5

Table 3 — Scheme for preparing dilutions series of water-insoluble antifungal agents to be used in broth dilution susceptibility tests

Antifungal solution										
Step	Concentration	Source	Volume	+	Solvent (e.g. DMSO) [a]	=	Intermediate concentration	=	Final concentration at 1:100	Log_2
	mg/l		ml		ml		mg/l		mg/l	
1	1600	Stock					1600		16	4
2	1600	Stock	0,5		0,5		800		8,0	3
3	1600	Stock	0,5		1,5		400		4,0	2
4	1600	Stock	0,5		3,5		200		2,0	1
5	200	Step 4	0,5		0,5		100		1,0	0
6	200	Step 4	0,5		1,5		50		0,5	-1
7	200	Step 4	0,5		3,5		25		0,25	-2
8	25	Step 7	0,5		0,5		12,5		0,125	-3
9	25	Step 7	0,5		1,5		6,25		0,0625	-4
10	25	Step 7	0,5		3,5		3,13		0,0313	-5

[a] Dimethyl sulfoxide.

3.3.4 Preparation of broth microdilution trays for tests to be read visually

3.3.4.1 Visual reading pathway

Working solutions are dispensed into 10 wells of each row of microdilution trays at 100 µl per well with double the desired final concentrations of antifungal agent in 96 well round-bottom disposable plastic trays.

At least one well per row, containing 100 µl of antimicrobial agent-free medium, should be included as a growth control for each strain tested. Likewise, a well containing 200 µl of antifungal agent-free medium should be included as an uninoculated negative control well for each strain tested.

3.3.5 Preparation of microdilution trays for tests to be read by spectrophotometer

3.3.5.1 Spectrophotometric reading pathway

Working solutions are dispensed into microdilution trays at 100 µl per well with double the desired final concentrations of antifungal agent in double strength medium in 96 well flat-bottom disposable plastic trays.

At least one well per row, containing 100 µl of antifungal agent-free medium, should be included as a growth control for each strain tested. Likewise, a well containing 200 µl of antifungal agent-free medium should be included as an uninoculated negative control well for each strain tested.

3.4 Storage of microdilution trays

Filled trays may be used immediately or may be stored in sealed plastic bags and immediately placed in a freezer (CLSI method, visual read: – 70 °C for up to 6 months; EUCAST method, spectrophotometer read: –70 °C or below for up to 6 months, or at –20 °C for not more than 1 month. Allowable storage period will be determined based upon drug manufacturer's instructions for individual compounds and conformance with acceptable QC ranges. Trays shall not be stored in a self-defrosting freezer and thawed. Antifungal solutions shall not be refrozen, as repeated freeze–thaw cycles accelerate the degradation of some antifungal agents.

3.5 Preparation of inoculum — General

3.5.1 General

Standardization of the inoculum is essential for accurate and reproducible broth dilution susceptibility tests.

All isolates should be subcultured onto a non-inhibitory agar medium to ensure purity and viability.

3.5.2 Preparation of inoculum for visual test reading

The inoculum should be prepared by picking five colonies approximately 1 mm in diameter from 20 (±2) h-old cultures of *Candida* spp. or 46 (±2) h-old cultures of *C. neoformans*. The colonies should be suspended in 5 ml of sterile 0,85 % saline or sterile water. Note that *C. neoformans* have a slow growth rate. The optimal growth temperature of *C. neoformans* is 30 °C.

The resulting suspension should be vortexed for 15 s and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline or sterile water to achieve an optical density equivalent to that produced by a 0,5 McFarland standard (see Appendix B) at 530 nm wavelength. This procedure will yield a yeast suspension of 10^6 to 5×10^6 CFU/ml.

Mix the adjusted yeast suspension with a vortex mixer, dilute 1:50 with the appropriate version of RPMI-1640 broth medium, and further dilute 1:20 with medium to obtain the two times the final test inoculum (10^3 to 5×10^3 CFU/ml). Dilute the (double strength) inoculum 1:1 when the wells are inoculated with 100 µl of the inoculum that will result in the desired final inoculum size of $0,5 \times 10^3$ to 2.5×10^3 CFU/ml.

3.5.3 Preparation of inoculum for spectrophotometric test reading

The inoculum should be prepared by picking five colonies approximately 1 mm in diameter from 18 to 24 h-old cultures of *Candida* spp. or 46 (±2) h-old cultures of *C. neoformans*. The colonies should be suspended in 5 ml of sterile distilled water.

The resulting suspension should be vortexed for 15 s and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline or sterile water to achieve an optical density equivalent to that produced by a 0,5 McFarland standard (see Appendix B) at 530 nm wavelength. This procedure will yield a yeast suspension of 10^6 to 5×10^6 CFU/ml.

Mix the adjusted yeast suspension with a vortex mixer, dilute 1:10 with sterile distilled water to obtain the double strength the test inoculum (10^5 to 5×10^5 CFU/ml). Dilute the (double strength) inoculum 1:1 when the wells are inoculated with 100 µl of the inoculum that will result in the desired final inoculum size of $0,5 \times 10^5$ to $2,5 \times 10^5$ CFU/ml.

3.6 Inoculation of microdilution trays

The trays shall be inoculated within 30 min of standardizing the inoculum suspension, in order to maintain viable cell number concentration. To each well containing 100 µl of diluted antifungal agent in broth (see 3.5.2 and 3.5.3), a volume of 100 µl of yeast suspension is added.

Viable counts shall be performed periodically on the positive control well of a microdilution tray to ensure that test wells contain the appropriate CFU based upon the method used for MIC reading. This shall be done by removing 10 µl from the growth control well immediately after inoculation and diluting it in 1 ml of broth or saline (for the visual reading method) or 2 ml of sterile distilled water (for the spectrophotometer reading method). 100 µl of the diluted suspension is spread over the surface of a suitable agar plate, which is then incubated overnight. An acceptable test suspension would yield 5-125 colonies.

3.7 Incubation of microdilution trays

3.7.1 General

Microdilution trays should be sealed in polyethylene bags or fitted with a tight lid before incubation, or another method that prevents desiccation. In order to avoid uneven heating, microdilution trays should not be stacked more than five high.

Microdilution trays are incubated at 35 ± 2 °C in ambient air for (22 ± 2) h for most antifungal agent-yeast combinations. Some isolates of *Cryptococcus* may not grow sufficiently unless the incubation temperature is lowered to 30 °C.

3.7.2 Visual pathway

Incubation times will vary based upon the yeast species and antifungal agent being tested. Many tests can be read following a 24 h incubation. See Appendix C for specific incubation times. For updates to this information please refer to the current edition of CLSI document M27[1].

3.7.3 Spectrophotometric pathway

MIC determinations should be performed after 24 ± 2 h readings if the absorbance of the positive control well is ≥ 0,2. If the absorbance is < 0,2, tests may be reincubated for 12-24 h. Failure to reach an absorbance of 0,2 after 48 h constitutes a failed test.

3.8 Reading MIC results

3.8.1 General

Results shall only be read when there is sufficient growth of the test organism (i.e. obvious button or acceptable turbidity/absorbance in the positive growth control), when there is no growth in the uninoculated or negative growth control (where present) and when purity of the inoculum has been established.

3.8.2 Visual reading method

With some antifungal drugs and some isolates, trailing growth (partial inhibition of growth over an extended range of antifungal concentrations) can occur. It is estimated to occur with fluconazole in about 5 % of isolates[11]. This trailing growth can make an isolate that appears susceptible after 24 h appear completely resistant if a 48 h reading is performed. For this reason, 24 h readings are preferred.

For the visual determination of MICs, the wells of the tray should be examined from the bottom side using a mirror reading device. It may prove helpful to gently agitate the growth in the tray prior to reading end points. For flucytosine, the azoles and echinocandin agents, the amount of growth in each well is compared with that in the positive growth control, and the MIC recorded is the lowest concentration of the agent that inhibits growth substantially (at least 50 %) as compared to the control. With amphotericin B, the MIC is the concentration that provides complete inhibition of growth.

3.8.3 Spectrophotometric reading method

For the spectrophotometric determination of MICs, the microdilution panels are read with a microdilution plate reader using a wavelength between 405 and 530 nm. The readings of the background medium control well should be subtracted from the readings of the other wells. For flucytosine, the azoles and echinocandin agents, the amount of growth in each well is compared with that in the positive growth control, and the MIC recorded is the lowest concentration of the agent that inhibits growth substantially (at least 50 %) as compared to the control. With amphotericin B, the MIC is the concentration that provides inhibition ≥ 90 % of growth compared to the control.

3.9 Interpretation of MICs

The clinical interpretation of MICs generated by either pathway of this standard should be based upon the current approved breakpoints of the respective standards body that formed the basis for the testing method. Thus, interpretation of MICs of antifungal agents determined by visual reading of end points should be based upon the latest published guidelines from the CLSI (www.CLSI.org)[1,7] and MICs determined by spectrophotometric readings should be interpreted using the latest breakpoints available from EUCAST (www.EUCAST.org)[2].

4 Quality control (QC)

The quality of test results is monitored by the concomitant use of control strains (see Tables 4 and 5). Stock control strains should be stored lyophilised or frozen (–70 °C for visual read method; –60 °C for spectrophotometric method). Prepare working cultures by subculture of stock strains on a non-inhibitory agar medium. Further subcultures may be made, from the first working culture only. Replace them regularly with new slants prepared from the freezer supply at least every two weeks. When available, at least two relevant QC strains should be tested every day that testing is carried out. Test colonies of control cultures are processed in the same way as routine cultures. MICs of antifungal agents for control organisms should be within the ranges given in Tables 4 and 5[2,7]. If out of control values are encountered, the testing should first be repeated to determine if the essential steps of the procedure are now well controlled. If continued out of control values are observed, a careful examination of all aspects of the procedure should be made, and further reference testing should be suspended until control values are again within the correct ranges.

Table 4 — Recommended 24 h and 48 h MIC limits for two QC strains for broth microdilution tests read visually[12,13]

MIC (mg/l) ranges for visual read microdilution tests							
Organism	**Antifungal agent**	**24 h range mode**		**% within range**	**48 h range mode**		**% within range**
Candida parapsilosis ATCC® 22019	Amphotericin B	0,25-2,0	0,5	97,1	0,5-4,0	2,0	91,7
	Anidulafungin	0,25-2,0	1,0	95,0	0,5-2,0	1,0	95,0
	Caspofungin	0,25-1,0	0,5	96,7	0,5-4,0	1,0	92,9
	Flucytosine (5-FC)	0,06-0,25	0,12	99,2	0,12-0,5	0,25	97,9
	Fluconazole	0,5-4,0	2,0	98,2	1,0-4,0	2,0	98,1
	Itraconazole	0,12-0,5	0,25	95,8	0,12-0,5	0,25	97,5
	Ketoconazole	0,03-0,25	0,06/0,12	97,5	0,06-0,5	0,12	98,3
	Micafungin	0,5-2	1	100,0	0,5-4,0	1	100,0
	Posaconazole	0,06-0,25	0,12	96,7	0,06-0,25	0,12	98,8
	Ravuconazole	0,016-0,12	0,06	95,8	0,03-0,25	0,06	98,3
	Voriconazole	0,016-0,12	0,06	100,0	0,03-0,25	0,06	100,0

NOTE 1 ATCC® is a registered trademark of the American Type Culture Collection.

NOTE 2 Data are reprinted with permission from the American Society for Microbiology and the authors.

Table 4 *(continued)*

MIC (mg/l) ranges for visual read microdilution tests							
Organism	**Antifungal agent**	**24 h range mode**		**% within range**	**48 h range mode**		**% within range**
Candida krusei ATCC® 6258	Amphotericin B	0,5-2,0	1,0	100,0	1,0-4,0	2,0	100,0
	Anidulafungin	0,03-0,12	0,06	97,9	0,03-0,12	0,06	97,5
	Caspofungin	0,12-1,0	0,5	98,8	0,25-1,0	0,5	97,5
	Flucytosine (5-FC)	4,0-16	8,0	97,5	8,0-32	16	99,6
	Fluconazole	8,0-64	16	100,0	16-128	32	100,0
	Itraconazole	0,12-1,0	0,5	95,8	0,25-1,0	0,5	100,0
	Ketoconazole	0,12-1,0	0,5	95,4	0,25-1,0	0,5	99,6
	Micafungin	0,12-0,5	0,25	99,6	0,12-0,5	0,25	99,0
	Posaconazole	0,06-0,5	0,25	100,0	0,12-1,0	0,5	99,6
	Ravuconazole	0,06-0,5	0,25	93,3	0,25-1,0	0,5	100,0
	Voriconazole	0,06-0,5	0,25	98,3	0,12-1,0	0,5	100,0

NOTE 1 ATCC® is a registered trademark of the American Type Culture Collection.

NOTE 2 Data are reprinted with permission from the American Society for Microbiology and the authors.

Table 5 — Recommended MIC limits for QC strains for broth microdilution tests read spectrophotometrically[2,14]

Organism	Antifungal agent	MIC limits (mg/l)
Candida parapsilosis ATCC® 22019	Amphotericin B	0,12-1,0
	Anidulafungin	0,25-1,0
	Caspofungin	NA
	Flucytosine (5-FC)	0,12-0,5
	Fluconazole	0,5-2,0
	Itraconazole	0,03-0,12
	Micafungin	NA
	Posaconazole	0,015-0,06
	Voriconazole	0,015-0,06
Candida krusei ATCC® 6258	Amphotericin B	0,12- 1,0
	Anidulafungin	≤ 0,06
	Caspofungin	NA
	Flucytosine (5-FC)	1,0-4,0
	Fluconazole	16,0-64,0
	Itraconazole	0,03-0,12
	Micafungin	NA
	Posaconazole	0,015-0,06
	Voriconazole	0,03-0,25

NOTE 1 ATCC® is a registered trademark of the American Type Culture Collection.

NOTE 2 NA denotes "not available".

[a] Yeast collection of Spanish National Center of Microbiology.

Table 5 *(continued)*

Organism	Antifungal agent	MIC limits (mg/l)
Candida albicans CL-CNM [a] F 8555	Amphotericin B	0,06-0,5
	Anidulafungin	NA
	Caspofungin	NA
	Flucytosine (5-FC)	0,06-0,25
	Fluconazole	32,0-128,0
	Itraconazole	0,25-1,0
	Micafungin	NA
	Posaconazole	0,12-0,5
	Voriconazole	0,5-2,0
Candida krusei CL-CNM [a] CL3403	Amphotericin B	0,25-1,0
	Anidulafungin	NA
	Caspofungin	NA
	Flucytosine (5-FC)	2,0-8,0
	Fluconazole	16,0-64,0
	Itraconazole	0,12-0,5
	Micafungin	NA
	Posaconazole	0,06-0,25
	Voriconazole	0,12-0,5

NOTE 1 ATCC® is a registered trademark of the American Type Culture Collection.

NOTE 2 NA denotes "not available".

[a] Yeast collection of Spanish National Center of Microbiology.

Annex A
(informative)

RPMI-1640 medium

A.1 General

RPMI-1640 medium buffered with 0,165 mol/l MOPS, 1 l.

10,4 g powdered RPMI-1640 medium (with glutamine and phenol red, without bicarbonate).

34,53 g MOPS (3-[N-morpholino] propanesulfonic acid) buffer.

Dissolve powdered medium in 900 ml distilled H_2O. Add MOPS (final concentration of 0,165 mol/l) and stir until dissolved. While stirring, adjust the pH to 7,0 at 25 °C using 1 mol/l sodium hydroxide. Add additional water to bring medium to a final volume of 1 l. Filter sterilize and store at 4 °C until use.

Table A.1 — Composition of RPMI-1640 medium (with glutamine and phenol red but without bicarbonate)

Constituent	g/ml water
L-arginine (free base)	0,200
L-aspargine (anhydrous)	0,050
L-aspartic acid	0,020
L-cystine • 2HCl	0,065 2
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	0,028 83
L-valine	0,020
Biotin	0,0002
D-pantothenic	0,000 25
Choline chloride	0,003
Folic acid	0,001

Table A.1 *(continued)*

Constituent	g/ml water
Myoinositol	0,035
Niacinamide	0,001
PABA (para-aminobenzoic acid)	0,001
Pyridoxine HCl	0,001
Riboflavin	0,000 2
Thiamine HCl	0,001
Vitamin B_{12}	0,000 005
Calcium nitrate × H_2O	0,100
Potassium chloride	0,400
Magnesium sulfate (anhydrous)	0,048 84
Sodium chloride	6,000
Sodium phosphate, dibasic (anhydrous)	0,800
D-glucose (CLSI method – visual read)	2,000
D-glucose (EUCAST method – spectrophotometer read)	20,00
Glutathione, reduced	0,001
Phenol red, Na	0,005 3

Table A.2 — Components of RPMI-1640 2 % glucose

Component	1x concentration	2x concentration
Distilled water	900 ml	900 ml
RPMI-1640 [a]	10,4 g	20,8 g
MOPS [b]	34,53 g	69,06 g
Glucose	18 g	36 g

[a] See Table 1.

[b] 3-(N-morpholino) propanesulphonic acid.

A.2 References for Annex A

a) Clinical and Laboratory Standards Institute (2008). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; 3rd Informational Supplement.* M27-S3. Wayne, PA.

b) European Committee on Antimicrobial Susceptibility Testing. (2007), *EUCAST Definitive Document EDef 7.1: Method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts.* Clinl Microbiol Infect; 14: 398-405, 2008.

Annex B
(informative)

McFarland 0,5 barium sulfate turbidity standard

To standardize the inoculum density, use a $BaSO_4$ turbidity standard (0,5 McFarland standard).

The procedure consists of the following steps:

a) Prepare this turbidity standard by adding 0,5 ml of 0,048 mol/l $BaCl_2$ (1,175 % w/v $BaCl_2 \times 2H_2O$) to 99,5 ml of 0,18 mol/l H_2SO_4 (volume fraction of 1 %).

b) Verify the correct density of the turbidity standard by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0,08 to 0,13 for the 0,5 McFarland standard.

c) Distribute 4 ml to 6 ml into screw-cap tubes of the same size as those used in growing or diluting the broth culture inoculum.

d) Tightly seal these tubes and store them in the dark at room temperature.

e) Vigorously agitate this turbidity standard on a mechanical vortex mixer just before use.

f) Replace standards or recheck their densities three months after preparation.

Annex C
(informative)

Acceptable reading times for MIC interpretations using the visual MIC reading procedure

Constituent IF "x_+3" "<Tbl_large_10>" "" <Tbl_large_10> IF "x_-3" "</Tbl_large_10>" "" </Tbl_large_10>	Acceptable time of MIC reading if growth is adequate	
	24 h	48 h
Amphotericin B	Yes	Yes
Echinocandins	Yes	No
Fluconazole	Yes	Yes[a]
Flucytosine	Yes	Yes
Itraconazole	No	Yes
Posaconazole	No	Yes
Ravuconazole	No	Yes
Voriconazole	No	Yes

[a] See 3.8.1 for discussion regarding disparities between 24 h and 48 h readings.

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