

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF YEASTS:

A. Principle

Antifungal drug dilutions are used to determine the MIC or MFC for an isolate when grown in the presence of an antifungal agent. Serial dilutions of antifungal agents are dispensed into appropriately labeled tubes. Each tube is then inoculated with a standardized nutrient broth suspension of the yeast being tested. The primary advantage of the broth dilution test is that it permits a quantitative estimate of both the inhibitory and fungicidal activities of the antifungal agent. Antifungal susceptibility tests should be performed (i) when the patient is failing therapy with an antifungal agent(s) that is known to be active against the infecting organism; (ii) to learn of potentially efficacious alternative drugs when the pathogenic yeast is one with well-known resistance to the drug of choice, e.g., *Candida lusitanae* and amphotericin B (AMB); (iii) when one is treating with 5-fluorocytosine (5-FC), an agent to which yeasts may be innately resistant or to which resistance rapidly develops; (iv) to ascertain antifungal activity with new agents for which no substantial or previously published data base exists; (v) for yeasts recovered from severely immunocompromised hosts with systemic disease, e.g., those with neutropenia; and (vi) for prospective studies of *in vitro* or *in vivo* correlation. The methods described here are those currently being used in multicenter collaborative studies by the Subcommittee on Antifungal Susceptibility Testing of the NCCLS. To date, no NCCLS standards have been published, but this procedure is based upon the December 1992 proposed standards by the NCCLS.

B. Specimen: Five isolated colonies of similar colony morphology at least 1 mm in diameter grown for 24-48 h on Sabouraud dextrose agar (SDA).

C. Materials: Indicate the expiration date of reagents on the container label and in the work record, or on the manufacturer's label. Label all reagents and media when first placed in use.

1. Media and Reagents (storage conditions; shelf life)

- a. SDA plates, Emmons modification, pH 7 (2-8°C)
- b. SDA slants, Emmons modification, pH 7 (2-8°C)
- c. RPMI-1640 medium buffered with 0.165 M MOPS [3-(*N*-morpholino) propanesulfonic acid] containing L-glutamine and lacking sodium bicarbonate; supplied dehydrated by American Biorganics, Inc., Niagara Falls, N.Y.; catalog no. R63165 (2 to 8°C; shelf life, 12 months); dehydrated RPMI 1640; sterile distilled reagent-grade water
 - (1) Add 1 package of RPMI-1640 to 1l reagent-grade sterile water and then stir to solubilize.
 - (2) Filter sterilize through two 500-ml-capacity filtration units with 0.22- μ -pore-size Millipore filters.
 - (3) Store the RPMI-1640 until needed (2 to 8°C; shelf life, 3 months).
- d. Sterile distilled reagent-grade water (25°C)
- e. Sterile 0.85% NaCl (25°C)
- f. Dimethyl sulfoxide (DMSO) (25°C), dimethylformamide (DMF) (25°C), and polyethylene glycol (PEG) (25°C)

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- g. Macrobrotth dilution tubes (sterile, 12x75 mm, polystyrene, snap cap) containing 0.1 ml volumes of the following antimicrobial agents (diluted in RPMI-1640) except for amphotericin B which requires M-3) at 10 times the suggested final concentrations (see Section V) maintained at -70°C
 - (1) Amphotericin B (AMB), 0.03 to 16 µg/ml
 - (2) Fluconazole (FLU), 0.125 to 64 µg/ml
 - (3) Fluorocytosine (5-FC), 0.125-64 µg/ml
 - (4) Ketoconazole (KETO), 0.03 to 16 µg/ml
 - (5) Miconazole (MON), 0.03-16 µg/ml
 - (6) Itraconazole (ITRA) 0.03-16 µg/ml
 - (7) Clotrimazole (CLOT), 0.25-16 µg/ml
 - (8) Nystatin (NYS), 0.7-18.5 µg/ml
 - (9) Natamycin (NATA), 0.15-19.2 µg/ml
- h. Potato Dextrose Agar Slants (PDA) (2-8°C)
- i. Bacto Antibiotic Medium 3 (M3) (2-8°C). Follow instructions on label for preparation.
- j. 25% ethanol
- k. 0.5N NaOH
- l. 0.5N HCl

2. Supplies

- a. Sterile wooden applicator sticks or sterile swabs
- b. Sterile test tubes
 - (1) 12x75 mm, polystyrene, snap cap
 - (2) 13x100 mm, polystyrene, screw cap
 - (3) 50 ml conical centrifuge
- c. 500 ml capacity filtration units with 0.22-µm-pore-size Millipore filters
- d. Sterile 1, 5, and 10 ml disposable serological pipettes and pipette bulb

3. Equipment

- a. Spectrophotometer (Coleman Model 35)
- b. Vortex mixer
- c. Micropipette, 100 µl capacity with sterile disposable tips
 - (1) Repetitive dispensing pipette, 100 µl capacity with sterile disposable syringes
 - (2) Calibrated loop, 0.001 ml
 - (3) 35°C ambient-air incubator

4. Quality Control

- a. QC strain
 - (1) *Candida albicans* (ATCC 90028)
 - (2) Maintain permanent stock cultures at -70°C on an PDA slant.

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- (3) Prepare working stock culture.
 - (a) With sterile wooden applicator stick, chip off a small portion of the frozen surface of an SDA slant, and streak for isolation.
 - (b) Weekly working stock cultures are prepared by subculturing three to five colonies of similar colony morphology from the previous week's working stock plate to a fresh SDA plate.
- (4) Subculture from working stock plate to another SDA plate on the day before use, and test as would be done for patient isolates.
- (5) See below for expected results.

***Candida albicans* (ATCC 90028)**

AMB	0.25-1.0 µg/ml	ITRA	0.3-0.5 µg/ml
5-FC	1.0-4.0 µg/ml	CLOT	0.25-2.0 µg/ml
KETO	≤ 0.03-0.125	NYS	0.57-4.6 µg/ml
FLU	≤ 0.125-0.5	MON	N/A

- (6) Record results on QC form.
 - b. Positive growth control should demonstrate good growth and be free of contaminating organisms.
 - c. Negative growth control should be free of growth.
 - d. Inoculum count verification plate should show 100 to 500 colonies.
 - e. Test considered in control:
 - (1) MIC's for QC strains are within acceptable limits.
 - (2) Growth and inoculum controls show appropriate growth.
 - (3) Results are appropriate for isolate tested (see table above).
 - f. See troubleshooting tips section below.

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5. Procedure

- a. Preparation of antifungal drugs and dilution schemes: Because antifungal drug preparation is a critical step in the performance of reproducible assays, commercially prepared macrobroth dilution tubes are unavailable, and techniques for preparation of the tubes vary from those generally employed with antibacterial antimicrobial agents, we have outlined this procedure in detail. Potency of pure assayed powder may vary from lot to lot.

- (1) **Amphotericin B** (AMB) - AMB is a polyene antifungal agent. Polyene drugs form complexes with ergosterol which open channels in the fungal membrane that cause leakage of critical intracellular constituents and subsequent cell death.

(a) Formulation

5 mg of Amphotericin B
1 ml dimethylsulfoxide (DMSO)
30 ml M-3
 $C_1 \times V_1 = C_2 \times V_2$
 $5000 \mu\text{g/ml} \times 1 \text{ ml} = 160 \mu\text{g/ml} \times V_2$
 $V_2 = (5000 \mu\text{g/ml} \times 1 \text{ ml})/160 \mu\text{g/ml} = 31.25 \text{ ml}$

(b) Preparation

- i) Weigh out 5 mg of amphotericin B powder using the Mettler balance.
- ii) Dissolve the powder with 1 ml of DMSO.
NOTE: Keep the amphotericin B in darkness as much as possible because light degrades this drug.
- iii) Dilute 1 ml (5000 $\mu\text{g/ml}$) amphotericin B in 30.25 ml of M-3 to make an initial concentration of 160.0 $\mu\text{g/ml}$.
NOTE: 30 ml of amphotericin B of 160 $\mu\text{g/ml}$ is enough to dispense into 15 racks of tubes. However, instructions are for setting up 12 racks.
NOTE: Fungizone is also available as an alternative for the powder. Dissolve a 50 mg lyophilized cake with 10 ml of sterile water. Aliquot into 1 ml portions. Add 1 ml to 30.25 ml of M-3 and continue as with the powder.
- iv) Prepare 12 racks of 12x75 Falcon tubes labeled 16-0.03 $\mu\text{g/ml}$ (i.e., 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03). Label tube 16 with the name of the drug → AmB
- v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled at 160 $\mu\text{g/ml}$. Place 8 ml of M-3 in tube 2 through tube 10. Place 16 ml of 160 $\mu\text{g/ml}$ drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2, continue the serial dilution to tube 10.

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- vi) Starting with the Falcon centrifuge tube containing the 0.03 µg/ml concentration of amphotericin B, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 5.0 ml of the concentrate with the Eppendorf repeating pipette into the 5.0 ml tip. Starting with the 0.03 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.03 tubes receive 100 µl. Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.06 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 µl of the desired concentrations.
 - vii) Place the tube racks into the -70°C freezer.
- (c) Storage conditions: -70°C freezer
 - (d) Shelf life: 6 months
 - (e) Quality control:
 - i) Sterility
 - ii) Each set of tubes should demonstrate an MIC of 0.25 µg/ml to 1.0 µg/ml range for *Candida albicans* (ATCC 90028).
- (2) **Fluconazole (FLU)** - FLU is a new difluorophenyl *bis*-triazole derivative that appears to possess a broader spectrum of activity than MON or KETO and is additionally much more water soluble. This latter attribute results in the attainment of substantial levels of FLU in biological fluids in contrast to other azole antifungal agents.
- (a) Formulation

10 mg of Fluconazole
1 ml dimethylformamide (DMF)
15.6 ml RPMI-1640 medium

$$C_1 \times V_1 = C_2 \times V_2$$
$$10,000 \mu\text{g/ml} \times 1 \text{ ml} = 640 \mu\text{g/ml} \times V_2$$
$$V_2 = (10,000 \mu\text{g/ml} \times 1 \text{ ml}) / 640 \mu\text{g/ml} = 15.63 \text{ ml}$$
 - (b) Preparation
 - i) Weigh out 10 mg of fluconazole using the Mettler balance.
 - ii) Dissolve with 1 ml of sterile DMF.
 - iii) Dilute 10,000 µg/ml fluconazole in 14.63 ml of RPMI medium to make an initial concentration of 640.0 µg/ml.
NOTE: This will dispense 11 racks of fluconazole MIC tubes.
 - iv) Make up 11 racks of 12x75 Falcon tubes labeled 64-00.125 µg/ml (i.e., 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125). Label tube 64 with the name of the drug → FLUC.
 - v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled as 640 µg/ml, tube 2 labeled as 32 µg/ml, and so on to tube 10 labeled at 0.125 µg/ml. Place 8 ml of RPMI in tube 2 through tube 10. Place 16 ml of 640 µg/ml drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2, continue the serial dilution to tube 10.

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- vi) Starting with the Falcon centrifuge tube containing the 0.125 µg/ml concentration of fluconazole, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 2.5 ml of the concentrate with the Eppendorf repeating pipette into the 2.5 ml tip. Starting with the 0.125 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.125 µg/ml tubes receive 100 µl. Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.25 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 µl of the desired concentrations.
 - vii) Place the tube racks into the -70°C freezer.
- (c) Storage conditions: -70°C freezer
- (d) Shelf life: 6 months
- (e) Quality control:
- i) Sterility
 - ii) Each set of tubes should demonstrate an MIC of ≤0.125 µg/ml to 0.5 µg/ml for *Candida albicans* (ATCC 90028).
- (3) **Fluorocytosine (5-FC)** - 5-FC (fluorocytosine; Ancobon; Roche Laboratories, Nutley, N.J.) is a fluorinated pyrimidine that acts as a true antimetabolite. In addition to inhibiting cellular multiplication, 5-FC may exert a fungicidal effect dependent on the drug concentration and period of exposure. Cytosine permease transports 5-FC across the fungal membrane and into the fungal cell, where it is deaminated by cytosine deaminase to 5-fluorouracil. Fungi that lack cytosine deaminase are resistant to 5-FC. 5-Fluorouracil results in faulty protein synthesis and has an effect on fungal nucleic acids. The 5-FC used in susceptibility testing is pure assayed powder with a potency of 100% unless specified otherwise.
- (a) Formulation
- 5 mg of 5-Fluorocytosine
1 ml sterile water
7.8 ml of RPMI-1640 medium
- $$C_1 \times V_1 = C_2 \times V_2$$
- $$5000 \mu\text{g/ml} \times 1 \text{ ml} = 640 \mu\text{g/ml} \times V_2$$
- $$V_2 = (5000 \mu\text{g/ml} \times 1 \text{ ml}) / 640 \mu\text{g/ml} = 7.81 \text{ ml}$$
- (b) Preparation
- i) Weigh out 5 mg of 5-fluorocytosine powder using the Mettler balance.
 - ii) Dissolve in 1 ml of sterile water.
NOTE: When placed in a 56°C water bath, it will dissolve faster and more efficiently.
 - iii) Dilute 1 ml (5000 µg/ml) 5-FC in 6.8 ml of RPMI medium to make an initial concentration of 640.0 µg/ml.
 - iv) Prepare 6 racks of 12x75 Falcon tubes labeled 64-0.125 µg/ml (i.e., 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125). Label tube 64 µg/ml with the name of the drug → 5-FC.

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- v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled as 640 µg/ml, tube 2 labeled as 320 µg/ml and so on to tube 10 labeled as 0.125 µg/ml. Place 7.8 ml of 640 µg/ml drug concentration in tube 1. Perform a serial dilution of 4 ml from tube 1 to tube 2; continue the serial dilution to tube 10.
 - vi) Starting with the Falcon centrifuge tube containing the 0.125 µg/ml concentration of 5-FC, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 2.5 ml of the concentrate with the Eppendorf repeating pipette into the 2.5 ml tip. Starting with the 0.125 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.125 tubes receive 100 µl. Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.25 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 µl of the desired concentrations.
 - vii) Place the tube racks into the -70°C freezer.
- (c) Storage conditions: -70°C freezer
 - (d) Shelf life: 6 months
 - (e) Quality control:
 - i) Sterility
 - ii) Each set of tubes should demonstrate an MIC of 1.0 µg/ml to 4.0 µg/ml range for *Candida albicans* (ATCC 90028).
- (4) Ketoconazole (KETO) - KETO is a dioxolane-imidazole derivative with broad-spectrum antifungal activity. Like MON, its mode of action is the inhibition of ergosterol synthesis. Additional mechanisms of action have also been identified, e.g., respiration effects involving cytochrome P-450. KETO (Janssen Pharmaceutical, Beerse, Belgium) is available as a pure assayed powder. Store in a desiccator at 2 to 8°C.

(a) Formulation

5 mg of Ketoconazole
1 ml of dimethylsulfoxide (DMSO)
31 ml of RPMI-1640 medium
 $C_1 \times V_1 = C_2 \times V_2$
 $5000 \mu\text{g/ml} \times 1 \text{ ml} = 160 \mu\text{g/ml} \times V_2$
 $V_2 = (5000 \mu\text{g/ml} \times 1 \text{ ml}) / 160 \mu\text{g/ml} = 31.25 \text{ ml}$

(b) Preparation

- i) Weigh out 5 mg of ketoconazole powder (USPA) using the Mettler balance.
- ii) Dissolve in 1 ml of DMSO.
- iii) Dilute 1 ml (5000 µg/ml) ketoconazole in 30.25 ml of RPMI 1640 to make an initial concentration of 160.0 µg/ml.
- iv) Prepare 12 racks of 12x75 Falcon tubes labeled 16-0.03 µg/ml (i.e., 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03). Label tube 16 with the name of the drug → KETO.

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- v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled as 160 $\mu\text{g/ml}$, tube 2 labeled as 80 $\mu\text{g/ml}$ and so on to tube 10 labeled at 0.03 $\mu\text{g/ml}$. Place 8 ml of RPMI in tube 2 through tube 10. Place 16 ml of 160 $\mu\text{g/ml}$ drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2; continue the serial dilution to tube 10.
 - vi) Starting with the Falcon centrifuge tube containing the 0.03 $\mu\text{g/ml}$ concentration of ketoconazole, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 μl (0.1 ml). Draw up 5 ml of the concentrate with the Eppendorf repeating pipette into the 5 ml tip. Starting with the 0.03 $\mu\text{g/ml}$ culture tubes, uncap one culture tube and dispense 100 μl into the culture tube; recap, and continue until all the 0.03 tubes receive 100 μl . Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.06 $\mu\text{g/ml}$ into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 μl of the desired concentrations.
 - vii) Place the tube racks into the -70°C freezer.
- (c) Storage conditions: -70°C freezer
 - (d) Shelf life: 6 months
 - (e) Quality control:
 - i) Sterility
 - ii) Each set of tubes should demonstrate an MIC of 0.03 $\mu\text{g/ml}$ to 0.125 $\mu\text{g/ml}$ for *Candida albicans* (ATCC 90028).
- (5) Miconazole (MON) - Miconazole is one of several synthetic antifungal compounds. The imidazoles have broad-spectrum activity. MON is a phenethyl imidazole derivative whose mode of action with the synthesis of ergosterol via inhibition of sterol C-14 demethylation, thus exerting major effects on fungal cell membranes. Additional mechanisms of action involve cytochrome P-450.

(a) Formulation

5 mg of Miconazole
1 ml of dimethylsulfoxide (DMSO)
18 ml of RPMI-1640 medium
 $C_1 \times V_1 = C_2 \times V_2$
 $2900 \mu\text{g/ml} \times 1 \text{ ml} = 160 \mu\text{g/ml} \times V_2$
 $V_2 = (2900 \mu\text{g/ml} \times 1 \text{ ml}) / 160 \mu\text{g/ml} = 18.13 \text{ ml}$

(b) Preparation

- i) Weigh out 2.9 mg of miconazole powder with the Mettler balance.
- ii) Dissolve the powder in 1 ml of DMSO.
- iii) Dilute 1 ml (2900 $\mu\text{g/ml}$) miconazole with 17.13 ml of RPMI-1640 to make an initial concentration of 160.0 $\mu\text{g/ml}$.
- iv) Prepare 12 racks of 12x75 Falcon tubes labeled 16-0.03 $\mu\text{g/ml}$ (i.e., 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03). Label tube 16 with the name of the drug \rightarrow MON.

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- v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled as 160 $\mu\text{g/ml}$, tube 2 labeled as 80 $\mu\text{g/ml}$ and so on to tube 10 labeled as 0.03 $\mu\text{g/ml}$. Place 4 ml of RPMI in tube 2 through tube 10. Place 8 ml of 160 $\mu\text{g/ml}$ drug concentration in tube 1. Perform a serial dilution of 4 ml from tube 1 to tube 2; continue the serial dilution to tube 10.
 - vi) Starting with the Falcon centrifuge tube containing the 0.03 $\mu\text{g/ml}$ concentration of miconazole, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 μl (0.1 ml). Draw up 2.5 ml of the concentrate with the Eppendorf repeating pipette into the 2.5 ml tip. Starting with the 0.03 $\mu\text{g/ml}$ culture tubes, uncap one culture tube and dispense 100 μl into the culture tube; recap, and continue until all the 0.03 tubes receive 100 μl . Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.06 $\mu\text{g/ml}$ into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 μl of the desired concentrations.
 - vii) Place the tube racks into the -70°C freezer.
- (c) Storage conditions: -70°C freezer
 - (d) Shelf life: 6 months
 - (e) Quality control:
 - i) Sterility
 - ii) There is no set MIC.

(6) Itraconazole (ITRA)

(a) Formulation

5 mg of Itraconazole

1 ml of polyethylene glycol (PEG)

30 ml of RPMI-1640 medium

$$C_1 \times V_1 = C_2 \times V_2$$

$$5000 \mu\text{g/ml} \times 1 \text{ ml} = 160 \mu\text{g/ml} \times V_2$$

$$V_2 = (5000 \mu\text{g/ml} \times 1 \text{ ml}) / 160 \mu\text{g/ml} = 31.25 \text{ ml}$$

(b) Preparation

- i) Weigh out 5 mg of itraconazole powder with the Mettler balance.
- ii) Dissolve in 1 ml of PEG.
NOTE: Heat in 56°C water bath to dissolve powder. Add drug to RPMI at room temperature.
- iii) Dilute 1 ml (5000 $\mu\text{g/ml}$) itraconazole in 30.25 ml of RPMI medium to make an initial concentration of 160.0 $\mu\text{g/ml}$.
- iv) Make up 12 racks of 12x75 Falcon tubes labeled 16-0.03 $\mu\text{g/ml}$ (i.e., 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03). Label tube 16 with the name of the drug \rightarrow ITRA.

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- v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled as 160 µg/ml, tube 2 labeled as 80 µg/ml and so on to tube 10 labeled as 0.03 µg/ml. Place 8 ml of RPMI in tube 2 through tube 10. Place 16 ml of 160 µg/ml drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2; continue the serial dilution to tube 10.
 - vi) Starting with the Falcon centrifuge tube containing the 0.03 µg/ml concentration of itraconazole, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 5 ml of the concentrate with the Eppendorf repeating pipette into the 5 ml tip. Starting with the 0.03 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.03 tubes receive 100 µl. Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.06 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 µl of the desired concentrations.
 - vii) Place the tube racks into the -70°C freezer.
- (c) Storage conditions: -70°C freezer
 - (d) Shelf life: 6 months
 - (e) Quality control:
 - i) Sterility
 - ii) Each set of tubes should demonstrate an MIC of ≤ 0.03 µg/ml to 0.5 µg/ml range for *Candida albicans* (ATCC 90028).

(7) Clotrimazole (CLOT)

- (a) Formulation
2.9 mg of Clotrimazole
1 ml of ethanol (25%)
 $C_1 \times V_1 = C_2 \times V_2$
 $2900 \mu\text{g/ml} \times 1 \text{ ml} = 160 \mu\text{g/ml} \times V_2$
 $V_2 = (2900 \mu\text{g/ml} \times 1 \text{ ml}) / 160 \mu\text{g/ml} = 18.13 \text{ ml}$
- (b) Preparation - **Follow instructions for Miconazole.**

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(8) Nystatin (NYS)

(a) Formulation

$$\text{Weigh (mg)} = \frac{\text{Potency Factor} \times \text{Concentration } (\mu\text{g/ml})}{1000 \mu\text{g/ml}}$$

(b) Sufficient material is weighed to prepare a solution of 5000 $\mu\text{g/ml}$.

- i) 1 ml DMSO
- ii) RPMI-1640

$$\begin{aligned} C_1 \times V_1 &= C_2 \times V_2 \\ 5000 \mu\text{g/ml} \times 1 \text{ ml} &= 185 \mu\text{g/ml} \times V_2 \\ V_2 &= (5000 \mu\text{g/ml} \times 1 \text{ ml}) / 185 \mu\text{g/ml} = 27 \text{ ml} \end{aligned}$$

(c) Preparation

- i) Weigh enough drug to make a 5000 $\mu\text{g/ml}$ using solution.
- ii) Dissolve in 1 ml DMSO.
- iii) Dilute 1 ml (5000 $\mu\text{g/ml}$) nystatin in 27 ml of RPMI medium to make an initial concentration of 185.0 $\mu\text{g/ml}$.
- iv) Prepare 6 racks of 12x75 Falcon tubes labeled 18.5-0.07 $\mu\text{g/ml}$ (i.e., 18.5, 9.25, 4.6, 2.3, 1.15, 0.58, 0.29, 0.14, 0.07). Label tube 18.5 with the name of the drug \rightarrow NYS.
- v) Label 10-50 ml Falcon centrifuge tubes starting with tube 1 labeled as 185 $\mu\text{g/ml}$, tube 2 labeled as 9.25 $\mu\text{g/ml}$ and so on to tube 10 labeled as 0.07 $\mu\text{g/ml}$. Place 4 ml of RPMI in tube 2 through tube 10. Place 8 ml of 160 $\mu\text{g/ml}$ drug concentration in tube 1. Perform a serial dilution of 4 ml from tube 1 to tube 2; continue the serial dilution to tube 10.
- vi) Starting with the Falcon centrifuge tube containing the 0.07 $\mu\text{g/ml}$ concentration of nystatin, pour the concentration into a reservoir. Set the Eppendorf repeating pipette to dispense 100 μl (0.1 ml). Draw up 5 ml of the concentrate with the Eppendorf repeating pipette into the 5 ml tip. Starting with the 0.07 $\mu\text{g/ml}$ culture tubes, uncap one culture tube and dispense 100 μl into the culture tube; recap, and continue until all the 0.07 tubes receive 100 μl . Discard the reservoir. Pour out the contents of the centrifuge tube containing 0.14 $\mu\text{g/ml}$ into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 μl of the desired concentrations.
- vii) Place the tube racks into the -70°C freezer.

- (d) Storage conditions: -70°C freezer
- (e) Shelf life: 6 months

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- (f) Quality control:
 - i) Sterility
 - ii) Each set of tubes should demonstrate an MIC of 0.57 µg/ml to 4.6 µg/ml range for *Candida albicans* (ATCC 90028).

(9) Natamycin

(a) Formulation

- i) Determine the amount of Natamycin to weight by using the following formula:

$$\text{Weigh (mg)} = \frac{\text{Potency Factor} \times \text{Concentration } (\mu\text{g/ml})}{1000 \mu\text{g/ml}}$$

EXAMPLE:

Desired volume = 3 ml

Desired concentration = 6400 µg/ml

Potency = whatever is on the label of the powdered drug

X = [3 ml x 6400 µg/ml]/960 µg/ml

- a) NaOH (0.5N)
- b) Ncl (0.5N) x 20 mg (0.020 g)

(b) Preparation

- i) Weigh out 20 mg of natamycin.
- ii) Dissolve in 30 mls NaOH (0.5N).
- iii) Within 30 minutes, neutralize with 0.5N HCl to a pH of 6.0 to 7.0.
- iv) Bring the volume to 10 ml with sterile water. Resulting concentration is 1922 µg/ml. Store at 4°C away from light.
- v) Dilute 1:10 (1 ml of solution and 9 ml sterile water) for a concentration of 192 µg/ml.
- vi) Prepare racks of 12x75 mm Falcon tubes labeled 19.2, 9.6, 4.8, 2.4, 1.2, 0.6, 0.3, and 0.15. Write on tube 19.2, the name of the drug → NATA.
- vii) Label 8-50 ml Falcon centrifuge tubes starting with tube 1 labeled 19.2, tube 2 labeled 9.6, and continuing until tube 8 is labeled 0.15. Add 4 ml of RPMI to tubes 2-8.
- viii) To tube 1, add 1 ml of 192 µg/ml solution to 10 mls of RPMI; serial dilute by transferring 4 mls.
- ix) Dispense 0.1 ml of antifungal solution in the 12x75 Falcon tubes labeled with appropriate concentration.

(c) Storage conditions: -70°C freezer for 1 year

- NOTE: (1) Sensitive to light.
(2) Solubility in H₂O and most organic solvents is extremely low.
(3) At pH 3 or 9, natamycin solution is less stable.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF YEASTS: (cont'd)

b. Inoculum Preparation

- (1) Streak QC strain *C. albicans* and test isolates on SDA. Incubate overnight in an ambient-air incubator at 35°C, and check for purity.
- (2) Pick three to five isolated colonies of similar colony morphology, and passage again to SDA. Use this plate for the initial inoculum preparation.
- (3) Using the tip of a sterile applicator stick, pick five isolated colonies of similar colony morphology at least 1 mm in diameter, and add to 5 ml of sterile 0.85% NaCl.
- (4) Vortex for 15 to 20 s.
- (5) Adjust the suspension to 85% transmittance at 530 nm using a spectrophotometer by adding sterile 0.85% NaCl as necessary. Resulting suspension = 1×10^6 to 5×10^6 CFU/ml
- (6) Add 1 ml of suspension to 9 ml of RPMI-1640. Resulting suspension equals 1×10^5 to 5×10^5 CFU/ml.
- (7) Tubes of this suspension may be held at 2 to 8°C for up to 3 h.

c. Inoculation and Incubation

- (1) Remove appropriate number of previously prepared drug concentration tubes from -70°C storage, and allow them to thaw at 25°C.
- (2) Arrange drug concentration tubes for each antifungal agent in ascending order, with the highest concentration on the left.
- (3) Include two empty tubes designated as the positive and negative growth controls, at the far right.
- (4) Calculate the volume of standardized inoculum-broth suspension needed for the QC strain and each isolate (1 ml of 1×10^5 - to 5×10^5 -CFU/ml suspension for every 9 ml of RPMI required, or a 1:10 dilution). Final concentration is 1×10^4 to 5×10^4 CFU/ml.
- (5) Using a 5-ml serological pipette, add 0.9 ml of the final inoculum to each drug concentration tube. This dilutes the drug concentration 1:10 to obtain the concentration indicated on the tube. After setting up each ten tubes, vortex the inoculum suspension to resuspend the yeast.
- (6) Growth controls:
 - (a) For positive growth control, add 0.9 ml of final inoculum to 0.1 ml of broth.
 - (b) For negative growth control, add 1 ml of broth to tube.
 - (c) Purity plate (inoculum count verification)
 - (d) Using a 100 ul pipette, place 0.01 ml (10 µl) of broth inoculum on an SDA plate, and streak evenly over the entire surface.
 - (e) Invert plate, and incubate it at 35°C for 24 to 48 h, or until colonies are visible for accurate counting. Record the colony count on worksheet.
- (7) Solvent control: Place 0.01 ml of inoculum into tube containing solvent diluted in RPMI or M-3.
- (8) Incubate purity plates and MIC tubes in 35°C ambient-air incubator. Shake the tubes after inoculating them.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF YEASTS: (cont'd)

d. Reading MICs

- (1) Read MICs at 24 and 48 h, and score as follows.
 - (a) 0 = optically clear
 - (b) 1+ = slightly hazy
 - (c) 2+ = prominent reduction in turbidity compared with that of the drug-free growth control
 - (d) 3+ = slight reduction in turbidity compared with that of the drug-free growth control
 - (e) 4+ = no reduction in turbidity compared with that of the drug-free growth control
- (2) Beginning with the lowest concentration and working toward the highest concentration for each drug, grasp the drug-free control plus one or two drug-inoculum tubes by the caps, and hold them up to view by transmitted light.
- (3) Using the thumb and forefinger of the opposite hand, gently flick each tube, and determine its score. Using a vortex will help homogenize the organism suspension.
 - (a) The MICs for AMB, NATA, and NYS are the lowest concentrations with a score of 0.
 - (b) The MICs for all other drugs are the lowest concentrations with a score of 2+. This will correspond to 50%-80% inhibition.
 - (c) Record results on the worksheet.

e. Plating and reading MFCs

- (1) Using a micropipette, remove separate 100- μ l samples from the MIC tube, each higher-concentration tube, and the positive growth control tube.
- (2) Using the tip of the micropipette, spread each 100- μ l sample over half the surface of an SDA plate. Incubate the plates at 35°C.
- (3) Read plates when colonies on the growth control plate are visible, usually 24 h, and again at 48 h.
 - (a) Any plate with five colonies or fewer is negative.
 - (b) The lowest concentration for which the subculture is negative is the MFC.

6. Results

- a. Interpretation - Providing QC isolate MIC values is acceptable, interpret results accordingly.
- b. Reporting - Providing QC is acceptable, report accordingly.

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF YEASTS: (cont'd)

7. Procedure Notes

a. Principle:

- (1) As infections caused by yeasts continue to increase, resistance to antifungal agents continues to occur, *in vitro* antifungal susceptibility testing will continue to increase in a significant manner. Critical factors influencing test variability include inoculum preparation, medium composition, pH, length of incubation, and the method of endpoint determination. This macrobroth dilution antifungal susceptibility testing protocol incorporates data regarding these variables.
- (2) It is advisable for most antifungal testing to be done at reference laboratories.
- (3) The majority of clinically significant yeast isolates are amenable to testing by this procedure. One exception is *Malassezia furfur*, which requires an oil overlay for growth, precludes testing by this method.

b. QC:

- (1) Controls are set up with each drug tested to ensure that the test is providing reproducible data. Each susceptibility test must include a QC strain that provides known and reproducible MICs. The laboratorian must monitor the MIC results with the QC strain as a means to monitor drug potency.
 - (a) The growth control tube ensures that the medium is functioning appropriately and the inoculum is actively growing.
 - (b) The negative broth control provides evidence that the medium is sterile.
 - (c) The inoculum verification plate confirms that the inoculum is properly standardized.
 - (d) Out-of-control results indicate that the test must be repeated.
- (2) The method of Pasarell and McGinnis (108) of storing isolates at -70°C is a simple, effective method for the long-term preservation of most fungi.

c. Procedures:

- (1) Actively growing yeast colonies 14 to 48 hrs old are required for testing. Most clinical isolates have adequate growth within 24 h, but *Cryptococcus neoformans* var. *neoformans* may require 48 h.
- (2) To provide an adequate volume of inoculum plus RPMI at the desired final concentration (1×10^4 to 5×10^4) for inoculation of all drug and control tubes, count the tubes to be used and divide by 9 to determine the amount of RPMI plus standardized inoculum required.
- (3) The test should be incubated at 35°C . Incubation at a lower temperature often results in lower MICs.
- (4) A yeast that does not produce adequate growth after 24 h of incubation is read at 48 and 72 h.
- (5) When reading antifungal MFCs, the cutoff of five colonies or fewer is roughly equivalent to 99.9% killing.
- (6) Report results based upon 48 h of incubation.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF YEASTS: (cont'd)

8. Limitations of the Procedure

- a. The methods described here are similar to those being used in multicenter collaborative studies by the Subcommittee on Antifungal Susceptibility Testing of the NCCLS. To date, no NCCLS standards have been published.
- b. Problems with trailing endpoints for the azoles in this macrobroth dilution procedure exist. Agar dilution procedures reportedly eliminate this problem, but MFCs cannot be determined by that method.

II. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS:

A. Principle:

Antifungal drug dilutions are used to determine the minimal inhibitory concentration (MIC) for antifungal agents. Serial dilutions of antifungal agents are dispensed into appropriately labeled tubes. Each tube is then inoculated with a standardized nutrient broth suspension of the fungus being tested. Susceptibility testing of moulds is being evaluated in a multicenter collaborative study by the Subcommittee on Antifungal Susceptibility Testing of the NCCLS. This procedure could be used for dermatophytes only if an appropriate control isolate is used. No proposed method has been developed.

- B. *Specimen*: Growth from three to five isolated colonies of similar colony morphology grown for 3-5 days on potato dextrose agar (PDA) slants.

- C. *Materials*: Date and label all reagents and media when prepared and placed in use.

1. Media and Reagents (storage conditions)

- a. SDA plates, Emmons modification, pH 7 (2-8°C).
- b. SDA slants, Emmons modification, pH 7 (2-8°C).
- c. RPMI-1640 medium buffered with 0.165 M MOPS [3-(*N*-morpholino) propanesulfonic acid] containing L-glutamine and lacking sodium bicarbonate; supplied dehydrated by American Biorganics, Inc., Niagara Falls, N.Y.; catalog no. R63165 (2 to 8°C; shelf life, 12 months).
- d. Bacto Antibiotic Medium 3 (M-3) (2-8°C; 3 months)
- e. Sterile distilled reagent-grade water (25°C).
- f. Sterile 0.85% NaCl (25°C).
- g. Dimethyl sulfoxide (DMSO) (25°C), polyethylene glycol (PEG) (MW 200), and dimethylformamide (DMF).
- h. Macrobroth dilution tubes (sterile, 12x75 mm, polystyrene, snap cap) containing 0.1 ml volumes of the following antimicrobial agents (diluted in RPMI-1640 except AMB which is in M-3) at 10 times the final concentrations (see Section V) maintained at -70°C.
 - (1) Amphotericin B (AMB), 0.03 to 16 µg/ml
 - (2) Fluconazole (FLU), 0.125 to 128 µg/ml
 - (3) Itraconazole (ITRA) 0.03 to 32 µg/ml
 - (4) Ketoconazole (KETO), 0.3 to 16 µg/ml
 - (5) Miconazole (MON), 0.03 to 16 µg/ml

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. *IN VITRO* ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS: (cont'd)

i. Potato Dextrose Agar Slants (PDA) (2-8°C)

2. Supplies

- a. Sterile wooden applicator sticks or sterile swabs
- b. Sterile test tubes

- (1) 12x75 mm, polystyrene, snap cap
- (2) 13x100 mm, polystyrene, screw cap
- (3) 50 ml conical centrifuge

- c. 500 ml capacity Millipore filtration units with 0.22-µm-pore-size
- d. Sterile 1, 5, and 10 ml disposable serological pipettes and pipette bulb
- e. McFarland 0.5 standard

3. Equipment

- a. Spectrophotometer (Coleman Model 35)
- b. Vortex mixer
- c. Micropipette, 100 µl capacity with sterile disposable tips
- d. Repetitive dispensing pipette, 100 µl capacity with sterile disposable syringes
- e. Mettler balance
- f. 35°C ambient-air incubator

D. *Quality Control*:

1. QC strains

- a. *Candida albicans* (ATCC 90028) and *Paecilomyces variotii* (ATCC 36257)
- b. Maintain permanent stock cultures at -70°C on PDA slants.
- c. Prepare working stock culture.

- (1) With sterile wooden applicator stick, chip off a small portion of the frozen surface of an SDA slant and streak for isolation.
- (2) Weekly working stock cultures are prepared by subculturing three to five colonies of similar colony morphology from the previous week's working stock plate to a fresh SDA plate. Moulds are subcultured to a fresh PDA slant.

- d. Subculture from working stock is plated to another SDA plate or PDA slant on the day before use.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS: (cont'd)

e. See below for expected results.

<i>Candida albicans</i> (ATCC 90028)		<i>Paecilomyces variotii</i> (ATCC36257)	
AMB	0.25-1.0 µg/ml	AMB	0.25-1.0 µg/ml
FLU	≤ 0.125-0.5 µg/ml	FLU	1.0-4.0 µg/ml
ITRA	≤0.3-0.5 µg/ml	ITRA	0.03-0.5 µg/ml
KETO	≤0.03-0.125 µg/ml	KETO	0.06-0.5 µg/ml
MON	NA	MON	0.03-0.5 µg/ml

f. Record results on QC form.

2. Positive growth control should demonstrate good growth and be free of contaminating organisms.
3. Negative growth control should be free of growth.
4. Inoculum count verification plate should show several colonies.
5. Solvent growth control should demonstrate good growth.
6. Test considered in control when:
 - a. MIC's for QC strains are within acceptable limits.
 - b. Controls show appropriate growth.
 - c. Results are appropriate for isolate tested.

E. Procedure:

1. Preparation of antifungal drugs and dilution schemes:

a. Amphotericin B (AMB)

(1) Formulation

5 mg of Amphotericin B
1 ml dimethylsulfoxide (DMSO)
31 ml M-3 medium
 $C_1 \times V_1 = C_2 \times V_2$
 $5000 \mu\text{g/ml} \times 1 \text{ ml} = 160 \mu\text{g/ml} \times V_2$
 $V_2 = (5000 \mu\text{g/ml} \times 1 \text{ ml}) / 160 \mu\text{g/ml} = 31.25 \text{ ml}$

(2) Preparation

- (a) Weigh out 5 mg of amphotericin B powder using the Mettler balance.
- (b) Dissolve the powder with 1 ml of DMSO.
NOTE: Keep the amphotericin B in darkness as much as possible because light degrades the amphotericin B.
- (c) Dilute 1 ml (5000 µg/ml) amphotericin B in 30.25 ml of M-3 medium to make an initial concentration of 160.0 µg/ml.
NOTE: 30 ml of amphotericin B at 160 µg/ml is enough to dispense into 15 racks of tubes. However, instructions are for setting up 12 racks.
- (d) Make up 12 racks of 12x75 Falcon tubes labeled 16-0.03 µg/ml (i.e., 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03). Label tube 16 with the name of the drug → AMB.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS: (cont'd)

- (e) Label 10, 50 ml Falcon centrifuge tubes starting with tube 1 labeled as 160 µg/ml, tube 2 labeled as 80 µg/ml, and so on to tube 10. Place 8 ml of M-3 in tube 2 through tube 10. Place 16 ml of 160 µg/ml drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2, continue the serial dilution to tube 10.
 - (f) Starting with the Falcon centrifuge tube containing the 0.3 µg/ml concentration of amphotericin B, pour the drug solution into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 5.0 ml of the concentrate with the Eppendorf repeating pipette into the 5.0ml tip. Starting with the 0.03 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.03 tubes receive 100 µl. Discard the reservoir. Pour out the content of the centrifuge tube containing 0.6 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all ten sets of tubes receive 100 µl of the desired concentrations.
 - (g) Place the tube racks into the -70°C freezer.
- (3) Storage conditions: -70°C freezer
 - (4) Shelf life: 6 months
 - (5) Quality control:
 - (a) Sterility
 - (b) Each set of tubes should demonstrate an MIC for *C. albicans* (ATCC 90028) of 0.25 µg/ml to 1.0 µg/ml and *P. variotii* (ATCC 36257) of 0.25 µg/ml to 1.0 µg/ml.

b. Fluconazole (FLU)

(1) Formulation

20 mg of Fluconazole
1 ml dimethylformamide (DMF)
15.6 ml RPMI-1640 medium
 $C_1 \times V_1 = C_2 \times V_2$
 $20,000 \mu\text{g/ml} \times 1 \text{ ml} = 1280 \mu\text{g/ml} \times V_2$
 $V_2 = (20,000 \mu\text{g/ml} \times 1 \text{ ml}) / 1280 \mu\text{g/ml} = 15.63 \text{ ml}$

(2) Preparation

- (a) Weigh out 20 mg of fluconazole using the Mettler balance.
- (b) Dissolve with 1 ml of DMF.
- (c) Dilute 1 ml (20,000 µg/ml) fluconazole in 14.63 ml of RPMI to make an initial concentration of 1280 µg/ml.
NOTE: This will dispense 11 racks of FLU MIC tubes.
- (d) Make up 11 racks of 12x75 Falcon tubes labeled 128-0.125 µg/ml (i.e., 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125). Label tube 128 with the name of the drug → FLU.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS: (cont'd)

- (e) Label 11, 50 ml Falcon centrifuge tubes starting with tube 1 as 1280 µg/ml, tube 2 labeled at 640 µg/ml, and so on to tube 11 labeled at 1.25 µg/ml. Place 8 ml of RPMI in tube 2 through tube 11. Place 16 ml of 1280 µg/ml drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2, continue the serial dilution to tube 11.
 - (f) Starting with the Falcon centrifuge tube containing the 1.25 µg/ml concentration of Fluconazole, pour the drug solution into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 2.5 ml of the concentrate with the Eppendorf repeating pipette into the 2.5 ml tip. Starting with the 0.125 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.125 tubes receive 100 µl. Discard the reservoir. Pour out the content of the centrifuge tube containing 0.25 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all 11 sets of tubes receive 100 µl of the desired concentrations.
 - (g) Place the tube racks into the -70°C freezer.
- (3) Storage conditions: -70°C freezer
 - (4) Shelf life: 6 months
 - (5) Quality control:
 - (a) Sterility (should remain uncontaminated)
 - (b) Each set of tubes should demonstrate an MIC for *C. albicans* (ATCC 90028) of ≤0.125 µg/ml to 0.5 µg/ml and *P. variotii* (ATCC 36257) of 1.0 µg/ml to 4.0 µg/ml.

c. Itraconazole (ITRA)

(1) Formulation

10 mg of Itraconazole
1 ml of polyethylene glycol (PEG)
30 ml of RPMI-1640 medium
 $C_1 \times V_1 = C_2 \times V_2$
 $10,000 \mu\text{g/ml} \times 1 \text{ ml} = 320 \mu\text{g/ml} \times V_2$
 $V_2 = (10,000 \mu\text{g/ml} \times 1 \text{ ml}) / 320 \mu\text{g/ml} = 31.25 \text{ ml}$

(2) Preparation

- (a) Weigh out 10 mg of itraconazole powder using the Mettler balance.
- (b) Dissolve in 1 ml of PEG.
- (c) Dilute 1 ml (10,000 µg/ml) itraconazole in 30.25 ml of RPMI to make an initial concentration of 320 µg/ml.
- (d) Make up 12 racks of 12x75 Falcon tubes labeled 32-0.03 µg/ml (i.e., 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03). Label tube 32 with the name of the drug → ITRA.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS: (cont'd)

- (e) Label 11, 50 ml Falcon centrifuge tubes starting with tube 1 labeled as 320 µg/ml, tube 2 labeled as 160 µg/ml and so on to tube 11 labeled as 0.3 µg/ml. Place 8 ml of RPMI in tube 2 through tube 11. Place 16 ml of 320 µg/ml drug concentration in tube 1. Perform a serial dilution of 8 ml from tube 1 to tube 2; continue the serial dilution to tube 11.
 - (f) Starting with the Falcon centrifuge tube containing the 0.3 µg/ml concentration of ITRA, pour the drug solution into a reservoir. Set the Eppendorf repeating pipette to dispense 100 µl (0.1 ml). Draw up 5 ml of the concentrate with the Eppendorf repeating pipette into the 5 ml tip. Starting with the 0.03 µg/ml culture tubes, uncap one culture tube and dispense 100 µl into the culture tube; recap, and continue until all the 0.03 tubes receive 100 µl. Discard the reservoir. Pour out the content of the centrifuge tube containing 0.6 µg/ml into a clean reservoir and repeat the process. Continue repeating the process until all 11 sets of tubes receive 100 µl of the desired concentrations.
 - (g) Place the tube racks into the -70°C freezer.
- (3) Storage conditions: -70°C freezer
 - (4) Shelf life: 6 months
 - (5) Quality control: Each set of tubes should demonstrate an MIC for *C. albicans* (ATCC 90028) of ≤0.03 µg/ml to 0.5 µg/ml and *P. variotii* (ATCC 36257) of 0.03 µg/ml to 0.5 µg/ml.

d. Ketoconazole and Miconazole (KETO and MON)

- (1) The tests are conducted as outlined in *In Vitro* Antifungal Susceptibility Testing of Yeasts.
- (2) *Paecilomyces variotii* (ATCC 36257) and *C. albicans* (ATCC 90028) are used as controls.

2. Inoculum Preparation

- a. Streak QC strain of *C. albicans* on SDA. Incubate overnight in an ambient-air incubator at 35°C and check for purity. Test isolates and *P. variotii* (ATCC 36257) will be grown on PDA slants. Two slants per mould are required. Some isolates of *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* may need to be subcultured to Emmons modification of SDA slants, rather than PDA slants in order to obtain better growth.
- b. Pick three to five isolated colonies of similar colony morphology and subculture again to SDA. Use this plate for the initial inoculum preparation. The QC isolate *C. albicans* is 24-48 h old, the test moulds are 48-72 h old. Slow growing moulds should be incubated up to 7 days before testing.
- c. Using the tip of a sterile applicator stick, pick five isolated colonies of similar colony morphology and add to 5 ml of sterile 0.85% NaCl. For moulds growing on slants, add 5 ml of sterile 0.85% NaCl to each tube, rub surface with a sterile wooden applicator stick, and then transfer suspension to a sterile tube.
- d. Vortex for 15 to 20 s.
- e. Adjust suspension to a McFarland 0.5 turbidity standard.
- f. Add 1 ml of suspension to 9 ml of RPMI 1640 or M-3 for amphotericin B (1:10 dilution).
- g. Tubes of this suspension may be held at 2 to 8°C for up to 3 h.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

I. *IN VITRO* ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND MOULDS: (cont'd)

3. Inoculation and Incubation

- a. Remove appropriate number of previously prepared drug concentration tubes from -70°C storage, and allow them to thaw at room temperature.
- b. Arrange drug concentration tubes for each antifungal agent in ascending order, with the highest concentration on the left. Include two empty tubes, designated as the positive and negative growth controls, at the far right, as well as 2 tubes labeled DMF, DMSO, or PEG control.
- c. Calculate the volume of standardized inoculum-broth suspension needed for QC strains and each isolate (1 ml McFarland 0.5 standard suspension for every 9 ml of either RPMI or M-3 is required).
- d. Using a 5-ml serological pipette, add 0.9 ml of the final inoculum to each drug concentration tube. This dilutes the drug concentration 1:10 to obtain the concentration indicated on the tube. Uncap each tube one at a time and recap. Every ten tubes vortex the inoculum suspension to resuspend the mould in the solution. Never leave the inoculum tube uncapped.
- e. Growth controls:
 - (1) For positive growth control, add 0.9 ml of final inoculum to 0.1 ml of broth.
 - (2) For negative growth control, add 1 ml of broth to tube.
 - (3) For DMF, DMSO, or PEG controls, add 0.1 ml of the 0.5% McFarland calibrated inoculum.
- f. Purity plate (inoculum count verification)
 - (1) Using a 100 μl pipette, place 0.01 ml (10 μl) of broth inoculum on an SDA plate, and streak evenly over the entire surface.
 - (2) Invert plate, and incubate it at 35°C for 48 to 96 h (or until colonies are visible for accurate counting). Record the colony count on worksheet.
- g. Incubate purity plates and MIC tubes in 35°C ambient-air incubator. Shake the tubes after inoculating them.

4. Reading MICs

- a. The test is read when the growth control shows adequate growth, which is typically 24-48 hours for most moulds, but it could be up to 96 hours.
- b. Read MICs the first day that the growth control shows growth and then 24 hours later. Score as follows.
 - (1) 0 = optically clear
 - (2) 1+ = slightly hazy
 - (3) 2+ = prominent reduction in turbidity compared with that of the drug-free growth control
 - (4) 3+ = slight reduction in turbidity compared with that of the drug-free growth control
 - (5) 4+ = no reduction in turbidity compared with that of the drug-free growth control
- c. Beginning with the lowest concentration and working toward the highest concentration for each drug, grasp the drug-free control plus one or two drug-inoculum tubes by the caps, and hold them up to view by transmitted light.

I. *IN VITRO* ANTIFUNGAL SUSCEPTIBILITY TESTING OF DIMORPHIC FUNGI AND

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

MOULDS: (cont'd)

- d. Using the thumb and forefinger of the opposite hand, gently flick each tube, and determine its score.
 - (1) The MICs for AMB are the lowest concentrations with a score of 0.
 - (2) The MICs for azoles are the lowest concentrations with a score of 2+. This will correspond to 50-80% inhibition.
 - (3) Record results on the worksheet.

F. Results:

1. Interpretation - Providing QC is acceptable, interpret results accordingly.
2. Reporting - Providing QC is acceptable, report accordingly in the worksheets.

G. Procedure Notes:

1. Principle - Although testing is currently nonstandardized, preliminary data being generated from multicenter studies by the Subcommittee on Antifungal Susceptibility Testing of the NCCLS suggest that acceptable inter- and intralaboratory agreements can be obtained. Critical technical factors influencing test variability include inoculum preparation, medium composition, pH, length of incubation, and the method of endpoint determination.
2. QC:
 - a. Controls are set up for each drug tested on a daily basis to ensure that the test is providing reproducible data.
 - b. Many methods for maintaining stock culture collections of fungi can be used. The method of Pasarell and McGinnis (108) of storing isolates at -70°C is an effective method for the preservation of fungi.
3. Procedures:
 - a. Actively growing fungal colonies are required for testing; thus the requirement for a 72- to 96-h subculture. Owing to slow growth rates, some moulds may require more than 96 h for adequate growth to be present.
 - b. The test described here is incubated at 35°C . Incubation at lower temperature (e.g., 30°C) often results in lower MICs. Lower incubation temperature may be required for moulds that do not actively grow at 35°C .
 - c. A fungus that does not produce adequate growth after 72 hrs of incubation may be encountered. When this occurs, read results at 96 h.
 - d. Report on the worksheet an initial (usually at 48 h) and second (72 h later) reading of MICs to provide information on antifungal activity over time. The optimum time for reading endpoints for various antifungal agents is currently being addressed by an NCCLS subcommittee.
 - e. The concentration marked on the tubes reflect the final drug concentration that they will contain.
4. Forms - See the following tables.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING

III. REFERENCES:

1. **McGinnis, M.R., and M.G. Rinaldi.** 1985. Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids, p.223-281. In V. Lorian (ed.), *Antibiotics in Laboratory Medicine*. The Williams & Wilkins Co., Baltimore.
2. **Rinaldi, M.G., and A.W. Howell.** 1988. Antifungal antimicrobics: laboratory evaluation, p. 325-356. In B. Wentworth (ed.), *Diagnostic Procedures for Mycotic and Parasitic Infections*, 7th ed. American Public Health Association, Washington, D.C.
3. **Walsh, T.J., G.P. Melcher, M.G. Rinaldi, J. Lecciones, D.A. McGough, P. Kelly, J. Lee, D. Callender, M. Rubin, and P.A. Pizzo.** 1990. *Trichosporon beigeli*, an emerging pathogen resistant to amphotericin B. *J. Clin. Microbiol.* **28**:1616-1622.
4. **Rex, J.H., C.R. Cooper, Jr., W.G. Merz, J.N. Galgiani, and E.J. Anaissie.** 1995. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. *Antimicrob. Agents Chemother.* **39**:906909.
5. **Pfaller, M.A., M. Bale, B. Buschelman, M. Lancaster, A. Espenel-Ingroff, J.H. Rex, M.G. Rinaldi, C.R. Cooper, and M.R. McGinnis.** 1995. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. *J. Clin. Microbiol.* **33**:1104-1107.

IV. SUPPLEMENTAL READING:

- Dick, J.D., B.R. Rosengard, W.G. Merz, R.K. Stuart, G.M. Hutchins, and R. Saral.** 1985. Fatal disseminated candidiasis due to amphotericin B-resistant *Candida guilliermondii*. *Ann. Intern. Med.* **102**:67-68.
- Doern, G.V., T.A. Tubert, K. Chopin, and M.G. Rinaldi.** 1986. Effect of medium composition on results of macrobroth dilution antifungal susceptibility testing of yeasts. *J. Clin. Microbiol.* **24**:507-511.
- Drutz, D.J., and R.I. Lehrer.** 1978. Development of amphotericin B-resistant *Candida tropicalis* in a patient with defective leukocyte function. *Am. J. Med. Sci.* **276**:77-92.
- Galgiani, J.N.** 1987. The need for improved standardization in antifungal susceptibility testing, p. 15-24. In R.A. Fromtling (ed.), *Recent Trends in the Discovery, Development and Evolution of Antifungal Agents*. J.R. Prous Science Publishers, S.A., Barcelona, Spain.
- Guinet, R., J. Chanas, A. Goullier, G. Bonnefoy, and P. Ambroise-Thomas.** 1983. Fatal septicemia due to amphotericin B-resistant *Candida lusitanae*. *J. Clin. Microbiol.* **18**:443-444.
- McGinnis, M.R.** 1980. Susceptibility testing and bioassay procedures, p. 412-446. In *Laboratory Handbook of Medical Mycology*. Academic Press, Inc., New York.
- Merz, W.G.** 1984. *Candida lusitanae*: frequency of recovery, colonization, infection, and amphotericin B resistance. *J. Clin. Microbiol.* **20**:1194-1195.
- NCCLS.** 1986. *Antifungal Susceptibility Testing*. Committee report, vol. 5, no. 17. NCCLS, Villanova, Pa.

X. SUPPLEMENTAL READING: (cont'd)

Pasarell, L. and M.R. McGinnis. 1992. Viability of Fungal Cultures Maintained at -70°C. *J. Clin. Microbiol.* 30(4): 1000-1004

Pfaller, M.A., Burmeister, M.S. Bartlett and M.G. Rinaldi. 1988. Multicenter evaluation of four methods of yeast inoculum preparation. *J. Clin. Microbiol.* **26**:1437-1441.

Pfaller, M.A., M.G. Rinaldi, J.N. Galgiani, M.S. Bartlett, B.A. Body, A. Espinel-Ingroff, R.A. Fromtling, G.S. Hall, C.E. Hughes, F.C. Odds, and A.M. Sugar. 1990. Collaborative investigation of variables in antifungal susceptibility testing of yeasts. *Antimicrob. Agents Chemother.* **34**:1648-1654.

Powderly, W.G., G.S. Kobayashi, G.P. Herzig, and G. Medoff. 1988. Amphotericin B-resistant yeast infection in severely immunocompromised patients. *Am. J. Med.* **84**:826-832.

ANTIFUNGAL SUSCEPTIBILITY TESTING WORKSHEET

Patient _____ Organism _____
 Sample # _____ Source of Isolate _____
 RPMI 1640 Lot # _____ pH _____ Date Prep _____
 M-3 Lot # _____ pH _____ Date Prep _____
 Date Rec'd _____ Tech _____ Date Report _____

INOCULUM STANDARDIZATION (1-5 x 10⁴ CFU/ml)

0.5 McFarland Std Spectrophotometric Inoculum Verification
 Wavelength _____ Colony Count _____
 % Transmittance _____ (Acceptable range 100-500)

MACRO BROTH DILUTION

Antifungal Agent		MIC			Drug "Set" Lot No.
Set Up Date	Reading Dates	(µg/ml)			
		24 h	48 h	72 h	
Amphotericin B					
Fluconazole					
5-Fluorocytosine					
Ketoconazole					
Miconazole					
Itraconazole					
Natamycin					
Clotrimazole					
Natamycin					
OTHER:					

Reviewed by: _____

