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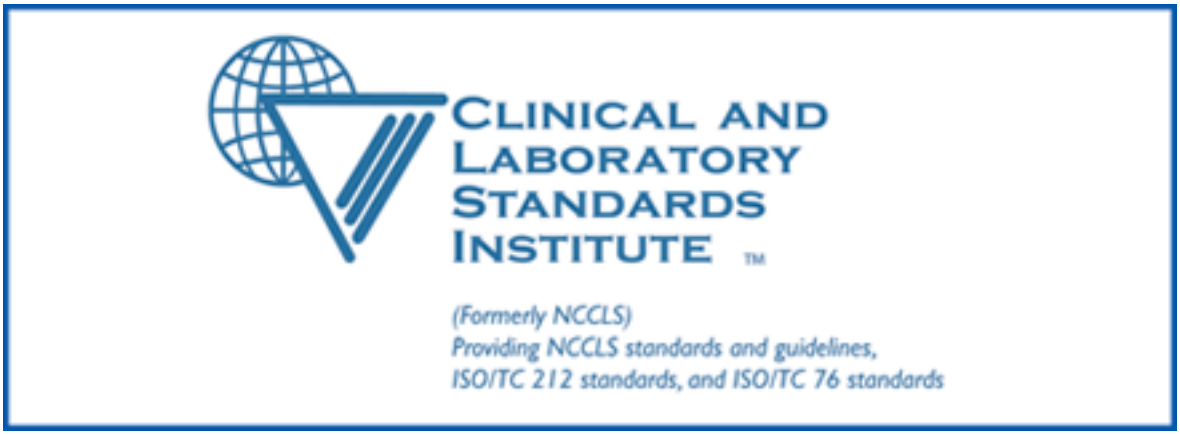
Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition



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.

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

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

Abstract

NCCLS document M27-A2—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition* describes a method for testing the susceptibility of antifungal agents to yeast that cause invasive fungal infections, including *Candida* species (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.

NCCLS. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition*. NCCLS document M27-A2 (ISBN 1-56238-469-4). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.

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Volume 22 Number 15

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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 NCCLS Area Committee for Microbiology formed the Subcommittee on Antifungal Susceptibility Testing. In 1985, this subcommittee published its first report[1] 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 NCCLS membership whose hospitals had greater than 200 beds were 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.[2,3,4,5] 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,[6,7] and broth microdilution procedures paralleling the broth macrodilution reference procedure became available.[5,8,9,10] 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,[11] 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.[12] The results of these studies are included in the current M27-A2 document.

Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR

1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections.*

Key Words

Antifungal, broth macrodilution, broth microdilution, susceptibility testing, yeasts

The Quality System Approach

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

QSEs

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

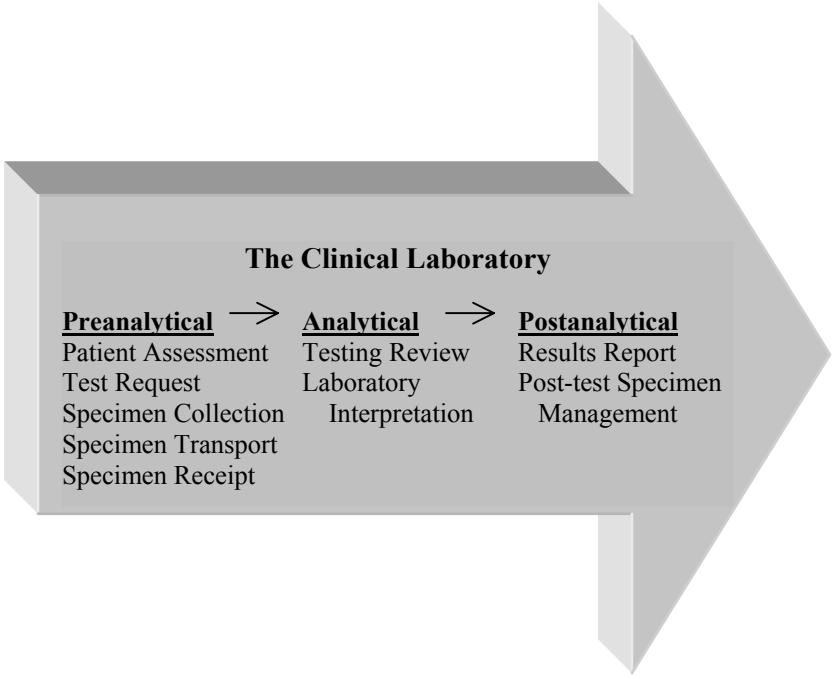
M27-A2 Addresses the Following Quality System Essentials (QSEs):

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

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

Path of Workflow

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



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

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

M27-A2 Addresses the Following Steps Within the Clinical Laboratory Path of Workflow

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

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

1 Introduction

The method described in this document is intended for testing yeasts that cause invasive infections. These yeasts encompass *Candida* species (including *Candida glabrata)* and *Cryptococcus neoformans*. The method has not been used in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma 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 NCCLS document M38—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*.[13,14]

M27-A2 is a "reference" standard being 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 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-A2.

1.1 Scope

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

The subcommittee has focused on developing relevant breakpoints for available antifungal agents,[11] and reference MIC ranges for microdilution testing of both established and newly introduced antifungal agents.[12]

1.2 Definitions[a]

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

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

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

2 Antifungal Agents

2.1 Source

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

2.2 Weighing Antifungal Powders

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

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

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

or

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

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

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

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

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

[b] In the U.S., antifungal or reference powders can also be obtained from the U.S. Pharmacopoeia (12001 Twinbrook Parkway, Rockville, MD 20852).

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

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

2.3 Preparing Stock Solutions

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

2.3.1 Use of Solvents Other Than Water

Some drugs must be dissolved in solvents other than water (see Table 1). Information on the solubility of an antifungal compound should be included with the drug. Such drugs should be dissolved at concentrations at least 100 times higher than the highest desired test concentration. Commonly used agents include analytical grade quality: 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 µg/mL, first weigh 4.8 mg (assuming 100% potency) of the antifungal powder and dissolve it in 3.0 mL DMSO. This will provide a stock solution at 1,600 µg/mL. Next, prepare further dilutions of this stock solution in DMSO. (See Tables 2 and 3.) The solutions in DMSO will be diluted tenfold in test medium (see Section 3.2.2) and a further tenfold when inoculated (see Section 3.3), 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 2.2 should be applied.

2.3.2 Filtration

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

2.3.3 Storage

Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at -60 °C or below but never at a temperature greater than -20 °C). Vials are to be removed as needed and used the same day. Any unused drug is to be discarded at the end of the day. Stock solutions of most antifungal agents can be stored at -60 °C or below for six months or more without significant loss of activity.[15] In all cases, any directions provided by the drug manufacturer

are to be considered as a part of these general recommendations and should supercede any other directions that differ. Any significant deterioration of an antifungal agent may be ascertained. This should be reflected in the results of susceptibility testing using quality control strains such as those in Table 4.

2.4 Number of Concentrations Tested

The concentrations to be tested should encompass the breakpoint concentrations and the expected results for the quality control 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; and new triazoles, 0.0313 to 16 µg/mL.

2.5 Selection of Antifungal Agents for Routine Testing and Reporting

Although breakpoints are now available for some organism-drug combinations; 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.

2.5.1 Generic Names

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

2.5.2 Number of Agents Tested

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

2.5.3 Guidelines for Selective Reporting

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 (e.g., when the infection is due to a non-*C. albicans* isolate). Interpretive breakpoints are available only for *Candida* spp. versus fluconazole, itraconazole, and flucytosine, and 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.

3 Test Procedures

3.1 Broth Medium

3.1.1 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) was found at least as satisfactory as several

other synthetic media and has been used to develop the standard.[3,4] The formula for this medium is provided in Table 6, and the preparation of the medium from powder is outlined in Appendix A.

3.1.2 Buffers

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

3.2 Preparing Diluted Antifungal Agents

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

(1) Use sterile, 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.

3.2.1 Water-Soluble Antifungal Agents

When twofold dilutions of a water-soluble antifungal agent are to be used, they may be prepared volumetrically in broth (Table 2). 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 3 is recommended.

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

Many persons find working with 1:10 dilutions (as shown in Table 2) easy and convenient. However, some automated pipets 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 that the same final concentrations of drug are obtained.

3.2.2 Water-Insoluble Antifungal Agents

For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, itraconazole, or the new triazoles, a dilution series of the agent should be prepared first at 100x final strength in an appropriate solvent (see Section 2.3.1). 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 1,600 to 3.13 µg/mL should have been prepared first in DMSO (see Section 2.3.1). To prepare 1-mL volumes of diluted antifungal agent (sufficient for ten tests), first pipet 0.9-mL volumes of RPMI 1640 broth into each of 11 sterile test tubes. Now, using a single pipet, 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 tenfold more concentrated than the final concentrations.

3.3 Inoculum Preparation

The steps for preparation of inoculum are as follows:

(1) All organisms should be subcultured from sterile vials 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* species 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).

(3) The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline 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 x 10^6 to 5 x 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 x 10^2 to 2.5 x 10^3 cells per mL.[2]

3.4 Inoculating RPMI-1640 Medium

Before adjusting the inoculum, 0.1 mL of the various antifungal concentrations are placed 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), 0.9 mL of the adjusted inoculum is added to each tube in the dilution series and mixed. This results in a 1:10 dilution of each antifungal concentration and a 10% dilution of the inoculum.

3.5 Incubation

With the exception of *C. neoformans*, tubes are incubated (without agitation) at 35 °C for 46 to 50 hours in ambient air. When testing *C. neoformans*, tubes should be incubated for a total of 70 to 74 hours before determining results.

3.6 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 as follows:

3.6.1 Amphotericin B

For amphotericin B, end points are typically well defined, and the MIC is easily read as the lowest drug concentration that prevents any discernible growth. Trailing end points with amphotericin B are usually not encountered.

3.6.2 Flucytosine and Azole Antifungals

For flucytosine and especially for azoles such as fluconazole and ketoconazole, end points are typically less well defined than that described for amphotericin B which may contribute to a significant source of variability. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement and also discriminates between putatively susceptible and resistant isolates. When turbidity persists, it is often identical for all drug concentrations above the MIC. The amount of allowable turbidity can be estimated by diluting 0.2 mL of drug-free control growth with 0.8 mL of media, producing an 80% inhibition standard.[5,16] 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.

3.7 Interpretation of Results

Interpretive breakpoints have been established at present only for some organism-drug combinations (see Appendix C). The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarized as follows:

3.7.1 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. Current work suggests that testing with Antibiotic Medium 3 supplemented with 2% glucose (dextrose) permits more reliable detection of resistant isolates.[17,18] However, the reproducibility of this method is still under study,[19] 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®): *C. lusitaniae* ATCC® 200950; ATCC® 200951, ATCC® 200952, ATCC® 200953, ATCC® 200954; *C. albicans* ATCC® 200955; *C. tropicalis* ATCC® 200956.

3.7.2 Flucytosine

Based largely on historical data and partially on the drug's pharmacokinetics, interpretive breakpoints for *Candida* spp. and flucytosine have been established (see Appendix C).

3.7.3 Fluconazole

Based on a large data package presented by fluconazole's manufacturer,[11] interpretive breakpoints for *Candida* spp. and fluconazole have been established (see Appendix C). These data are principally drawn from studies of oropharyngeal candidiasis and of invasive infections due to *Candida* spp. in non-neutropenic patients, and their clinical relevance in other settings is uncertain. In addition, these interpretive breakpoints are not applicable to *C. krusei*, and thus identification to the species level is

required in addition to MIC determination. The utility of testing isolates of *C. neoformans* is currently under intense study, and recent data do suggest a correlation between elevated MIC and clinical failure.[20]

3.7.4 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.

3.7.5 Itraconazole

Based on a large data package presented by itraconazole's manufacturer,[11] interpretive breakpoints for *Candida* spp. and itraconazole have been established (see Appendix C). 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 3 can lead to substantial errors due to dilution artifacts.

3.7.6 New Triazoles

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

3.8 Broth Microdilution Modifications

A substantial body of data has now been presented that documents excellent concordance between results obtained by the broth macrodilution methodology described above and a broth microdilution adaptation.[5,8-10,21-24] 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 that are relevant to the broth microdilution test are discussed in detail.

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

The broth microdilution test is performed by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells). The 2x drug concentrations are dispensed into the wells of Rows 1 to 10 of the microdilution plates in 100-µL volumes with a multichannel pipet. Row 1 contains the highest (either 64 or 16 µg/mL) drug concentration, and Row 10 contains the lowest drug concentration (either 0.12 or 0.03 µg/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. Each well of a microdilution tray is inoculated on the day of the test with 100 µL of the corresponding 2x diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentrations mentioned above. The growth control wells contain 100 µL of sterile, drug-free medium and are inoculated with 100 µL of the corresponding diluted (2x) inoculum suspensions. The QC organisms are tested in the same manner and are included each time an

isolate is tested. Row 12 of the microdilution plate can be used to perform the sterility control (drug-free medium only).

The microdilution plates are incubated at 35 °C and observed for the presence or absence of visible growth. Agitation of the plates may simplify reading of the end points. The microdilution wells are scored with the aid of a reading mirror; the growth in each well is compared with that of the growth control (drug-free) well. A numerical score, which ranges from 0 to 4, is given to each well using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 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.[24,25] The MIC for amphotericin B is defined as the lowest concentration in which a score of 0 (optically clear) is observed and, for 5-FC and the azoles, as the lowest concentration in which a score of 2 (prominent decrease in turbidity) is observed. A prominent decrease in turbidity corresponds to approximately 50% inhibition in growth as determined spectrophotometrically. The microdilution MICs read at 48 hours (72 hours for most *C. neoformans*) provide the best agreement with the reference broth macrodilution method.[23,24,26]

3.9 Impact of Time Reading: 24 Hours versus 48 Hours

The M27-A2 methodology for *Candida* recommends an end point reading at 48 hours. For most isolates, the difference between reading at 24 hours versus 48 hours is minimal and will not alter the interpretative category (i.e., does not change whether the isolate would be categorized as "susceptible" or "resistant"). However, recent work has begun to include 24-hour readings, because (a) MICs can often be read at 24 hours; and (b) readings taken at 24 hours may be more clinically relevant for some isolates. Isolates for which the earlier reading is important 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 in about 5% of isolates,[27] 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[27,28] 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[29] 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.[30] In light of these observations, both 24-hour and 48-hour microdilution MIC ranges are provided for the two QC strains and eight systemic antifungal agents (Table 5).

3.10 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 7. These modifications are not part of the current methodology, but interested laboratories may wish to explore their clinical relevance.

4 Quality Control

4.1 Purpose

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

- the precision and accuracy of the susceptibility test procedure;
- the performance of reagents, testing conditions, and instructions used in the test; 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 quality control and reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.[6]

4.2 Quality Control Responsibilities

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

Manufacturers are responsible for the following:

- antifungal stability;
- antifungal identification;
- potency of antifungal stock solutions;
- compliance with good manufacturing practices;
- integrity of the product; and
- accountability and traceability to the consignee.

4.2.2 Laboratory (User)

The laboratorian is responsible for the following:

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

4.2.3 Mutual Responsibility

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

4.3 Selecting Reference Strains

Ideal reference strains for quality control of dilution methods have MICs that fall near the midrange of the concentration for all antifungal agents tested. An ideal control strain is inhibited at the fifth dilution of a nine-dilution $\log_2$ series, but strains with MICs between the third and seventh dilution are acceptable. Before a strain is accepted as a reference, it is to be tested for as long as is necessary to demonstrate that its antifungal susceptibility pattern is genetically stable. NCCLS document M23—*Development of* In Vitro *Susceptibility Testing Criteria and Quality Control Parameters*, provides guidelines for the selection of appropriate quality control strains and the determination of acceptable MIC ranges. The strains listed in Tables 4 and 5 were selected in accordance with these criteria.[6,7,12]

4.4 Storing Reference Strains

4.4.1 Methods for Prolonged and Short-term Storage

Reference strains are stored in a way that minimizes the possibility of mutation in the 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 °C as described by Pasarell and McGinnis.[31] Alternatively, reference strains (but not *Cryptococcus* species) can be preserved by suspending fungal cells in 50% glycerol solution in small vials and freezing and storing them at -70 °C.

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

4.4.2 Sources for Reference Strains

Reference strains are obtained from a source that is able to provide information on the origination of the culture (for example, from the American Type Culture Collection [ATCC®],[c] from commercial sources with documented culture history, or from reference institutions with demonstrated ability to store and use the organisms consistently with minimal contamination).

4.4.3 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, potato dextrose agar, or soybean casein digest agar.

(2) Select growth from several colonies and perform the appropriate susceptibility tests to demonstrate that they give the expected MIC results (see Table 4 for expected MICs of 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 freezer maintained as in Section 2.3.3 or in liquid nitrogen.

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

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

4.5 Routine Use of Reference Strains

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

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

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

(3) Subculture the mixture onto potato dextrose agar plates and incubate them at 35 °C for 24 hours for *Candida* species, 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 them at 2 to 8 °C.

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

(7) Always perform susceptibility tests on colonies from overnight plates.

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

4.6 Batch of Medium and Lot of Plasticware Control

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

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

(2) Incubate at least one uninoculated tube 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 3.1.2).

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

4.7 Quality Control Frequency

4.7.1 MIC Ranges

MIC accuracy ranges for a single control test are listed in Tables 4 and 5.[6,7,12] In general, 1 out of every 20 MIC values in a series of 20 consecutive tests might be out of control (i.e., outside the stated range) due to random variation of the test. Two consecutive out-of-control results or any more than 2 out-of-control results in 20 consecutive control tests requires corrective action. Any time corrective action is taken, the count of 20 begins again.

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

4.7.2 Frequency of Testing

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

(2) For each drug-microorganism combination, no more than 3 of the 30 MIC values (i.e., MIC values obtained from one drug-microorganism combination for 30 consecutive test days) may be outside the accuracy ranges stated in Tables 4 and 5.

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

(3) 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 (i.e., MIC values obtained from one drug-microorganism combination for five consecutive test days) must be within the accuracy ranges stated in Tables 4 and 5.

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

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

4.8 Other Control Procedures

4.8.1 Growth Control

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

4.8.2 Purity Control

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

4.8.3 End Point Interpretation Control

End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. The results are recorded and compared to the results obtained by an experienced reader. Specific reference strains with predetermined MICs are particularly helpful for this purpose, especially with fluconazole.[6,7, 12]

4.9 Quality Control Strains (see also Section 4.3)

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

Tables 4 and 5 list expected ranges for strains found to be acceptable as quality control strains. Also shown are additional strains that can be useful for conducting reference studies.[6,7, 12]

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Appendix A. RPMI 1640 Medium

RPMI 1640 medium buffered with 0.165 mol/L MOPS, 1 L. (See Table 6.)

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

Dissolve powdered medium in 900 mL distilled H_2O. Add MOPS (final concentration of 0.165 mol/L) and stir until dissolved. While stirring, adjust the pH to 7.0 at 25 °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.

Appendix B. McFarland 0.5 Barium Sulfate Turbidity Standard

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

The procedure consists of the following steps:

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

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

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

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

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

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

Appendix C. Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida* Species

Antifungal Agent	Susceptible (S)	Susceptible-dose dependent (S-DD)[‡]	Intermediate (I)[§]	Resistant (R)
Fluconazole[*]	≤ 8	16-32	-	≥ 64
Itraconazole[†]	≤ 0.125	0.25-0.5	-	≥ 1
Flucytosine	≤ 4	-	8-16	≥ 32

Shown are the breakpoints (µg/mL) for *Candida* species against the indicated agents. If MICs are measured using a scale that yields results falling between categories, the next higher category is implied. Thus, an isolate with a fluconazole MIC of 12.5 µg/mL would be placed in the S-DD category.[¶]

Footnotes

[*] For fluconazole, these guidelines are based substantially on experience with mucosal infections, but are consistent with the limited information for invasive infections due to *Candida* spp. Isolates of *C. krusei* are assumed to be intrinsically resistant to fluconazole, and their MICs should not be interpreted using this scale. It is also pertinent that the 8-µg/mL upper boundary for the susceptible range of fluconazole is not known with certainty—the data would permit selection of either 4 or 8 µg/mL for this cut-off.

[†] For itraconazole, the data are based entirely on experience with mucosal infections, and data supporting breakpoints for invasive infections due to *Candida* spp. are not available.

[‡] Susceptibility is dependent on achieving the maximal possible blood level. For fluconazole, doses of 400 mg/day or more may be required in adults with normal renal function and body habitus. For itraconazole, measures to assure adequate drug absorption and plasma itraconazole concentrations of >0.5 µg/mL may be required for optimal response.

[§] The susceptibility of these isolates is not certain, and the available data do not permit them to be clearly categorized as either "susceptible" or "resistant. "

[¶] These breakpoints were adopted at a meeting of the subcommittee held June 1, 1996 in Reston, VA. These breakpoints are considered tentative for one year and are open for comments.

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

Antifungal Agent	Solvent* (Full Strength and Intermediate Solutions)	Diluent (Final Concentrations)
Amphotericin B	DMSO†	Medium
Ketoconazole	DMSO†	Medium
Itraconazole	DMSO†	Medium
Posaconazole	DMSO†	Medium
Ravuconazole	DMSO†	Medium
Voriconazole	DMSO†	Medium
Fluconazole	Water	Medium
Flucytosine (5-FC)	Water	Medium

* These compounds are potentially toxic. Consult the material safety data sheets (MSDS) available from the product manufacturer before using any of these materials.

† Dimethyl sulfoxide

Table 2. Scheme for Preparing Dilutions of Water-Soluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests

Antimicrobial Solution							
Step	Concentration (µg/mL)	Source	Volume + (mL)	Medium = (mL)	Intermediate = Concentration (µg/mL)	Final Concentration at 1:10 (µg/mL)	Log_2
1	5120	Stock	1 mL	7	640 µg/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

Table 3. Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests

Antimicrobial Solution							
Step		Source	Volume + (mL)	Solvent = (mL) (e.g., DMSO)*	Intermediate = Concentration (µg/mL)	Final Concentration at 1:100 (µg/mL)	Log_2
1	1,600	Stock			1,600 µg/mL	16	4
2	1,600	Stock	0.5	0.5	800	8.0	3
3	1,600	Stock	0.5	1.5	400	4.0	2
4	1,600	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

*Dimethyl sulfoxide

Table 4. Recommended 48-hour MIC Limits for Two Quality Control and Four Reference Strains for Broth Macrodilution Procedures. (From Pfaller MA, et al. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. *J Clin Microbiol* 1995;33:1104-1107; and Rex JH, et al. Quality control guidelines for National Committee for Clinical Laboratory Standards-recommended broth macrodilution testing of ketoconazole and itraconazole. *J Clin Microbiol* 1996;34:816-817.)

Organism	Purpose	Antifungal Agent	MIC Range (μg/mL)	% of MICs Within Range
Candida parapsilosis ATCC® 22019	QC	Amphotericin B Fluconazole Itraconazole Ketoconazole 5FC	0.25-1.0 2.0-8.0 0.06-0.25 0.06-0.25 0.12-0.5	99.1 99.1 99.0 99.0 98.6
Candida krusei ATCC® 6258	QC	Amphotericin B Fluconazole Itraconazole Ketoconazole 5FC	0.25-2.0 16-64 0.12-0.5 0.12-0.5 4.0-16	99.5 99.1 94.0 100.0 96.8
Candida albicans ATCC® 90028	Reference	Amphotericin B Fluconazole 5FC	0.5-2.0 0.25-1.0 0.5-2.0	91.9 97.3 95.0
Candida albicans ATCC® 24433	Reference	Amphotericin B Fluconazole 5FC	0.25-1.0 0.25-1.0 1.0-4.0	99.5 95.9 91.9
Candida parapsilosis ATCC® 90018	Reference	Amphotericin B Fluconazole 5FC	0.5-2.0 0.25-1.0 ≤0.12-0.25	96.4 98.2 99.5
Candida tropicalis ATCC® 750	Reference	Amphotericin B Fluconazole 5FC	0.5-2.0 1.0-4.0 ≤0.12-0.25	93.7 95.5 99.5

NOTE: ATCC® is a registered trademark of the American Type Culture Collection.

Table 5. Recommended 24- and 48-hour MIC Limits for Two Quality Control Strains for Broth Microdilution. (From Barry AL, et al. Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. *J Clin Microbiol.* 2000;38:3457-3459. Used with permission from the American Society for Microbiology and AL Barry.)

		MIC (µg/mL) ranges for microdilution tests					
Organism	**Antifungal Agent**	**Range**	**24-h Mode**	**% within Range**	**Range**	**48-h Mode**	**% within Range**
Candida parapsilosis ATCC® 22019	Amphotericin B	0.25-2.0	0.5	97	0.5-4.0	2.0	92
	5FC	0.06-0.25	0.12	99	0.12-0.5	0.25	98
	Fluconazole	0.5-4.0	2.0	98	1.0-4.0	2.0	99
	Itraconazole	0.12-0.5	0.25	96	0.12-0.5	0.25	98
	Ketoconazole	0.03-0.25	0.06/0.12	98	0.06-0.5	0.12	98
	Voriconazole	0.016-0.12	0.06	100	0.03-0.25	0.06	100
	Ravuconazole	0.016-0.12	0.06	96	0.03-0.25	0.06	98
	Posaconazole	0.06-0.25	0.12	97	0.06-0.25	0.12	99
Candida krusei ATCC® 6258	Amphotericin B	0.5-2.0	1.0	100	1.0-4.0	2.0	100
	5FC	4.0-16	8.0	98	8.0-32	16	99
	Fluconazole	8.0-64	16	100	16-128	32	100
	Itraconazole	0.12-1.0	0.5	96	0.25-1.0	0.5	100
	Ketoconazole	0.12-1.0	0.5	96	0.25-1.0	0.5	99
	Voriconazole	0.06-0.5	0.25	98	0.25-1.0	0.5	100
	Ravuconazole	0.06-0.5	0.25	93	0.25-2.0	0.5	100
	Posaconazole	0.06-0.5	0.25	100	0.12-1.0	0.5	99

NOTE: ATCC® is a registered trademark of the American Type Culture Collection.

Table 6. Composition of RPMI 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-aspargine (anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine • 2HCI	0.0652	Folic acid	0.001
L-glutamic acid	0.020	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_{12}	0.000005
L-lysine • HCI	0.040	Calcium nitrate × H_2O	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulfate (anhydrous)	0.04884
L-proline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate, dibasic (anhydrous)	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathione, reduced	0.001
L-tyrosine • 2Na	0.02883	Phenol red, Na	0.0053
L-valine	0.020		

Table 7. Modifications for Special Circumstances[*]

Drug	Organism	Modification	Reference
Amphotericin B	*Candida* spp	Use of Antibiotic Medium 3 may enhance detection of resistance, but this medium is not standardized and substantial lot-to-lot variability is possible.	See Section 3.7.1 [†,‡]
All drugs	*C. neoformans*	Use of Yeast Nitrogen Base may enhance the growth of *C. neoformans* and improve the clinical relevance of antifungal MICs.	[§, ¶]
All drugs	All organisms	Supplementation of the test medium so that it contains glucose at a final concentration of 20 g/L may simplify end point determination.	[#]

[*] These modifications are not a part of the formal M27-A 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 M27-A that may enhance its utility under specific circumstances.

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

Summary of Comments and Subcommittee Responses

M27-A: *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard*

General

1. With reference to pages 6 and 8, the yeast stock inoculum suspension in sterile saline is diluted 1:100 and 1:20 or 1:50 and 1:20 in RPMI 1640 broth for the macrodilution and microdilution respectively. Alternatively it would be more convenient, i.e., 1:100 or 1:50 instead of RPMI 1640 broth. Our comparison shows no difference in the MICs using either diluent.

- **We agree. There is no reason to change the procedures.**

2. In some of the NCCLS documents such as M7 and M27 there are tables that tell people how to dissolve various antimicrobials. Some of the materials that are being used as solvents (methanol, DMSO, ethanol) can be dangerous. DMSO, for example, is considered as an allergen, an irritant, and, if heated, emits toxic vapors. More importantly, it is a compound, which is absorbed through the skin carrying with it anything dissolved in it (1). These documents have the "Universal Precautions" statement but no chemical safety statement. I suggest that a chemical safety statement be put into these documents. Preferably this statement should put in the section where the recommendation to use the material is made.

- **The statement has been added as a footnote to Table 1**.

Summary of Delegate Comments and Subcommittee Responses

M27-A2: *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition*

General+

1. The instructions for determining the MIC for fluconazole, itraconazole, and 5FC are ambiguous. In one area it is suggested that a dilution = 80% inhibition be made using the growth control, and in another area it states the MIC = the well with the sharpest drop-off or decrease of growth. It has been our experience that determining the MIC as = the well with the sharpest drop-off is much more reproducible (both intra- and interobserver).

- **There are differences in determining the endpoints for these agents when tested by macro- vs microdilution methods. The macrodilution method may be read as the first tube in which an 80% inhibition relative to the growth control is seen. It is not possible to tell the difference between 50% and 80% inhibition by eye when using the microdilution method, thus the alternative wording of "sharp decrease...". These distinctions are explained within the document. The reading of the microdilution MICs for the azoles and 5FC is difficult, and after much discussion and investigation the subcommittee arrived at the description that the endpoint is best approximated by the first "sharp or obvious" decrease in growth. We have also stated that this visual reading approximates what one would find if one used a 50% inhibition spec reading. These definitions are now well described in the literature as well, including numerous publications from the subcommittee.**

Section 2.2

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

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

Section 2.3.2

3. If filtration is employed, it would be appropriate to indicate which media can be used based on drugs and diluent.

- **The subcommittee does not stress filtration, but if used, there are no exclusions regarding medium, especially since the "standard" is RPMI.**

Sections 3.7.3 and 3.7.5

4. Lack of clinical correlation is well stated. However, there may be a hidden intimation that susceptibility testing should be performed for oral candidacies. I'm not sure how often that should be recommended.

- **The subcommittee has been careful in describing clinical correlates, as well as those instances where testing may be helpful. The subcommittee believes that testing of oral isolates may be useful in selected instances involving suboptimal response to therapy.**

Section 3.8

5. Wells in rows 1 to 10 contain drug dilutions. Wells in row 11 are used for growth controls. Wells in row 12 (not 11) may be used as sterility controls. What about DMSO controls?

- **The dilution scheme described provides assurance that DMSO is less than 1% in any given well and will not affect growth.**

6. "Prominent decrease in turbidity" seems arbitrary. Are there any electronic ways to measure?

- **It is stated that a "prominent" decrease in turbidity determined visually will correlate with a 50% reduction in turbidity determined spectrophotometrically.**

Section 4.8.3

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

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

Related NCCLS Publications*

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

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

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

M23-A2 **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Second Edition (2001).** 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-T2 **Antimycobacterial Susceptibility Testing; Tentative Standard—Second Edition (2000).** This document contains recommendations for common culture media, standardization of drug concentrations, a method for standardizing inoculum dilutions, and clearly defined end points in susceptibility testing of tuberculosis-like organisms.

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

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.

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

NOTES

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