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# Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard


This document addresses the selection of antifungal agents, preparation of antifungal stock solutions and dilutions for testing implementation and interpretation of test procedures, and quality control requirements for susceptibility testing of filamentous fungi (moulds) that cause invasive fungal infections.

A standard for global application developed through the NCCLS consensus process.

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# Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard

## Abstract

NCCLS document M38-A—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard* describes a method for testing the susceptibility of filamentous fungi (moulds) that cause invasive fungal infections, including *Aspergillus* species, *Fusarium* species, *Rhizopus arrhizus, Pseudallescheria boydii (Scedosporium apiospermum),* and *Sporothrix schenckii* and other opportunistic pathogenic moulds to antifungal agents. Selection of antifungal agents, preparation of antifungal stock solutions and dilutions for testing, implementation, and interpretation of test procedures, and the purpose and implementation of quality control procedures are discussed. A careful examination of the responsibilities of the manufacturer and the user in quality control is also presented.

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# Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard

Volume 22 Number 16

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## Contents

## Contents (Continued)

## Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory methods to guide in the selection of antifungal therapy have gained greater attention. The NCCLS Area Committee on Microbiology formed the Subcommittee on Antifungal Susceptibility Testing and data were collected for testing yeasts in a series of collaborative studies. As a result, NCCLS document M27—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast,* was published with the establishment of MIC ranges and the development of breakpoints.

Based on these achievements, the subcommittee concluded that it would be useful to work toward a reproducible reference testing procedure for the antifungal susceptibility testing of filamentous fungi (moulds). A working group on filamentous fungi was formed and charged with the responsibility of carrying out studies to collect data and to refine the methodology to perform susceptibility testing of these fungal species. As a result of two collaborative studies, agreement within the subcommittee was achieved regarding testing conditions that included inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria for MIC determination.[1,2] An additional study has indicated some degree of correlation between *in vitro* test results and response to treatment in animal models. [2,3]

Because of its suitability for antifungal susceptibility testing of yeasts, synthetic RPMI-1640 medium was the test medium that the subcommittee evaluated as the potential reference medium for moulds.[1,2] The subcommittee has evaluated other medium formulations, but the standard RPMI medium facilitated a more consistent identification of itraconazole resistance in *Aspergillus* spp. in eight laboratories.[4] Drug stock solution preparation and dilution procedures previously developed for antifungal testing of yeasts procedures (M27) also were adopted.

### Standard Precautions

Because it is often impossible to know what might be infectious, all human blood, fluid, or tissue specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl. 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

### Key Words

Antifungal, broth microdilution, filamentous fungi or moulds, susceptibility testing

## The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS document HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

**QSEs**

| | |
|---|---|
| Documents & Records | Information Management |
| Organization | Occurrence Management |
| Personnel | Assessment |
| Equipment | Process Improvement |
| Purchasing & Inventory | Service & Satisfaction |
| Process Control | Facilities & Safety |

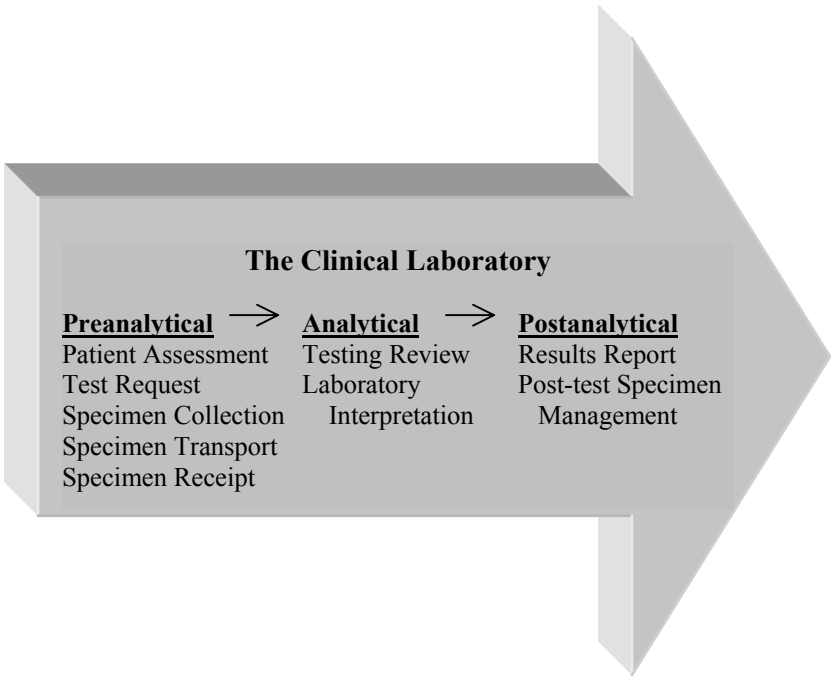
**M38-A Addresses the following Quality System Essentials (QSEs)**

| Documents & Records | Organization | Personnel | Equipment | Purchasing & Inventory | Process Control | Information Management | Occurrence Management | Assessment | Process Improvement | Service & Satisfaction | Facilities & Safety |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **X** | | | | | | |

Adapted from NCCLS document HS1-A—*A Quality System Model for Health Care*

### Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely, quality laboratory information. The arrow depicts the sequence, from left to right, that any clinical laboratory follows. In addition, the necessary steps or subprocesses are listed below them.




Adapted from NCCLS document HS1-A—*A Quality System Model for Health Care*

Most of NCCLS's documents relate to the clinical laboratory, so the most common path of workflow will be that depicted above. The path of workflow for other healthcare activities, e.g., respiratory services, imaging services, etc., or for other types of organizations, e.g., medical device manufacturers, will differ from that of the clinical laboratory. All such paths of workflow describe the sequence of activities necessary to produce an organization's or an entity's specific product or services. For those documents that relate to other paths of workflow, the icon will reflect different process steps.

If the document is specific to clinical laboratory processes or procedures, the following chart will indicate which process step(s) are included within the specific document.

**M38-A Addresses the Following Steps Within the Clinical Laboratory Path of Workflow**

| **Preanalytical** | | | | | **Analytical** | | **Postanalytical** | |
|---|---|---|---|---|---|---|---|---|
| Patient Assessment | Test Request | Specimen Collection | Specimen Transport | Specimen Receipt | Testing Review | Laboratory Interpre-tation | Results Report | Post-test Specimen Management |
| | | | | | **X** | **X** | **X** | **X** |

# Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard

## 1 Introduction

The method described in this document is intended for testing the more common filamentous fungi or moulds that cause invasive infections. These moulds encompass *Aspergillus* species, *Fusarium* species, *Rhizopus* species, *Pseudallescheria boydii*, and the mycelial form of *Sporothrix schenckii*. Although other opportunistic monilaceous and dematiaceous moulds have been evaluated,[5] caution should be used when interpreting the MIC results from other mould/drug combinations. The method has not been used in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis*, or *Coccidioides immitis*, *Histoplasma capsulatum* variety *capsulatum*, or *Penicillium marneffei*, or *S. schenckii*.

NCCLS document M38-A is a "reference" standard developed through a consensus process to facilitate agreement among laboratories in measuring the susceptibility of moulds to antifungal agents. It is to be emphasized that the relationship between *in vitro* versus *in vivo* data has only been attempted in animal models.[3] An important use of a reference method is to provide a standard basis from which other methods can be developed, which also will result in interlaboratory agreement within specified ranges. Such methods might have particular advantages, such as ease of performance, economy, or more rapid results; therefore, their development could be highly desirable. To the extent that any method produces concordant results with this reference method, it would be considered to be in conformity with NCCLS document M38-A.

### 1.1 Scope

This document describes a method for testing the susceptibility of filamentous fungi (moulds) that cause invasive fungal infections, including *Aspergillus* species, *Fusarium* species, *Rhizopus arrhizus, Pseudallescheria boydii,* and *Sporothrix schenckii* and other pathogenic moulds to antifungal agents. Addressed in this document are testing conditions including inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria for MIC determination.

This standard focuses on the fully defined synthetic medium RPMI-1640 for testing of moulds because of examples of the suitability of this test medium for antifungal susceptibility testing of yeast.[1,2]

NCCLS document M27—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast,* is referenced for drug stock solution preparation and dilution procedures.

### 1.2 Definitions[a]

**Antibiogram**, *n* – Overall profile of antimicrobial susceptibility results of a microbial species to a battery of antimicrobial agents.

**Minimal inhibitory concentration (MIC),** *n* – The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents.* For complete definitions and detailed source information, please refer to the most current edition of that document.

# 2 Antifungal Agents

## 2.1 Source

Antifungal standards or reference powders can be obtained commercially, directly from the drug manufacturer, or from the United States Pharmacopeia (12601 Twinbrook Parkway, Rockville, MD 20852). Pharmacy stock or other clinical preparations **should not** be used. Acceptable powders bear a label that states the drug's generic name, its assay potency (usually expressed in micrograms [µg] or International Units per mg of powder), and its expiration date. The powders are to be stored as recommended by the manufacturers, or at -20 °C or below, in a desiccator, preferably in a vacuum. When the desiccator is removed from the freezer, it is to be allowed to come to room temperature before it is opened (to avoid condensation of water).

## 2.2 Weighing Antifungal Powders

All antifungal agents are assayed for standard units of activity. The assay units can differ widely from the actual weight of the powder and often differ within a drug production lot. Thus, a laboratory must standardize its antifungal solutions based on assays of the lots of antifungal powders that are being used.

Either of the following formulae may be used to determine the amount of powder or diluent needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL) x Concentration (µg/mL)}}{\text{Assay Potency (µg/mg)}} \quad (1)$$

or

$$\text{Vol. (mL)} = \frac{\text{Weight (mg) x Assay Potency (µg/mg)}}{\text{Concentration (µg/mL)}} \quad (2)$$

The antifungal powder should be weighed on an analytical balance that has been calibrated with National Institute of Standards and Technology (NIST [Gaithersburg, MD]) weights or other approved reference weights. Usually, it is advisable to accurately weigh a portion of the antifungal agent in excess of that required and to calculate the volume of diluent needed to obtain the concentration desired.

***Example***: To prepare 100 mL of a stock solution containing 1280 µg of antifungal agent per mL with antifungal powder that has a potency of 750 µg/mg, use the first formula to establish the weight of powder needed:

$$\underset{\text{(mg)}}{\text{Weight}} = \frac{\underset{\text{(Target Vol.)}}{100\text{mL}} \overset{\text{x}}{} \underset{\text{(Desired Conc.)}}{1280\ \mu\text{g/mL}}}{\underset{\text{(Potency)}}{750\ \mu\text{g/mg}}} = 170.7\ \text{mg} \quad (3)$$

Because it is advisable to weigh a portion of the powder in excess of that required, powder was deposited on the balance until 182.6 mg was reached. With that amount of powder weighed, formula (2) above is used to determine the amount of diluent to be measured:

$$\frac{\text{Volume}}{(\text{mL})} = \frac{(\text{Powder Weight}) \times (\text{Potency})}{(\text{Desired Concentration})} = \frac{182.6\ \text{mg} \times 750\ \mu\text{g/mg}}{1280\ \mu\text{g/mL}} = 107.0\ \text{mL} \quad (4)$$

Therefore, the 182.6 mg of the antifungal powder is to be dissolved in 107.0 mL of diluent.

## 2.3 Preparing Stock Solutions

Antifungal stock solutions are to be prepared at concentrations of at least 1280 µg/mL or ten times the highest concentration to be tested, whichever is greater. There are some antifungal agents of limited solubility, however, that can require lower concentrations. In all cases, information provided by the drug manufacturer should be considered as part of determining solubility.

### 2.3.1 Use of Solvents Other Than Water

Some drugs must be dissolved in solvents other than water (see Table 1). Information on the solubility of an antifungal compound should be included with the drug. Such drugs should be dissolved at concentrations at least 100 times higher than the highest desired test concentration. Commonly used agents include: analytical grade dimethyl sulfoxide (DMSO), ethyl alcohol, polyethylene glycol, and carboxy methyl cellulose. When such solvents are used, a *series of dilutions* at 100 times the final concentration should be prepared from the antifungal stock solution in the same solvent. Each intermediate solution should then be further diluted to final strength in the test medium (see Table 1). This procedure avoids dilution artifacts that result from precipitation of compounds with low solubility in aqueous media.

For example, to prepare for a broth microdilution test series containing a water-insoluble drug that can be dissolved in DMSO, for which the highest desired test concentration is 16 µg/mL, first weigh 4.8 mg (assuming 100% potency) of the antifungal powder and dissolve it in 3.0 mL DMSO. This will provide a stock solution at 1,600 µg/mL.Next, prepare further dilutions of this stock solution in DMSO (see Table 2) The solutions in DMSO will be diluted 1:50 in test medium (see Section 3.2), and a further twofold when inoculated (see Section 3.4); reducing the final solvent concentration to 1% DMSO at this concentration (without drug) should be used in the test as a diluent control.

The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations in Section 2.2 should be applied.

### 2.3.2 Filtration

Normally, stock solutions do not support contaminating microorganisms and they can be assumed to be sterile. If additional assurance of sterility is desired, they are to be filtered through a membrane filter. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antifungal agents, are *not* to be used. Whenever filtration is used, it is important that the absence of adsorption is documented by results of appropriate assay procedures.

### 2.3.3 Storage

Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at -60 °C or below but never at a temperature greater than -20 °C). Vials are to be removed as needed and used the same day. Any unused drug is to be discarded at the end of the day. Stock solutions of most antifungal agents can be stored at -60 °C or below for six months or

more without significant loss of activity.[6] In all cases, any directions provided by the drug manufacturer are to be considered as a part of these general recommendations and should supercede any other directions that differ. Any significant deterioration of an antifungal agent may be ascertained. This should be reflected in the results of susceptibility testing using quality control strains or reference strains such as those in Table 4.

## 2.4 Number of Concentrations Tested

The concentrations to be tested should encompass the expected results for the available quality control strains. Based on previous studies, the following drug concentration ranges may be relevant: amphotericin B, 0.0313 to 16 µg/mL; flucytosine, 0.125 to 64 µg/mL; ketoconazole, 0.0313 to 16 µg/mL; itraconazole and new traizoles, 0.0313 to 16 µg/mL; and fluconazole, 0.125 to 64 µg/mL.

## 2.5 Selection of Antifungal Agents for Routine Testing and Reporting

Routine testing is not recommended. At each institution, the decision to perform testing of fungi is best made as a collaborative effort of infectious diseases practitioners, the pharmacy and therapeutics committee, clinical microbiology personnel, and the infection control committee.

### 2.5.1 Generic Names

To minimize confusion, all antifungal agents should be referred to by official nonproprietary (i.e., generic) names.

### 2.5.2 Number of Agents Tested

For laboratories performing antifungal testing on moulds, testing with amphotericin B and itraconazole would be appropriate to provide practical information, to minimize errors, and to fit into a practical schedule. Testing with other agents, e.g., the new triazoles, also would be appropriate as they become available.

### 2.5.3 Guidelines for Selective Reporting

Testing may be warranted under certain selected circumstances such as the following: as part of periodic batch surveys that establish antibiograms for collections of pathogenic isolates obtained from within an institution; and to aid in the management of invasive infections due to filamentous fungi when the utility of the azole antifungal agents is uncertain. Interpretive breakpoints are not available for any species of filamentous fungi versus any antifungal agent, and the clinical relevance of testing any organism-drug combination remains uncertain. Specimens for culture and other procedures should be obtained before initiation of antifungal therapy.

# 3 Test Procedures

## 3.1 Broth Medium

### 3.1.1 Synthetic Medium

The completely synthetic medium RPMI-1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) was found satisfactory for testing the filamentous fungi and has been used to develop the proposed standard.[1,2] The formula for this medium is provided in Table 5, and the preparation of the medium from powder is outlined in Appendix A.

### 3.1.2 Buffers

Media should be buffered to a pH of 7.0 ± 0.1 at 25 °C. A buffer should be selected that does not antagonize antifungal agents. Tris buffer is unsatisfactory because it antagonizes the activity of flucytosine. Zwitterion buffers are preferable to buffers that readily traverse the cell membrane, such as phosphate buffers, because, theoretically, the latter can produce unexpected interactions with antifungal agents. One buffer that has been found to be satisfactory for antifungal testing is MOPS (3-[N-morpholino] propanesulfonic acid) at a final concentration of 0.165 mol/L for pH 7.0. The pH of each batch of medium is to be checked with a pH meter immediately after the medium is prepared; the pH should be between 6.9 and 7.1 at room temperature (25 ºC). MIC performance characteristics of each batch of broth are evaluated using a standard set of quality control organisms (see Section 4).

## 3.2 Preparing Diluted Antifungal Agents

The steps for preparation and storage of diluted antifungal agents are as follows:

(1) Use sterile, plastic test tubes to prepare drug dilutions and sterile, disposable, multiwell microdilution plates (96 U-shaped wells) to perform the tests.

(2) Use a growth control well containing RPMI-1640 medium without antifungal agents (but with nonaqueous solvent where necessary) for each organism tested.

When twofold dilutions of a water-soluble antifungal agent are to be used, they may be prepared volumetrically in broth (Table 3). The procedure for antifungals that are not soluble in water is different from that for water-soluble agents and is described below. When running a small number of tests, consulting the schedule in Table 2 is recommended.

The total volume of each dilution to be prepared depends on the number of tests to be performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL will be adequate for about eight tests (one microdilution tray), allowing for pipetting. A single pipet is used for measuring all diluents and then for adding the stock antifungal solution to the first tube. A separate pipet is used for each remaining dilution in that set. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are two times more concentrated than the final concentrations.

For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, or itraconazole, a dilution series of the agent should be prepared first at 100 x final strength in an appropriate solvent (see Section 2.3.1). Each of these nonaqueous solutions should now be diluted 1:50 in RPMI-1640 medium.

For example, if a dilution series with final concentrations in the range 16 µg/mL to 0.0313 µg/mL is desired, a concentration series from 1,600 to 3.13 µg/mL should have been prepared first in DMSO (see Section 2.3.1 and Table 2). To prepare 5 mL volumes of diluted antifungal agent (sufficient for 45 tests), first pipet 4.9 mL volumes of RPMI-1640 medium into each of ten sterile test tubes. Now, using a single pipet, add 0.1 mL of DMSO alone to one 4.9 mL lot of medium (control medium), then 0.1 mL of the lowest (3.13 µg/mL) drug concentration in DMSO, then 0.1 mL of the 6.25 µg/mL concentration and continue in sequence up the concentration series, each time adding 0.1 mL volumes to 4.9 mL medium. These volumes can be adjusted according to the total number of tests required. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are twofold more concentrated than the final concentrations.

### 3.3 Inoculum Preparation

When the risk of substantial spatter or aerosolization is present, the manipulation should be performed in a Class IIA or IIB biological safety cabinet. Details are father outlined in NCCLS document M29–*Protection of Laboratory Workers from Occupationally Acquired Infections.*

Initial work demonstrated that reliable nongerminated conidial or sporangiospore suspensions could be prepared by a spectrophotometric procedure,[7,8,9] and that concentrations of viable conidial or sporangiospore test inocula in a range of approximately 0.4 x $10^4$ to 5 x $10^4$ CFU/mL provided the most reproducible MIC data.[1,2] To induce conidium and sporangiospore formation, most fungi (*Aspergillus* species, *P. boydii, R. arrhizus* and *S. schenckii*) should be grown on potato dextrose agar for 7 days at 35 °C. *Fusarium* species should be incubated for 48 to 72 hours at 35 °C and then until day 7 at 25 to 28 °C. Cover seven-day-old colonies with approximately 1 mL of sterile 0.85% saline, and prepare a suspension by gently probing the colonies with the tip of a transfer pipette. Addition of one drop (approximately 0.01 mL) of Tween 20 will facilitate the preparation of *Aspergillus* inocula. The resulting mixture of conidia or sporangiospores and hyphal fragments is withdrawn and transferred to a sterile tube. After heavy particles are allowed to settle for 3 to 5 minutes, the upper homogeneous suspension is transferred to a sterile tube, the cap tightened and mixed with a vortex mixer for 15 seconds. (CAUTION: Remove the cap carefully as liquid adhering to the cap may produce aerosols upon opening.) The densities of the conidial or sporangiospore suspensions are read and adjusted to an optical density (OD) that all range from 0.09 to 0.11 (80 to 82% transmittance) for *Aspergillus* species and *S. schenckii* and 0.15 to 0.17 (68 to 70% transmittance) for *Fusarium* species, *P. boydii*, and *R. arrhizus*. These suspensions will be diluted 1:50 in the standard medium. Inoculum suspensions of *P. boydii* may require a lower (50%) dilution factor. The 1:50 inoculum dilutions will correspond to 2x the density needed of approximately 0.4 x $10^4$ to 5 x $10^4$ CFU/mL. The test inoculum will be made in sufficient volume to directly inoculate each well with 0.1 mL of the corresponding diluted inoculum suspension.

Inoculum quantitation can be performed by plating 0.01 mL of a 1:100 dilution of the adjusted inoculum on Sabouraud glucose (dextrose) agar to determine the viable number of CFU per milliliter.[1,2,4,7] The plates will be incubated at 28 to 30 °C and observed daily for the presence of fungal colonies. Colonies should be counted as soon as possible after growth becomes visible, especially for isolates of *R. arrhizus*. The incubation times will range from 24 hours or less (*R. arrhizus*) to 5 days (*P. boydii*).

### 3.4 Inoculating RPMI-1640 Medium

Each well will be inoculated on the day of the test with 0.1 mL of the 2x conidial or sporangiospore inoculum suspension. This step will dilute the drug concentrations, inoculum densities, and solvent if used to the final desired test concentrations. The growth control wells will contain 0.1 mL of the corresponding diluted inoculum suspension and 0.1 mL of the drug diluent (2%) without antifungal agent (See Section 3.2). QC and reference organisms are tested in the same manner and are included each time an isolate is tested.

### 3.5 Incubation

All microdilution trays are incubated at 35 °C without agitation. Trays containing *Rhizopus* species are examined after 21 to 26 hours of incubation before determining MIC results. Most other opportunistic filamentous fungi, including: *Fusarium* spp., *Aspergillus* spp. and *Sporothrix schenkii* are evaluated after 46 to 50 hours of incubation. *P. boydii* is examined after 70 to 74 hours.

### 3.6 Reading Results

The MIC is the lowest concentration of an antifungal agent that substantially inhibits growth of the organism as detected visually. For the conventional microdilution procedure, the growth in each MIC well

is compared with that of the growth control with the aid of a reading mirror. Each microdilution well is then given a numerical score as follows: 4-no reduction in growth; 3-slight reduction in growth or approximately 75% of the growth control (drug-free medium); 2-prominent reduction in growth or approximately 50% of the growth control; 1-slight growth or approximately 25% of the growth control; and 0-optically clear or absence of growth.

### 3.6.1 Amphotericin B

For amphotericin B, end points are typically well defined and the MIC is easily read as the lowest drug concentration that prevents any discernible growth (score 0). Trailing end points with amphotericin B are usually not encountered. Such a pattern may reflect clinically relevant drug resistance.

### 3.6.2 Flucytosine, Fluconazole, and Ketoconazole

For flucytosine, and especially for azoles such as fluconazole and ketoconazole, end points are typically less well defined than that described for amphotericin B which may contribute to a significant source of variability. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 50% (half or more) reduction in growth, of the growth in the control well (drug-free medium). When this turbidity persists, it is often identical for all drug concentrations above the MIC. Reference strains of defined susceptibility can be used in the training of new personnel.

### 3.6.3 Itraconazole

For itraconazole and the new triazoles, posaconazole, ravuconazole, and voriconazole, end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth (100% inhibition, or numerical score of 0). Trailing end points with these agents against *Aspergillus* spp. and most other opportunistic pathogenic moulds are not usually encountered. It is possible that such a pattern could reflect clinically relevant drug resistance as it has been demonstrated for *A. fumigatus* strains that have been clinically resistant to itraconazole.[4,10,11]

## 3.7 Interpretation of Results

Interpretive breakpoints have not been established at present. The clinical relevance of testing this group of fungal pathogens remains uncertain.

### 3.7.1 Amphotericin B

Experience to date using the procedures described in this standard indicates that amphotericin B MICs for most opportunistic filamentous fungi isolates are clustered between 0.5 and 2.0 µg/mL. However, amphotericin B MICs for some species (*A. terreus*, *Acremonium strictum*, *S. apiospermum* and *S. prolificans*) can be above 2 µg/mL (MIC ranges of 2 to 16 µg/mL).[5] Although very little data are available regarding correlation between MIC and outcome of treatment with amphotericin B for the filamentous fungi, MICs above 2 µg/mL have been associated with treatment failures and MICs below 2 µg/mL with clinical cure among 28 patients treated with amphotericin B for invasive aspergillosis caused by *A. fumigatus* (8 cases), *A. flavus* (12 cases) and *A. terreus* (9 cases). [12]

### 3.7.2 Flucytosine

Filamentous fungi are usually not susceptible to flucytosine and most MICs are >64 µg/mL for these isolates. The exceptions are some isolates of *Aspergillus* species and phaeoid (dematiaceous) fungi.

### 3.7.3 Fluconazole

Filamentous fungi are usually not susceptible to fluconazole and most MICs are >64 µg/mL for these isolates. The exceptions are some isolates of the dimorphic fungi and dermatophytes.

### 3.7.4 Ketoconazole

Experience to date using the procedures described in this standard indicates that MICs for moulds vary between 0.0313 and 16 µg/mL. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with ketoconazole.

### 3.7.5 Itraconazole and New Triazoles

The importance of proper preparation of drug dilutions for this insoluble compound cannot be over-emphasized.[7] (See NCCLS document M27—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts.)* Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 2 can lead to substantial errors due to dilution artifacts. As for ketoconazole, experience to date using the procedures described in this standard indicates that MICs for moulds vary between 0.0313 and 16 µg/mL. However, preliminary data indicate that high itraconazole MICs (>8 µg/mL) are associated with clinical resistance to this agent[10,11] when MICs are determined by the M38-A microdilution method after 48 hours of incubation.[4] Data are not yet available to indicate a correlation between MIC and outcome of treatment with the new triazoles.

## 3.8 Broth Macrodilution Modifications

Data have been published that document good concordance between results obtained by the broth microdilution methodology described above and a broth macrodilution adaptation.[1,2] Some clinical laboratories may choose to implement broth macrodilution rather than the broth microdilution method primarily because of safety issues. The steps and testing conditions that are relevant to the broth macrodilution test are discussed in detail.

The 100-fold drug dilutions described for the broth microdilution procedure should be diluted 1:10 with RPMI to achieve the tenfold strength needed for the broth macrodilution test. The stock inoculum suspensions are prepared and adjusted, as described, under the broth microdilution test. The stock conidia or sporangiospore suspension is mixed for 15 seconds with a vortex, diluted 1:100 with medium to obtain the test inoculum ($0.4 \times 10^4$ to $5 \times 10^4$ CFU/mL).

The 10x drug concentrations are dispensed into 12 x 75-mm sterile tubes in 0.1 mL volumes. These tubes may be sealed in plastic bags and stored frozen at –70 ºC for up to six months without deterioration of drug potency. Each tube is inoculated on the day of the test with 0.9 mL of the corresponding diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentrations mentioned for the microdilution method. The growth control receives 0.1 mL of 10-fold of the drug diluent without antifungal agent and is inoculated with 0.9 mL of the corresponding diluted inoculum suspensions. The QC organisms are tested in the same manner and are included each time an isolate is tested.

Tubes are incubated at 35 °C (without agitation) and observed for the presence or absence of visible growth. The tubes are scored and MICs determined as described for the broth microdilution procedure.

## 3.9 Other Modifications

Preliminary data have demonstrated that determination of MICs using a colorimetric end point enhances the interlaboratory agreement of itraconazole MICs.[2,9] This procedure can be performed by adding 2x

colorimetric indicator (modified resazurin) to a 2x concentration of the standard RPMI medium and following the steps described above for either the microdilution test or its modification.

For the colorimetric procedure, the wells are examined for a change in color from blue (indicating no growth) to purple (indicating partial inhibition) or to red (indicating growth). The MIC of an azole is the drug concentration that shows a slight color change from blue to purple and of amphotericin B, the drug concentration that shows no color change or the first well that remains blue.

# 4 Quality Control

## 4.1 Purpose

The goals of a quality control program are to monitor the following:

- The precision and accuracy of the susceptibility test procedure
- The performance of reagents, testing conditions, and instructions used in the test
- The performance of persons who conduct the tests and read the results.

The goals are best realized by, but not limited to, the use of quality control and reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.[13,14,15]

## 4.2 Quality Control Responsibilities

### 4.2.1 Manufacturers (Commercial and/or "In-House" Products)

Manufacturers are responsible for the following:

- Antifungal stability
- Antifungal identification
- Potency of antifungal stock solutions
- Compliance with good manufacturing practices
- Integrity of the product
- Accountability and traceability to the consignee.

### 4.2.2 Laboratory (User)

The laboratorian is responsible for the following:

- Storage (drug deterioration)
- Operator proficiency
- Adherence to procedure (e.g., inoculum effect, incubation conditions [time and temperature]).

### 4.2.3 Mutual Responsibility

Manufacturers of commercial products should design and recommend a quality control program that allows the user to evaluate those variables (e.g., inoculum levels, storage/shipping conditions) that most likely will cause user performance problems and to determine that the assay is performing correctly when carried out according to directions for use.

## 4.3 Selecting Reference Strains

Ideal reference strains for quality control of dilution methods have MICs that fall near the mid-range of the concentration for all antifungal agents tested. An ideal control strain is inhibited at the fifth dilution of a nine-dilution -$\log_2$ series, but strains with MICs between the third and seventh dilution are acceptable. Before a strain is accepted as a reference, it is to be tested for as long as is necessary to demonstrate that its antifungal susceptibility pattern is genetically stable. NCCLS document M23—*Development of* In Vitro *Susceptibility Testing Criteria and Quality Control Parameters*, provides guidelines for the selection of appropriate quality control strains and the determination of acceptable MIC ranges. The QC strains listed in Table 4 were selected in accordance with the criteria in M23 and can be used as controls for the antifungal susceptibility testing of moulds until mould isolates are selected. In, addition, the reference mould isolates listed in Table 4 also can be used.

## 4.4 Storing Reference Strains

### 4.4.1 Methods for Prolonged and Short-term Storage

Reference strains are stored in a way that minimizes the possibility of mutation in the organisms.

- There are three preferred methods for prolonged storage of reference strains. Fungal isolates may be grown on potato dextrose agar and then frozen at -70 °C.[16] Alternatively, reference strains can be preserved by suspending fungal cells in 50% glycerol solution or in the cryogenic solution of commercial vials containing porous beads that have been demonstrated by the manufacturer to preserve fungi. Vials can be stored at either –70 °C, or in liquid nitrogen, or in the vapor of liquid nitrogen.[17]

- For short-term storage, working stock cultures can be grown on Sabouraud dextrose agar until sufficient growth is observed and stored at 2 to 8 ºC. Fresh slants are prepared at two-week intervals by serial transfer. To avoid mixed cultures, no more than three passages should be made after removal from frozen stock culture.

### 4.4.2 Sources for Reference Strains

Reference strains are obtained from a source that is able to provide information on the origination of the culture (for example, from the American Type Culture Collection [ATCC®],[b] from commercial sources with documented culture history, or from reference institutions with demonstrated ability to store and use the organisms consistently with minimal contamination). A new stock culture should be obtained whenever a significant deviation from the expected end point is observed.

[b] ATCC® is a registered trademark of the American Type Culture Collection.

### 4.4.3 Preparing Strains for Storage

To prepare strains for storage, it is necessary to do the following:

(1) Grow *Candida* species overnight on either Sabouraud dextrose agar or soybean casein digest agar. Grow moulds for seven days on potato dextrose agar.

(2) Select growth from several colonies and perform the appropriate susceptibility tests to demonstrate that they give the expected MIC results (see Table 4 for expected MICs of two reference strains). Moulds generally require a longer (five- to seven-day) incubation period.

(3) Subculture strains yielding expected results onto the same medium that was used for the primary culture and incubate long enough for sufficient growth to occur (usually from one to three days).

(4) Examine the resulting growth carefully to be sure it is a pure culture.

(5) Suspend the growth from the plate in the stabilizing fluid to make a heavy suspension (or if lyophilizing, suspend the growth in the appropriate medium).

(6) Distribute the turbid suspension in small volumes (one or two drops) into suitable sterile containers.

(7) Place these containers in a freezer maintained as in Section 2.3.3 or in liquid nitrogen.

Stocks prepared using the procedure just outlined can remain indefinitely without significant risk of alteration in antifungal susceptibility patterns. When the supply of containers is nearly exhausted, this process is repeated to make a new supply.

## 4.5 Routine Use of Reference Strains

For routine use of reference strains, it is necessary to do the following:

(1) Remove a container of the culture from the freezer or obtain a lyophilized vial.

(2) Let the frozen mixture thaw or rehydrate the lyophilized culture.

(3) For *Candida* species, transfer a portion of the mixture onto Sabouraud dextrose agar and incubate at 35 ºC for 24 hours. Subculture moulds on potato dextrose agar and incubate for seven days.

(4) Remove four to five colonies, subculture them to medium for the appropriate susceptibility tests, and then subculture them onto soybean casein digest agar slants.

(5) After incubating the strains overnight, store them at 2 to 8 ºC.

(6) Subculture from the slant to an agar plate.

(7) Always perform susceptibility tests on colonies from overnight plates (*Candida* species) or seven-day cultures (moulds).

The agar slants may be used as working stock cultures. They are replaced regularly with new slants prepared from the freezer supply at least every two weeks.

## 4.6 Batch of Medium and Lot of Plasticware Control

For batch or lot control, the procedural steps are as follows:

(1) Test each new batch of medium or lot of microdilution trays or macrodilution tubes with one of the quality control strains listed in Table 4 to determine if MICs fall within the expected range; if they do not, reject the batch or lot.

(2) Incubate at least one uninoculated tube from each batch for the same amount of time as required to complete the test to be sure of the medium's sterility.

(3) New lots of RPMI-1640 medium should be tested for acceptable performance before being used to test clinical isolates because recent studies have demonstrated that some lots do not perform adequately. The pH should be 6.9 to 7.1 (see Section 3.1.2).

(4) Record the lot numbers of all materials and reagents used in these tests.

## 4.7 Quality Control Frequency

### 4.7.1 MIC Ranges

MIC accuracy ranges for a single control test are listed in Table 4. In general, 1 out of every 20 MIC values in a series of 20 consecutive tests might be out of control (i.e., outside the stated range) due to random variation of the test. Two consecutive out-of-control results or any more than 2 out-of-control results in 20 consecutive control tests require corrective action. Any time corrective action is taken, the count of 20 begins again.

**NOTE:** Do not confuse this procedure with the procedure for establishing satisfactory performance of MIC tests for the purpose of performing quality control tests weekly instead of daily (see Section 4.7.2).

### 4.7.2 Frequency of Testing

To monitor the overall performance of the test system, include appropriate reference strains each day the test is performed. However, the frequency of test monitoring may be reduced if the laboratory can document satisfactory performance with daily control tests. For this purpose, satisfactory performance is defined as follows:

(1) Documentation that all reference strains were tested for 30 consecutive test days.

(2) For each drug-microorganism combination, no more than 3 of the 30 MIC values (i.e., MIC values obtained from one drug-microorganism combination for 30 consecutive test days) may be outside the accuracy ranges stated in Table 4.

**NOTE:** This procedure is only for establishing satisfactory performance of MIC tests for the purpose of performing quality control tests weekly instead of daily. Do not confuse this procedure with the steps that must be taken for corrective action defined in Section 4.7.1.

(3) The overall performance evaluation of the test system (as outlined above) should be restarted (i.e., monitored for 30 consecutive test days) each time a reagent component (new batch of stock drug or new batch of frozen QC organisms) is changed.

(4) When these conditions are fulfilled, each reference strain must be tested at least once per week. Whenever an MIC value outside the accuracy range is observed using the weekly accuracy

monitoring system, daily control tests must be reinstated long enough to define the source of the aberrant result and to document resolution of the problem. Resolution of the problem may be documented as follows:

(a) Test with appropriate reference strains for five consecutive test days.

(b) For each drug-microorganism combination, all of the five MIC values (i.e., MIC values obtained from one drug-microorganism combination for five consecutive test days) must be within the accuracy ranges stated in Table 4.

(5) If resolution of the problem cannot be documented (i.e., at least one of the five MIC values is observed to be outside the accuracy range) daily control testing must be continued. To return to weekly testing in the future will require documentation of satisfactory performance for another 30 consecutive test days as outlined in this section.

For some drugs, quality control tests must be done more frequently than once per week because of the relatively rapid degradation of the drug.

## 4.8 Other Control Procedures

### 4.8.1 Growth Control

Each broth microdilution or macrodilution series should include a growth control of RPMI 1640 medium without antifungal agent to assess viability of the test organisms. With the broth tests, the growth control also serves as a turbidity control for reading end points.

### 4.8.2 Purity Control

A sample of each inoculum is streaked on a suitable agar plate and incubated until there is sufficient visible growth to detect mixed cultures and to provide freshly isolated colonies in the event retesting proves necessary.

### 4.8.3 End Point Interpretation Control

End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. The results are recorded and compared to the results obtained by an experienced reader. Specific reference strains with predetermined MICs are particularly helpful for this purpose, especially with itraconazole.

## 4.9 Quality Control Strains (see also Section 4.3)

Ideal reference strains for quality control of dilution tests have MICs that consistently fall near the midpoint of the concentration range tested for all antifungal agents; e.g., an ideal control strain would be inhibited at the fourth dilution of a seven-dilution series, but strains with MICs at either the third or fifth dilution would also be acceptable.

Table 4 lists expected ranges for strains found to be acceptable as quality control strains for testing yeasts. Also shown are additional strains that can be useful for conducting reference studies.[2]

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## Appendix A. RPMI-1640 Medium

**RPMI-1640 medium buffered with 0.165 mol/L MOPS, 1 L**.

10.4 g powdered RPMI-1640 medium (with glutamine and phenol red, without bicarbonate)
34.53 g MOPS (3-[N-morpholino] propanesulfonic acid) buffer

Dissolve powdered medium in 900 mL distilled $H_2O$. Add MOPS (final concentration of 0.165 mol/L) and stir until dissolved. While stirring, adjust the pH to 7.0 at 25 $^oC$ using 1 mol/L sodium hydroxide. Add additional water to bring medium to a final volume of 1 L. Filter sterilize and store at 4 ºC until use.

## Appendix B. McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, a $BaSO_4$ turbidity standard is used (0.5 McFarland Standard).

The procedure consists of the following steps:

(1) Prepare this turbidity standard by adding 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \bullet 2H_2O$) to 99.5 mL of 0.18 mol/L (0.36 N) $H_2SO_4$ (1% v/v).

(2) Verify the correct density of the turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland Standard.

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

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

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

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

 

**Table 1. Solvents and Diluents for Preparation of Stock Solutions of Antifungal Agents**

| Antifungal Agent | Solvent (Full Strength and Intermediate Solutions) | Diluent (Final concentrations) |
|---|---|---|
| Amphotericin B | DMSO* | Medium |
| Ketoconazole | DMSO* | Medium |
| Itraconazole | DMSO* | Medium |
| Posaconazole | DMSO* | Medium |
| Ravuconazole | DMSO* | Medium |
| Voriconazole | DMSO* | Medium |
| Fluconazole | Water | Medium |
| Flucytosine | Water | Medium |

*Dimethyl sulfoxide

## Table 2. Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests

| Antimicrobial Solution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Step | Concentration (µg/mL) | Source | Volume (mL) | + Solvent (mL) (e.g., DMSO)* | = Intermediate Concentration (µg/mL) | = Final Concentration at 1:50(µg/mL)† | $Log_2$ |
| 1 | 1,600 | Stock | | | 1,600 µg/mL | 32 | 4 |
| 2 | 1,600 | Stock | 0.5 | 0.5 | 800 | 16 | 3 |
| 3 | 1,600 | Stock | 0.5 | 1.5 | 400 | 8.0 | 2 |
| 4 | 1,600 | Stock | 0.5 | 3.5 | 200 | 4.0 | 1 |
| 5 | 200 | Step 4 | 0.5 | 0.5 | 100 | 2.0 | 0 |
| 6 | 200 | Step 4 | 0.5 | 1.5 | 50 | 1.0 | -1 |
| 7 | 200 | Step 4 | 0.5 | 3.5 | 25 | 0.5 | -2 |
| 8 | 25 | Step 7 | 0.5 | 0.5 | 12.5 | 0.25 | -3 |
| 9 | 25 | Step 7 | 0.5 | 1.5 | 6.25 | 0.125 | -4 |
| 10 | 25 | Step 7 | 0.5 | 3.5 | 3.13 | 0.0625 | -5 |

* Dimethyl sulfoxide
† 2x concentrations

## Table 3. Scheme for Preparing Dilutions of Water-Soluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests

| Antimicrobial Solution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Step | Concentration (μg/mL) | Source | Volume (mL) | + Medium (mL) | = Intermediate Concentration (μg/mL) | = Final 2x Concentration at 1:5 (μg/mL) | $Log_2$ |
| 1 | 5120 | Stock | 1 mL | 7 | 640 μg/mL | 128 | 6 |
| 2 | 640 | Step 1 | 1.0 | 1.0 | 320 | 64 | 5 |
| 3 | 640 | Step 1 | 1.0 | 3.0 | 160 | 32 | 4 |
| 4 | 160 | Step 3 | 1.0 | 1.0 | 80 | 16 | 3 |
| 5 | 160 | Step 3 | 0.5 | 1.5 | 40 | 8 | 2 |
| 6 | 160 | Step 3 | 0.5 | 3.5 | 20 | 4 | 1 |
| 7 | 20 | Step 6 | 1.0 | 1.0 | 10 | 2 | 0 |
| 8 | 20 | Step 6 | 0.5 | 1.5 | 5 | 1.0 | -1 |
| 9 | 20 | Step 6 | 0.5 | 3.5 | 2.5 | 0.5 | -2 |
| 10 | 2.5 | Step 9 | 1.0 | 1.0 | 1.25 | 0.25 | -3 |
| 11 | 2.5 | Step 9 | 0.5 | 1.5 | 0.625 | 0.12 | -4 |
| 12 | 2.5 | Step 9 | 0.5 | 3.5 | 0.3125 | 0.0625 | -5 |

  

**Table 4. Recommended MIC Limits for Two Quality Control and Reference Strains for Broth Dilution Procedures.** (From Barry, et al. Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. *J Clin Microbiol.* 2000; 38: 3457-3459. Pfaller MA, et al. Quality control guidelines for National Committee for Clinical Laboratory Standards-recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. *J Clin Microbiol.* 1995; 33:1104-1107. Rex JH, et al. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of ketoconazole and itraconazole. *J Clin Microbiol.* 1996; 34:816-817. Espinel-Ingroff A, et al. Multicenter evaluation of proposed standardization procedure for antifungal susceptibility testing of filamentous fungi. *J Clin Microbiol.* 1997; 35:139-143. Espinel-Ingroff A, et al. Optimal susceptibility testing conditions for detection of azole resistance in Aspergillus spp.: NCCLS collaborative evaluation. *Antimicrob Agents Chemother.* 2001; 43: 1828-1835. Reprinted with permission from the authors and the American Society for Microbiology.)

| Organism | Purpose | Antifungal Agent | MIC Range* (µg/mL) | % of MICs Within Range |
|---|---|---|---|---|
| *Candida parapsilosis* ATCC® 22019 | QC | Amphotericin B<br>Fluconazole<br>Itraconazole<br>Ketoconazole<br>Posaconazole<br>Ravuconazole<br>Voriconazole<br>5FC | 0.5-4.0<br>1.0-4.0<br>0.12-0.5<br>0.06-0.5<br>0.06-0.25<br>0.03-0.25<br>0.03-0.25<br>0.12-0.5 | 99.1<br>99.1<br>99.0<br>99.0 |
| *Candida krusei* ATCC® 6258 | QC | Amphotericin B<br>Fluconazole<br>Itraconazole<br>Ketoconazole<br>Posaconazole<br>Ravuconazole<br>Voriconazole<br>5FC | 1.0-4.0<br>16-128<br>0.25-1.0<br>0.25-1.0<br>0.12-1.0<br>0.25-1.0<br>0.12-1.0<br>8.0-32 | 99.5<br>99.1<br>94.0<br>100.0 |
| *Aspergillus flavus* ATCC® 204304 | Reference | Amphotericin B<br>Itraconazole<br>Posaconazole<br>Ravuconazole<br>Voriconazole | 0.5-4<br>0.2-0.5<br>0.06-0.5<br>0.5-4<br>0.5-4 | 100.0<br>100.0<br>100.0<br>100.0<br>100.0 |
| *Aspergillus fumigatus* ATCC® 204305 | Reference | Amphotericin B<br>Itraconazole | 0.5-2<br>0.12-1.0 | 100.0<br>100.0 |

* **MIC ranges for *Candida* QC isolates are microdilution values after 48 hours of incubation;** MIC ranges also are available by the macrodilution method (48 hours only) and after 24 hours by the microdilution method (See above references).

 

## Table 5. Composition of RPMI-1640 Medium

| Constituent | g/L Water | Constituent | g/L Water |
|---|---|---|---|
| L-arginine (free base) | 0.200 | Biotin | 0.0002 |
| L-aspargine (anhydrous) | 0.050 | D-pantothenic | 0.00025 |
| L-aspartic acid | 0.020 | Choline chloride | 0.003 |
| L-cystine • 2HCI | 0.0652 | Folic acid | 0.001 |
| L-glutamic acid | 0.020 | Myo-inositol | 0.035 |
| L-glutamine | 0.300 | Niacinamide | 0.001 |
| Glycine | 0.010 | PABA | 0.001 |
| L-histidine (free base) | 0.015 | Pyridoxine HCl | 0.001 |
| L-hydroxyproline | 0.020 | Riboflavin | 0.0002<br>0.000005 |
| L-isoleucine | 0.050 | Thiamine HCl | 0.001 |
| L-leucine | 0.050 | Vitamin $B_{12}$ | 0.000005 |
| L-lysine • HCI | 0.040 | Calcium nitrate • $H_2O$ | 0.100 |
| L-methionine | 0.015 | Potassium chloride | 0.400 |
| L-phenylalanine | 0.015 | Magnesium sulfate (anhydrous) | 0.04884 |
| L-proline | 0.020 | Sodium chloride | 6.000 |
| L-serine | 0.030 | Sodium phosphate, dibasic (anhydrous) | 0.800 |
| L-threonine | 0.020 | D-glucose | 2.000 |
| L-tryptophan | 0.005 | Glutathione, reduced | 0.001 |
| L-tyrosine • 2Na | 0.02883 | Phenol red, Na | 0.0053 |
| L-valine | 0.020 | | |

  

**NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.**

## Summary of Comments and Subcommittee Responses

M38-P: *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Proposed Standard*

General

1. The term "conidium-forming" should be deleted, because the Zygomycetes do not produce conidia.

- **The term "conidium-forming" has been deleted.**

2. Please address safety precautions for working with filamentous fungi in general and specifically those used with the microbroth dilution method.

- **This issue has been addressed in the Standard Precautions statement and in Section 3.3.**

3. The solvents for itraconazole and ketoconazole recommended in the proposed standard are DimethylSulfoxide. I would like to mention that alternative solvents are: for ketoconazole – 0.2 N HC (Shadomy et al. 1985); for itraconazole – 0.2M HCI in ethanol (Espinel-Ingroff, et al. 1984. *In vitro* studies with R 51211(itraconazole). *Antimicrob Ag Chemoth*. 26:5-9.)

- **As described in Section 2.3.1, the preparation procedure used for drug dilutions avoids dilution artifacts that result from precipitation of itraconazole and other agents of limited solubility.**

4. The overall methodology of this document is well prepared. This method has been recommended for *Aspergillus*, *Fusarium*, *P. boydii* (*Scedosporium apiospermum)* and *Sporothrix schenckii*. Was this method tried on the mycelial phase of *Histoplasma*, *Blastomyces*, *Coccidioides* and *Penicillium marneffei*?

- **Although the M38 method has been evaluated for other opportunistic and emerging pathogenic moulds and data have been published for the dimorphic fungi by following the standard conditions described in this document, collaborative studies have not been conducted for the latter group.**

5. This document does not say if the method was also tried on saprobic moulds such as *Paecilomyces*. Although *Paecilomyces* is considered a normal environmental contaminant, however *Paecilomyces lilacinus* has been documented a pathogen in the literature that is resistant to amphotercin B.

- **Among other emerging mould pathogens, the subcommittee evaluated three isolates of *Paecilomyces lilacinus* under a collaborative study (Reference 5). Amphotericin B MICs were ≥8 micrograms/mL in the three participant laboratories by the M38-A testing conditions.**

6. Since conidia are tested using the method to determine MIC of the fungal agents, some filamentous fungi produce very little or no conidia especially when they become atypical. *Aspergillus fumigatus* is one of the species that may revert to pleomorphic and become atypical. These white strains of

 

*Aspergillus fumigatus* often fail to produce conidia and therefore would pose a problem in antifungal testing methodology.

- **Some isolates may need a longer incubation, but most of them would eventually produce sufficient conidia for MIC determination, or they can be tested using hyphal particles as the inoculum.**

7. Inoculum preparation requires nonpigmented conidial or sporangiospore suspension. Therefore this method may not be suitable for dematiaceous mould since dark pigment may be interfering in the solution. Many dark pigmented fungi remain nonsporulating for a long time, hence this method may not be suitable for this group of fungi.

- **The subcommittee has evaluated this method with a few species of dematiaceous fungi in three laboratories. Interlaboratory agreement was above 90% when testing both established and investigational agents (Reference 5).**

8. Inoculum accuracy may also be challenged by the presence of fungal hyphae that are easily transferred with conidia and conidiogenous cells. If the number of conidia is the key factor in the performance of this method, it will be difficult to calculate from the turbidity of the solution to know the exact concentration of the fungal hyphae, spore or other elements.

- **The preparation of hyphal suspensions may provide unsuitable inoculum suspensions. However, the density of the inoculum should be verified by colony counts.**

9. Some slow growing conidia may also pose problem to reach to the end point of testing. Can the incubation period by extended in any circumstances? Fungi isolated from patients that have been on antifungal treatment may appear inhibited in the test method. Therefore, more data is needed to evaluate the test results.

- **Collaborative studies have not been conducted regarding this issue. Longer incubation may be needed for such fungi in order to induce sufficient growth for MIC determination.**

10. Incubation period is variable depending on the type of fungal species involved; however, incubation beyond 96 hours may be required for slow growing organisms. In this case, if microdilution wells are used, there is a risk for these wells to dry up during this time. Early test score may produce false values.

- **Section 3.8 describes a macrodilution modification, which could be more suitable for such isolates.**

11. Reading results is done by numerical score method ranging from zero to four. The only successful value that appears desirable from the score range is if it falls between zero and one. This method of measuring end point determination is very subjective.

- **Section 3.6 describes the conventional criterion of reading the MICs of itraconazole, amphotericin B and the three new triazoles: 100% inhibition or the first optically clear well. This criterion would facilitate the end point determination.**

12. Data indicating a correlation between MIC and outcome of treatment when available would provide the information about the success or the failure of the procedure.

- **Studies are currently under development at this time.**

13. Color modification has been suggested for the broth dilution method. It has been noticed that during the broth dilution testing of yeast against antifungal agent, the dye used for the color contrast gave shades of color that confused the reader to call positive or negative. Therefore, the addition of a dye to improve visual clarity should be done carefully. More data on this is necessary.

- **The procedure of MIC determination is based upon inhibition of growth or turbidity instead of pH change.**

14. The procedure to perform antifungal susceptibility testing should remain under the control of the reference laboratories until enough data is obtained to determine the credibility of this very important procedure. However, other laboratories capable of performing antifungal susceptibility testing should be allowed. This would provide the different laboratories the opportunity of matching their results with one another and open the doors to commute enough data for analysis.

- **To perform antifungal susceptibility testing should be the decision of each individual institution/organization.**

Section 2.3.2

15. Would it be appropriate to mention 0.22 μm as an appropriate filter type?

- **The subcommittee agrees that it is not necessary to be this specific.**

Section 2.5.2

16. Please include guidelines for "reasonable." In addition it would be helpful to address the following issues to help justify expenses of "bringing up" this kind of testing. . .(1) a minimum number of tests per week or month necessary to maintain proficiency, (2) the minimum frequency of testing to ensure a turnaround time that is clinically relevant, and (3) the level to which "dedicated" personnel are needed as opposed to training "everyone."

- **The first two issues are already addressed in Section 4 of the document and the last issue should be the decision of each individual institution/organization.**

Section 3.1.1

17. Can Antibiotic Medium #3 be used for amphotericin B?

- **The standard RPMI-1640 medium is suitable for testing amphotericin B against the filamentous fungi.**

Section 3.2

18. A sentence acknowledging that host-adapted non- or poorly sporulating *Aspergillus fumigatus* isolates cannot be used to prepare inoculum in this manner would be appropriate.

- **This issue has been addressed. (See Comment 9).**

19. You specify RPMI-1640 here. I think that instead you should just refer to incubation medium (Section 3.1.1).

- **RPMI-1640 is the testing medium.**

 

Section 3.3

20. Does "modified" Sabouraud glucose (dextrose) agar mean Emmons modification?

- **The term "modified" has been deleted.**

21. Is it necessary to use seven-day cultures for rapidly growing organisms such as *Rhizopus* and *Aspergillus*?

- **The subcommittee believes this is necessary for the purpose of standardization.**

22. Is the recommendation to do colony counts on every test?

- **Colony counts validate the inoculum size; the frequency of this procedure should be an individual decision.**

Section 3.4

23. Section 3.4 refers to Section 3.2.2 and there is no Section 3.2.2.

- **This has been corrected.**

Section 3.5

24. Regarding the general issue of "most other opportunistic filamentous fungi": Since data have been collected primarily on the 5 organisms/organism groups listed on page 1, what criteria will be used for establishing incubation time and/or other technical issues when testing less frequently isolated opportunistic pathogens?

- **Data have been collected now for other species of both moniliaceous and dematiaceous fungi and among these common pathogens, only *Cladophialophora bantiana* and *P. lilacinus* required more than 48 hours of incubation (72 hours) (See Reference 5).**

25. As some filamentous fungi grow slower at higher temperatures (35 °C), is it always appropriate to perform susceptibility tests at this, nonoptimal temperature?

- **Very few isolates of pathogenic moulds do not grow at 35 °C, and they can be tested at 30 °C.**

26. Is 72-hour incubation sufficient for *P. boydii*?

- **Most isolates of *P. boydii* produce enough growth for MIC determination after 72 hours.**

Section 3.8

27. Usually the macrodilution is the standard with the microdilution as a modification. The macrodilution should be used for dimorphic organisms and for slower growing organisms, which may require more than 72 hours incubation.

- **Both methods have been described in Sections 3.2 and 3.8 of the document.**

28. Could you clarify what constitute the frequency of QC testing.

- **At least one QC strain should be included each time testing is performed.**

Appendix A

29. Would it be appropriate to mention filter size?

- **The subcommittee agrees that it is not necessary to be this specific.**

Tables 2 and 3

30. The final columns of these tables work with 5x or 50x "Final concentrations." Why don't we instead show the 2x or 20x concentrations that are most relevant to setting up for the final 1:1 mix of drug and bug in a microdilution tray?

- **The columns show the 2x concentrations needed for the microdilution test.**

 

## Summary of Delegate Comments and Subcommittee Responses

M38-A: *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard*

General

1. Why is the tube dilution test still in use and not "Etest"?

- **Since Etest is a commercial system, we cannot make recommendations for testing by that method.**

Abstract

2. With regards to *Rhizopus arrhizus*, why is the species name used? Most laboratories do not make specie-level identifications. Can one use these standards for any *Rhizopus* isolate? Also why not reference *Scedosporium* instead of *Pseudallescheria* since most laboratories recover the anamorphic form?

- **This standard will apply to any species of *Rhizopus* as long as it has the same sporangiospore size. As suggested, *Scedosporium* has been referenced along with *Pseudallescheria* in the Abstract.**

Section 2.2

3. The exact figure of 182.6 mg to weigh out in excess of that needed seems strange. What is the reason for not rounding off to 182 or 180?

- **This is merely an example and is stated as such.**

Section 3.3

4. Regarding inoculum preparation, I have a problem in how to create a standard inoculum from strain to strain of clinical isolates. Should there be any instructions on making a microscopic exam to be sure that the optical density (OD) turbidity truly represents spores? How well does OD always relate to spore concentrations?

- **The inoculum quantitation procedure referenced in this section is the best way to check OD versus CFU/mL. Additional references have been added to data from studies that demonstrate the use of OD with 90% of the inocula being within the expected CFU/mL range.**

Section 3.6

5. Should a wet mount be prepared and examined microscopically to be sure the "slight" and "prominent" visual assessment is due to granulation?

- **The density of the inoculum should be verified as instructed in Section 3.3.**

Section 3.7.1

6. Are laboratories required to make exact species identification (e.g., *A. terreus, Acremonium strictum*) and limit antifungal testing to those?

- **In certain cases (e.g., sterile body sites) it is necessary to speciate to determine if a significant pathogen has been isolated.**

Section 4.3

7. Are there ATCC reference strain recommendations for moulds?

- **ATCC reference strain recommendations for moulds can be found in Table 4.**

Section 4.8.3

8. With regard to "end point interpretation," are there any photographs available to show various reactions so that observer interpretation differences are minimized? How about a CD-ROM program?

- **The subcommittee considered this but has decided not to include photos at this time. There are a number of new agents where this may be necessary; these will be considered for inclusion at a later time**.

 

## Related NCCLS Publications*

**M2-A7** **Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Seventh Edition (2000).** This revised standard contains updated recommended techniques, interpretive criteria and quality control parameters for disk susceptibility testing.

**M7-A5** **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Fifth Edition (2000).** This revised standard provides updated reference methods for the determination of minimal inhibitory concentrations (MICs) for aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

**M11-A5** **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Fifth Edition (2001).** Provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

**M23-A2** **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Second Edition (2001).** Addresses the required and recommended data needed for the selection of appropriate interpretative standards and quality control guidelines for new antimicrobial agents.

**M24-T2** **Antimycobacterial Susceptibility Testing; Tentative Standard—Second Edition (2000).** This document contains recommendations for common culture media, standardization of drug concentrations, a method for standardizing inoculum dilutions and clearly defined end points in susceptibility testing of tuberculosis-like organisms.

**M27-A2** **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard (2002).** This document addresses the selection and preparation of antifungal agents, implementation and interpretation of test procedures, and quality control requirements for susceptibility testing of yeasts that cause invasive fungal infections.

**M29-A2** **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

**NOTES**

 

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