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M27

## Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts

This standard includes 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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## Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts

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### Abstract

Clinical and Laboratory Standards Institute document M27—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts* describes a method for testing the susceptibility to antifungal agents of yeast that cause invasive fungal infections, including *Candida* spp. 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.

Clinical and Laboratory Standards Institute (CLSI). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. 4th ed. CLSI standard M27. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2015.

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## Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory guidance for selecting 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. 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**. Due to examples of drug antagonism by some complex media for certain antifungal agents, 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
- Incubation temperature
- Incubation duration
- 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 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 QC 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 minimal inhibitory concentration ranges for **microdilution testing** of both established and newly introduced antifungal agents.<sup>12</sup> The study results are included in this document and the current edition of CLSI document **M27/M44S**.<sup>13</sup>

**NOTE:** Current fungal taxonomy is under revision. Many genera have both a teleomorph (sexual state) and an anamorph (asexual state) name. In this document, the traditional *Candida* anamorph names are used to provide continuity to both past procedures and associated documents such as the current edition of M27-S.

## Overview of Changes

### General

Formatting and organization of document revised to reflect the new CLSI quality system essential/path-of-workflow template and revised CLSI style.

References to the information supplement updated to reflect the new combined supplement for broth dilution and disk diffusion testing of yeasts.

Added references to epidemiological cut off values and CLSI documents M57 and the current edition of M57-S.

Commented [MH1]: Is this still true?

Commented [MH2]: Should this be broth macrodilution?

Commented [MH3]: Sheryl, change this to a reference to M27/M44S

Commented [LS(4)]: How about something like this?

Commented [MH5]: A list of changes made since the last edition needs to be added

I reviewed M27-S3 and have started to add some of the things that have been changed in this edition. Please add or revise as appropriate

#### **Subchapter 1.4.2 (Definitions)**

Revised the definitions of interpretive breakpoints for consistency with other antimicrobial susceptibility testing documents.

Added a definition for breakpoint/interpretive criteria.

Added a definition for multiple drug resistant.

#### **Subchapter 1.4.3 (Abbreviations/Acronyms)**

Added the abbreviation for Royal Park Memorial Institute (culture medium)(RPMI)

#### **Chapter 3 (Antifungal Testing Process)**

Added an antifungal testing process flow chart.

Replaced procedural text with step/action tables.

Added text regarding the deletion of breakpoints for itraconazole and flucytosine.

#### **Chapter 4 (Quality System Essential: Process Management – Quality Control)**

Added text regarding the need for laboratories in the United States to develop an individualized quality control plan.

**NOTE:** The findings and conclusions in this document are those of the authors and do not necessarily represent the views of the organizations they represent.

#### **Key Words**

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



## Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts

### Chapter 1: Introduction

This chapter includes:

- Document scope and applicable exclusions
- Background information pertinent to the document content
- Standard precautions information
- “Note on Terminology” that highlights particular use and/or variation in use of terms and/or definitions
- Terms and definitions used in the document
- Abbreviations and acronyms used in the document

#### 1.1 Scope

This document describes a method for testing susceptibility to antifungal agents of yeasts that cause infections, including *Candida* spp. and *Cryptococcus neoformans*. The target audience for this document is laboratory personnel who will be performing antifungal susceptibility testing on yeasts. The focus is on the developing relevant breakpoints for available antifungal agents,<sup>11</sup> and reference minimal inhibitory concentration (MIC) ranges for broth dilution testing of both established and newly introduced antifungal agents.<sup>12</sup> For interpretive MIC breakpoints and MIC ranges for QC isolates refer to the current edition of CLSI document [M27/M44S](#).

Commented [MH6]: Sheryl, add reference to M27/M44S

This method has not been extensively validated for the yeast forms of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum* variety *capsulatum*.

#### 1.2 Background

The broth dilution (micro- and macrobroth) methods described are intended for testing yeasts that cause invasive infections including *Candida* spp. 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 standardization problems not addressed by this procedure. For an antifungal broth dilution susceptibility testing reference method for filamentous fungi, refer to CLSI document M38.<sup>14-16</sup>

This document provides a “reference” standard developed through a consensus process to facilitate the agreement among laboratories in measuring yeast susceptibility 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 results in interlaboratory agreement within specified ranges. For example, broth microdilution methods using an indicator dye to facilitate breakpoint determinations have been configured to produce results paralleling those obtained by the broth microdilution reference method. To the extent that any method

produces concordant results with this reference method, it would be considered to be in conformity with this document.

### 1.3 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 known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of bloodborne pathogens. The Centers for Disease Control and Prevention address this topic in published guidelines that address the daily operations of diagnostic medicine in humans and animals while encouraging a culture of safety in the laboratory. [Miller JM, Astles JR, Baszler T, et al.; National Center for Emerging and Zoonotic Infectious Diseases, CDC. Guidelines for safe work practices in human and animal medical diagnostic laboratories. *MMWR Surveill Summ.* 2012;61 Suppl:1-102] For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29 [CLSI. Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Fourth Edition. CLSI document M29-A4. Wayne, PA: Clinical and Laboratory Standards Institute; 2014].

### 1.4 Terminology

#### 1.4.1 A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, ISO, and European Committee for Standardization (CEN) documents; and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. In light of this, CLSI’s consensus process for development and revision of standards and guidelines focuses on harmonization of terms to facilitate the global application of standards and guidelines.

#### 1.4.2 Definitions

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

**antimicrobial susceptibility test interpretive category** – 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.

- 1) **susceptible (S)** – a category that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.
- 2) **susceptible-dose dependent (SDD)** – a category that implies that susceptibility of an isolate is dependent on the dosing regimen that is used in the patient. In order to achieve levels that are likely to be clinically effective against isolates for which the susceptibility testing results (either minimal inhibitory concentrations [MICs] or disk diffusion) are in the SDD category, it is necessary to use a dosing regimen (ie, higher doses, more frequent doses, or both) that results in higher drug exposure

**Commented [KL7]:** The following was proposed and agreed to at the Jan 2010 CLSI meeting, however bullet 3 needs revision to fit new IBPs if included:

MDR *Candida* defined as:

1. Resistant (not susceptible to at least one agent in two or more of the major antifungal classes;
2. would include both R and S-DD for fluconazole;
3. would classify those with echinocandin MIC > 2 as R;
4. would classify those with amphotericin B MIC > 1 as R. add JCM article to references and AAC reference (need to find.)

**Commented [MH87]:** 5/11: add multidrug resistant (see below). Do you want the MDR definition as part of this as a NOTE or should it be separate?

than the dose that was used to establish the susceptible breakpoint. Consideration should be given to the maximum approved dosage regimen, because higher exposure gives the highest probability of adequate coverage of an SDD isolate. The drug label should be consulted for recommended doses and adjustment for organ function.

- 3) **intermediate (I)** – a category that 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; **NOTE:** The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher than normal dosage of a drug can be used. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.
- 4) **resistant (R)** – a category that implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs that fall in the range in which specific microbial resistance mechanisms are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

**breakpoint/interpretive criteria** – minimal inhibitory concentration (MIC) value used to indicate susceptible, intermediate, and resistant, as defined above.

For example, for antifungal agent X with interpretive criteria of:

	MIC (µg/mL)
Susceptible	≤4
Intermediate	8–16
Resistant	≥32

“Susceptible breakpoint” is 4 µg/mL

“Resistant breakpoint” is 32 µg/mL

**epidemiological cut-off value (ECV)** – the highest susceptibility endpoint of the wild-type minimal inhibitory concentration population that detects emergence of *in vitro* resistance or that separates wild-type from non-wild-type isolates. These values are based solely on *in vitro* data and do not predict the clinical outcome to therapy as breakpoints do.

**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; **NOTE:** 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.

**multiple drug resistant** – *Candida* spp. that are resistant (not susceptible) to at least one agent in two or more of the major antifungal classes

**non-wild type** – isolates with mechanisms of resistance and reduced susceptibility of the agent being evaluated.

**quality control (QC)** – the operational techniques and activities that are used to fulfill requirements for quality; **NOTE 1:** In health care testing, the set of procedures designed to monitor the test method and the results to ensure test system performance; **NOTE 2:** QC includes testing control materials, charting the

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results and analyzing them to identify sources of error, and evaluating and documenting any remedial action taken as a result of this analysis.

**trailing growth** – reduced but persistent growth over an extended range of concentrations that increases with incubation time

**wild-type** – isolates without mechanisms of resistance.

#### 1.4.3 Abbreviations and Acronyms

ATCC®	American Type Culture Collection
DMSO	dimethylsulfoxide
MIC	minimal inhibitory concentration
MOPS	3-(N-morpholino) propanesulfonic acid
QC	quality control
RPMI	Royal Park Memorial Institute (culture medium)

Commented [MH10]: I will manage this list as per CLSI style

## Chapter 2: Indications for Performing Susceptibility Tests<sup>17-19</sup>

This chapter includes:

- Indications for performing susceptibility tests
- Selecting antifungal agents for routine testing and reporting

Susceptibility testing is indicated for any organism contributing 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:

- Altered drug targets
- Altered drug uptake or efflux
- 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 organism type 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 microorganism types should not be tested on the same susceptibility test plate or panel. The practice of performing susceptibility tests directly with patient material (eg, normally sterile body fluids and urine) should be avoided except in medical emergencies when the direct Gram stain suggests a single pathogen. When testing has been performed directly with patient material, results should be reported as preliminary, and the susceptibility test must be repeated using standardized methodology.

When the nature of the infection is not clear, the specimen contains mixed growth or normal flora, and 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 provide the antimicrobial agent concentration needed to inhibit the infecting organism at the infection site. However, the MIC does not represent an absolute value. The “true” *in vitro* MIC is somewhere between the lowest test concentration inhibiting the organism’s growth (ie, the MIC reading) and the next lower test concentration. For example, if twofold dilutions were used and the MIC is 16 µg/mL, the “true” MIC would be between 16 and 8 µg/mL. Even under controlled conditions, a dilution test may not yield the same end point each time it is performed. Generally, the acceptable test reproducibility is within one twofold dilution of the actual end point. To avoid greater variability, the dilution test must be standardized and carefully controlled.

Traditionally, MICs have been determined using concentrations derived 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 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; however, some are more difficult to control. When there is growth inhibition 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

**Commented [MH11]:** Sheryl move reference 17 from the header to here

Galgiani JN. Antifungal susceptibility tests. *Antimicrobial Agents Chemother.* 1987;31:1867-1870.

**Commented [MH12]:** Sheryl, move references 18 and 19 from the header to here

Rex JH, Pfaller MA. Has antifungal susceptibility testing come of age? *Clinical Infect Dis.* 2002;35:982-989.

Anhalt JP, Washington JA II. Preparation and storage of antimicrobial solutions. In: Balows A, et al, eds. *Manual of Clinical Microbiology.* 5th ed. Washington, DC: American Society for Microbiology; 1991:1199-1200.

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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 for directing therapy, an interpretive category (ie, susceptible, susceptible-dose dependent, intermediate, nonsusceptible, or resistant) should accompany the MIC result based on the criteria outlined in the current edition of CLSI document M27/M44S.<sup>13</sup> When 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.

## 2.1 Selecting Antifungal Agents for Routine Testing and Reporting

Although breakpoints are now available for many fungal microorganism-drug combinations (see the current edition of CLSI document M27/M44S),<sup>13</sup> routine testing is not recommended. The decision to perform antifungal susceptibility testing is best made as a collaborative effort of each institution's infectious disease practitioners, pharmacy committee, microbiology laboratory personnel, and the infection control committee.

Testing may be warranted under certain selected circumstances including:

- As part of periodic batch surveys that establish antibiograms for collections of pathogenic isolates obtained from within an institution
- To aid in the management of refractory *Candida* spp infections in patients who appear to be experiencing therapeutic failure of the standard agents at standard doses
- To aid in the management of invasive *Candida* spp infections when the utility of the azole antifungal agents or other classes are uncertain (eg, when the infection is due to a non-*C. albicans* isolate or resistance patterns change)

Interpretive breakpoints are available for *Candida* spp. vs anidulafungin, caspofungin, fluconazole, 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.

### 2.1.1 Generic Names

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

### 2.1.2 Number of Agents Tested

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

**Commented [MH13]:** Sheryl, please change this to a reference to the new supplement M27/M44.

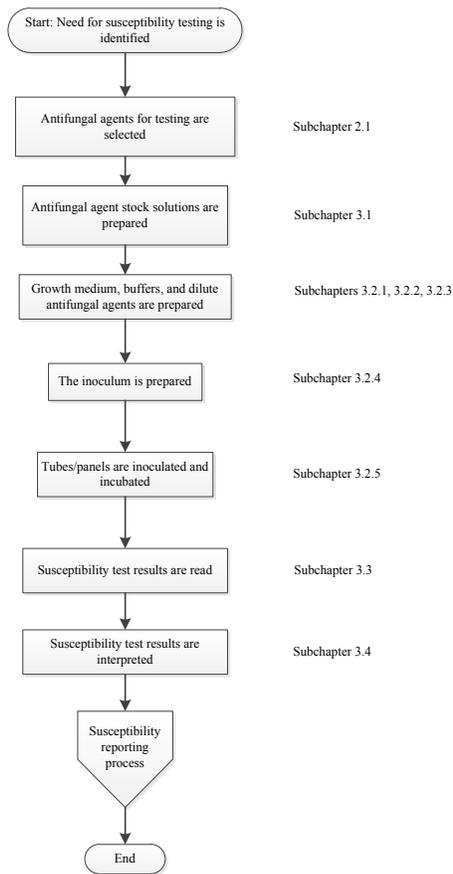
13 is for the previous version mentioned in the foreword. I have highlighted those Xref that also need to be adjusted

### Chapter 3: Antifungal Susceptibility Testing Process

This chapter includes:

- Overview of the antifungal susceptibility testing process
- Antifungal agent preparation
- Stock solution preparation
- Antifungal susceptibility testing procedures
- Reading results
- Interpreting results

Figure 1 shows the activities associated with the antifungal susceptibility testing process.



**Figure 1. Antifungal Susceptibility Testing Process**

### 3.1 Preparation of Antifungal Agents

#### 3.1.1 Source

Antifungal standards or reference powders can be obtained directly from the drug manufacturer or from commercial sources. 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. Powders should be stored as recommended by the manufacturers, or at –20 °C or below (never store in a self-defrosting freezer), in a desiccator, and preferably in a vacuum. When the desiccator is removed from the freezer, it should be allowed to adjust to room temperature before opening (to avoid water condensation).

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#### 3.1.2 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, laboratories need to standardize the antifungal solutions based on assays of the antifungal powders lots that are being used.

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

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

or

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

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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 needed and to calculate the diluent volume 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:

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

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

$$\text{Volume (mL)} = \frac{182.6 \text{ mg} \cdot 750 \mu\text{g/mg}}{128 \mu\text{g/mL (Desired Concentration)}} = 107.0 \text{ mL} \quad (4)$$

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

### 3.1.3 Preparing Stock Solutions

Antifungal stock solutions at concentrations of at least 1280 µg/mL or 10 times the highest concentration to be tested, whichever is greater, should be prepared. However, some antifungal agents having limited solubility may need lower concentrations.

**NOTE: In all cases, information provided by the drug manufacturer should be considered as part of determining solubility.**

#### 3.1.3.1 Using Solvents Other Than Water

Some drugs must be dissolved in solvents other than water (see CLSI document M27/M44S).<sup>15</sup> Antifungal compound solubility information 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
- 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 low solubility compounds precipitating in aqueous media.

For example, to prepare for a broth dilution test series containing a water-insoluble drug that can be dissolved in DMSO, for which the highest desired test concentration is 16 µg/mL:

Step	Action	Comment
1.	Weigh 4.8 mg of the antifungal powder	Assumes 100% potency. If the manufacturer indicates the powder is not at 100% potency, adjust the weight upward to compensate for the decrease in potency. See Subchapter 6.2 for the appropriate calculations.
2.	Dissolve the antifungal powder in 3.0 mL DMSO	This provides a stock solution at 1600 µg/mL.
3.	Prepare further dilutions of this stock solution in DMSO.	See Appendix A
4.	Dilute the solutions in DMSO tenfold in test medium and a further tenfold when inoculated	See Subchapters 7.2.2 and 7.3 This step reduces the final solvent concentration to 1%.
5.	Use DMSO at this concentration (without drug) in the test as a dilution control.	

Abbreviation: DMSO, dimethylsulfoxide

#### 3.1.3.2 Filtration

Normally, stock solutions do not support microorganism growth and can be assumed to be sterile. If additional sterility assurance is desired, the stock solutions should be passed through a membrane filter. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antifungal

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agents, should not be used. Whenever using filtration, the absence of adsorption by checking results of the appropriate QC assays should be documented.

#### 3.1.3.3 Storage

Small volumes of the sterile stock solutions should be dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at  $-60\text{ }^{\circ}\text{C}$  or below, but never at a temperature greater than  $-20\text{ }^{\circ}\text{C}$ ). Vials should be removed from storage as needed and used the same day. Any unused drug should be discarded at the end of the day. Most antifungal agent stock solutions can be stored at or below  $-60\text{ }^{\circ}\text{C}$  for six months or more without significant loss of activity.<sup>20</sup> In all cases, any directions provided by the drug manufacturer should be considered as a part of these general recommendations, which supersede the directions above. Any significant antifungal agent deterioration may be determined by reviewing the results susceptibility testing using QC strains (see the current edition of CLSI document [M27/M44S](#).<sup>15</sup>)

#### 3.1.3.4 Number of Concentrations Tested

The concentrations tested should incorporate the breakpoint concentrations and the expected results for the QC strains. Based on previous studies, the drug concentration ranges listed in Table 1 should be used.

**Table 1. Recommended Antifungal Agent Concentrations**

Antifungal Agent	Concentration ( $\mu\text{g/mL}$ )
amphotericin B	0.0313–16
anidulafungin	0.015–8
caspofungin	0.015–8
fluconazole	0.125–64
flucytosine	0.125–64
isavuconazole	0.0313–16
itraconazole	0.0313–16
ketoconazole	0.0313–16
micafungin	0.015–8
posaconazole	0.0313–16
ravuconazole	0.0313–16
voriconazole	0.0313–16

## 3.2 Testing Procedures

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### 3.2.1 Preparing Growth Medium

A completely synthetic medium should be used for susceptibility testing. Royal Park Memorial Institute culture medium (RPMI) 1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) has been found to be at least as satisfactory as several other synthetic media and has been used to develop the standard.<sup>3,4</sup> The medium's formula is provided in Appendix B and the steps for the medium's preparation from powder is provided in Appendix C. Alternative media may be advantageous for some organisms and some drugs. Appendix D provides modifications for special circumstances.

### 3.2.2 Preparing Buffers

Media should be buffered to  $\text{pH } 7.0 \pm 0.1$  at  $25\text{ }^{\circ}\text{C}$ . A buffer that does not antagonize antifungal agents should be selected. Tris buffer is unsatisfactory because it antagonizes flucytosine activity. Zwitterion buffers are preferable to buffers (eg, phosphate buffers) that readily traverse the cell membrane because, theoretically, the latter can produce unexpected interactions with antifungal agents. A buffer satisfactory for antifungal testing is 3-(N-morpholino) propanesulfonic acid (MOPS) (final concentration  $0.165\text{ mol/L}$  for  $\text{pH } 7.0$ ). The pH of each medium batch should be checked with a pH meter immediately after preparation

and should fall between 6.9 and 7.1 at room temperature (25 °C). MIC performance characteristics of each broth batch should be evaluated using a standard set of QC organisms (see Subchapter 8).

### 3.2.3 Preparing Diluted Antifungal Agents

The conditions for preparing and storing diluted antifungal agents are as follows:

- Sterile 12- × 75-mm plastic test tubes should be used to perform the tests.
- A growth control tube containing RPMI 1640 medium without antifungal agents (but with nonaqueous solvent where necessary) for each organism tested should be used.
- Tubes should be closed with loose screw-caps, or plastic or metal caps.

#### 3.2.3.1 Water-Soluble Antifungal Agents

For water-soluble antifungal agents, twofold dilutions may be prepared volumetrically in broth (see Appendix E). The procedure for insoluble antifungal agents differs from water-soluble agents and is described in Subchapter 3.2.3.2. When running a small number of tests, the schedule in Appendix E should be consulted.

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

Many users find working with 1:10 dilutions (as shown in Appendix E) easy and convenient. However, some automated pipettes deliver only 1.0 or 0.1 mL volumes; therefore, a ratio of 1:11 is preferable. If 1:11 dilutions are prepared, the dilution scheme should be altered so the same final drug concentrations are obtained.

#### 3.2.3.2 Water-Insoluble Antifungal Agents

For water-insoluble antifungal agents (eg, amphotericin B, anidulafungin, caspofungin, micafungin, itraconazole, ketoconazole, posaconazole, isavuconazole, and voriconazole), a dilution series of the agent should be prepared first at 100 times final strength in an appropriate solvent (see Subchapter 6.3.1). Each nonaqueous solution should then 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 first be prepared in DMSO (see Subchapter 6.3.1). The following steps should be used to prepare 1-mL volumes of diluted antifungal agent (sufficient for 10 tests):

1. Pipette 0.9-mL volumes of RPMI 1640 broth into each of 11 sterile test tubes.
2. 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.

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- Continue in sequence through 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 needed. 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.

### 3.2.4 Preparing the Inoculum

All organisms should be subcultured onto antimicrobial-free growth medium (eg, Sabouraud dextrose agar or potato dextrose agar) and passaged to ensure purity and viability. The incubation temperature throughout must be 35 °C. Use the steps below to prepare the inoculum.

Step	Action	Comment
1.	Pick 5 colonies that are at least ≈1 mm in diameter	Select colonies from 24-hr-old cultures of <i>Candida</i> spp. or 48-hr-old cultures of <i>C. neoformans</i> .
2.	Suspend the colonies in 5 mL of sterile 0.145-mol/L saline (8.5 g/L NaCl; 0.85% saline) or sterile water.	
3.	Vortex the resulting suspension for 15 seconds.	
4.	Adjust the cell density 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 F) 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.
5.	Make a working suspension by preparing a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium.	This procedure results in $5.0 \times 10^2$ to $2.5 \times 10^3$ cells per mL. <sup>2</sup>

Abbreviations: NaCl, sodium chloride; RPMI, Royal Park Memorial Institute (culture medium)

### 3.2.5 Inoculating and Incubating Susceptibility Tests

#### 3.2.5.1 Inoculating RPMI-1640 Medium

Before adjusting the inoculum, the following steps should be used to inoculate the tubes.

Step	Action	Comment
1.	Place 0.1 mL of the various antifungal concentrations in 12 × 75 mm tubes	
2.	Add 0.1 mL of drug diluent without antifungal agent to the growth control tube.	
3.	Add 0.9 mL of the adjusted inoculum to each tube in the dilution series.	This should be completed within 15 minutes after the inoculum has been standardized or within two hours if the inoculum is kept at 4 °C.
4.	Mix each tube.	This results in a 1:10 dilution of each antifungal concentration and a 10% dilution of the inoculum

### 3.2.5.2 Inoculating Broth Microdilution Plates

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. The following steps should be used to prepare the dilutions.

Step	Action	Comment
1.	Prepare and adjust the stock inoculum suspensions, as described for the broth macrodilution test.	See Subchapter
2.	Mix the stock yeast suspension by vortexing for 15 seconds.	
3.	Dilute the suspension 1:50, then to 1:20 with medium to obtain the two times test inoculum.	$1 \times 10^3$ to $5 \times 10^3$ CFU/mL
4.	Inoculate the wells	When the wells are inoculated, the (two times) inoculum 1:1 is diluted and the desired final inoculum size is achieved ( $0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/mL).

Abbreviations: CFU/mL, colony forming units per mL

To perform the broth microdilution test, use sterile, disposable, multiwell microdilution plates (96 U-shaped wells) and use the following steps.

Step	Action	Comment
1.	Using a multichannel pipette, dispense the two times drug concentrations into the wells of microdilution plates rows 1 to 10 in 100- $\mu$ L volumes.	Disperse in order of concentration with either the highest or lowest concentration in the first well, and then a gradient going up or down across the next row of wells. The trays may be sealed in plastic bags and stored frozen at $-70^\circ\text{C}$ for up to 6 months without the drug potency deteriorating.
2.	On testing day, inoculate each microdilution tray well with 100 $\mu$ L of the corresponding two times diluted inoculum suspension.	This brings the drug dilutions and inoculum densities to the final concentrations ( $0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/mL).
3.	Inoculate the growth control wells, with 100 $\mu$ L of the corresponding diluted (two times) inoculum suspensions	Growth control wells contain 100 $\mu$ L of sterile, drug-free medium.  Test the QC organisms (see Subchapter XX) in the same manner each time an isolate is tested.  Use Row 12 of the microdilution plate to perform the sterility control (drug-free medium only).

Microdilution plates and macrodilution tubes should be incubated (without agitation) and at  $35^\circ\text{C}$  in ambient air for 24 hours (or up to 48 hours for slow growers). When testing *C. neoformans*, tubes should be incubated for a total of 70 to 74 hours before determining results.

### 3.3 Reading Results

After the appropriate incubation time, the tubes and plates should be observed for the presence or absence of visible growth.

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### 3.3.1 Reading Time for Broth Macrodilution

The amount of growth in the tubes containing the agent should be compared visually with the amount of growth in the growth-control tubes (no antifungal agent) used in each set of tests and scored using the following numerical scale:

- 0 – optically clear
- 1 – slightly hazy
- 2 – prominent decrease (≈50%) in turbidity
- 3 – slight reduction in turbidity
- 4 – no reduction in turbidity.

### 3.3.2 Reading Time for Broth Microdilution

For broth microdilution, QC limits are established for all drugs at 24 hours and 48 hours (see the current edition of CLSI document M27/M44S).<sup>13 25</sup>

Microdilution MICs should be read at:<sup>23,24,26</sup>

- 24 hours for the echinocandins, amphotericin B, fluconazole, flucytosine, itraconazole, voriconazole, ravuconazole, and posaconazole
- 72 hours for most *C. neoformans* isolates against all drugs referenced above

**NOTE:** Current interpretive breakpoints are defined only for the 24 hour readings.

Agitating the plates is optional and may simplify reading the end points. Microdilution wells should be scored using a reading mirror and the growth in each well compared to the growth control (drug-free) well.

When clumping of an isolate hinders scoring, pipetting, vortexing, or other mixing techniques should be tried.<sup>24,27</sup> The amphotericin B MIC is defined as the lowest concentration in which there is no growth (optically clear). For the azoles, echinocandins, and flucytosine, the MIC is defined as the lowest concentration at which there is a 50% decrease in growth (prominent decrease in turbidity).

### 3.3.3 Trailing Growth and the Impact on Reading Time

For *Candida* spp. an end-point for both macro- and microdilution methods should be read at 24 or 48 hours. 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”). 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 growth inhibition over an extended range of antifungal concentrations). Estimated as occurring for fluconazole in about 5% of isolates,<sup>28</sup> this trailing growth can be so great as to make an isolate appear susceptible after 24 hours and appear completely resistant at 48 hours. Two independent *in vivo* investigations of this phenomenon using murine models of disseminated candidiasis showed that isolates with this behavior should be categorized as “susceptible” rather than “resistant.”<sup>28,29</sup> 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>30</sup> and by a clinical demonstration that oropharyngeal candidiasis due to such isolates responds to low doses of fluconazole typically used to treat susceptible isolates.<sup>31</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 current edition of CLSI document M27/M44S).<sup>13</sup>

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### 3.3.3.1 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 growth end points with amphotericin B are not usually seen.

### 3.3.3.2 Flucytosine and Azole Antifungal Agents

For flucytosine and especially for azoles, end points are typically less well defined than those described for amphotericin B, which may contribute to a significant source of variability. Applying a less stringent end point, an approximately 50% reduction in growth relative to the drug-free growth control, has improved interlaboratory agreement and also discriminates between presumed susceptible and resistant isolates. When turbidity persists, it is often identical for all drug concentrations above the MIC. Even dispersing clumps that can become evident after incubation can make end-point determination more reproducible. Reference strains with defined susceptibility can also be used when training new personnel.

### 3.3.3.3 Echinocandin Antifungal Agents

For echinocandins, MIC end points should be determined after 24 hours incubation and read as the lowest drug concentration to produce a prominent decrease in turbidity ( $\approx$  50% reduction). This translates to approximately a 50% reduction in growth relative to the drug-free growth control.

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## 3.4 Interpreting Results

Interpretive breakpoints have been established for only some organism-drug combinations (see the current edition of CLSI document M27/M44S).<sup>13</sup> The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarized as outlined in the following subchapters.

### 3.4.1 Amphotericin B

Experience with the procedures described in this document indicates that amphotericin B MICs for *Candida* spp. isolates are tightly clustered between 0.25 and 1.0  $\mu\text{g/mL}$ . When isolates appearing resistant to amphotericin B in animal models are tested using M27 methods, MIC values greater than 1  $\mu\text{g/mL}$  may be obtained. At present, the M27 methodology does not consistently detect such isolates; therefore, it can only be concluded that if an amphotericin B MIC of  $>1$   $\mu\text{g/mL}$  is obtained for a *Candida* spp. isolate, then that isolate is likely resistant to amphotericin B. Some research has suggested that testing with Antibiotic Medium 3 supplemented with 2% glucose (dextrose) and reading MICs after 24 hours incubation results in more reliably detects resistant isolates.<sup>32,33</sup> However, this method's reproducibility has been questioned,<sup>34</sup> and laboratories choosing this testing method need to carefully compare their results with those obtained for isolates with known responses to amphotericin B. Potentially useful reference isolates are available American Type Culture Collection (ATCC<sup>®</sup>): *Candida lusitanae* ATCC<sup>®</sup> 200950, ATCC<sup>®</sup> 200951, ATCC<sup>®</sup> 200952, ATCC<sup>®</sup> 200953, ATCC<sup>®</sup> 200954; *C. albicans* ATCC<sup>®</sup> 200955; and *Candida tropicalis* ATCC<sup>®</sup> 200956.

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

### 3.4.2 Flucytosine

Previously published interpretive breakpoints for flucytosine were established with minimal clinical data and emerging data now suggest that those breakpoints were not correct. Therefore, the previously published breakpoints should not be used.

### 3.4.3 Fluconazole

Species-specific interpretive breakpoints for *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* and fluconazole have been established (see the current edition of M27/M44S<sup>13</sup>).<sup>11</sup> These interpretive breakpoints are not applicable to *Candida krusei*; therefore, identification to the species level is necessary to accurately interpret and report MICs. In addition, when an isolate is identified as *C. glabrata* and the MIC is  $\leq 32$ , patients should receive the maximum fluconazole dosage regimen. Expert consultation on selecting a maximum dosage regimen may be useful. The utility of testing isolates of *C. neoformans* is currently being studied, and recent data suggest a correlation between elevated MIC and clinical failure.<sup>35</sup>

### 3.4.4 Ketoconazole

Experience testing with broth dilution methodology indicates that yeast MICs vary between 0.03 and 16  $\mu\text{g/mL}$ . However, data are not yet available to confirm a correlation between MICs and treatment outcome with ketoconazole.

### 3.4.5 Itraconazole

Previously published interpretive breakpoints for flucytosine were established with minimal clinical data and emerging data now suggest that those breakpoints were not correct. Therefore, the previously published breakpoints should not be used.

For *Candida* spp. without breakpoints, epidemiological cut off values (ECVs) that define the limit of the wild-type distribution have been established for *Candida* spp. and itraconazole and may be useful for distinguishing between wild-type and non-wild-type (isolates with intrinsic or acquired known resistance mechanisms) isolates (see the current edition of CLSI document M57-S[M57S]).

### 3.4.6 Voriconazole

Interpretive breakpoints for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. and voriconazole have been established (see the current edition of CLSI document M27/M44S).<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>36</sup> In other settings, the clinical relevance of breakpoints is uncertain.

### 3.4.7 New Triazoles

Experience testing posaconazole and ravuconazole with broth dilution methodology indicates that yeast MICs vary between 0.03 and 16  $\mu\text{g/mL}$  with the majority of isolates inhibited by  $\leq 1$   $\mu\text{g/mL}$  for both agents. For isavuconazole, yeast MICs range between 0.008 to 8  $\mu\text{g/mL}$  with the majority (90% of tested isolates having an equivalent or lower MIC) inhibited by 0.5  $\mu\text{g/mL}$ .<sup>37</sup> However, data are not yet available to indicate a correlation between MIC and treatment outcome with these agents.

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

Using procedures described in this document, MIC survey data for >2500 patient isolates of *Candida* spp. indicate that MICs vary between 0.007 and 8  $\mu\text{g/mL}$  with  $\geq 99\%$  of isolates inhibited by  $\leq 2$   $\mu\text{g/mL}$ .<sup>38,39</sup>

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However, isolates harboring *Fks* mutations, the major resistance mechanism to echinocandins, have been identified with MICs that fall below 2 µg/mL. Therefore, species-specific interpretive breakpoints have been established that allow for discrimination between potentially resistant mutants and susceptible strains (see the current edition of CLSI document M27/M44S).<sup>13</sup>

#### 3.4.9 Other Modifications

In addition to ongoing efforts to simplify the procedures described in this document, some more fundamental method modifications have been developed in response to specific problems and are described in Appendix D. These modifications are not part of the current methodology, but interested laboratories may wish to explore their clinical relevance.

## Chapter 4: Quality System Essential: Process Management – Quality Control

This chapter includes:

- Quality control responsibilities
- Reference strains selection
- Reference strain storage
- Media batches and plasticware lot control
- Quality control frequency
- Quality control strains
- Other quality control procedures

The goals of a QC program are to monitor:

- The susceptibility test procedure's precision (repeatability) and accuracy
- The performance of reagents, testing conditions, and instructions used in the test
- The performance of persons conducting the tests and reading the results

The goals are best realized by, but not limited to, the using QC and reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.<sup>6</sup>

### 4.1 Quality Control Responsibilities

Antifungal susceptibility test manufacturers and users have a shared responsibility for quality. The primary purpose of QC testing performed by manufacturers (laboratory developed reference methods or commercial methods) is to ensure that the testing materials and reagents have been appropriately manufactured. The primary purpose of QC testing performed by laboratories (users) is to ensure that the testing materials and reagents are maintained properly and testing is performed according to established procedures.

Manufacturers (commercial and/or laboratory developed products) are responsible for the following:

- Antifungal stability
- Antifungal labeling
- Antifungal stock solution potency
- Compliance with good manufacturing practices
- Product integrity
- Accountability and traceability to the consignee

Manufacturers should design and recommend a QC program that aids users in evaluating those variables (eg, inoculum levels, storage/shipping conditions) that will most likely cause user performance problems and to determine that the assay is performing correctly when directions for use are followed.

The laboratory (user) is responsible for the following:

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

Laboratories should familiarize themselves with regulatory and accreditation requirements for QC in their specific area.

## 4.2 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 QC strain is inhibited at the fifth dilution of a nine-dilution log<sub>2</sub> series, but strains for which the antifungal agent MICs are between the third and seventh dilution are acceptable. Before a strain is accepted as a reference, it should be tested for as long as necessary to demonstrate that its antifungal susceptibility pattern is genetically stable. CLSI document M23<sup>40</sup> provides guidelines for selecting appropriate QC strains and the determining acceptable MIC ranges. The strains listed in the current edition of CLSI document M27/M44S<sup>13</sup> were selected in accordance with these criteria.<sup>6,7,12</sup>

## 4.3 Storing Reference Strains

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

Reference strains should be stored so that the possibility of organism mutation is minimized.

There are two preferred methods for prolonged reference strain storage. Yeasts may be grown on potato dextrose agar and then frozen at  $-70^{\circ}\text{C}$ .<sup>41</sup> Alternatively, reference strains can be preserved by suspending fungal cells in 15% glycerol solution in small vials and freezing and storing them at  $-70^{\circ}\text{C}$ .

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  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$ . Fresh slants should be prepared 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, a new stock culture should be obtained.

### 4.3.2 Sources for Reference Strains

Reference strains should be obtained from a source that is can provide information on the culture's origination (eg, from commercial sources with documented culture history, or from reference institutions with demonstrated ability to store and use the organisms consistently with minimal contamination).

### 4.3.3 Preparing Strains for Storage

To prepare strains for storage, the steps below should be followed.

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Step	Action	Comment
1.	Grow the organisms overnight on Sabouraud dextrose agar or potato dextrose agar plates.	
2.	Select growth from several colonies and perform the appropriate susceptibility tests to demonstrate that they produce the expected MIC results	See the current edition of CLSI document M27/M44S <sup>13</sup> for expected MICs of some reference strains
3.	Subculture strains yielding expected results onto the same medium as the primary culture	
4.	Incubate subcultures long enough for sufficient growth to occur.	Usually from 1 to 3 days
5.	Examine the resulting growth carefully to ensure it is a pure culture.	
6.	Suspend the growth from the plate in 20% glycerol to make a heavy suspension.	If lyophilizing, suspend the growth in the appropriate medium.
7.	Distribute the turbid suspension in small volumes (1 to 2 drops) into several small containers.	
8.	Place the containers in a –70 °C freezer or in liquid nitrogen.	See Subchapter 6.3.3.

Abbreviations: MIC, minimal inhibitory concentration

Stocks prepared using this procedure can be stored indefinitely without significant risk of altering antifungal susceptibility patterns. When the supply of containers is nearly exhausted, repeat the process to prepare a new supply.

#### 4.3.4 Routine Use of Reference Strains

For routine use of reference strains, the steps below should be followed.

Step	Action	Comment
1.	Remove a container of the culture from the –70 °C freezer or obtain a lyophilized vial.	
2.	Thaw the frozen mixture or rehydrate the lyophilized culture.	
3.	Subculture the mixture onto potato dextrose agar plates and incubate.	35 °C for 24 hours for <i>Candida</i> spp. 35 °C for 48 hours for <i>C. neoformans</i>
4.	Remove 4 to 5 colonies and subculture them to the appropriate susceptibility test medium	
5.	Subculture the colonies onto potato dextrose agar slants.	
6.	Incubate the agar slants overnight.	
7.	Store the resulting cultures on agar slants at 2 °C to 8 °C.	The agar slants may be used as working stock cultures. At least every two weeks, replace them with new slants prepared from the freezer supply.
8.	Subculture growth from the stored slant to an agar plate for testing.	Always perform susceptibility testing on colonies from overnight plates.

#### 4.4 Controlling Media Batches and Plasticware Lots

For batch or lot control, the following should be performed:

- Each new batch of medium, macrodilution tube lot, or microdilution plate lot needs to be tested with one of the QC strains listed in the current edition of [CLSI document M27/M44S<sup>13</sup>](#) to determine if MICs fall within the expected range. If the MICs do not fall within the expected range, the batch or lot needs to be rejected.
- To ensure the medium’s sterility, at least one uninoculated tube or microdilution plate from each batch needs to be incubated for the same amount of time as needed to complete the test.
- New lots of RPMI 1640 medium need to be tested for acceptable performance before using to test patient isolates. Recent studies have demonstrated that some lots do not perform adequately. The pH should be 6.9 to 7.1 (see [Subchapter 7.1.2](#)).
- Lot numbers of all materials and reagents used in these tests need to be recorded.

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#### 4.5 Quality Control Frequency

##### 4.5.1 Minimal Inhibitory Concentration Ranges

MIC QC limits for a single control test are listed in the current edition of [CLSI document M27/M44S<sup>13</sup>](#).<sup>6,7,12</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 test variation. Two consecutive out-of-control results or any more than two out-of-control results in 20 consecutive control tests need 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 Subchapter 4.5.2).

##### 4.5.2 Frequency of Testing

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

- Testing all reference strains and documenting results for 30 consecutive test days.
- 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 the current edition of [CLSI document M27/M44S<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 [Subchapter 8.7.1](#).

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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 QC tests must be reinstated long enough to

define the source of the aberrant result and to document resolution of the problem. The problem may be resolved as follows:

- Testing with appropriate reference strains and documenting the results for five consecutive test days.
- For each drug-microorganism combination, all 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 the current edition of **CLSI document M27/M44S**.<sup>13</sup>

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. To return to weekly testing in the future, documenting satisfactory performance for another 30 consecutive test days is required.

For some drugs, QC tests need to be done more frequently than once per week because of the relatively rapid degradation of the drug.

#### 4.6 Quality Control Strains

Ideal reference strains for dilution test QC have MICs that consistently fall near the midpoint of the concentration range tested for all antifungal agents. An ideal control strain would be inhibited at the fourth dilution of a seven-dilution series; however, strains with MICs at either the third or fifth dilution are also acceptable.

The current edition of **CLSI document M27/M44S**<sup>13</sup> lists expected ranges for strains determined to be acceptable as QC strains. Also shown are additional strains that can be useful for conducting reference studies.<sup>6,7,12</sup>

Refer to Subchapter 8.3 for additional information.

#### 4.7 Other Quality Control Procedures

##### 4.7.1 Growth Control

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

##### 4.7.2 Purity Control

A sample of each inoculum should be streaked onto 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 is necessary.

##### 4.7.3 End-point Interpretation Control

End-point interpretation should be monitored periodically to minimize MIC end point variation among observers. All laboratory personnel performing these tests should independently read a selected set of dilution tests. The results should be recorded and compared to the results obtained by an experienced reader. QC and reference strains with predetermined MICs are particularly useful for this purpose, especially with fluconazole.<sup>6,7,12</sup>

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## Chapter 5: Conclusion

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## Chapter 6: Supplemental Information

This chapter includes:

- References
- Appendixes
- The Quality Management System Approach
- Related CLSI Reference Materials

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

Antifungal Solution							
Step	Concentration ( $\mu\text{g/mL}$ )	Source	Volume (mL)	+ Solvent (mL) (eg, DMSO)	= Intermediate Concentration ( $\mu\text{g/mL}$ )	= Final Concentration at 1:100 ( $\mu\text{g/mL}$ )	$\text{Log}_2$
1	1600	Stock			1600 $\mu\text{g/mL}$	16	4
2	1600	Stock	0.5	0.5	800	8.0	3
3	1600	Stock	0.5	1.5	400	4.0	2
4	1600	Stock	0.5	3.5	200	2.0	1
5	200	Step 4	0.5	0.5	100	1.0	0
6	200	Step 4	0.5	1.5	50	0.5	-1
7	200	Step 4	0.5	3.5	25	0.25	-2
8	25	Step 7	0.5	0.5	12.5	0.125	-3
9	25	Step 7	0.5	1.5	6.25	0.0625	-4
10	25	Step 7	0.5	3.5	3.13	0.0313	-5

Abbreviation: DMSO, dimethyl sulfoxide

**Appendix B. Composition of Royal Park Memorial Institute 1640 Medium (with glutamine and phenol red, but without bicarbonate)**

Constituent	g/L Water	Constituent	g/L Water
L-arginine (free base)	0.200	Biotin	0.0002
L-asparagine (anhydrous)	0.050	D-pantothenic acid	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine • 2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myoinositol	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
L-isoleucine	0.050	Thiamine HCl	0.001
L-leucine	0.050	Vitamin B <sub>12</sub>	0.000005
L-lysine • HCl	0.040	Calcium nitrate • H <sub>2</sub> O	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		

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### Appendix C. Preparing Royal Park Memorial Institute 1640 Medium

Royal Park Memorial Institute (RPMI) 1640 medium buffered with 0.165 mol/L, 3-[N-morpholino] propanesulfonic acid (MOPS), 1 L

#### Materials

10.4 g powdered RPMI 1640 medium (with glutamine and phenol red, without bicarbonate)  
34.53 g MOPS buffer

Step	Action	Comment
1.	Dissolve powdered medium in 900 mL distilled water	
2.	Add MOPS and stir until dissolved.	Final concentration of 0.165 mol/L
3.	While stirring, adjust the pH to 7.0 at 25 °C using 1 mol/L sodium hydroxide	
4.	Add additional water to bring medium to a final volume of 1 L	
5.	Filter sterilize and store at 4 °C until use	

Abbreviations: MOPS, 3-[N-morpholino] propanesulfonic acid

#### Appendix D. Modifications for Special Circumstances of Broth Dilution Testing

Drug	Organism	Modification
Amphotericin B <sup>1,2</sup>	<i>Candida</i> spp.	Use of Antibiotic Medium 3 and a 24-hour end point has enhanced detection of resistance in some reports, but this medium is not standardized, substantial lot-to-lot variability is possible, and experience has varied.
All drugs <sup>3,4</sup>	<i>C. neoformans</i>	Using Yeast Nitrogen Base may enhance the growth of <i>C. neoformans</i> and improve the clinical relevance of antifungal MICs.
All drugs <sup>5</sup>	All organisms	Supplementing the test medium so it contains glucose at a final concentration of 20 g/L may simplify end-point determination.

Abbreviation: MIC, minimal inhibitory concentration.

**NOTE:** These modifications are not a part of the formal reference methodology and the utility of each of these modifications remains to be established. This table is provided solely as a reference for laboratories that are interested in studying adaptations of the reference method that may enhance its utility under specific circumstances.

#### References for Appendix D

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**Appendix E. Scheme for Preparing Dilutions of Water-Soluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests**

Antifungal Solution										
Step	Concentration ( $\mu\text{g/mL}$ )	Source	Volume (mL)	+	Medium (mL)	=	Intermediate Concentration ( $\mu\text{g/mL}$ )	=	Final Concentration at 1:10 ( $\mu\text{g/mL}$ )	$\text{Log}_2$
1	5120	Stock	1 mL		7		640 $\mu\text{g/mL}$		64	6
2	640	Step 1	1.0		1.0		320		32	5
3	640	Step 1	1.0		3.0		160		16	4
4	160	Step 3	1.0		1.0		80		8	3
5	160	Step 3	0.5		1.5		40		4	2
6	160	Step 3	0.5		3.5		20		2	1
7	20	Step 6	1.0		1.0		10		1	0
8	20	Step 6	0.5		1.5		5		0.5	-1
9	20	Step 6	0.5		3.5		2.5		0.25	-2
10	2.5	Step 9	1.0		1.0		1.25		0.125	-3
11	2.5	Step 9	0.5		1.5		0.625		0.0625	-4
12	2.5	Step 9	0.5		3.5		0.3125		0.03125	-5

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## Appendix F. McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, use a barium sulfate (BaSO<sub>4</sub>) turbidity standard (0.5 McFarland standard).

The procedure for preparing a BaSO<sub>4</sub> turbidity standard (0.5 McFarland standard) is as follows:

Step	Action	Comment
1.	Add 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	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	Screw-cap tubes should be of the same size as those used in growing or diluting the broth culture inoculum.
4.	Tightly seal the tubes	Store the prepared tubes 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	

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### The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system (QMS) 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 QMS 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 as follows:

Organization	Personnel	Process Management	Nonconforming Event Management
Customer Focus	Purchasing and Inventory	Documents and Records	Assessments
Facilities and Safety	Equipment	Information Management	Continual Improvement

M27 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.

Organization	Customer Focus	Facilities and Safety	Personnel	Purchasing and Inventory	Equipment	Process Management	Documents and Records	Information Management	Nonconforming Event Management	Assessments	Continual Improvement

### Path of Workflow

A path of workflow is the description of the necessary processes to deliver the particular product or service that the organization or entity provides. A laboratory path of workflow consists of the sequential processes: preexamination, examination, and postexamination and their respective sequential subprocesses. All laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

M27 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

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M27

### Related CLSI Reference Materials\*

- M23**      **Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters. 3rd ed., 2008.** This document addresses the required and recommended data needed for the selection of appropriate interpretive criteria and quality control ranges for antimicrobial agents.
- M27/M44S**      **Reference Methods for Antifungal Susceptibility Testing of Yeasts; Draft Informational Supplement. 1<sup>st</sup> ed. 2016.** This document provides updated tables for the Clinical and Laboratory Standards Institute antifungal susceptibility testing documents M27 and M44.
- M29**      **Protection of Laboratory Workers From Occupationally Acquired Infections. 4th ed., 2014.** Based on US 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**      **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. 2nd ed., 2008.** 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 and cutaneous fungal infections.
- M57**      **Principles and Procedures for the Development of Epidemiological Cutoff Values for Antifungal Susceptibility Testing; Draft Guideline. 1<sup>st</sup> ed., 2016.** This document describes the criteria for development and use of epidemiological cutoff values for guiding clinical decisions for fungi/antifungal combinations for which there are no breakpoints.
- M57S**      **Principles and Procedures for the Development of Epidemiological Cutoff Values for Antifungal Susceptibility Testing; Draft Informational Supplement. 1<sup>st</sup> ed. 2016.** This document provides the epidemiological cutoff value tables for the Clinical and Laboratory Standards Institute antifungal susceptibility testing document M57.

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\* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.



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