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Clinical laboratory testing and in vitro diagnostic test systems — Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

Laboratoires d'analyses de biologie médicale et systèmes de diagnostic in vitro — Méthode de référence de microdilution en milieu liquide 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 procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 140, *In vitro diagnostic medical devices,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 16256:2012), which has been technically revised.

The main changes are as follows:

— addition of “broth micro-dilution” to the title;

— removal of 48 h reading for *Candida* species by the visual reading method;

— removal of definitions for susceptibility and resistance that are beyond the scope of this test performance document;

— inclusion of considerations for antifungal testing of yeast species with micro-dilution trays “treated” by manufacturers of the trays prior to use in the tests;

— updating of viable count testing methods for visual and spectrophotometer test pathways.

— addition of new antifungals (isavuconazole, rezafungin) to the testing and quality control range tables;

— detailed characterization of the components of one formulation of RPMI-1640 known to provide reproducible results of antifungal susceptibility tests for *Candida* species and *Cryptococcus neoformans*;

— reassigning of annexes;

— update of bibliography to more relevant information about performance of antifungal susceptibility testing for yeast fungi.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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 can 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 can 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 are required for intra- and inter-laboratory reproducibility, as results can 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 two-fold) increasing concentrations are inoculated with a known number of microorganisms.

Broth micro-dilution denotes the performance of the broth dilution test in micro-dilution trays.

The reference methods described in this document are intended for the testing of pure cultures of yeast fungi. The broth micro-dilution methods described in this document are the same as those described by the Clinical and Laboratory Standards Institute (CLSI)[1][5] and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[2][10]. These methods were initially shown to provide MICs of fluconazole that were similar, if not identical up to 2 mg/l[3]. Further the methods have been shown to provide MICs for two quality control strains of licensed antifungal agents that are similar as described in this document although quality control results for the spectrophotometer can trend slightly lower than for the visual reading method. The laboratory that wishes to use this document for conducting studies of newer antifungal agents, or as a reference method for comparison to MICs generated by a diagnostic device, can select which of the procedure options to use based upon the choice of MIC reading determined by visual inspection (CLSI method)[5] or by use of a spectrophotometer (EUCAST method)[2][10]. In either case, the procedural details for that option should be followed explicitly. In the first edition of this document, i.e. ISO 16256:2012, the reported quality control tests were performed using broth micro-dilution trays that were not treated in some way by the manufacturers of the plastic trays for either the visual or spectrophotometer method.

In this document the following verbal forms are used:

— “shall” indicates a requirement;

— “should” indicates a recommendation;

— ”may” indicates a permission;

— “can” indicates a possibility or a capability.

Clinical laboratory testing and in vitro diagnostic test systems — Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

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

1 Scope

This document 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 *Blastomyces dermatitidis* and/or *Histoplasma capsulatum* variety capsulatum. Moreover, testing filamentous fungi (moulds) introduces several additional problems in standardization not addressed by the current procedure. Those methods are beyond the scope of this document.

This document describes the broth micro-dilution reference method, which can be implemented by either of two pathways. One pathway involves visual determination of MICs (CLSI method)[1][5]; the second pathway involves spectrophotometric determination of MICs (EUCAST method)[2][10]. 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. 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 document; interpretive category breakpoints specific to the CLSI- and EUCAST-derived methods can be found by consulting the latest interpretive tables provided by the organizations[5][15]. Routine susceptibility testing methods or diagnostic test devices can be compared with this reference method in order to ensure comparable and reliable results for validation or registration purposes.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
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 1 to entry: Disinfectants, antiseptics and preservatives are not included in this definition.

3.2 Antifungal agents — properties

3.2.1
potency
active fraction of a test substance, determined in a bioassay against a reference powder of the same substance

Note 1 to entry: 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.

3.2.2
concentration
amount of an *antifungal agent* (3.1) in a specified volume of liquid

Note 1 to entry: The concentration is expressed as mg/l.

Note 2 to entry: mg/l = µg/ml but use of the unit µg/ml is not recommended.

3.3
stock solution
initial solution used for further dilutions

3.4
minimum inhibitory concentration
MIC
lowest *concentration* (3.2.2) that, under specified in vitro test conditions, reduces growth by an agreed amount within a specified period of time

Note 1 to entry: The MIC is expressed in mg/l.

3.5
wild type
absence of phenotypically-detectable acquired resistance mechanisms to the *antifungal agent* (3.1) in a given fungal strain

3.6
reference strain
catalogued, well-characterized fungal strain with stable, specified antifungal susceptibility phenotypes and/or genotypes

Note 1 to entry: Reference strains are kept as stock cultures, from which working cultures are derived. They are obtainable from culture collections and used for quality control.

3.7 Susceptibility testing method

3.7.1
broth dilution
technique in which containers are filled with appropriate volumes of an antifungal solution, employing incrementally (usually two-fold) increasing *concentrations* (3.2.2) of the *antifungal agent* (3.1) and appropriate volumes of *broth* (3.8) with a specified *inoculum* (3.9)

Note 1 to entry: The aim of this method is the determination of the *minimum inhibitory concentration* (3.4).

3.7.2
broth micro-dilution
performance of *broth dilution* (3.7.1) in micro-dilution trays with a capacity of ≤300 µl per well

3.8
broth
fluid medium used for the in vitro growth of yeast fungi

3.9
inoculum
number of colony-forming units of yeast in a suspension, calculated with respect to the final volume

Note 1 to entry: The inoculum is expressed as colony-forming units per millilitre (CFU/ml).

4 Test procedures

4.1 General

4.1.1 Trays and method

The tests are performed in plastic disposable micro-dilution 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.

4.1.2 Conditions for use of disposable micro-dilution trays

The tests were originally performed in broth micro-dilution trays that have had no additional treatment by the manufacturer. Quality control data by manufacturers of untreated trays (and on which this document was originally based) have shown that quality control results are consistently in specification for all antifungal agents tested. In some jurisdictions there has been a suggestion that results can be more consistent using treatment of the plastic trays. Treatment of the plastic, either by coating or corona discharge to impart an electrical charge to the plastic, is used in tissue culture studies and allows the tissue cells to adhere to the plastic. It is unknown if this process has been standardized for all micro-dilution tray manufacturers. It is known that with some antifungal agents the treated trays can result in elevated MICs compared to untreated trays. Such treatment can affect the reporting of results for those agents[13]. Those laboratories that use "treated" micro-dilution trays and read by spectrophotometer should ensure that the treated trays being utilized in testing provide the same quality control results as those indicated in Table 5. Those quality control ranges were originally performed with untreated trays. The data indicates that for almost all antifungal agents, the quality control ranges for the two standard strains listed in this document (*Candida parapsilosis* ATCC®[1)] 22019 and *Candida krusei* ATCC® 6258) are the within one log2 dilution for both testing/reading methods. Comparative quality control ranges for those strains for the spectrophotometer method are the same as originally reported using untreated trays[10] and for treated trays[2], with the exception of caspofungin (see Table 5). Comparative MIC observations for clinical isolates provided by the visual reading method[5] and those spectrophotometer readings using treated plates[2]for both testing methods should be interpreted with caution.

4.2 Medium

4.2.1 General

RPMI-1640 broth shall be used (see Table A.1 and Table A.2 for details for preparation of the two complete product versions of RPMI-1640 glucose broth) for both reading methods.

4.2.2 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.

1) ATCC is the registered trademark of a product supplied by the American Type Culture Collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.2.3 Spectrophotometric reading pathway

The RPMI-1640 medium should contain 2,0 % 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.

4.3 Antifungal agents

4.3.1 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.

4.3.2 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 Formulae (1) and (2) 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	DMSO [a]	Medium
Fluconazole	DMSO [a]	Medium
Isavuconazole	DMSO [a]	Medium
Itraconazole	DMSO [a]	Medium
Ketoconazole	DMSO [a]	Medium
Micafungin	DMSO [a]	Medium
Posaconazole	DMSO [a]	Medium
Ravuconazole	DMSO [a]	Medium
Rezafungin	DMSO [a]	Medium
Voriconazole	DMSO [a]	Medium

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

4.3.3 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 two-fold 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, it has been reported that serial dilutions of the more hydrophilic compounds can produce acceptable results[6]. 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 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	5 120	Stock	1,0		3,0		1 280		128	7
2	5 120	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		0,062 5	-4
13	2,5	Step 10	0,5		3,5		0,312 5		0,031 25	-5

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

Antifungal solution										
Step	Conc.	Source	Vol.	+	Solvent (DMSO) [a]	=	Intermediate conc.	=	Final conc. at 1:100	Log_2
	mg/l		ml		ml		mg/l		mg/l	
1	1 600	Stock					1 600		16	4
2	1 600	Stock	0,5		0,5		800		8,0	3
3	1 600	Stock	0,5		1,5		400		4,0	2
4	1 600	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		0,062 5	-4
10	25	Step 7	0,5		3,5		3,13		0,031 3	-5
[a] Dimethyl sulfoxide.										

4.4 Preparation of broth micro-dilution trays

4.4.1 Preparation for tests read visually – Visual reading pathway

Working solutions are dispensed into 10 wells of each row of micro-dilution 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.

4.4.2 Preparation for tests read by spectrophotometer - Spectrophometric reading pathway

Working solutions are dispensed into micro-dilution 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.

4.5 Storage of micro-dilution 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 allowing thawing. Antifungal solutions shall not be refrozen, as repeated freeze–thaw cycles accelerate the degradation of some antifungal agents.

4.6 Preparation of inoculum

4.6.1 General

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

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

4.6.2 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 *Cryptococcus 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 Annex 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 x 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 $2,5 \times 10^3$ CFU/ml.

4.6.3 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 *Cryptococcus 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 Annex 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 x 10^5 to $2,5 \times 10^5$ CFU/ml.

4.7 Inoculation of micro-dilution 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 4.4.1 and 4.4.2), a volume of 100 µl of yeast suspension is added.

Viable counts shall be performed periodically on the positive control well of a micro-dilution tray to ensure that test wells contain the appropriate CFU based upon the method used for MIC reading. For the visual reading pathway, this shall be done by removing 20 µl from the positive growth control well immediately after inoculation and spreading it over the surface of a suitable agar plate (e.g. Sabouraud dextrose agar). After 24 h of incubation at (35 ± 2) °C, the number of colonies, are counted and multiplied by 100. A correct inoculum will produce 10 colonies to 80 colonies. For the spectrophotometer reading method, 10 µl from the positive growth control is diluted into 2 ml of water; then 100 µl is spread over the surface of a suitable agar plate. After 24 h of incubation at (35 ± 2) °C, the number of colonies, are counted. An acceptable test suspension should yield 5 colonies to 125 colonies.

4.8 Incubation of micro-dilution trays

4.8.1 General

Micro-dilution 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, micro-dilution trays should not be stacked more than five high.

Micro-dilution trays are incubated at (35 ± 2) °C in ambient air for (24 ± 2) h for most antifungal agent-yeast combinations. Some isolates of *Cryptococcus neoformans* can not grow sufficiently unless the incubation temperature is lowered to 30 °C.

4.8.2 Visual pathway

Incubation times will vary based upon the yeast species and antifungal agent being tested. All tests can be read following a 24 h incubation with the exception of *Cryptococcus neoformans* that can require up to 48 h of incubation. For updates to this information, see CLSI M60 ED2:2020[5].

4.8.3 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 re-incubated for 12 h to 24 h. Failure to reach an absorbance of 0,2 after 48 h constitutes a failed test.

4.9 Reading MIC results

4.9.1 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.

4.9.2 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[3][7]. This trailing growth can make an isolate that appears susceptible after 24 h appear completely resistant if a 48 h reading is performed. It is also known that ergosterol content in *Candida albicans* correlates with 24 h visual test results[7]. For this reason, 24 h readings are standard, with the exception of the slower growing *Cryptococcus neoformans*.

For the visual determination of MICs, the wells of the tray should be examined from the bottom side using a mirror reading device. It can 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.

4.9.3 Spectrophotometric reading methods

For the spectrophotometric determination of MICs, the micro-dilution trays are read with a micro-dilution plate reader using a wavelength between 405 nm 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.

4.10 Interpretation of MICs

The clinical interpretation of MICs generated by either pathway of this document 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[16] and MICs determined by spectrophotometric readings should be interpreted using the latest guidelines available from EUCAST[17].

5 Quality Control (QC)

The quality of test results is monitored by the concomitant use of standard control strains (see Tables 4 and 5). Stock control strains should be stored lyophilised or frozen at or below −60 °C. Prepare working cultures by subculture of stock strains on a non-inhibitory agar medium. Further subcultures shall 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 the two standard 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[4][5][8][9][10][11][14]. 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. The quality control ranges established in this document for visual reading were performed using micro-dilution trays that were untreated by the manufacturer(s) of the plastic trays. For spectrophotometric reading the quality control ranges have been shown to be similar for both untreated trays and for the treated trays used to define the same ranges for the two quality strains, *Candida parapsilosis* ATCC® 22019 and *Candida krusei* ATCC® 6258 that have been used in all international quality control guides[2][5][10]. These ranges are similar to those produced by visual reading but tend to be one log2 dilution lower than for visual reading. Minor quality control range differences for any agents tested with treated trays[2] are shown in Table 5. Users are reminded that the electronic treatment of trays by manufacturers can not be identical (details as given in 4.1.2).

Additional quality control strains are used for certain antifungal agent – yeast mould species in some parts of the world (e.g. References [2] and [10]). It is recommended to consult the appropriate references as required since these strains have not been compared for both visual and spectrophotometric methods.

Table 4 — Recommended 24 h MIC ranges for two QC strains for broth micro-dilution tests read visually[1][5][8][9]

Organism	Antifungal agent	MIC Range (mg/l)
Candida parapsilosis ATCC® 22019	Amphotericin B	0,25 to 2,0
	Anidulafungin	0,25 to 2,0
	Caspofungin	0,25 to 1,0 Untreated N/A Treated
	Flucytosine (5-FC)	0,06 to 0,25
	Fluconazole	0,5 to 4,0
	Isavuconazole	0,015 to 0,06
	Itraconazole	0,12 to 0,5
	Ketoconazole	0,03 to 0,25
	Micafungin	0,5 to 2,0 Untreated N/A Treated
	Posaconazole	0,06 to 0,25
	Ravuconazole	0,015 to 0,12
	Rezafungin	0,25 to 2,0
	Voriconazole	0,015 to 0,12
Candida krusei ATCC® 6258	Amphotericin B	0,5 to 2,0
	Anidulafungin	0,03 to 0,12
	Caspofungin	0,12 to 1,0 Untreated N/A Treated
	Flucytosine (5-FC)	4,0 to 16
	Fluconazole	8,0 to 64
	Isavuconazole	0,06 to 0,5
	Itraconazole	0,12 to 1,0
	Ketoconazole	0,12 to 1,0
	Micafungin	0,12 to 0,5 Untreated N/A Treated
	Posaconazole	0,06 to 0,5
	Ravuconazole	0,06 to 0,5
	Rezafungin	0,015 to 0,12
	Voriconazole	0,06 to 0,5
NOTE 1 N/A denotes “not available”. This refers to quality control ranges NOTE 2 Micro-dilution trays for visual reading are supplied untreated from manufacturers. Quality control ranges for caspofungin, and micafungin are given for untreated trays.		

Table 5 — Recommended MIC ranges for 2 QC strains for broth micro-dilution tests read spectrophotometrically[2][10][11][14][12]

Organism	Antifungal agent	MIC range (mg/l)
Candida parapsilosis ATCC® 22019	Amphotericin B	0,12 to 1,0
	Anidulafungin	0,25 to 1,0
	Caspofungin	0,25 to 1,0 Untreated N/A Treated
	Flucytosine (5-FC)	0,12 to 0,5
	Fluconazole	0,5 to 2,0
	Isavuconazole	0,015 to 0,06 Untreated 0,008 to 0,03 Treated
	Itraconazole	0,03 to 0,12
	Micafungin	0,5 to 2,0
	Posaconazole	0,015 to 0,06
	Voriconazole	0,015 to 0,06
Candida krusei ATCC® 6258	Amphotericin B	0,12 to 1,0
	Anidulafungin	≤0,06
	Caspofungin	0,12 to 1,0 Untreated N/A Treated
	Flucytosine (5-FC)	1,0 to 4,0
	Fluconazole	16,0 to 64,0
	Isavuconazole	0,015 to 0,06
	Itraconazole	0,03 to 0,12
	Micafungin	0,03 to 0,12
	Posaconazole	0,015 to 0,06
	Voriconazole	0,03 to 0,25

NOTE N/A denotes "not available" for ranges tested with treated plates The QC ranges for untreated plates read spectrophotometrically (for caspofungin) are given as shown and were performed according to the appropriate method in an independent multi-laboratory unpublished study, but fall within the same range as visually read trays[12].

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.

The RPMI powder used has all components except MOPS buffer, sodium hydroxide (NaOH) and glucose. Verify the medium does not contain sodium bicarbonate. Dissolve the 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 (NaOH). Add glucose (depending on reading method). Add additional distilled H_2O 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

Constituent	g/l water
L-arginine (free base)	0,200
L-asparagine (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	0,000 2
D-pantothenic	0,000 25
(with glutamine and phenol red but without bicarbonate) Users should check that the concentrations of components of RPMI from suppliers are as described in the table.	

Table A.1 *(continued)*

Constituent	g/l water
Choline chloride	0,003
Folic acid	0,001
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
Glucose	2,0
Glutathione, reduced	0,001
Phenol red, Na	0,005 3
(with glutamine and phenol red but without bicarbonate) Users should check that the concentrations of components of RPMI from suppliers are as described in the table.	

Table A.2 — Components of RPMI-1640 with 0,2 % and 2 % glucose (Complete medium)[2][5]

Component	1x concentration Visual Reading 0,2 % glucose final	2x concentration Spectrophotometric Reading 2 % glucose final
Distilled H_2O	900 ml	900 ml
RPMI-1640 [a]	10,4 g	20,8 g
MOPS [b]	34,53 g	69,06 g
Glucose	No additional glucose (see 4.2.2)	36 g for final 2,0 % (see 4.2.3)
Additional Distilled H_2O	to 1,0 l	to 1,0 l

[a] See Table 1.

[b] 3-(N-morpholino) propanesulphonic acid. 0,165 mol/l final.