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Method for the determination of minimum inhibitory concentration (MIC) by broth dilution of fermentative yeasts

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INTRODUCTION

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing. In dilution tests, microorganisms are tested for their ability to produce visible growth in microtitration plate wells of broth (broth microdilution) containing serial dilutions of the antimicrobial agents. The MIC is defined as the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism. The method described in this document is intended for testing yeasts that cause clinically significant infections (primarily *Candida* species). This standard encompasses only those yeasts that are able to ferment glucose. Thus, testing the susceptibility of nonfermentative yeasts such as *Cryptococcus neoformans* var. neoformans cannot be determined by the current procedure nor is the methodology suitable for the yeast forms of dimorphic fungi.

PURPOSE

The standard method described herein is intended to provide a valid method for testing the susceptibility to antifungal agents of yeasts that ferment glucose. The method is intended primarily to facilitate an acceptable degree of conformity, i.e. agreement within specified ranges, between laboratories in measuring the susceptibility of yeasts to antifungal agents. The method is designed to be easy to perform, rapid, economic, and to be suitable for reading with microtitration plate readers, which allows direct transfer, storage and manipulation of data with a computer. The method is also intended to produce concordant results with the American National Committee for Clinical and Laboratory Standards (NCCLS) document on antifungal susceptibility testing of yeasts, *Approved Standard M27-A2* [1].

BROTH MEDIUM

Medium for testing all antifungal agents

A completely synthetic medium, RPMI 1640 supplemented with glutamine and a pH indicator but without bicarbonate, is recommended [2,3]. However, RPMI 1640 medium (Table 1) contains only 2 g of glucose per litre (0.2%), a concentration that is lower than commonly used for culturing yeasts. Supplementing medium to a final concentration of 20 g per litre (2%) glucose 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 antagonizes the activity of flucytosine, and phosphate buffer and may give unexpected interactions with antifungal agents. 3-(N-morpholino)

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Table 1 Composition of RPMI 1640 Medium

Constituent	g/L water
L-arginine (free base)	0.200
L-aspargine (anhydrous)	0.050
L-aspartic acid	0.020
L-cystine 2HCl	0.0652
L-glutamic acid	0.020
L-glutamine	0.300
Glycine	0.010
L-histidine (free base)	0.015
L-hydroxyproline	0.020
L-isoleucine	0.050
L-leucine	0.050
L-lysine HCl	0.040
L-methionine	0.015
L-phenylalanine	0.015
L-proline	0.020
L-serine	0.030
L-threonine	0.020
L-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
PABA	0.001
Pyridoxine HCl	0.001
Riboflavin	0.0002
Thiamine HCl	0.001
Vitamin B_{12}	0.000005
Calcium nitrate H_20	0.100
Potassium chloride	0.400
Magnesium sulfate (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	0.053

[a]Note: this medium has a 0.2% glucose concentration.

propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L, pH 7.0, is satisfactory for RPMI 1640 media. The recommended medium – RPMI 2% G – is prepared as follows:

1 Add components to 900 mL of distilled water (see Table 2).

Table 2 Preparation of RPMI medium

	(1 × concentration)	(2 × concentration)
RPMI 1640 (Table 1)	10.4 g	20.8 g
MOPS	34.53 g	69.06 g
Glucose	18 g	36 g

2 Stir until dissolved completely.
3 With stirring, adjust the pH to 7.0 at 25 °C with 1M sodium hydroxide.
4 Add water to a final volume of 1 litre.
5 Filter sterilize with a 0.22-μm pore size filter.
6 Store at 4 °C.
7 For quality control purposes one aliquot of the sterilized medium is used for sterility checks, for retesting the pH (6.9–7.1 is acceptable) and as a growth control with a control strain.

Medium for testing amphotericin B

No specific medium can be recommended for testing amphotericin B. The nonsynthetic broth Antibiotic Medium 3 (AM3), supplemented to a final concentration of 2% glucose, has been evaluated for detecting resistance to amphotericin B [5–8]. However, there is batch-to-batch variation and differences in performance of the medium from different manufacturers. Preliminary results also indicate that an inoculum size of 0.5–2.5 $\times 10^5$ CFU/mL is too high for testing amphotericin B in AM3 [5].

Antifungal drugs

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 commercial sources. Clinical preparations must not be used. Powders must be supplied with the drug's generic name, a lot number, potency (expressed in μg or International Units per mg of powder, or as a percentage active ingredient), expiry date and recommended storage conditions. Store powders in sealed containers at −20 °C or below with a desiccant unless otherwise recommended by the manufacturers. Ideally, hygroscopic agents should be dispensed into aliquots, one of which is used on each occasion. Allow containers to warm to room temperature before opening them to avoid condensation of water on the powder.

Preparation of stock solutions

Antifungal drug solutions must be prepared taking into account the potency of the lot of antifungal drug powder that is being used. The amount of

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powder or diluent required to prepare a standard solution may be calculated as follows:

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

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

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 more than 100 mg of powder is weighed.

Antifungal drug stock solutions should be prepared at concentrations 100 times the highest concentration to be tested. Solvents other than water are required to dissolve some antifungal drugs (Table 3). Information on the solubility of antifungal compounds should be provided, by the supplier, with the drug. Dimethyl sulfoxide (DMSO) is suitable for dissolving ketoconazole, itraconazole and flucytosine. The latter drug can also be dissolved in 50 : 50 acetone : water. Water is also a suitable solvent for flucytosine and fluconazole.

Sterilization of stock solutions is not normally necessary. If required, sterilization can be achieved by membrane filtration. Other filter materials must not be used as they may adsorb significant amounts of drug. If filtration is used, samples before and after filtration must be assayed to confirm that there has been no adsorption 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 may be stored at −70 °C for at least 6 months without significant loss of activity [9]. Remove vials when required and use them the same day. Discard any drug left over on that day. Significant deterioration of an antifungal drug will be seen in the results of testing the susceptibility of quality control strains (Table 4). If necessary, the drug can be assayed to determine the potency.

Table 3 Solvents and diluents for preparation of stock solutions of antifungal agents requiring solvents other than water

Antifungal agent	Solvent (full strength)	Diluent
Amphotericin B	DMSO[a]	Medium
Ketoconazole	DMSO[a]	Medium
Itraconazole	DMSO[a]	Medium
Flucytosine	DMSO or 50 : 50 acetone:water	Medium

[a]Dimethyl sulfoxide.

Table 4 Reference strains for quality control (data generated in seven laboratories where each MIC was determined nine times)

Strain	Antifungal agent	Geometric mean MIC (mg/L)	Modal MIC (mg/L)	Median MIC (mg/L)	Minimum MIC (mg/L)	Maximum MIC (mg/L)	Quality control (QC) ranges[a]	% MICs within QC range	NCCLS range[b]
Candida krusei	Flucytosine	2.6	2.0	2.0	1.0	8.0	1.0–4.0	97	4.0–16.0
ATCC 6258	Fluconazole	25.7	16.0	32.0	16.0	32.0	8.0–32.0	100	16.0–64.0
	Itraconazole	0.12	0.12	0.12	0.03	0.50	0.06–0.25	95	0.12–0.50
Candida parapsilosis	Flucytosine	0.25	0.25	0.25	0.25	0.50	0.12–0.50	100	0.12–0.50
ATCC 22019	Fluconazole	1.51	2.0	2.0	1.0	4.0	1.0–4.0	100	2.0–8.0
	Itraconazole	0.07	0.06	0.06	0.03	0.25	0.03–0.12	97	0.06–0.25

[a]the mode ± one two-fold dilution [b]NCCLS ranges included for comparison.

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Table 5 Appropriate concentration ranges for antifungal agents

Antifungal drug	Range (mg/L)
Flucytosine	0.125–64
Fluconazole	0.125–64
Itraconazole	0.015–8
Ketoconazole	0.015–8

Range of concentrations tested

The range of concentrations tested will depend on the organism and the antifungal drug being tested but a two-fold dilution series based on 1 mg/L is conventionally used. As a guideline, the range of concentrations should encompass the breakpoint, when one exists, as well as the expected results for the quality control strains. Based on previous studies the drug concentration ranges in Table 5 are appropriate.

Preparation of microtitration plates

Sterile plastic, disposable, microtitration plates with 96 flat-bottom wells are used. The medium used in the plates is prepared at double the final strength to allow for a 50% dilution once the inoculum is added. This approach allows the inoculum to be prepared in distilled water, which permits the absorbance to be determined using a spectrophotometer without interference from colored media.

Preparation of plates for hydrophilic antifungal agents

The method for preparing these plates is as follows:

Step 1 (Figure 1a)

1 Column 1 of the microtitration tray is filled with 200 μL of double-strength culture medium (RPMI 2% G) containing twice the final drug concentration (128 mg/L for flucytosine and fluconazole).
2 Columns 2–12 are filled with 100 μL of double-strength medium (RPMI 2% G).

Step 2 (Figure 1b)

1 100-μL amounts are taken from wells in column 1 and diluted two-fold by transferring them to column 2 with a multichannel pipette (± 2% coefficient of variation). 100 μL samples are then removed from column 2 and transferred to column 3, and so on through to column 10. The last 100 μL of diluted drug is then discarded.
2 Thus, each well in columns 1–10 will contain 100 μL of double-strength RPMI 2% G containing twice the final antifungal drug concentrations.

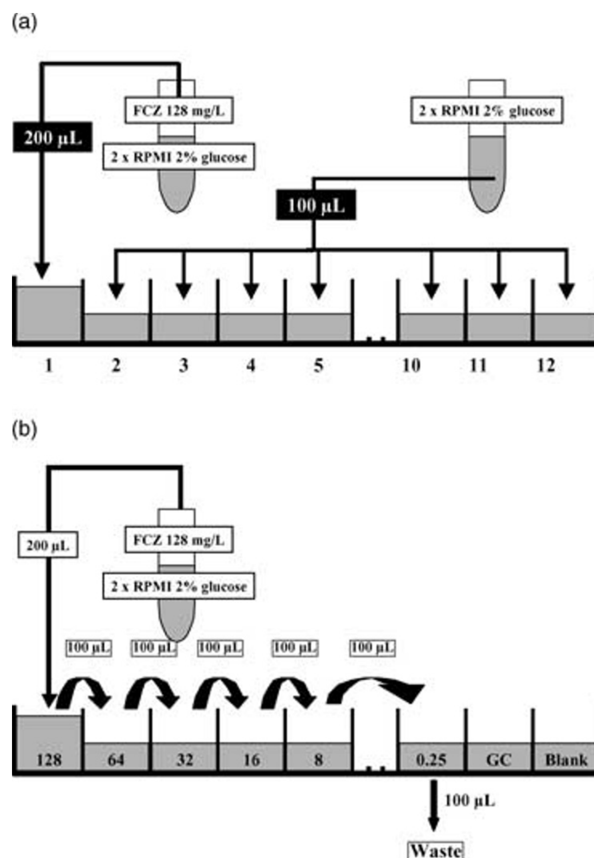

Figure 1 Preparation of microtitration plates with antifungal agents. (a) Step 1 (b) Step 2. FCZ, Fluconazole.

Step 3

The trays can be sealed in plastic bags or aluminum foil and stored frozen at −70 °C 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.

Preparation of plates for hydrophobic antifungal agents

The dilutions of the hydrophobic antifungal agents, ketoconazole and itraconazole, must be prepared in a different way to that described above.

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Step 1

1 Take an antifungal drug stock tube from the −70 °C freezer (itraconazole and ketoconazole 1600 mg/L).
2 Fill nine further tubes with 150 μL of DMSO.
3 Take 150 μL from the stock solution and perform 1 : 2 dilutions in tubes 2–10.
4 This will produce a tube dilution series from 1600 mg/L to 3 mg/L.

Step 2

1 Fill 10 tubes with 9.9 mL of RPMI 2% glucose double strength.
2 Take 100 μL from each of the tubes with antifungal drug and DMSO and transfer to each of the 10 tubes with 9.9 mL of culture medium (1 : 100 dilution). The concentration of DMSO in each of the culture medium tubes is 1% (Figure 2a).
3 Alternatively, use a 12-well-pipette reservoir. Normally, each well of the reservoir holds 5 mL (Figure 2b).

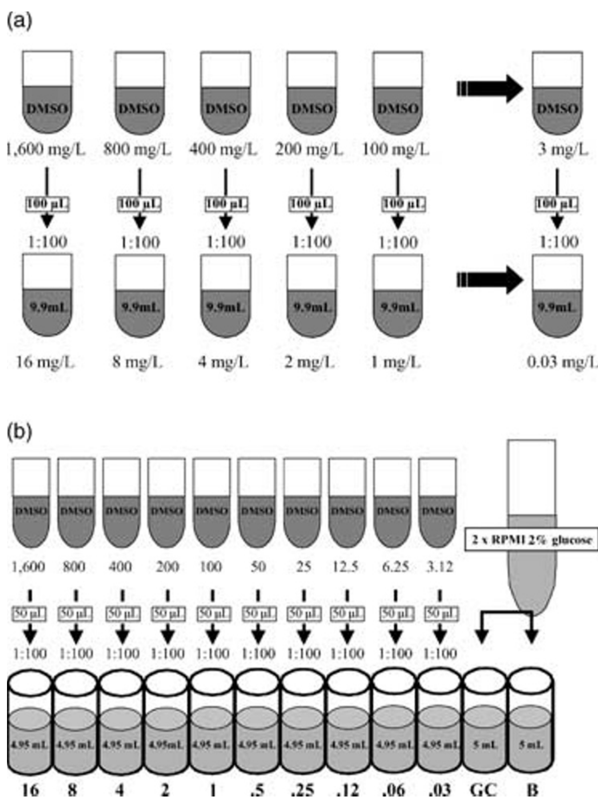

Figure 2 Preparation of dilutions for plates with hydrophobic antifungal agents. (a) Tube method (b) Alternative method with 12-well-pipette reservoir.

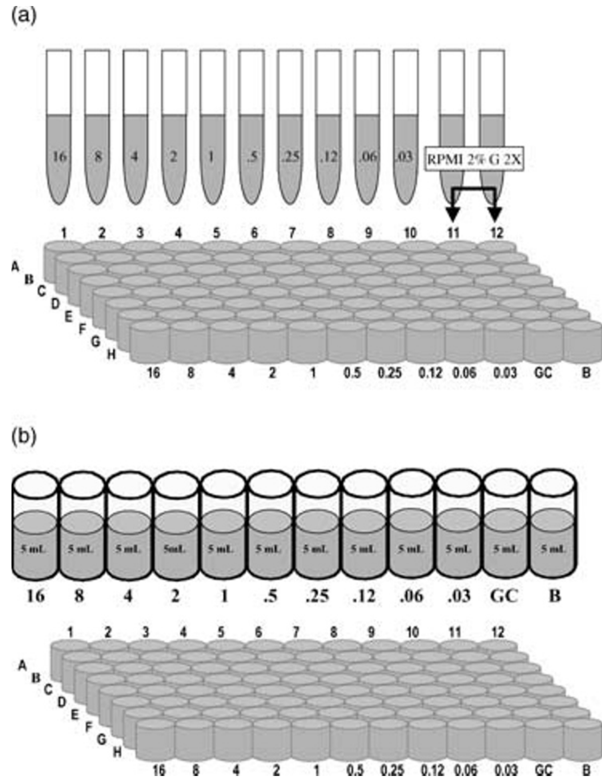

Figure 3 (a) Preparation of microtitration plates with hydrophobic antifungal agents. (a) Dispensing agents from dilutions in tubes (b) Alternative method dispensing agents from 12-well-pipette reservoir with a multichannel pipette.

Step 3

1 Fill each column – from 1 to 10 – of the microtitration plate with 100 μL from each of the tubes containing the corresponding concentration. Thus, fill column 1 from the tube containing 16 mg/L, column 2 from the tube containing 8 mg/L, and so on until column 10 is filled from the tube containing 0.03 mg/L.
2 Each well of column 11 and 12 should be filled with 100 μL of RPMI 2% glucose double strength.
3 Thus, each well in columns 1–10 will contain 100 μL of twice the final antifungal drug concentrations in double-strength RPMI 2% G with 1% DMSO. Columns 11 and 12 will contain RPMI 2% G (Figure 3a).
4 Alternatively, use a multichannel pipette to fill the microtitration plate from the 12-well pipette reservoir (Figure 3b).

Step 4

The trays of itraconazole, and ketoconazole can be sealed in plastic bags or aluminum foil and stored

Table 6 Preparation of McFarland 0.5 turbidity standard

Step	Procedure
1	Add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2{\cdot}2H_20$) to 99.5 mL of 0.18 mol/L (0.36 N) H_2S0_4 (1% v/v) and mix thoroughly.
2	Check the density with a spectrophotometer having a 1-cm light path and matched cuvette. The absorbance at 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.

frozen at −70 °C 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.

Preparation of inoculum

Preparation of inoculum is as follows:

1 Culture all yeasts in ambient air at 35–37 °C on recovery medium (Sabouraud's dextrose agar or peptone dextrose agar) for 18–24 h before testing.

2 Prepare the inoculum by picking five distinct colonies of diameter = 1 mm from 18 to 24 h cultures and suspending them in 5 mL of sterile distilled water.

3 Completely suspend the inoculum by vigorous shaking on a vortex mixer for 15 s. The cell density is then adjusted to the density of a 0.5 McFarland standard (Table 6) by adding sterile distilled water and measuring absorbance in a spectrophotometer at a wavelength of 530 nm. This will give a yeast suspension of 1–5 × 10^6 cfu/mL. Prepare a working suspension by a 1 in 10 further dilution of the stock suspension in sterile distilled water to yield 1–5 × 10^5 cfu/mL.

Inoculation of microtitration Plates (Figure 4)

Each well of a microtitration tray is inoculated with 100 μL of 1–5 × 10^5 cfu/mL yeast suspension, which will give the required drug concentration and inoculum density (final inoculum = 0.5–2.5 × 10^5 CFU/mL). The growth control wells (column 11), which contained 100 μL of sterile drug-free medium, are also inoculated with 100 μL of the same inoculum suspension. Column 12 of the microtitration plate is filled with 100 μL

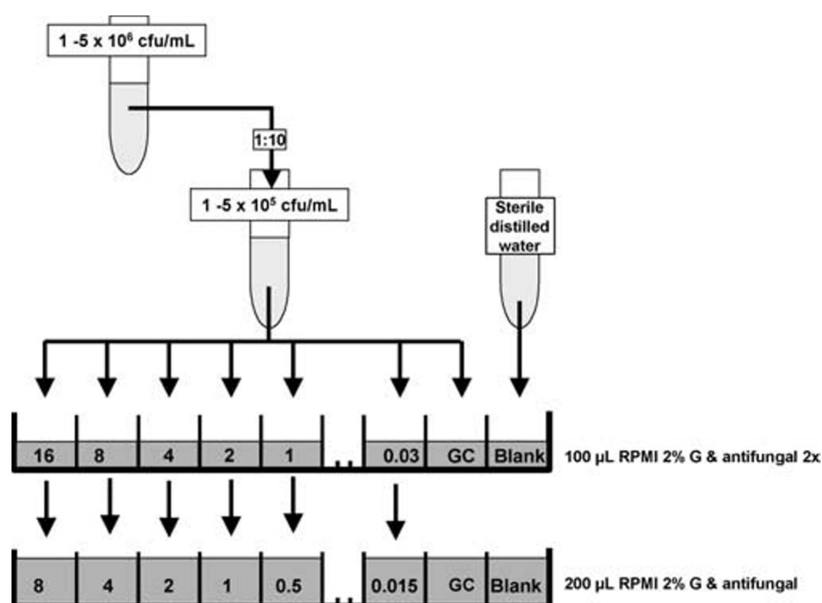

Figure 4 Inoculation of microtitration plates.

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of sterile distilled water from the lot used to prepare the inoculum as a sterility control for media and distilled water (drug-free medium only). Quality control organisms are tested by the same method and are included each time an isolate is tested.

Incubation of microtitration plates

The microtitration plates are incubated without agitation at 35–37 °C in ambient air for 24 h. An absorbance of <0.5 indicates poor growth and occurs most commonly amongst strains of *Candida parapsilosis* and *Candida guilliermondii*. Such plates should be re-incubated a further 12–24 h and then read. Failure to reach an absorbance of 0.5 after 48 h constitutes a failed test.

Reading results

The microtitration plates must be read with a microtitration plate reader. The recommended wavelength for measuring the absorbance of the plate is 530 nm, although others can be used, e.g. 405 nm or 450 nm. The value of the blank (background column 12) should be subtracted from readings for the rest of the wells.

If required, microtitration plates may be agitated using a microtitration plate shaker before reading to ensure uniform turbidity and to resuspend any yeast cells that may have sedimented. Microdilution plate shakers vary and the device should be adjusted so that visible homogeneity of the cultures is achieved in the growth control wells.

Flucytosine and azole antifungal agents

The MIC of flucytosine (5-flucytosine) and the azole antifungal drugs is the lowest drug concentration that gives rise to an inhibition of growth equal to or greater than 50% of that of the drug-free control. This will be called MIC 50%.

Interpretation of results

Interpretative breakpoints have yet to be established and the clinical relevance of testing remains uncertain. However, the NCCLS has recommended breakpoints for flucytosine, fluconazole and itraconazole [1] that are based on limited data and may need to be revised [10,11].

Quality control

Control procedures are the means by which the quality of results is assured and are described in detail by the NCCLS [1]. The routine quality of test results is monitored by the use of control strains.

Control strains

Control strains should exhibit MICs close to the middle of the range of the $\log_2$ series tested, and antifungal drug susceptibility patterns of control strains must be genetically stable. The recommended control strains are shown in Table 4 and additional strains that may be useful for conducting reference studies are currently being sought. Guidelines for the selection of appropriate quality control strains are further discussed in NCCLS document M23-A [12]. The recommended strains were selected in accordance with these criteria [13,14] and will be used to ensure concordance with NCCLS methods [1].

Control strains should be obtained from a reliable source such as the American Type Culture Collection (ATCC®), National Collection for Pathogenic Fungi (NCPF®), Centraal Bureau voor Schimmelcultures (CBS®), commercial suppliers or members of the EUCAST AFST Subcommittee.

Storage of control strains

Yeasts may be stored for extended periods by growth on potato dextrose agar followed by freezing at −70 °C [15], or by subculturing loopfuls of yeast in 50% glycerol solution and freezing at −70 °C [16]. Cultures can be stored short-term on Sabouraud's agar or peptone dextrose agar slopes at 2–8 °C, with new cultures prepared every two weeks.

Routine use of control strains

For routine use of control strains, fresh cultures on a recovery agar plate (e.g. Sabouraud's dextrose agar or peptone dextrose agar) must be prepared from agar slopes or frozen cultures.

Control strains must be included each day the test is performed and the MICs should be within the control ranges given in Table 4. If more than one test in 20 is out of range the source of error must be investigated.

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Each test must include a well of medium without antifungal drug to demonstrate growth of the test organisms and provide a turbidity control for reading end points.

Subculture inocula on a suitable agar medium (preferably a chromogenic medium) to ensure purity and to provide fresh colonies if re-testing is required.

Test each new batch of medium, lot of microtitration trays, and lot of RPMI 1640 2% G broth with one of the quality control strains listed in Table 4 to ensure that MICs fall within the expected range.

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