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# Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition

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.

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A standard for global application developed through the Clinical and Laboratory Standards Institute consensus process.



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*Advancing Quality in Health Care Testing*

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### Abstract

Clinical and Laboratory Standards Institute document M27-A3—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition* describes a method for testing the susceptibility of antifungal agents to yeast that cause invasive fungal infections, including *Candida* spp. (and *Candida glabrata*), and *Cryptococcus neoformans*. Selection and preparation of antifungal agents, 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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## Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory aids to guide in the selection of antifungal therapy have gained greater attention. In 1982, the CLSI Area Committee for Microbiology formed the Subcommittee on Antifungal Susceptibility Testing. In 1985, this subcommittee published its first report<sup>1</sup> in which the results of a questionnaire and a small collaborative study were presented. These results are summarized as follows:

- Approximately 20% of the responding CLSI membership whose hospitals had greater than 200 beds was performing antifungal testing.
- Most testing involved broth dilution methodology.
- Most strains tested were *Candida albicans* or other species of yeasts.
- Most centers tested only a few isolates per year.
- Agreement in minimal inhibitory concentration (MIC) results among several laboratories that participated in a collaborative study was unacceptably low.

Based on these findings, the subcommittee concluded that it would be useful to work toward a more reproducible reference testing procedure.

Agreement already existed regarding several elements of the procedure. To facilitate further analysis of various test conditions, the reference method should be a broth macrodilution procedure. Because of examples of drug antagonism by some complex media for certain antifungals, the subcommittee restricted its interest only to fully defined synthetic media. Drug stock solution preparation and dilution procedures previously developed for antibacterial testing procedures were adopted with minor modifications.

Despite agreement in some areas, other factors required additional data to be resolved. These included inoculum preparation; inoculum size; choice among several synthetic media; temperature of incubation; duration of incubation; and end-point definition. These factors were the focus of a series of collaborative studies.<sup>2-5</sup> As a result, agreement within the subcommittee was achieved on all of the factors and led to the publication of M27-P in 1992. In the next four years (1992-1996), reference MIC ranges were established for two quality control strains for the available antifungal agents,<sup>6,7</sup> and broth microdilution procedures paralleling the broth macrodilution reference procedure became available.<sup>5,8-10</sup> This information was included in a revised standard in 1995 (M27-T). In further revising the document, the subcommittee focused its attention on developing relevant breakpoints for available antifungal agents,<sup>11</sup> included in M27-A (1997). Since then, the subcommittee has developed 24- and 48-hour reference MIC ranges for microdilution testing of both established and newly introduced antifungal agents.<sup>12</sup> The results of these studies are included in the current M27-A3 and M27-S3 (Informational Supplement)<sup>13</sup> documents.

## Key Words

antifungal, broth macrodilution, broth microdilution, susceptibility testing, yeasts

## Updated Information in This Edition

### Definitions (Section 4)

Modified definition:

Minimal inhibitory concentration (MIC)

Added definition:

Antimicrobial susceptibility test interpretive category

Quality control

### Additional Section

Indications for performing susceptibility tests (Section 5)

Time of reading (Section 7.8.1)

### Data Inclusion/Exclusion

Established numerical scale criteria for visual comparison of the amount of growth in the control tubes (Section 7.6)

Established guide for reading and interpretation of results of Echinocandin antifungals (Sections 7.6.3 and 7.7.8)

Expanded recommendations and explanations on acceptable time of reading for antifungal agents when growth is adequate (Sections 7.8.1 and 7.9)

### Tables

All related tables were updated and compiled separately as M27-S3, Informational Supplement instead of a document Appendix. Updates on each table include:

#### **Table 1: Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida* spp.**

Added new column on “nonsusceptible (NS)” criteria for interpretive guidelines.

Added breakpoints criteria for the following antifungal agents:

Anidulafungin

Caspofungin

Micafungin

Voriconazole (first added in M27-S2, published February 2006)

Provided additional footnote information for Flucytosine, Anidulafungin, Caspofungin, and Micafungin.

#### **Table 2: Solvents and Diluents for Preparation of Stock Solutions of Antifungal Agents**

Added solvents and diluents recommendations for the following antifungal agents:

Anidulafungin

Caspofungin

Micafungin

#### **Table 5: Recommended 48-Hour MIC Limits for Two Quality Control and Four Reference Strains for Broth Macrodilution Procedures**

Added information on *Issatchenkia orientalis* as the known sexual form of *Candida krusei*.

#### **Table 6: Recommended 24- and 48-Hour MIC Limits for Two Quality Control Strains for Broth Microdilution**

Added the following antifungal agents:

Anidulafungin (first added in M27-S2, published February 2006)

Caspofungin (first added in M27-S2, published February 2006)

Micafungin

## Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition

### 1 Scope

This document describes a method for testing the susceptibility to antifungal agents of yeasts, including *Candida* spp. and *Cryptococcus neoformans*, that cause infections. This method has not been extensively validated for the yeast forms of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum* variety *capsulatum*.

The subcommittee has focused on developing relevant breakpoints for available antifungal agents,<sup>11</sup> and reference MIC ranges for microdilution testing of both established and newly introduced antifungal agents.<sup>12</sup> Interpretive minimal inhibitory concentration (MIC) breakpoints and MIC ranges for quality control (QC) isolates are summarized in an Informational Supplement<sup>13</sup> to this document.

### 2 Introduction

The broth macrodilution method described in this document is intended for testing yeasts that cause invasive infections. These yeasts encompass *Candida* spp. (including *Candida glabrata*) and *C. neoformans*. The method has not been used in studies of the yeast forms of dimorphic fungi, such as *B. dermatitidis* and/or *H. capsulatum* variety *capsulatum*. Moreover, testing filamentous fungi (moulds) introduces several additional problems in standardization not addressed by the current procedure. A reference method for broth dilution antifungal susceptibility testing of filamentous fungi has been developed and is now available as CLSI document M38.<sup>14-16</sup>

M27-A3 is a “reference” standard developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of yeasts to antifungal agents. 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. For example, broth microdilution methods, described in this document, have been configured to produce results paralleling those obtained by the broth macrodilution reference method. 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 M27-A3.

### 3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention.<sup>17</sup> For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to CLSI document M29.<sup>18</sup>

### 4 Definitions

**antibiogram** – overall profile of antimicrobial susceptibility results of a microbial species to a battery of antimicrobial agents.

**antimicrobial susceptibility test interpretive category** – **1)** a classification based on an *in vitro* response of an organism to an antimicrobial agent at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent; **2) susceptible antimicrobial susceptibility test interpretive category** – a category that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection; **3) susceptible-dose dependent antimicrobial susceptibility test interpretive category** – the “susceptible-dose dependent” category implies clinical efficacy when higher than normal dosage of a drug can be used and maximal possible blood level achieved; **4) intermediate antimicrobial susceptibility test interpretive category** – the “intermediate” category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates and/or available data do not permit them to be clearly categorized as either “susceptible” or “resistant.” This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations; **5) resistant antimicrobial susceptibility test interpretive category** – a category that implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules; **6) nonsusceptible** – this category is used for organisms that currently have only a susceptible interpretive category, but not intermediate or resistant interpretive categories (ie, susceptible-only interpretive category) and is often given to new antimicrobial agents for which no resistant isolates have yet been encountered.

**minimal inhibitory concentration (MIC)** – the lowest concentration of an antimicrobial agent that causes a specified reduction in visible growth in an agar or broth dilution susceptibility test. The magnitude of reduction in visible growth is assessed using the following numerical scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in visible growth; 3, slight reduction in visible growth; and 4, no reduction in visible growth.

**quality control** – the operational techniques that are used to ensure accuracy and reproducibility.

## 5 Indications for Performing Susceptibility Tests<sup>19-21</sup>

Susceptibility testing is indicated for any organism that contributes to an infectious process warranting antimicrobial chemotherapy if its susceptibility cannot be reliably predicted from knowledge of the organism’s identity. Susceptibility tests are most often indicated when the causative organism is thought to belong to a species capable of exhibiting resistance to commonly used antimicrobial agents. Mechanisms of resistance include alteration of drug targets, altered drug uptake or efflux, and absence of microbial enzymes to metabolize drug to active form. Some organisms have predictable susceptibility to antimicrobial agents, and empiric therapy for these organisms is widely accepted. Susceptibility tests are also important in studies of the epidemiology of resistance and in studies of new antimicrobial agents.

Isolated colonies of each type of organism that may be pathogenic should be selected from primary agar plates and tested for susceptibility. Identification procedures are often performed at the same time. Mixtures of different types of microorganisms should not be tested on the same susceptibility test plate or panel. The practice of conducting susceptibility tests directly with clinical material (eg, normally sterile body fluids and urine) should be avoided except in clinical emergencies when the direct gram stain suggests a single pathogen. When testing has been carried out directly with the clinical material, results should be reported as preliminary, and the susceptibility test must be repeated using the standardized methodology.

When the nature of the infection is not clear and the specimen contains mixed growth or normal flora, in which the organisms probably bear little relationship to the infectious process being treated, susceptibility tests are often unnecessary, and the results may be misleading.

The MIC obtained using a dilution test may tell a physician the concentration of antimicrobial agent required at the site of infection to inhibit the infecting organism. The MIC, however, does not represent an

absolute value. The “true” *in vitro* MIC is somewhere between the lowest test concentration that inhibits the organism’s growth (that is, the MIC reading) and the next lower test concentration. If, for example, twofold dilutions were used and the MIC is 16 µg/mL, the “true” MIC would be between 16 and 8 µg/mL. Even under the best of controlled conditions, a dilution test may not yield the same end point each time it is performed. Generally, the acceptable reproducibility of the test is within one twofold dilution of the actual end point. To avoid greater variability, the dilution test must be standardized and carefully controlled as described herein.

MICs have been determined using concentrations derived traditionally from serial twofold dilutions indexed to the base 2 (eg, 1, 2, 4, 8, 16 µg/mL). Other dilution schemes have also been used, including use of as few as two widely separated or “breakpoint” concentrations or concentrations between the usual values (eg, 4, 6, 8, 12, 16 µg/mL). The results from these alternative methods may be equally useful clinically; however, some are more difficult to control. When there is inhibition of growth at the lowest concentration tested, the true MIC value cannot be accurately determined and should be reported as equal to or less than the lowest concentration tested. To apply interpretive criteria when concentrations between the usual dilutions are tested, results falling between serial twofold dilutions should be rounded up to the next highest concentration (eg, an MIC of 6 µg/mL would become 8 µg/mL).

Whenever MIC results are reported to clinicians to direct therapy, an interpretive category (ie, susceptible, susceptible-dose dependent, intermediate, nonsusceptible, or resistant) should accompany the MIC result based on the criteria outlined in Table 1 of the M27 Informational Supplement.<sup>13</sup> When tests in which four or fewer consecutive concentrations are tested or when nonconsecutive concentrations are tested, an interpretive category result must be reported. The MIC result may also be reported, if desired.

## 6 Antifungal Agents

### 6.1 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. Store the powders as recommended by the manufacturers, or at –20 °C or below (never store in a self-defrosting freezer), in a desiccator, preferably in a vacuum. When the desiccator is removed from the freezer, allow it to come to room temperature before opening (to avoid condensation of water).

### 6.2 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 formulas used determines the amount of powder or diluent needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (}\mu\text{g/mL)}}{\text{Assay Potency (}\mu\text{g/mg)}} \quad (1)$$

or

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

The antifungal powder should be weighed on an analytical balance that has been calibrated by approved reference weights from a national metrology organization. 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  $\mu\text{g}$  of antifungal agent per mL with antifungal powder that has a potency of 750  $\mu\text{g/mg}$ , use the first formula to establish the weight of powder needed:

$$\text{Weight (mg)} = \frac{100 \text{ mL} \cdot 1280 \mu\text{g/mL}}{750 \mu\text{g/mg}} = 170.7 \text{ mg} \quad (3)$$

(Potency)

Because it is advisable to weigh a portion of the powder in excess of that required, deposit powder on the balance until approximately 180 mg is reached. With that amount of powder weighed, use formula (2) above to determine the amount of diluent to be measured:

$$\frac{\text{Volume (mL)}}{\text{(mL)}} = \frac{182.6 \text{ mg} \cdot 750 \mu\text{g/mg}}{1280 \mu\text{g/mL}} = 107.0 \text{ mL} \quad (4)$$

(Desired Concentration)

Therefore, dissolve the 182.6 mg of the antifungal powder in 107.0 mL of diluent.

### 6.3 Preparing Stock Solutions

Prepare antifungal stock solutions at concentrations of at least 1280  $\mu\text{g/mL}$  or 10 times the highest concentration to be tested, whichever is greater. Some antifungal agents, however, of limited solubility can require lower concentrations. In all cases, information provided by the drug manufacturer should be considered as part of determining solubility.

#### 6.3.1 Use of Solvents Other Than Water

Some drugs must be dissolved in solvents other than water (see Table 2, M27 Informational Supplement).<sup>13</sup> 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. This procedure avoids dilution artifacts that result from precipitation of compounds with low solubility in aqueous media.

For example, to prepare for a broth macrodilution test series containing a water-insoluble drug that can be dissolved in DMSO, for which the highest desired test concentration is 16  $\mu\text{g/mL}$ , 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 1600 µg/mL. Next, prepare further dilutions of this stock solution in DMSO. (See Table 4, M27 Informational Supplement.)<sup>13</sup> Dilute the solutions in DMSO tenfold in test medium (see Section 7.2.2) and a further tenfold when inoculated (see Section 7.3), reducing the final solvent concentration to 1%. DMSO at this concentration (without drug) should be used in the test as a dilution control.

The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations in Section 6.2 should be applied.

### 6.3.2 Filtration

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

### 6.3.3 Storage

Dispense small volumes of the sterile stock solutions into sterile polypropylene or polyethylene vials, carefully seal, and store (preferably at -60 °C or below, but never at a temperature greater than -20 °C). Remove vials as needed and use the same day. Discard any unused drug 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.<sup>22</sup> In all cases, consider any directions provided by the drug manufacturer as a part of these general recommendations, which supersede 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 (QC) strains such as those in Table 5 of the M27 Informational Supplement.<sup>13</sup>

## 6.4 Number of Concentrations Tested

The concentrations tested should encompass the breakpoint concentrations and the expected results for the QC strains. Based on previous studies, the following drug concentration ranges should be used: amphotericin B, 0.0313 to 16 µg/mL; flucytosine, 0.125 to 64 µg/mL; ketoconazole, 0.0313 to 16 µg/mL; itraconazole, 0.0313 to 16 µg/mL; fluconazole, 0.125 to 64 µg/mL; posaconazole, ravuconazole, and voriconazole, 0.0313 to 16 µg/mL; and anidulafungin, caspofungin, and micafungin, 0.015 to 8 µg/mL.

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

Although breakpoints are now available for some organism-drug combinations (see Table 1, M27 Informational Supplement),<sup>13</sup> 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 committee, clinical microbiology personnel, and the infection control committee.

### 6.5.1 Generic Names

To minimize confusion, all antifungal agents should be referred to by international nonproprietary (ie, generic) names.

### 6.5.2 Number of Agents Tested

To make routine susceptibility tests relevant and practical, usually a limited number of antibacterial agents are tested. Although this is not an immediate issue for antifungal agents, the same principle would apply.

### 6.5.3 Guidelines for Selective Testing

Testing may be warranted under certain selected circumstances such as the following: (a) as part of periodic batch surveys that establish antibiograms for collections of pathogenic isolates obtained from within an institution; (b) to aid in the management of refractory oropharyngeal infections due to *Candida* spp. in patients who appear to be experiencing therapeutic failure of the standard agents at standard doses; and (c) to aid in the management of invasive infections due to *Candida* spp. when the utility of the azole antifungal agents is uncertain (eg, when the infection is due to a non-*C. albicans* isolate). Interpretive breakpoints are available for *Candida* spp. vs anidulafungin, caspofungin, fluconazole, flucytosine, itraconazole, micafungin, and voriconazole. The clinical relevance of testing any other organism-drug combination remains uncertain. Specimens for culture and other procedures should be obtained before antifungal therapy is initiated.

## 7 Test Procedures

### 7.1 Broth Medium

#### 7.1.1 Synthetic Medium

A completely synthetic medium is recommended for susceptibility tests. RPMI 1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) has been found at least as satisfactory as several other synthetic media and has been used to develop the standard.<sup>3,4</sup> The formula for this medium is provided in Table 7 of the M27 Informational Supplement,<sup>13</sup> and the preparation of the medium from powder is outlined in Appendix A. Alternative media may be advantageous for some organisms and some drugs. Table 8 of the M27 Informational Supplement<sup>13</sup> lists modifications for special circumstances.

Investigators preparing work for publication are encouraged to provide information regarding modification to the standard method used in their work.

#### 7.1.2 Buffers

Media should be buffered to a pH of  $7.0 \pm 0.1$  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] (final concentration 0.165 mol/L for pH 7.0). Check the pH of each batch of medium with a pH meter immediately after preparing the medium; the pH should be between 6.9 and 7.1 at room temperature (25 °C). Evaluate MIC performance characteristics of each batch of broth using a standard set of QC organisms (see Section 8).

### 7.2 Preparing Diluted Antifungal Agents

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

- (1) Use sterile, 12- x 75-mm plastic test tubes to perform the tests.
- (2) Use a growth control tube containing RPMI 1640 medium without antifungal agents (but with nonaqueous solvent where necessary) for each organism tested.
- (3) Close the tubes with loose screw-caps, or plastic or metal caps.

### 7.2.1 Water-Soluble Antifungal Agents

When twofold dilutions of a water-soluble antifungal agent are used, they may be prepared volumetrically in broth (see Table 3, M27 Informational Supplement).<sup>13</sup> 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 4 of the M27 Informational Supplement<sup>13</sup> is recommended.

The total volume of each dilution prepared depends on the number of tests performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL will be adequate for about nine tests, allowing for pipetting. Use a single pipette for measuring all diluents and then for adding the stock antifungal solution to the first tube. Use a separate pipette for each remaining dilution in that set. Because there will be a 1:10 dilution of the drugs when combined with the inoculum, the working antifungal solutions are 10 times more concentrated than the final concentrations.

Many persons find working with 1:10 dilutions (as shown in Table 3, M27 Informational Supplement)<sup>13</sup> easy and convenient. However, some automated pipettes deliver only 1.0 or 0.1 mL volumes; therefore, a ratio of 1:11 would be preferable. It is unimportant whether the final test volume is 1.0 mL or 1.1 mL. If 1:11 dilutions are made, the dilution scheme should be altered so the same final concentrations of drug are obtained.

### 7.2.2 Water-Insoluble Antifungal Agents

For antifungal agents that cannot be prepared as stock solutions in water, such as amphotericin B, anidulafungin, itraconazole, ketoconazole, posaconazole, and voriconazole, a dilution series of the agent should be prepared first at 100 times final strength in an appropriate solvent (see Section 6.3.1). Each of these nonaqueous solutions should now be diluted tenfold in RPMI 1640 broth.

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 1600 to 3.13 µg/mL should have been prepared first in DMSO (see Section 6.3.1). To prepare 1-mL volumes of diluted antifungal agent (sufficient for 10 tests), first pipette 0.9-mL volumes of RPMI 1640 broth into each of 11 sterile test tubes. Then, using a single pipette, add 0.1 mL of DMSO alone to one 0.9-mL lot of broth (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 0.9 mL broth. These volumes can be adjusted according to the total number of tests required. Because there will be a 1:10 dilution of the drugs when combined with the inoculum, the working antifungal solutions are 10 times more concentrated than the final concentrations.

## 7.3 Inoculum Preparation

The steps for preparation of inoculum are as follows:

- (1) All organisms should be subcultured onto Sabouraud dextrose agar or potato dextrose agar and passaged to ensure purity and viability. The incubation temperature throughout must be 35 °C.
- (2) The inoculum should be prepared by picking five colonies of ~1 mm in diameter from 24-hour-old cultures of *Candida* spp. or 48-hour-old cultures of *C. neoformans*. The colonies should be suspended in 5 mL of sterile 0.145-mol/L saline (8.5 g/L NaCl; 0.85% saline) or sterile water.
- (3) The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline or sterile water to increase the transmittance to that produced by a 0.5 McFarland standard (see Appendix B) at 530 nm wavelength. This

procedure will yield a yeast stock suspension of  $1 \times 10^6$  to  $5 \times 10^6$  cells per mL. A working suspension is made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium, which results in  $5.0 \times 10^2$  to  $2.5 \times 10^3$  cells per mL.<sup>2</sup>

## 7.4 Inoculating RPMI-1640 Medium

Before adjusting the inoculum, place 0.1 mL of the various antifungal concentrations in 12- x 75-mm tubes. The growth control receives 0.1 mL of drug diluent without antifungal agent. Within 15 minutes after the inoculum has been standardized (up to two hours if inoculum is kept at 4 °C), add 0.9 mL of the adjusted inoculum to each tube in the dilution series and mix. This results in a 1:10 dilution of each antifungal concentration and a 10% dilution of the inoculum.

## 7.5 Incubation

With the exception of *C. neoformans*, incubate tubes (without agitation) at 35 °C for 24 to 48 hours in ambient air. When testing *C. neoformans*, tubes should be incubated for a total of 70 to 74 hours before determining results.

## 7.6 Reading Results

The amount of growth in the tubes containing the agent is compared visually with the amount of growth in the growth-control tubes (no antifungal agent) used in each set of tests using the following numerical scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity.

### 7.6.1 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 of 0). Trailing end points with amphotericin B are usually not encountered.

### 7.6.2 Flucytosine (5-FC) and Azole Antifungals

For flucytosine and especially for azoles, 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 (score of 2, prominent decrease in turbidity), which translates to an approximately 50% reduction in growth relative to the drug-free growth control, has improved interlaboratory agreement and also discriminates between putatively susceptible and resistant isolates. When turbidity persists, it is often identical for all drug concentrations above the MIC. Even dispersion of clumps that can become evident after incubation can make end-point determination more reproducible. Reference strains of defined susceptibility can also be used in the training of new personnel.

### 7.6.3 Echinocandin Antifungals

For echinocandins, determine MIC end points after 24 hours incubation and read as the lowest drug concentration to produce a prominent decrease in turbidity (score of 2), which translates to an approximately 50% reduction in growth relative to the drug-free growth control.

## 7.7 Interpretation of Results

Interpretive breakpoints have been established at present only for some organism-drug combinations (see Table 1, M27 Informational Supplement).<sup>13</sup> The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarized as follows:

### 7.7.1 Amphotericin B

Experience to date using the procedures described in this standard indicates that amphotericin B MICs for *Candida* spp. isolates are tightly clustered between 0.25 and 1.0 µg/mL. When isolates that appear resistant to amphotericin B in animal models are tested by M27 methods, MIC values greater than 1 µg/mL may be obtained. Unfortunately, the M27 methodology does not consistently permit detection of such isolates, and all that can at present be concluded is that if an amphotericin B MIC of >1 µg/mL is obtained for a *Candida* spp. isolate, then that isolate is likely resistant to amphotericin B. Some work has suggested that testing with Antibiotic Medium 3 supplemented with 2% glucose (dextrose) and reading MICs after 24 hours incubation permits more reliable detection of resistant isolates.<sup>23,24</sup> However, the reproducibility of this method has been questioned,<sup>25</sup> and laboratories that choose to do this testing must carefully compare their results with those obtained for isolates with known responses to amphotericin B. A collection of potentially useful reference isolates has been deposited in the American Type Culture Collection (ATCC®)<sup>a</sup>: *Candida lusitanae* ATCC® 200950, ATCC® 200951, ATCC® 200952, ATCC® 200953, ATCC® 200954; *C. albicans* ATCC® 200955; and *Candida tropicalis* ATCC® 200956.

### 7.7.2 Flucytosine (5-FC)

Based largely on historical data and partially on the drug's pharmacokinetics, interpretive breakpoints for *Candida* spp. and flucytosine have been established (see Table 1, M27 Informational Supplement).<sup>13</sup>

### 7.7.3 Fluconazole

Based on a large data package presented by fluconazole's manufacturer,<sup>11</sup> interpretive breakpoints for *Candida* spp. and fluconazole have been established (see Table 1, M27 Informational Supplement).<sup>13</sup> These data are principally drawn from studies of oropharyngeal candidiasis and of invasive infections due to *Candida* spp. in nonneutropenic patients, and their clinical relevance in other settings is uncertain. These interpretive breakpoints are not applicable to *Candida krusei*, and thus identification to the species level is required for accurate interpretation and reporting of MICs. In addition, when an isolate is identified as *C. glabrata* and the MIC is ≤32, patients should receive a maximum dosage regimen of fluconazole. Expert consultation on selection of a maximum dosage regimen may be useful. The utility of testing isolates of *C. neoformans* is currently under intense study, and recent data suggest a correlation between elevated MIC and clinical failure.<sup>26</sup>

### 7.7.4 Ketoconazole

Experience to date using the procedures described in this standard indicates that MICs for yeast vary between 0.03 and 16 µg/mL. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with ketoconazole.

### 7.7.5 Itraconazole

Based on a large data package presented by itraconazole's manufacturer,<sup>11</sup> interpretive breakpoints for *Candida* spp. and itraconazole have been established (see Table 1, M27 Informational Supplement).<sup>13</sup> These data are entirely from studies of oropharyngeal candidiasis, and their clinical relevance in other settings is uncertain. In addition, the importance of proper preparation of drug dilutions for this insoluble compound cannot be over-emphasized. Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 4 of the M27 Informational Supplement<sup>13</sup> can lead to substantial errors due to dilution artifacts.

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<sup>a</sup> ATCC is a registered trademark of the American Type Culture Collection.

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### 7.7.6 Voriconazole

Based on a large data package presented by voriconazole's manufacturer, interpretive breakpoints for *Candida* spp. and voriconazole have been established (see Table 1, M27 Informational Supplement).<sup>13</sup> These data are drawn from *in vitro* tests, animal models, and six clinical trials with the majority taken from a clinical trial in nonneutropenic patients with candidemia,<sup>27</sup> and their clinical relevance in other settings is uncertain.

### 7.7.7 New Triazoles

Experience to date with posaconazole and ravuconazole, using procedures described in this standard, indicates that yeast MICs vary between 0.03 and 16  $\mu\text{g}/\text{mL}$  with the majority of isolates inhibited by  $\leq 1$   $\mu\text{g}/\text{mL}$  for both agents. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with these agents.

### 7.7.8 Echinocandins (Anidulafungin, Caspofungin, and Micafungin)

Using procedures described in this standard, MIC survey data for >2500 clinical isolates of *Candida* spp. indicate that MICs vary between 0.007 and 8  $\mu\text{g}/\text{mL}$  with  $\geq 99\%$  of isolates inhibited by  $\leq 2$   $\mu\text{g}/\text{mL}$ .<sup>28,29</sup> MICs of  $\leq 2$   $\mu\text{g}/\text{mL}$  are predictive of susceptibility (see M27 Informational Supplement).<sup>13</sup> However, the datasets contain too few outcomes of treatment of infection with higher MICs to permit creation of a resistant category.

## 7.8 Broth Microdilution Modifications

A substantial body of data has been presented that documents excellent concordance between results obtained by the broth macrodilution methodology described above and a broth microdilution adaptation.<sup>5,8-10,30-33</sup> The ease of performance of broth microdilution tests is very attractive, and most clinical laboratories will probably choose to implement this method, rather than the broth macrodilution method. The steps and testing conditions relevant to the broth microdilution test are discussed in detail.

The tenfold drug dilutions described for the broth macrodilution procedure should be further diluted 1:5 with RPMI to achieve the two times strength needed for the broth microdilution test. Prepare and adjust the stock inoculum suspensions, as described under the broth macrodilution test. Mix the stock yeast suspension for 15 seconds with a vortex, dilute 1:50, and further dilute 1:20 with medium to obtain the two times test inoculum ( $1 \times 10^3$  to  $5 \times 10^3$  CFU/mL). Dilute the (two times) inoculum 1:1 when the wells are inoculated and the desired final inoculum size is achieved ( $0.5 \times 10^3$  to  $2.5 \times 10^3$  CFU/mL).

Perform the broth microdilution test by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells). Dispense the two times drug concentrations into the wells of Rows 1 to 10 of the microdilution plates in 100- $\mu\text{L}$  volumes with a multichannel pipette. Row 1 contains the highest (either 64 or 16  $\mu\text{g}/\text{mL}$ ) drug concentration and Row 10 contains the lowest drug concentration (either 0.12 or 0.03  $\mu\text{g}/\text{mL}$ ). These trays may be sealed in plastic bags and stored frozen at  $-70$  °C for up to six months without deterioration of drug potency. Inoculate each well of a microdilution tray on the day of the test with 100  $\mu\text{L}$  of the corresponding two times diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentrations mentioned above. Inoculate the growth control wells, which contain 100  $\mu\text{L}$  of sterile, drug-free medium, with 100  $\mu\text{L}$  of the corresponding diluted (two times) inoculum suspensions. Test the QC organisms in the same manner and include each time an isolate is tested. Row 12 of the microdilution plate can be used to perform the sterility control (drug-free medium only).

Incubate the microdilution plates at 35 °C and observe for the presence or absence of visible growth. Agitation of the plates is optional and may simplify reading of the end points. Score the microdilution wells with the aid of a reading mirror; compare the growth in each well with that of the growth control (drug-free) well. Give a numerical score to each well using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity (approximately 50% as determined visually or spectrophotometrically); 3, slight reduction in turbidity; and 4, no reduction of turbidity. When clumping of an isolate hinders scoring of wells, try pipetting, vortexing, or other mixing techniques.<sup>33,34</sup> The MIC for amphotericin B is defined as the lowest concentration in which a score of 0 (optically clear) is observed. For the azoles, echinocandins, and flucytosine, the MIC is defined as the lowest concentration in which a score of 2 (prominent decrease in turbidity) is observed.

### 7.8.1 Time of Reading

The original work with M27 focused on reading the MIC after 48 hours incubation. As reading after only 24 hours offers a clear advantage for patient care, QC limits were established for all drugs at 24 hours and 48 hours (see Table 6, M27 Informational Supplement).<sup>13</sup> In parallel with this work, it also became apparent that reading at 24 hours was both possible and in some cases, preferable (see also Section 7.9). For example, echinocandin MICs should be read at 24 hours (see Section 7.6.3).<sup>35</sup>

It is known at present that microdilution MICs read at 24 hours for the echinocandins; 24 or 48 hours for amphotericin B and fluconazole; 48 hours for flucytosine, itraconazole, voriconazole, ravuconazole, and posaconazole; and 72 hours for most *C. neoformans* isolates against all above referenced drugs provide agreement with the reference broth macrodilution method.<sup>32,33,36</sup> The following times of reading are thus now supported:

Antifungal Agent	Acceptable Time of MIC Reading if Fungal Growth Is Adequate	
	24 h	48 h
Amphotericin B	Yes (1)	Yes (1)
Echinocandins	Yes (2)	No
Fluconazole	Yes (3)	Yes
Flucytosine	No (4)	Yes
Itraconazole	No (4)	Yes
Posaconazole	No (4)	Yes (1)
Ravuconazole	No (4)	Yes (1)
Voriconazole	No (5)	Yes

### Notes

1. No approved interpretive breakpoints are available.
2. Current interpretive breakpoints are defined only for the 24 hours reading.
3. The current fluconazole interpretive breakpoints were defined based on MIC readings obtained with the reference method at 48 hours. Recent analyses have shown that these same interpretive breakpoints provide similar clinical predictive correlation when used with MICs read after 24 hours incubation. When reading fluconazole MICs at 24 hours, it is especially important to be aware of the comment regarding *C. glabrata* found in the footnote of Table 1, M27 Informational Supplement (“Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida spp.*”): When an isolate is identified as *C. glabrata* and the MIC is  $\leq 32$ , patients should receive a maximum dosage of fluconazole.
4. Evaluation of the utility of a 24-hour MIC reading has not as yet been undertaken for these drugs.
5. Work to evaluate the utility of a 24-hour MIC reading for voriconazole is currently underway.

## 7.9 Trailing Growth and the Impact of Time of Reading

The M27 methodology for *Candida* recommends an end-point reading at 48 hours for both macro- and microdilution methods. For most isolates, the difference between readings at 24 hours vs 48 hours is minimal, and will not alter the interpretive category (ie, does not change whether the isolate would be categorized as “susceptible” or “resistant”). Thus, as noted in Section 7.8.1, microdilution readings at both 24 hours and 48 hours provide agreement with the reference methodology for most drugs. However, readings taken at 24 hours may be more clinically relevant for some isolates. Antimicrobial agents for which the earlier reading is important (eg, echinocandins) show a dramatic rise in MIC between 24 hours and 48 hours, due to significant trailing growth (partial inhibition of growth over an extended range of antifungal concentrations). Estimated as occurring for fluconazole in about 5% of isolates,<sup>37</sup> this trailing growth can be so great as to make an isolate that appears susceptible after 24 hours appear completely resistant at 48 hours. Two independent *in vivo* investigations of this phenomenon that employed murine models of disseminated candidiasis<sup>37,38</sup> have shown that isolates with this behavior should be categorized as “susceptible” rather than “resistant.” This concept has been corroborated by a demonstration that trailing growth can be eliminated by lowering the pH of the test medium to 5 or less,<sup>39</sup> and by a clinical demonstration that oropharyngeal candidiasis due to such isolates respond to a low dose of fluconazole used to treat typical susceptible isolates.<sup>40</sup> In light of these observations, both 24-hour and 48-hour microdilution MIC ranges are provided for the two QC strains and multiple systemic antifungal agents (see Table 6, M27 Informational Supplement).<sup>13</sup>

## 7.10 Other Modifications

In addition to ongoing efforts to simplify the procedures described in this standard, some more fundamental modifications of the method have been developed in response to specific problems and are described in Table 8 of the M27 Informational Supplement.<sup>13</sup> These modifications are not part of the current methodology, but interested laboratories may wish to explore their clinical relevance.

# 8 Quality Control

## 8.1 Purpose

The goals of a QC program are to monitor the following:

- the precision (repeatability) and accuracy of the susceptibility test procedure;
- the performance of reagents, testing conditions, and instructions used in the test; and
- 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 QC and reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.<sup>6</sup>

## 8.2 QC Responsibilities

### 8.2.1 Manufacturers (Commercial and/or “In-House” Products)

Manufacturers are responsible for the following:

- antifungal stability;
- antifungal labeling;
- potency of antifungal stock solutions;

- compliance with good manufacturing practices;
- integrity of the product; and
- accountability and traceability to the consignee.

### 8.2.2 Laboratory (User)

The laboratorian is responsible for the following:

- storage (drug deterioration);
- operator proficiency; and
- adherence to procedure (eg, inoculum effect, incubation conditions [time and temperature]).

### 8.2.3 Mutual Responsibility

Manufacturers should design and recommend a QC program that allows the user to evaluate those variables (eg, 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.

## 8.3 Selecting Reference Strains

Ideal reference strains for QC of dilution methods have MICs that fall near the midrange of the concentration for all antifungal agents tested. An ideal control strain is inhibited at the fifth dilution of a nine-dilution log<sub>2</sub> series, but strains for which the antimicrobial agent MICs are between the third and seventh dilution are acceptable. Before a strain is accepted as a reference, test it for as long as necessary to demonstrate that its antifungal susceptibility pattern is genetically stable. CLSI/NCCLS document M23<sup>41</sup> provides guidelines for the selection of appropriate QC strains and the determination of acceptable MIC ranges. The strains listed in Tables 5 and 6 of the M27 Informational Supplement<sup>13</sup> were selected in accordance with these criteria.<sup>6,7,12</sup>

## 8.4 Storing Reference Strains

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

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

- There are two preferred methods for prolonged storage of reference strains. Yeasts may be grown on potato dextrose agar and then frozen at  $-70\text{ }^{\circ}\text{C}$  as described by Pasarell and McGinnis.<sup>42</sup> Alternatively, reference strains can be preserved by suspending fungal cells in 15% glycerol solution in small vials and freezing and storing them at  $-70\text{ }^{\circ}\text{C}$ .
- For short-term storage, working stock cultures can be grown on Sabouraud dextrose agar or peptone dextrose agar slants until sufficient growth is observed, and stored at  $2\text{ }^{\circ}\text{C}$  to  $8\text{ }^{\circ}\text{C}$ . Prepare fresh slants at two-week intervals by serial transfer from frozen stocks. To avoid mixed cultures, no more than three passages should be made after removal from frozen stock culture. Whenever aberrant results occur, obtain a new stock culture.

### 8.4.2 Sources for Reference Strains

Obtain reference strains from a source that is able to provide information on the origination of the culture (for example, from the American Type Culture Collection [ATCC<sup>®</sup>], from commercial sources with

documented culture history, or from reference institutions with demonstrated ability to store and use the organisms consistently with minimal contamination).

### 8.4.3 Preparing Strains for Storage

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

- (1) Grow the organisms overnight on petri dishes containing Sabouraud dextrose agar or 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 5, M27 Informational Supplement,<sup>13</sup> for expected MICs of some reference strains).
- (3) Subculture strains yielding expected results onto the same medium that was used for 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 several small containers.
- (7) Place these containers in a  $-70^{\circ}\text{C}$  freezer maintained as in Section 6.3.3, 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, repeat this process to make a new supply.

### 8.5 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  $-70^{\circ}\text{C}$  freezer or obtain a lyophilized vial.
- (2) Let the frozen mixture thaw or rehydrate the lyophilized culture.
- (3) Subculture the mixture onto potato dextrose agar plates and incubate them at  $35^{\circ}\text{C}$  for 24 hours for *Candida* spp., or for 48 hours for *C. neoformans*.
- (4) Remove four to five colonies, subculture them to medium for the appropriate susceptibility tests, and then subculture them onto potato dextrose agar slants.
- (5) After incubating the strains overnight, store on agar slants at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$ .
- (6) Subculture from the slant to an agar plate for testing.
- (7) Always perform susceptibility tests on colonies from overnight plates.

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

## 8.6 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, lot of macrodilution tubes, or lot of microdilution plates, with one of the QC strains listed in Table 5 of the M27 Informational Supplement<sup>13</sup> 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 or microdilution plate 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 7.1.2).
- (4) Record the lot numbers of all materials and reagents used in these tests.

## 8.7 QC Frequency

### 8.7.1 MIC Ranges

MIC QC limits for a single control test are listed in Tables 5 and 6 (see M27 Informational Supplement).<sup>6,7,12,13</sup> In general, 1 out of every 20 MIC values in a series of 20 consecutive tests might be out of control (ie, outside the stated range) due to random variation of the test. Two consecutive out-of-control results or any more than two 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 QC tests weekly instead of daily (see Section 8.7.2).

### 8.7.2 Frequency of Testing

The overall performance of the test system should be monitored by testing 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) Document 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 (ie, MIC values obtained from one drug-microorganism combination for 30 consecutive test days) may be outside the accuracy ranges stated in Tables 5 and 6 of the M27 Informational Supplement.<sup>13</sup>

**NOTE:** This procedure is only for establishing satisfactory performance of MIC tests for the purpose of performing QC tests weekly instead of daily. Do not confuse this procedure with the steps that must be taken for corrective action defined in Section 8.7.1.

- (3) When these conditions are fulfilled, each reference strain must be tested at least once per week and whenever any reagent component is changed. 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 (ie, MIC values obtained from one drug-microorganism combination for five consecutive test days) must be within the accuracy ranges stated in Tables 5 and 6 of the M27 Informational Supplement.<sup>13</sup>
- (4) If resolution of the problem cannot be documented (ie, at least one of the five MIC values is observed to be outside the accuracy range), daily control testing must be continued. Returning 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, QC tests must be done more frequently than once per week because of the relatively rapid degradation of the drug.

## **8.8 Other Control Procedures**

### **8.8.1 Growth Control**

Each broth macrodilution and microdilution 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.

### **8.8.2 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.

### **8.8.3 End-point Interpretation Control**

Monitor end-point interpretation 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. Record the results and compare to the results obtained by an experienced reader. QC and reference strains with predetermined MICs are particularly helpful for this purpose, especially with fluconazole.<sup>6,7,12</sup>

## **8.9 QC Strains (see also Section 8.3)**

Ideal reference strains for QC of dilution tests have MICs that consistently fall near the midpoint of the concentration range tested for all antifungal agents (eg, 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).

Tables 5 and 6 of the M27 Informational Supplement<sup>13</sup> list expected ranges for strains found to be acceptable as QC strains. Also shown are additional strains that can be useful for conducting reference studies.<sup>6,7,12</sup>

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## Appendix A. RPMI 1640 Medium

**RPMI 1640 medium buffered with 0.165 mol/L MOPS, 1 L.** (See Table 7, M27 Informational Supplement.)<sup>1</sup>

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<sub>2</sub>O. Add MOPS (final concentration of 0.165 mol/L) and stir until dissolved. While stirring, adjust the pH to 7.0 at 25 °C using 1 mol/L sodium hydroxide. Add additional water to bring medium to a final volume of 1 L. Filter sterilize and store at 4 °C until use.

### Reference for Appendix A

<sup>1</sup> CLSI. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Informational Supplement*. CLSI document M27-S3. Wayne, PA; Clinical and Laboratory Standards Institute; 2008.

## Appendix B. McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, use a BaSO<sub>4</sub> turbidity standard (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<sub>2</sub> (1.175% w/v BaCl<sub>2</sub> × 2H<sub>2</sub>O) to 99.5 mL of 0.18 mol/L H<sub>2</sub>SO<sub>4</sub> (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 0.13 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 monthly after preparation.

**Clinical and Laboratory Standards Institute consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact CLSI or visit our website at [www.clsi.org](http://www.clsi.org).**

## Summary of Delegate Comments and Committee Responses

M27-A3: *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition*

### Section 1, Scope

1. First paragraph, first sentence: “This document describes a method for testing the susceptibility to antifungal agents of yeasts, ~~that cause infections~~, including *Candida spp.* and *Cryptococcus neoformans*, that cause infections.”
  - **The sentence has been revised as suggested.**
2. First paragraph, second sentence: “This method has not been extensively validated for the yeast forms of dimorphic...”
  - **The sentence has been revised as suggested.**

### Section 2, Introduction

3. First paragraph, third sentence: “The method has not been used in studies of the yeast forms of dimorphic fungi, such as *B. dermatitidis* ~~or~~ and/or *H. capsulatum* variety *capsulatum*.”
  - **The sentence has been revised as suggested.**
4. First paragraph, fourth sentence: “Moreover, testing filamentous fungi (~~molds~~ moulds) introduces several additional...” (same as in M27-A2).
  - **The sentence has been revised as suggested.**
5. First paragraph, last sentence: CLSI document M38-A.
  - **The document citation for M38 in this sentence follows the editorial policy for all CLSI documents. No change has been made to the document.**

### Section 6.2, Weighing Antifungal Powders

6. Third paragraph, first sentence: (NIST<sub>2</sub>, Gaithersburg, MD)...
  - **The text the commenter is referring to has since been removed from the document.**
7. Fifth paragraph, first sentence: “Because it is advisable to obtain a volume of a stock solution in excess of that required, weigh a portion of the powder in excess ~~of that required~~, deposit powder on the balance until 182.6 mg is reached.”
  - **To maintain consistency throughout all CLSI antifungal documents, the wording in the document will remain unchanged.**

Section 7.8.1, Time of Reading

8. In Section 7.8.1, posaconazole and ravuconazole are missing from both the table and text. I would urge the committee to reconsider, as posaconazole is approved for OPC, and laboratories will want to test the drug even if they are not 100% sure what the MIC value means.
- **The table in Section 7.8.1 has been updated to add posaconazole and ravuconazole. An additional note for both antifungal agents is also provided for further clarification.**

Section 7.9, Trailing Growth and the Impact of Time of Reading

9. Fifth sentence: “Antimicrobial agents for which the earlier reading is important (eg, echinocandins) show a dramatic rise...”
- **The sentence has been revised as suggested.**

Section 8.4.1, Methods for Prolonged and Short-term Storage

10. Second bullet: Provide formulation for peptone dextrose agar slants through the addition of an Appendix C.
- **CLSI prefers to avoid recommending specific suppliers. These slants are routinely available from several sources. No change has been made to the document.**

Section 8.4.3, Preparing Strains for Storage

11. Number 1: Provide formulation for soybean casein digest agar through the addition of an Appendix D.
- **CLSI prefers to avoid recommending specific suppliers. These slants are routinely available from several sources. No change has been made to the document.**

Appendix B. McFarland 0.5 Barium Sulfate Turbidity Standard

12. Since step 2's absorbance is in the range of 0.08 to 0.13, ie, a region of very slight slope on the absorbance curve (turbidity [concentration] on x-axis is plotted vs absorbance on y-axis), the verification might be improved by revising the protocol to prepare initially a more concentrated Barium Sulfate Turbidity Standard, followed by verification of its correct density by using a spectrophotometer, and then diluting the verified Barium Sulfate Turbidity Standard to obtain the 0.5 Barium Sulfate Turbidity Standard.
- **These instructions appear in several CLSI documents and should be consistent. Practically, most laboratories will not go to the trouble of preparing their own barium sulfate standards, but purchase the 0.5 standard from their usual distributor. No change has been made to the document.**

**NOTES**

## The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management 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. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*. The quality management system approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any health care service’s path of workflow (ie, operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The QSEs are:

- |                                  |                                                  |                                                                              |                                                          |
|----------------------------------|--------------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------|
| Documents & Records Organization | Equipment Purchasing & Inventory Process Control | Information Management Occurrence Management Assessments—External & Internal | Process Improvement Customer Service Facilities & Safety |
|----------------------------------|--------------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------|

M27-A3 addresses the QSEs indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment—External & Internal	Process Improvement	Customer Service	Facilities & Safety
M7					X M2 M7 M11 M23 M24 M29 M38						M29

Adapted from CLSI/NCCLS document HS1—*A Quality Management 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, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow, which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

M27-A3 addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Preexamination				Examination			Postexamination	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
			M24	X M24 M38	X M2 M7 M11 M24 M38	X M2 M7 M11 M24 M38	X M2 M7 M11 M24 M38	X M24 M38

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

## Related CLSI Reference Materials\*

- M2-A9**      **Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition (2006).** This document contains the current Clinical and Laboratory Standards Institute-recommended methods for disk susceptibility testing, criteria for quality control testing, and updated tables for interpretive zone diameters.
- M7-A7**      **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition (2006).** This document addresses reference methods for the determination of minimal inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.
- M11-A7**      **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Seventh Edition (2007).** This standard provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by agar dilution and broth microdilution.
- M23-A2**      **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Second Edition (2001).** This document addresses the required and recommended data needed for the selection of appropriate interpretative standards and quality control guidelines for new antimicrobial agents.
- M24-A**      **Antimycobacterial Susceptibility Testing; Approved Standard (2003).** This standard provides protocols and related quality control parameters and interpretive criteria for the susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.
- M29-A3**      **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on U.S. regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.
- M38-A**      **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard (2002).** 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.

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\* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most current editions.

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Fresenius Medical Care/Spectra East (NJ)  
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Jackson Health System (FL)  
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Jacobi Medical Center (NY)  
John C. Lincoln Hospital (AZ)  
John Muir Medical Center (CA)  
John T. Mather Memorial Hospital (NY)  
Johns Hopkins Medical Institutions (MD)  
Johns Hopkins University (MD)  
Johnson City Medical Center (TN)  
JPS Health Network (TX)  
Kadlec Medical Center (WA)  
Kaiser Permanente (CA)  
Kaiser Permanente (MD)  
Kaiser Permanente (OH)  
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King Hussein Cancer Center  
Kings County Hospital Center (NY)  
Kingston General Hospital (Canada)  
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Laboratory Alliance of Central New York (NY)  
LabPlus Auckland Healthcare Services Limited (New Zealand)  
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Massachusetts General Hospital Division of Laboratory Medicine (MA)  
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Mayo Clinic (MN)  
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Memorial Hospital Laboratory (CO)  
Memorial Medical Center (IL)  
Memorial Medical Center (PA)  
Memorial Regional Hospital (FL)  
Mercy Franciscan Mt. Airy (OH)  
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Mercy Medical Center (CO)  
Mercy Medical Center (OR)  
Methodist Hospital (MN)  
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Methodist Hospital Pathology (NE)  
MetroHealth Medical Center (OH)  
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Metropolitan Medical Laboratory, PLC (IA)  
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Middletown Regional Hospital (OH)  
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Center (NV)  
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(CO)  
St. Anthony's Hospital (FL)  
St. Barnabas Medical Center (NJ)  
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St. John's Regional Health Center (MO)  
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St. Joseph Mercy Hospital (MI)  
St. Joseph's Hospital (FL)  
St. Joseph's Hospital & Health  
Center (ND)  
St. Joseph's Medical Center (CA)  
St. Joseph's Regional Medical  
Center (NJ)  
St. Jude Children's Research Hospital  
(TN)  
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St. Luke's Hospital (FL)  
St. Luke's Hospital (IA)  
St. Luke's Hospital (PA)  
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South Dakota State Health Laboratory  
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Southern Health Care Network  
(Australia)  
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Southwest Nova District Health  
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Speare Memorial Hospital (NH)

Spectrum Health - Blodgett Campus  
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State of Hawaii Department of Health (HI)  
State of Washington-Public Health Labs (WA)  
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Stillwater Medical Center (OK)  
Stony Brook University Hospital (NY)  
Stormont-Vail Regional Medical Center (KS)  
Sudbury Regional Hospital (Canada)  
Suncoast Medical Clinic (FL)  
Sunnybrook Health Science Center  
(ON, Canada)  
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(NV)  
Swedish Medical Center (CO)  
Sydney South West Pathology Service  
(Australia)  
T.J. Samson Community Hospital (KY)  
Taipei Veterans General Hospital (Taiwan)  
Taiwan Society of Laboratory Medicine  
Tallaght Hospital  
Tartu University Clinics (Tartu)  
Temple Univ. Hospital - Parkinson  
Pav. (PA)  
Texas Children's Hospital (TX)  
Texas Department of State Health Services  
Thomason Hospital (TX)  
Timmins and District Hospital  
(Canada)  
The Toledo Hospital (OH)  
Touro Infirmary (LA)  
Tri-Cities Laboratory (WA)  
Trident Medical Center (SC)  
Trinity Medical Center (AL)  
Tripler Army Medical Center (HI)  
Tufts New England Medical Center  
Hospital (MA)  
Tulane Medical Center Hospital & Clinic  
(LA)  
Turku University Central Hospital  
UCI Medical Center (CA)  
UCLA Medical Center  
Clinical Laboratories (CA)  
UCSD Medical Center (CA)  
UCSF Medical Center China Basin (CA)  
UMass Memorial Medical Center (MA)  
UMC of Southern Nevada (NV)  
UNC Hospitals (NC)  
Union Clinical Laboratory (Taiwan)  
United Christian Hospital (Hong Kong)  
United Clinical Laboratories (IA)  
Unity HealthCare (IA)  
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(Belgium)  
University College Hospital (Ireland)  
University Medical Center at Princeton  
(NJ)  
University of Alabama-Birmingham  
Hospital (AL)  
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(AR)  
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Center (CO)  
University of Colorado Hospital  
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(IA)  
University of Kentucky Med. Ctr. (KY)  
University of Maryland Medical System  
(MD)  
University of Medicine & Dentistry, NJ  
University Hosp. (NJ)  
University of Miami (FL)  
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Fairview  
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