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

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.

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

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Abstract

Clinical and Laboratory Standards Institute document M38-A2—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition* describes a method for testing the susceptibility of filamentous fungi (moulds) that cause invasive (*Aspergillus* spp., *Fusarium* spp., *Rhizopus oryzae (R. arrhizus)*, *Pseudallescheria boydii [Scedosporium apiospermum]*, *S. prolificans*, *Sporothrix schenckii*, and other opportunistic pathogenic moulds) and cutaneous (dermatophyte, *Trichophyton*, *Microsporum*, and *Epidermophyton* spp.) fungal infections to antifungal agents. Selection of antifungal agents; preparation of antifungal stock solutions and dilutions for testing, implementation, and interpretation of test procedures; and the purpose and implementation of quality control procedures are discussed. A careful examination of the responsibilities of the manufacturer and the user in quality control is also presented.

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Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory methods to guide the selection of antifungal therapy have gained greater attention. The CLSI Area Committee on Microbiology formed the Subcommittee on Antifungal Susceptibility Testing, and data for testing filamentous fungi were collected in a series of collaborative studies. As a result, CLSI document M27[1] was published with the establishment of quality control MIC ranges and the development of breakpoints.

Based on these achievements, the subcommittee concluded that it would be useful to work toward a reproducible reference testing procedure for the antifungal susceptibility testing of filamentous fungi (moulds). A working group on filamentous fungi was formed and charged with the responsibility of carrying out studies to collect data and to refine the methodology to perform susceptibility testing of these fungal species. As a result of several collaborative studies, agreement within the subcommittee was achieved regarding testing conditions for the nondermatophyte moulds that included inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria for MIC determination.[2-5] This consensus method was published in 2002 as M38-A.

In M38-A2, supplemental material (QC data for mould isolates as well as echinocandin testing guidelines) has been incorporated.[6-9] In addition, methods for testing dermatophyte moulds are provided, based on a series of consensus studies.[10,11]

Because of its suitability for antifungal susceptibility testing of yeasts, synthetic RPMI-1640 medium was the test medium that the subcommittee evaluated as the potential reference medium for moulds including the dermatophytes.[2,3,10,12] The subcommittee has evaluated other media formulations, but the standard RPMI medium facilitated more consistent identification of itraconazole resistance in *Aspergillus* spp. in eight laboratories.[5] Drug stock solution preparation and dilution previously developed for antifungal testing of yeasts procedures (CLSI document M27)[1] also were adopted.

Key Words

antifungal, broth microdilution, dermatophytes, filamentous fungi or moulds, susceptibility testing

Updated Information in This Edition

Definitions (Section 4)

Added definition for:
- Minimal effective concentration (MEC)
- Quality control

Additional Sections and Appendix

Inoculum quantitation (Section 6.3.1)

MIC and MEC reading results for:
- Echinocandins (Section 6.6.5)
- Ciclopirox (Section 6.6.6)
- Grieseofulvin (Section 6.6.7)
- Terbinafine (Section 6.6.8)

Interpretation of results for:
- Echinocandins (Section 6.7.6)
- Ciclopirox (Section 6.7.7)
- Griseofulvin (Section 6.7.8)
- Terbinafine (Section 6.7.9)

Appendix A. MECs of Caspofungin and Anidulafungin:
- A1: MECs for Caspofungin
- A2: MECs for Anidulafungin

Data Inclusion/Exclusion

Expanded list of relevant drug concentration to include echinocandins and other suitable drug concentration ranges for testing dermatophytes (Section 5.4)

Expanded recommendations on inoculum preparation of dermatophyte species (Section 6.3)

Additional recommendations on incubation of *Alternaria* spp. and echinocandins (Section 6.5)

Modified section on reading results to include new information on echinocandins antifungal agents and MIC and MEC comparison (Section 6.6.1)

Expanded recommendations on reading results of itraconazole and new triazoles (Section 6.6.4)

Tables

All related tables are incorporated at the end of the document. Updates on each table include:

Table 1: Solvents and Diluents for Preparation of Stock Solutions of Antifungal Agents
Added solvents and diluent recommendations for the following:

Anidulafungin	Griseofulvin
Caspofungin	Micafungin
Ciclopirox	Terbinafine

Updated Information in This Edition (Continued)

Tables 2 and 2A: Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests
Expansion of the table to separate scheme for preparing dilution series of water insoluble antifungal agents for nondermatophyte isolates (Table 2) and dermatophyte isolates (Table 2A)

Deletion of Log_2 column

Table 3: Scheme for Preparing Dilutions of Water-Soluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests
Deletion of Log_2 column

Table 4: Recommended MIC or MEC Limits for QC and Reference Strains for Broth Dilution Procedures
Addition of Mode and Incubation Time column

Addition of/updated data on the following organisms:

Paecilomyces variotii ATCC® MYA-3630
Candida parapsilosis ATCC® 22019: Micafungin
Candida krusei ATCC® 6258: Micafungin
Aspergillus flavus ATCC® 204304
Aspergillus fumigatus ATCC® MYA-3627
Aspergillus flavus ATCC® MYA-3631
Aspergillus terreus ATCC® MYA-3633
Fusarium moniliforme ATCC® MYA-3629
Fusarium solani ATCC® 3636
Scedosporium apiospermum ATCC® MYA-3635
Scedosporium apiospermum ATCC® MYA-3634
Trichophyton mentagrophytes MRL 1957 ATCC® MYA-4439[10]
Tinea rubrum MRL 666 ATCC® MYA-4438[10]

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

1 Scope

This document describes a method for testing the susceptibility of filamentous fungi (moulds) that cause invasive (*Aspergillus* spp., *Fusarium* spp., *Rhizopus oryzae* [*R. arrhizus*], *Pseudallescheria boydii* [*Scedosporium apiospermum*], *Sporothrix schenckii,* and other pathogenic moulds) and cutaneous (the dermatophytes *Trichophyton*, *Microsporum,* and *Epidermophyton* spp.) fungal infections to antifungal agents.[2-5,10] Addressed in this document are testing conditions including inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria end-point determination.[7-9] Quality control (QC) reference ranges are also provided.[6,11]

This standard focuses on the fully defined synthetic medium RPMI-1640 for testing of moulds because of the suitability of this test medium for antifungal susceptibility testing of yeasts.[2,3,11,12]

Refer to CLSI document M27[1] for drug stock solution preparation and dilution procedures.

2 Introduction

The method described in this document is intended for testing common filamentous fungi or moulds, including the dermatophytes, which cause invasive and cutaneous infections, respectively. These moulds encompass *Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp., *P. boydii (S. apiospermum), S. prolificans*, the mycelial form of *S. schenckii*, other Zygomycetes and opportunistic monilaceous and dematiaceous moulds,[4,12] as well as the dermatophyte *Trichophyton*, *Microsporum,* and *Epidermophyton* spp.[10] Caution should be used when interpreting the minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) results for any mould/drug combination. The method has not been used in studies of the yeast or mould form of dimorphic fungi, such as *Blastomyces dermatitidis*, *Coccidioides immitis*, *Coccidioides posadasii, Histoplasma capsulatum* variety *capsulatum*, *Penicillium marneffei*, or *S. schenckii*. The method also has not been used in studies of dermatophytes with the echinocandins or nondermatophyte moulds with ciclopirox, griseofulvin, or terbinafine.

This document is a "reference" standard developed through a consensus process to facilitate agreement among laboratories in measuring the susceptibility of moulds to antifungal agents. It is emphasized that the relationship between *in vitro* vs *in vivo* data has only been evaluated in animal models.[12] An important use of a reference method is to provide a standard basis from which other methods can be developed, which also will result in interlaboratory agreement within specified ranges. Such methods might have particular advantages, such as ease of performance, economy, or more rapid results; therefore, their development could be highly desirable. To the extent that any method produces concordant results with this reference method, it would be considered to be in conformity with M38-A2.

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention.[13] For specific precautions for preventing the laboratory transmission

of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to CLSI document M29.[14]

4 Definitions

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

minimal effective concentration (MEC) – the lowest concentration of an antimicrobial agent that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well; **NOTE:** This terminology is currently used only with respect to testing of the echinocandin antifungal agents (see Appendix A).

minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that causes a specified reduction in visible growth of a microorganism in an agar or broth dilution susceptibility test.

quality control (QC) – the operational techniques that are used to ensure accuracy and reproducibility.

5 Antifungal Agents

5.1 Source

Antifungal standards or reference powders can be obtained commercially, directly from the drug manufacturer. Pharmacy stock or other clinical preparations should not be used. Acceptable powders bear a label that states the drug's generic name, its assay potency (usually expressed in micrograms [µg] or International Units per mg of powder), and its expiration date. Store the powders as recommended by the manufacturers, or at −20 °C or below (never in a self-defrosting freezer), in a desiccator, preferably in a vacuum. When the desiccator is removed from the freezer, allow it to come to room temperature before opening (to avoid condensation of water).

5.2 Weighing Antifungal Powders

Assay all antifungal agents 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 used.

Use either of the following formulas to determine the amount of powder or diluent needed for a standard solution:

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

or

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \bullet \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}} \quad (2)$$

The antifungal powder should be weighed on an analytical balance that has been calibrated by approved reference weights from a national metrology organization. Usually, it is advisable to accurately weigh a portion of the antifungal agent in excess of that required and to calculate the volume of diluent needed to obtain the concentration desired.

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

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

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

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

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

5.3 Preparing Stock Solutions

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

5.3.1 Use of Solvents Other Than Water

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

For example, to prepare for a broth microdilution test series containing a water-insoluble drug that can be dissolved in DMSO, for which the highest desired test concentration is 16 µg/mL, first weigh 4.8 mg (assuming 100% potency) of the antifungal powder and dissolve it in 3.0 mL DMSO. This will provide a stock solution at 1600 µg/mL. Then prepare further dilutions of this stock solution in DMSO (see Table 2). Dilute the solutions in DMSO 1:50 in test medium (see Section 6.2), and a further 2x (twofold) dilution will occur when inoculated (see Section 6.4), reducing the final solvent concentration to 1% DMSO at each drug concentration, as well as in the growth control (drug-free medium) used in the test as a solvent control.

The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations in Section 5.2 should be applied.

5.3.2 Filtration

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

5.3.3 Storage

Dispense small volumes of the sterile stock solutions into sterile polypropylene or polyethylene vials, carefully seal, and store (preferably at −60 °C or below, but never at a temperature greater than −20 °C). Remove vials as needed and use the same day. Discard any unused drug at the end of the day. Stock solutions of most antifungal agents can be stored at −60 °C or below for six months or more without significant loss of activity.[15] In all cases, consider any directions provided by the drug manufacturer as a part of these general recommendations, and those directions should supersede any other directions that differ. Any significant deterioration of an antifungal agent may be ascertained. This should be reflected in the results of susceptibility testing using QC strains or reference strains such as those in Table 4.

5.4 Number of Concentrations Tested

The concentrations tested should encompass the expected results for the available QC strains. Based on previous studies for nondermatophyte moulds, the following drug concentration ranges may be relevant: amphotericin B, 0.0313 to 16 μg/mL; flucytosine, 0.125 to 64 μg/mL; ketoconazole, 0.0313 to 16 μg/mL; itraconazole and new triazoles (posaconazole, ravuconazole, and voriconazole), 0.0313 to 16 μg/mL; fluconazole, 0.125 to 64 μg/mL; and echinocandins (anidulafungin, caspofungin, and micafungin), 0.015 to 8 μg/mL. Suitable drug concentration ranges for testing dermatophytes are: ciclopirox, 0.06 to 32 μg/mL; griseofulvin, 0.125 to 64 μg/mL; itraconazole, 0.001 to 0.5 μg/mL; posaconazole, 0.004 to 8 μg/mL; voriconazole, 0.001 to 0.5 μg/mL; fluconazole, 0.125 to 64 μg/mL; and terbinafine, 0.001 to 0.5 μg/mL.[10]

5.5 Selection of Antifungal Agents for Routine Testing and Reporting

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

5.5.1 Generic Names

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

5.5.2 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 principal would apply.

5.5.3 Guidelines for Selective Testing

Testing may be warranted under certain selected circumstances such as the following: as part of periodic batch surveys that establish antibiograms for collections of pathogenic isolates obtained from within an institution; and to aid in the management of invasive and cutaneous infections due to filamentous fungi

when the utility of the azole antifungal agents is uncertain. Interpretive breakpoints are not available for any species of filamentous fungi vs any antifungal agent, and the clinical relevance of testing any organism-drug combination remains uncertain. Specimens for culture and other procedures should be obtained before initiation of antifungal therapy.

6 Test Procedures

6.1 Broth Medium

6.1.1 Synthetic Medium

The completely synthetic medium RPMI-1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) is satisfactory for testing the filamentous fungi, and has been used to develop the proposed standard.[2,3,10] The formula for this medium is provided in Table 5, and the preparation of the medium from powder is outlined in Appendix B.

6.1.2 Buffers

Media should be buffered to a pH of 7.0 ± 0.1 at 25 °C. A buffer should be selected that does not antagonize antifungal agents. Tris buffer is unsatisfactory because it antagonizes the activity of flucytosine. Zwitterion buffers are preferable to buffers that readily traverse the cell membrane, such as phosphate buffers, because, theoretically, the latter can produce unexpected interactions with antifungal agents. One buffer that has been found to be satisfactory for antifungal testing is MOPS (3-[N-morpholino] propanesulfonic acid) at a final concentration of 0.165 mol/L at pH 7.0. Check the pH of each batch of medium 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). Evaluate MIC performance characteristics of each batch of broth using a standard set of QC organisms (see Section 7 and Table 4).

6.2 Preparing Diluted Antifungal Agents

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

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

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

When 2x (twofold) dilutions of a water-soluble antifungal agent are used, they may be prepared volumetrically in broth (see Table 3). The procedure for antifungal agents 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 prepared depends on the number of tests performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL will be adequate for about eight tests (one microdilution tray), allowing for pipetting. Use a single pipette for measuring all diluents and then for adding the stock antifungal solution to the first tube. Use a separate pipette for each remaining dilution in that set. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are two times more concentrated than the final concentrations.

For antifungal agents that cannot be prepared as stock solutions in water, such as amphotericin B, anidulafungin, ciclopirox, griseofulvin, itraconazole, ketoconazole, posaconazole, ravuconazole, terbinafine, or voriconazole (see Table 1 for solvents), a dilution series of the agent should be prepared at

first 100 times the final strength in an appropriate solvent (see Section 5.3.1). Then, each of these nonaqueous solutions should be diluted 1:50 in RPMI-1640 medium.

For example, if a dilution series with final concentrations in the range 16 µg/mL to 0.0313 µg/mL is desired, a concentration series from 1600 to 3.13 µg/mL should have been prepared first in DMSO (see Section 5.3.1 and Table 2). To prepare 5-mL volumes of diluted antifungal agent (sufficient for 45 tests), first pipette 4.9-mL volumes of RPMI-1640 medium into each of 10 sterile test tubes. Then, using a single pipette, add 0.1 mL of DMSO alone to one 4.9-mL lot of medium (control medium), then 0.1 mL of the lowest (3.13 µg/mL) drug concentration in DMSO, then 0.1 mL of the 6.25-µg/mL concentration, and continue in sequence up the concentration series, each time adding 0.1-mL volumes to 4.9 mL medium. These volumes can be adjusted according to the total number of tests required. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are twofold more concentrated than the final concentrations.

6.3 Inoculum Preparation

When the risk of substantial spatter or aerosolization is present, the manipulation should be performed in a Class IIA or IIB biological safety cabinet. Details are further outlined in CLSI document M29.[14]

Nondermatophyte species – Initial work demonstrated that reliable nongerminated conidial or sporangiospore suspensions could be prepared by a spectrophotometric procedure,[12,16-18] and that concentrations of viable conidial or sporangiospore test inocula in a range of approximately 0.4×10^4 to 5×10^4 CFU/mL provided the most reproducible MIC data.[2,3] To induce conidium and sporangiospore formation, most fungi should be grown on potato dextrose agar for seven days at 35 °C or until good sporulation is obtained; good sporulation may be obtained after 48 hours of incubation for some isolates (eg, Zygomycete and *Aspergillus* spp.). *Fusarium* spp. may need to be incubated for 48 to 72 hours at 35 °C and then until day seven at 25 °C to 28 °C. Cover sporulating colonies with approximately 1 mL of sterile 0.85% saline, and prepare a suspension by gently probing the colonies with the tip of a transfer pipette. Addition of one drop (approximately 0.01 mL) of Tween 20 will facilitate the preparation of *Aspergillus* spp. inocula. The resulting mixture of conidia or sporangiospores and hyphal fragments is withdrawn and transferred to a sterile tube. After allowing heavy particles to settle for three to five minutes, transfer the upper homogeneous suspension to a sterile tube, tighten the cap, and mix with a vortex mixer for 15 seconds. (**CAUTION:** Remove the cap carefully, as liquid adhering to the cap may produce aerosols upon opening.) Read and adjust the densities of the conidial or sporangiospore suspensions to an optical density (OD) at 530 nm that ranges from 0.09 to 0.13 for *Aspergillus* spp., *Paecilomyces lilacinus, P. variotii, Exophiala dermatitidis,* and *S. schenckii*; 0.15 to 0.17 for *Fusarium* spp., *S. apiospermum*, *Ochroconis gallopava*, *Cladophialophora bantiana*, *R. oryzae,* and other zygomycetous species; and 0.25 to 0.3 for *Bipolaris* spp. and *Alternaria* spp.[19] Dilute these suspensions 1:50 in the standard medium. Inoculum suspensions of *S. apiospermum, Bipolaris* spp., and *Alternaria* spp. may require a lower (50%) dilution factor. The 1:50 inoculum dilutions will be 2x (twofold) more concentrated than the density needed or approximately 0.4×10^4 to 5×10^4 CFU/mL. Make the test inoculum in sufficient volume to directly inoculate each well with 0.1 mL of the corresponding diluted inoculum suspension.

Dermatophyte species – Most dermatophyte isolates produce sufficient conidia on potato dextrose agar. However, conidium formation by *Trichophyton rubrum* is very poor on standard fungal media including potato dextrose agar. Because of that, the use of oatmeal agar (see Appendix D for preparation instructions) has been recommended as the optimal growth medium for inducing conidium formation in *T. rubrum* isolates.[10,20] Dermatophyte isolates should be grown on potato dextrose agar or oatmeal agar (*T. rubrum* isolates only) at 30 °C for four to five days or until good conidial growth is present. Cover colonies with approximately 1 mL of sterile 0.85% saline, and prepare a suspension by gently probing the colonies with the tip of a transfer pipette or sterile swab. Allow the resulting suspension to settle for five to 10 minutes, count conidia with a hemacytometer, and adjust the concentration as needed. The final

suspension should be made 2x more concentrated than the density needed for testing (1×10^3 to 3×10^3 CFU/mL).[10]

6.3.1 Inoculum Quantitation

The accuracy of the final inoculum may be verified as follows:

Inoculum quantitation of nondermatophyte moulds – This step can be performed by plating 0.01 mL of a 1:10 dilution of the adjusted inoculum on Sabouraud glucose (dextrose) agar to determine the viable number of colony-forming units (CFU) per milliliter.[2,3,5,16] Incubate the plates at 28 °C to 30 °C and observe daily for the presence of fungal colonies. Colonies should be counted as soon as possible after growth becomes visible, especially for isolates of *R. oryzae*. The incubation times will range from 24 hours or less (*R. oryzae*) to five days (*S. apiospermum*).

Inoculum quantitation of dermatophytes – This step can be performed by plating 0.01 mL dilution of the adjusted inoculum on Sabouraud glucose agar to determine the viable number of CFU per milliliter. Incubate the plates at 28 °C to 30 °C and observe daily for the presence of fungal colonies.

6.4 Inoculating RPMI-1640 Medium

Inoculate each well on the day of the test with 0.1 mL of the 2x conidial or sporangiospore inoculum suspension. This step will dilute the drug concentrations, inoculum densities, and solvent, if used, to the final desired test concentrations. The growth control wells will contain 0.1 mL of the corresponding diluted inoculum suspension and 0.1 mL of the drug diluent (2%) without antifungal agent (see Section 6.2). Test QC and reference organisms in the same manner and include each time an isolate is tested.

6.5 Incubation

Incubate all microdilution trays at 35 °C without agitation; some isolates of *Alternaria* spp. may not grow at this incubation temperature and incubation at 30 °C is more suitable. Trays containing *Rhizopus* spp. are examined after 21 to 26 hours of incubation before determining MIC results. Evaluate most other opportunistic filamentous fungi, including *Fusarium* spp., *Aspergillus* spp., and *S. schenckii,* after 46 to 50 hours of incubation. Examine *Scedosporium* spp. after 70 to 74 hours. For the echinocandins, evaluate isolates after 21 to 26 hours (eg, *Aspergillus* spp. and *Paecilomyces variotii*) and 46 to 72 hours (*Scedosporium* spp.), or the first day when sufficient growth (confluent growth covering the bottom of the well) is present in the growth control well (drug-free medium) for MEC determination.

Evaluate trays containing dermatophyte isolates after four days of incubation.[10]

6.6 MIC and MEC Reading Results

6.6.1 General

The MIC is the lowest concentration of an antifungal agent that substantially inhibits growth of the organism, as detected visually when testing most antifungal agents. For the conventional microdilution procedure, compare the growth in each MIC well with that of the growth control with the aid of a reading mirror.

When testing echinocandin antifungal agents, the evaluation of the MEC has been found to provide more consistent and reproducible susceptibility data than the conventional MIC reading.[7-9] The MEC is the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well (see Appendix A). For evaluating the

MEC, compare the growth in each well with that of the growth control (drug-free medium) with the aid of a reading mirror.

Reference strains of defined susceptibility can be used in the training of new personnel.

6.6.2 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 (100% inhibition). Trailing end points with amphotericin B are usually not encountered. Such a pattern may reflect clinically relevant drug resistance.

6.6.3 Fluconazole, Flucytosine, and Ketoconazole

For fluconazole, flucytosine, and ketoconazole, end points are typically less well defined than for amphotericin B, a problem which may be a significant source of variability. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 50% or more (nondermatophyte isolates) to 80% or more (dermatophyte isolates) reduction in growth compared to the growth in the control well (drug-free medium). When this turbidity persists, it is often identical for all drug concentrations above the MIC.

6.6.4 Itraconazole, Posaconazole, Ravuconazole, and Voriconazole

For these azoles, end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth (100% inhibition). Trailing end points with these agents against *Aspergillus* spp. and most other opportunistic pathogenic moulds are not usually encountered. It is possible that such a pattern could reflect clinically relevant drug resistance as it has been demonstrated for *Aspergillus fumigatus* strains that have been clinically resistant to itraconazole.[5,12]

However, when testing dermatophyte isolates against voriconazole, posaconazole, and itraconazole, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).

6.6.5 Echinocandins (anidulafungin, caspofungin, micafungin)

For echinocandins, end points are also typically less well defined than that described for amphotericin B, and application of the MEC end point has improved reproducibility.[7-9] The MEC is read as the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well (see Appendix A).

6.6.6 Ciclopirox

For ciclopirox, end points are typically less well defined than that described for amphotericin B. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).

6.6.7 Griseofulvin

For griseofulvin, end points are typically less well defined than that described for amphotericin B. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).

6.6.8 Terbinafine

For terbinafine, end points are typically less well defined than that described for amphotericin B. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).

6.7 Interpretation of Results

Breakpoints have not been established for mould testing. However, working breakpoints were assigned for analytical purposes by the error bounding method during a collaborative study to evaluate the performance of a new agar disk diffusion method in identifying resistant mould isolates to itraconazole, posaconazole, voriconazole, amphotericin B, and caspofungin.[19] As MICs below 1 µg/mL are usually reported for most *Aspergillus* spp. with the five agents; for *S. apiospermum* and *P. lilacinus* with posaconazole and voriconazole; for *Alternaria* spp. and *Bipolaris spicifera* with the three triazoles; and for some Zygomycete isolates with posaconazole and amphotericin B; isolates were grouped as susceptible (MIC or MEC ≤1 µg/mL), intermediate (MIC or MEC 2 µg/mL), and resistant (MIC or MEC ≥ 4 µg/mL) for all five drugs. This grouping was based on reported *in vitro* data obtained with a large number of isolates. It must be emphasized that these were working breakpoints for analytical purposes only. **The clinical relevance of testing this group of fungal pathogens remains uncertain, and breakpoints with proven relevance have yet to be identified or approved by CLSI or any regulatory agency.**

6.7.1 Amphotericin B

Experience to date using the procedures described in this standard indicates that amphotericin B MICs for most nondermatophyte opportunistic filamentous fungi isolates are clustered between 0.5 and 2.0 µg/mL. However, amphotericin B MICs for some species (*Aspergillus terreus*, *Acremonium strictum*, *P. lilacinus*, *S. apiospermum,* and *Scedosporium prolificans*) can be above 2 µg/mL (MIC ranges of 2 to 16 µg/mL).[12,18] Although very little data are available regarding correlation between MIC and outcome of treatment with amphotericin B for the filamentous fungi, MICs above 2 µg/mL have been associated with treatment failures and MICs below 2 µg/mL with clinical cure among 29 patients treated with amphotericin B for invasive aspergillosis caused by *A. fumigatus* (eight cases), *Aspergillus flavus* (12 cases), and *A. terreus* (nine cases).[21]

6.7.2 Flucytosine

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

6.7.3 Fluconazole

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

6.7.4 Ketoconazole

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

6.7.5 Itraconazole, Posaconazole, Ravuconazole, and Voriconazole

The importance of proper preparation of drug dilutions for water-insoluble compounds such as these cannot be over-emphasized.[4] (See CLSI document M27.)[1] Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 2 can lead to substantial errors due to dilution artifacts. As for ketoconazole (see previous paragraph), experience to date using the procedures described in this standard indicates that MICs for nondermatophyte moulds vary between 0.0313 and 16 µg/mL. However, preliminary data indicate that high itraconazole MICs (>8 µg/mL) are associated with clinical resistance to this agent[22,23] when MICs are determined by the M38-A2 microdilution method after 48 hours of incubation.[5] Data are not yet available to indicate a correlation between MIC and outcome of treatment with the new triazoles vs nondermatophyte moulds.

MICs of the azoles, including fluconazole, itraconazole, posaconazole, ravuconazole, and voriconazole are usually low against dermatophytes, but high fluconazole MICs (≥16 µg/mL) have been reported.[24] Correlation between *in vitro* triazole MICs for dermatophyte isolates with clinical outcome remains to be determined.

6.7.6 Echinocandins (anidulafungin, caspofungin, micafungin)

Work to date has focused principally on testing of *Aspergillus* isolates and little information exists to guide work with other moulds. MECs for *Aspergillus* isolates are usually ≤1 µg/mL.[12] Correlation of the MEC with clinical outcome remains to be determined.

6.7.7 Ciclopirox

Most ciclopirox MICs are ≤1 µg/mL for the dermatophytes. Correlation of MIC with clinical outcome has yet to be determined.[10]

6.7.8 Griseofulvin

Most griseofulvin MICs are ≤1 µg/mL for the dermatophytes. Correlation of MIC with clinical outcome has yet to be determined.[10]

6.7.9 Terbinafine

Most terbinafine MICs are ≤0.25 µg/mL for the dermatophytes, but MICs ≥0.5 µg/mL have been reported for *T. rubrum*.[25] Correlation of MIC with clinical outcome has yet to be determined.

6.8 Broth Macrodilution Modifications

Published data document good concordance between results obtained by the broth microdilution methodology described above and a broth macrodilution adaptation.[2,3] Some clinical laboratories may choose to implement broth macrodilution rather than the broth microdilution method, primarily because of safety issues. The steps and testing conditions relevant to the broth macrodilution test are discussed in detail; this method has not been evaluated with the echinocandins or for the dermatophytes.

The 100 times final strength drug dilutions described for the broth microdilution procedure should be diluted 1:10 with RPMI-1640 to achieve the 10x (10-fold) strength needed for the broth macrodilution test. Prepare and adjust the stock inoculum suspensions, as described under the broth microdilution test. Mix the stock conidia or sporangiospore suspension for 15 seconds with a vortex, and dilute 1:100 with medium to obtain the test inoculum (0.4×10^4 to 5×10^4 CFU/mL).

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

Incubate tubes at 35 °C (without agitation) and observe for the presence or absence of visible growth. Score the tubes and determine MICs as described for the broth microdilution procedure.

6.9 Other Modifications

Preliminary data have demonstrated that determination of MICs using a colorimetric end point enhances the interlaboratory agreement of itraconazole MICs.[2,18] This procedure can be performed by adding 2x colorimetric indicator (modified resazurin) to a 2x concentration of the standard RPMI-1640 medium and following the steps described above for either the microdilution test or its modification.

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

7 QC

7.1 Purpose

The goals of a QC program are to assist in monitoring the following:

- the precision (repeatability) and accuracy of the susceptibility test procedures;
- the performance of reagents, testing conditions, and instructions used in the test; and
- the performance of persons who conduct the tests and read the results.

The goals are best realized by, but not limited to, the use of QC and reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.[6,9,11,26-29]

7.2 QC Responsibilities

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

Manufacturers are responsible for the following:

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

7.2.2 Laboratory (User)

The laboratorian is responsible for the following:

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

7.2.3 Mutual Responsibility

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

7.3 Selecting Reference Strains

Ideal reference strains for QC of dilution methods have MICs that fall near the mid-range of the concentration for all antifungal agents tested. An ideal control strain is inhibited at the fifth dilution of a nine-dilution-$\log_2$ series, but strains with MICs between the third and seventh dilution are acceptable. Before a strain is accepted as a reference, test it for as long as necessary to demonstrate that its antifungal susceptibility pattern is genetically stable. CLSI/NCCLS document M23[30] provides guidelines for the selection of appropriate QC strains and the determination of acceptable MIC or MEC ranges. The QC strains listed in Table 4 were selected in accordance with the criteria in CLSI/NCCLS document M23[30] and can be used as controls for the antifungal susceptibility testing of moulds until mould isolates are selected. In addition, the reference mould isolates listed in Table 4 also can be used.

7.4 Storing Reference Strains

7.4.1 Methods for Prolonged and Short-term Storage

Store reference strains in a way that minimizes the possibility of mutation in the organisms.

- There are three preferred methods for prolonged storage of reference strains. Fungal isolates may be grown on potato dextrose agar and then frozen at −70 °C.[31] Alternatively, reference strains can be preserved by suspending fungal cells in 10% glycerol solution or in the cryogenic solution of commercial vials containing porous beads that have been demonstrated by the manufacturer to preserve fungi. Vials can be stored at either −70 °C, or in liquid nitrogen, or in the vapor of liquid nitrogen.[32,33]

- For short-term storage, working stock cultures can be grown on Sabouraud dextrose agar until sufficient growth is observed and stored at 2 °C to 8 °C. Prepare fresh slants 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.

7.4.2 Sources for Reference Strains

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

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

7.4.3 Preparing Strains for Storage

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

(1) Grow moulds for seven days on potato dextrose agar or dermatophytes on Sabouraud glucose agar or oatmeal agar (*T. rubrum* isolates) for seven days at 28 °C.

(2) Select growth from several colonies and perform the appropriate susceptibility tests to demonstrate that they give the expected MIC or MEC results (see Table 4 for expected MICs for QC and/or reference strains).

(3) Subculture strains yielding expected results onto the same medium that was used for the primary culture and incubate long enough for sufficient growth to occur (usually from one to seven 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 (see Section 7.4.1) to make a heavy suspension (or if lyophilizing, suspend the growth in the appropriate medium).

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

(7) Place these containers in a freezer maintained as in Section 7.4.1 or in liquid nitrogen. Stocks prepared using the procedure just outlined can remain indefinitely without significant risk of alteration in antifungal susceptibility patterns. When the supply of containers is nearly exhausted, repeat this process to make a new supply.

7.5 Routine Use of Reference Strains

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

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

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

(3) For *Candida* spp., transfer a portion of the mixture onto Sabouraud dextrose agar and incubate at 35 °C for 24 hours. Subculture moulds on potato dextrose agar or oatmeal agar (*T. rubrum* isolates) and incubate four or five days or until good conidial growth is present (dermatophyte isolates) to seven days (nondermatophyte isolates).

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

(5) After incubating the strains, store them at 2 °C to 8 °C.

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

(7) Always perform susceptibility tests on colonies from overnight plates (*Candida* spp.), seven-day cultures (nondermatophyte isolates), or four- to five-day cultures (dermatophyte isolates).

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

7.6 Batch of Medium and Lot of Plasticware Control

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

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

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

(3) New lots of RPMI-1640 medium should be tested for acceptable performance before being used to test clinical isolates, because recent studies have demonstrated that some lots do not perform adequately. The pH should be 6.9 to 7.1 (see Section 6.1.2).

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

7.7 QC Frequency

7.7.1 MIC or MEC Ranges

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

NOTE: Do not confuse this procedure with the procedure for establishing satisfactory performance of MIC tests for the purpose of performing QC tests weekly instead of daily (see Section 7.7.2).

7.7.2 Frequency of Testing

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

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

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

NOTE: This procedure is only for establishing satisfactory performance of MIC or MEC tests for the purpose of performing QC tests weekly instead of daily. Do not confuse this procedure with the steps that must be taken for corrective action defined in Section 7.7.1.

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

(4) When these conditions are fulfilled, each reference strain must be tested at least once per week. Whenever an MIC or MEC 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 or MEC values (ie, MIC or MEC values obtained from one drug-microorganism combination for five consecutive test days) must be within the accuracy ranges stated in Table 4.

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

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

7.8 Other Control Procedures

7.8.1 Growth Control

Each broth microdilution or macrodilution series should include a growth control of RPMI-1640 medium without antifungal agent (water-soluble agents) or RPMI-1640 medium without antifungal agent plus 1% of the solvent used (nonwater-soluble agents) to assess viability of the test organisms. With the broth tests, the growth control also serves as a turbidity control for reading end points.

7.8.2 Purity Control

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

7.8.3 End-point Interpretation Control

Periodically monitor end-point interpretation to minimize variation in the interpretation of MIC or MEC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. Record the results and compare to the results obtained by an experienced reader. Specific reference strains with predetermined MICs are particularly helpful for this purpose, especially with itraconazole.

7.9 QC Strains (see also Section 7.3)

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

Table 4 lists expected ranges for strains found to be acceptable as QC. Also shown are additional strains that can be useful for conducting reference studies.[2,6,9,11,26-28]

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
Anidulafungin	DMSO	Medium
Caspofungin	Water	Medium
Ciclopirox	DMSO	Medium
Fluconazole	Water	Medium
Flucytosine	Water	Medium
Griseofulvin	DMSO	Medium
Itraconazole	DMSO	Medium
Ketoconazole	DMSO	Medium
Micafungin	Water	Medium
Posaconazole	DMSO	Medium
Ravuconazole	DMSO	Medium
Terbinafine	DMSO	Medium
Voriconazole	DMSO	Medium

DMSO = Dimethyl sulfoxide

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

Antimicrobial Solution							
Step	Concentration (μg/mL)	Source	Volume (mL)	+ Solvent (mL) (eg, DMSO)*	= Intermediate Concentration (μg/mL)	= Final Concentration at 1:50(μg/mL)†	
1	1600	Stock			1600 μg/mL	32	
2	1600	Stock	0.5	0.5	800	16	
3	1600	Stock	0.5	1.5	400	8.0	
4	1600	Stock	0.5	3.5	200	4.0	
5	200	Step 4	0.5	0.5	100	2.0	
6	200	Step 4	0.5	1.5	50	1.0	
7	200	Step 4	0.5	3.5	25	0.5	
8	25	Step 7	0.5	0.5	12.5	0.25	
9	25	Step 7	0.5	1.5	6.25	0.125	
10	25	Step 7	0.5	3.5	3.13	0.0625	

* Dimethyl sulfoxide
† 2x (twofold) concentrations

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

Antimicrobial Solution							
Step	Concentration (μg/mL)	Source	Volume (mL)	+ Solvent (mL) (eg, DMSO)*	= Intermediate Concentration (μg/mL)	=	Final Concentration at 1:50(μg/mL)†
1	6400	Stock			6400		128
2	6400	Stock	0.5	0.5	3200		64
3	6400	Stock	0.5	1.5	1600		32
4	6400	Stock	0.5	3.5	800		16
5	800	Step 4	0.5	0.5	400		8
6	800	Step 4	0.5	1.5	200		4
7	800	Step 4	0.5	3.5	100		2
8	100	Step 7	0.5	0.5	50		1
9	100	Step 7	0.5	1.5	25		0.5
10	100	Step 7	0.5	3.5	12.5		0.25
11	12.5	Step 10	0.5	0.5	6.25		0.125
12	12.5	Step 10	0.5	1.5	3.125		0.0625
13	12.5	Step 10	0.5	3.5	1.56		0.0313
14	1.56	Step 13	0.5	0.5	0.78		0.0156
15	1.56	Step 13	0.5	1.5	0.39		0.0078
16	1.56	Step 13	0.5	3.5	0.195		0.0039
17	0.195	Step 16	0.5	0.5	0.0975		0.0019

* **Dimethyl sulfoxide**
† **2X (twofold) concentrations**

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

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

*2X (twofold) concentrations

Table 4. Recommended MIC or MEC Limits for QC and Reference Strains for Broth Dilution Procedures. (Reprinted with permission from the authors and the American Society for Microbiology.)

Organism	Purpose	Antifungal Agent	MIC Range (µg/mL)	Mode	% of MICs Within Range	Incubation Times
Paecilomyces variotii **ATCC® MYA-3630** [6,9] **(see note 4)**	**QC**	**Amphotericin B**	**1-4**	**2.0**	**100.0**	**48 hours**
		Itraconazole	**0.06-0.5**	**0.12**	**100.0**	**48 hours**
		Voriconazole	**0.015-0.12**	**0.06**	**100.0**	**48 hours**
		Posaconazole	**0.03-0.25**	**0.06**	**99.5**	**48 hours**
	Reference (MEC)	**Anidulafungin**	**≤0.015**	**N/A**	**100.0**	**24 hours**
Candida parapsilosis ATCC® 22019 [27,29]	QC	Amphotericin B	0.5-4.0	2.0	91.7	48 hours
		5FC	0.12-0.5	0.25	97.9	48 hours
		Fluconazole	1.0-4.0	2.0	98.1	48 hours
		Itraconazole	0.12-0.5	0.25	97.5	48 hours
		Ketoconazole	0.06-0.5	0.12	98.3	48 hours
		Voriconazole	0.03-0.25	0.06	100.0	48 hours
		Ravuconazole	0.03-0.25	0.06	98.3	48 hours
		Posaconazole	0.06-0.25	0.12	98.8	48 hours
		Anidulafungin	0.5-2.0	1.0	95.0	48 hours
		Caspofungin	0.5-4.0	1.0	92.9	48 hours
		Micafungin	**0.5-4.0**	**1.0**	**100.0**	**48 hours**
Candida krusei ATCC® 6258 [27,29]	QC	Amphotericin B	1.0-4.0	2.0	100.0	48 hours
		5FC	8.0-32	16	99.6	48 hours
		Fluconazole	16-128	32	100.0	48 hours
		Itraconazole	0.25-1.0	0.5	100.0	48 hours
		Ketoconazole	0.25-1.0	0.5	99.6	48 hours
		Voriconazole	0.12-1.0	0.5	100.0	48 hours
		Ravuconazole	0.25-1.0	0.5	100.0	48 hours
		Posaconazole	0.12-1.0	0.5	99.6	48 hours
		Anidulafungin	0.03-0.12	0.06	97.5	48 hours
		Caspofungin	0.25-1.0	0.5	97.5	48 hours
		Micafungin	**0.12-0.5**	**0.25**	**99.0**	**48 hours**
Aspergillus flavus ATCC® 204304 [2,4] **(see note 3)**	Reference	**Amphotericin B**	**0.5-4**	**ND**	**100.0**	**48 hours**
		Itraconazole	**0.25-0.5**	**ND**	**100.0**	**48 hours**
		Voriconazole	**0.5-4**	**ND**	**100.0**	**48 hours**
		Ravuconazole	**0.5-4**	**ND**	**100.0**	**48 hours**
		Posaconazole	**0.06-0.5**	**ND**	**100.0**	**48 hours**
Aspergillus fumigatus ATCC® MYA-3626[6,9] **(see note 4)**	Reference	**Amphotericin B**	**0.5-4.0**	**2.0**	**98.7**	**48 hours**
		Itraconazole	**0.25-2.0**	**1.0**	**95.7**	**48 hours**
		Voriconazole	**0.25-1.0**	**0.5**	**100.0**	**48 hours**
	Reference (MEC)	**Anidulafungin**	**≤0.015**	**N/A**	**100.0**	**24 hours**
Aspergillus fumigatus ATCC® MYA-3627[6]	Reference	**Amphotericin B**	**0.5-4.0**	**2.0**	**99.2**	**48 hours**
		Itraconazole	**≥ 16**	**>16**	**95.0**	**48 hours**
		Voriconazole	**0.25-1.0**	**0.5**	**99.2**	**48 hours**
Aspergillus flavus ATCC® MYA-3631[6]	Reference	**Amphotericin B**	**1.0-8.0**	**2.0**	**98.8**	**48 hours**
		Voriconazole	**0.5-2.0**	**1.0**	**98.3**	**48 hours**
		Posaconazole	**0.12- 1.0**	**0.5**	**97.1**	**48 hours**

Table 4. (Continued)

Organism	Purpose	Antifungal Agent	MIC Range (μg/mL)	Mode	% of MICs Within Range	Incubation Times
Aspergillus terreus ATCC® MYA-3633[6,9]	Reference	**Amphotericin B**	**2.0-8.0**	**4.0**	**98.3**	**48 hours**
		Voriconazole	**0.25-1.0**	**0.5**	**99.2**	**48 hours**
(see note 4)	**Reference (MEC)**	**Anidulafungin**	**≤0.015**	**N/A**	**99.6**	**24 hours**
Fusarium moniliforme ATCC® MYA-3629[6,9]	Reference	**Amphotericin B**	**2.0-8.0**	**4.0**	**99.6**	**48 hours**
		Itraconazole	**>16**	**>16**	**97.9**	**48 hours**
		Voriconazole	**1.0-4.0**	**2.0**	**100.0**	**48 hours**
		Posaconazole	**0.5-2.0**	**1.0**	**98.1**	**48 hours**
(see note 4)	**Reference (MIC)**	**Anidulafungin**	**>8**	**N/A**	**97.5**	**48 hours**
***F. solani*[9]** ATCC® 3636 **(see note 4)**	**Reference (MIC)**	**Anidulafungin (MIC)**	**>8**	**N/A**	**96.7**	**48 hours**
Scedosporium apiospermum ATCC® MYA-3635[6]	**Reference**	**Amphotericin B**	**4.0-16**	**8.0**	**98.8**	**72 hours**
		Voriconazole	**0.5-2.0**	**1.0**	**100.0**	**72 hours**
		Posaconazole	**1.0-4.0**	**2.0**	**98.3**	**72 hours**
***Scedosporium apiospermum* ATCC® MYA-3634[9] (see note 4)**	**Reference (MEC)**	**Anidulafungin**	**1-4**	**2**	**96.7**	**48-72 hours**
Trichophyton mentagrophytes MRL 1957 ATCC® MYA-4439[10] **(see note 6)**	**Reference**	**Ciclopirox**	**0.5-2**	**1.0**	**97.5**	**4 days**
		Griseofulvin	**0.12-0.5**	**0.25**	**96.3**	**4 days**
		Itraconazole	**0.03-0.25**	**0.06**	**96.2**	**4 days**
		Posaconazole	**0.03-0.25**	**0.06**	**95.2**	**4 days**
		Terbinafine	**0.002-0.008**	**0.004**	**97.9**	**4 days**
		Voriconazole	**0.03-0.25**	**0.06**	**95.2**	**4 days**
T. rubrum MRL 666 ATCC® MYA-4438[10] **(see note 6)**	**Reference**	**Ciclopirox**	**0.5-2**	**1.0**	**97.5**	**4 days**
		Fluconazole	**0.5-4**	**1.0**	**95.2**	**4 days**
		Voriconazole	**0.008-0.06**	**0.015**	**96.1**	**4 days**

ND = not determined; N/A = not applicable

NOTE 1: Information in boldface type is considered tentative for one year.

NOTE 2: MIC ranges correspond only to the indicated time of incubation. In some cases, MIC ranges also are available by the macrodilution method (48 hours only) and after 24 hours by the microdilution method (see references 23, 24, and 25). One of the QC isolates should be used per standard QC testing procedures (see Section 7.7).

Table 4 (Continued)

NOTE 3: The MIC ranges for *A. flavus* ATCC® 204304 are based on data from a collaborative study that were not obtained according to the CLSI/NCCLS document M23[30] process. However, this is the only mould for which reproducible reference limits were established for ravuconazole and it is included in the table for this reason.

NOTE 4: Although some of the anidulafungin MEC (various moulds) and ≥ 50% inhibition MIC (*Fusarium* isolates only) ranges are off-scale, these isolates could aid in the identification of potential resistance or the determination of the novel MEC end point (see Appendix A). The anidulafungin concentration range in the study was 0.015 µg/mL to 32 µg/mL, but off-scale MICs of >32 from that study are reported in Table 4 as >8 to be consistent with the recommended routine testing range for this compound.[8]

NOTE 5: As *Issatchenkia orientalis* is now known to be the sexual form (the teleomorph) of *C. krusei*, it would be technically correct to use *I. orientalis* as the name for this fungus. However, this change would confuse most users and the far more widely used name *Candida krusei* is retained.

NOTE 6: Four days or until good growth (confluent hyphal growth covering the bottom of the well) is obtained in the growth control well.[11]

Table 5. Composition of RPMI-1640 Medium

Constituent	g/L Water	Constituent	g/L Water
L-arginine (free base)	0.200	Biotin	0.0002
L-aspargine (anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine • 2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myo-inositol	0.035
L-glutamine	0.300	Niacinamide	0.001
Glycine	0.010	PABA	0.001
L-histidine (free base)	0.015	Pyridoxine HCl	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002 0.000005
L-isoleucine	0.050	Thiamine HCl	0.001
L-leucine	0.050	Vitamin B_{12}	0.000005
L-lysine • HCl	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		

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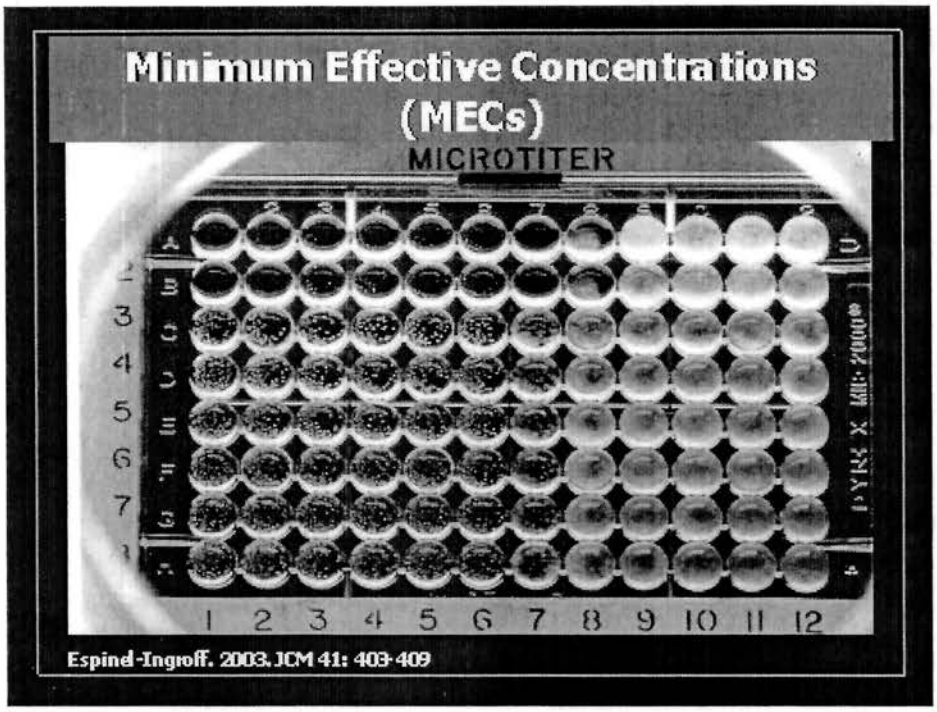
Appendix A. MECs of Caspofungin and Anidulafungin

A1. Minimal Effective Concentrations (MECs) of Caspofungin (Figure below reprinted with permission from the American Society for Microbiology and the author.)



Shown are dilution series of caspofungin (column 12 is the drug-free growth control, and columns 11 to 1 contain drug concentrations that ascend in two-fold steps from 0.007 in column 11 to 8 µg/mL in column 1) vs eight *Aspergillus* isolates after 24 hours of incubation. The MECs are the lowest concentrations of caspofungin that led to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well (column 12).[1] MECs for rows A and B (*Aspergillus niger*) are the wells of column 7 (0.12 µg/mL), and for rows C to H (*A. flavus*, *A. terreus* and *A. fumigatus*) are the wells of column 6 (0.25 µg/mL).

A2. MECs of Anidulafungin

Isolate	MEC	0.015	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	Growth control
S. apiospermum ATCC® MYA-3634	4													
F. solani ATCC® MYA-3636	≥32													
A. fumigatus ATCC® MYA-3727	≤0.015													
A. flavus ATCC® MYA-3626	≤0.015													

Appendix A. (Continued)

Shown are dilution series of anidulafungin vs different mould isolates after 24 hours (*Aspergillus* isolates), 48 hours (*Fusarium solani* isolate), and 72 hours (*S. apiospermum*) of incubation. The MECs are the lowest concentrations of anidulafungin that led to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control wells.[2] One might wish to read the *Scedosporium* isolate's MEC as 2 µg/mL, but at 4 µg/mL, the change in morphology is more defined and all wells have the same trailing.

References for Appendix A

[1] Espinel-Ingroff A. Evaluation of broth microdilution testing parameters and agar diffusion Etest procedure for testing susceptibilities of *Aspergillus* spp. to caspofungin acetate (MK-0991). *J Clin Microbiol.* 2003;41:403-409.

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Appendix B. RPMI-1640 Medium

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

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

Dissolve powdered medium in 900 mL distilled H_2O. Add MOPS (final concentration of 0.165 mol/L) and stir until dissolved. While stirring, adjust the pH to 7.0 at 25 °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 C. McFarland 0.5 Barium Sulfate Turbidity Standard

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

The procedure consists of the following steps:

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

(2) Verify the correct density of the turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.

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

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

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

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

Appendix D. Oatmeal Agar

To 1 L of distilled water, add:

100 g baby oatmeal cereal
15 g granulated agar
0.03 g gentamicin

Mix well. Dispense 500 mL into liter autoclavable beakers (tends to boil over). Autoclave at 121 °C for 20 minutes. Immediately pour into petri plates and allow to cool. Store at 4 °C to 6 °C.

QC:

Positive – *Trichophyton rubrum* – conidia formation
Negative – none
Sterility – No growth

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

Summary of Delegate Comments and Subcommittee Responses

M38-A2: *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition*

General

1. My only problem is there are no interpretations for many of the drugs that are listed in the document, so providing a method to test the MIC of these different drugs for various moulds in the clinical laboratory is basically implying that the test is valid and the MIC is useful (when it may not be at all). In other words, the document should say that only certain, clinically validated drug/bug MIC combinations should be reported. For all others, no MIC should be reported, as there are no clinical correlation data. Adding a statement that there are no interpretations for the MIC doesn't help, as medical doctors will essentially ignore that.

- **The current document has focused on providing a consistent tool for determining MICs without which MIC-outcome correlations cannot be sought. The absence of a correlation is expected for this stage of evolution of the work of the committee in this area. Therefore, no changes have been made to the document.**

2. Global context: The text states that reference standards can be obtained from different sources, and then specifically mentions the USP. I suggest revising to include other sources in other countries comparable to USP, or delete USP as a specifically named source.

- **USP as a specific named source has been deleted.**

3. The document M38-A2 developed by CLSI's Subcommittee on Antifungal Susceptibility Testing clearly communicates materials and procedures essential for performance of the "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi" for not only etiologic agents of invasive, opportunistic mycoses (eg, *Aspergillus* spp., *Fusarium* spp., and *Rhizopus oryzae*), but also etiologic agents of cutaneous mycoses (eg, *Trichophyton, Microsporum,* and *Epidermophyton* spp.). Furthermore, the document conveys quality control (QC) data for filamentous fungal isolates and criteria for echinocandin testing. Thus, M38-A2 advances CLSI's previous document M38-A (2002). I submit the following suggestions for consideration by CLSI's Subcommittee on Antifungal Susceptibility Testing:

   Abstract: First paragraph, line five: ~~superficial~~ cutaneous (dermatophyte, *Trichophyton, Microsporum, Epidermophyton* spp.) fungal infections…

- **The sentence in the abstract has been revised as suggested.**

Section 2, Introduction

4. First paragraph, second sentence: ~~S.~~ *Scedosporium prolificans.*

- **For consistency, only the species name is spelled out within this sentence.**

Section 3, Standard Precautions

5. Reference at the end of the paragraph: CLSI document M29-A... please check the CLSI document in the reference that is referred to in the superscript.

- **The Standard Precautions section is a standard boiler plate for all CLSI documents. No change has been made to the document.**

Section 5.2, Weighing Antifungal Powders

6. Global context: Remove NIST and state to use reference weights from a national metrology organization. It may be useful to include a few national metrology organizations in different countries, but only NIST is not appropriate.

- **NIST as an example of an approved reference weight has been deleted.**

Section 6.3, Inoculum Preparation

7. Fourth sentence, second paragraph, line 10: Addition of one drop (approximately 0.01 mL) of Tween 20 will facilitate the preparation of *Aspergillus* spp. inocula.

- **The sentence has been revised as suggested.**

Section 6.7.5, Itraconazole, Posaconazole, Ravuconazole, and Voriconazole

8. First paragraph, second sentence: (See CLSI document M27-A2.)

- **The version of each CLSI document is not mentioned to encourage the readers to use the most recently published version.**

Section 7.9, QC Strains (see also Section 7.3)

9. Table 4 (Continued), NOTE 3, first sentence: CLSI document M23-A3... please check the CLSI document in reference 30 that is referred to in the superscript.

- **The version of each CLSI document is not mentioned to encourage the readers to use the most recently published version.**

NOTES

The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.* The quality management system approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any health care service's path of workflow (ie, operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The QSEs are:

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

M38-A2 addresses the QSEs indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessments—External & Internal	Process Improvement	Customer Service	Facilities & Safety
M7					X M2 M7 M11 M23 M24 M27 M29						M29

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

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

M38-A2 addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Preexamination				**Examination**			**Postexamination**	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
			M24	X M24 M27	X M2 M7 M11 M24 M27	X M2 M7 M11 M24 M27	X M2 M7 M11 M24 M27	X M24 M27

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

Related CLSI Reference Materials*

M2-A9 **Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition (2006).** This document contains the current Clinical and Laboratory Standards Institute-recommended methods for disk susceptibility testing, criteria for quality control testing, and updated tables for interpretive zone diameters.

M7-A7 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition (2006).** This document addresses reference methods for the determination of minimal inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

M11-A7 **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Seventh Edition (2007).** This standard provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by agar dilution and broth microdilution.

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

M24-A **Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard (2003).** This standard provides protocols and related quality control parameters and interpretive criteria for the susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.

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

M29-A3 **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** 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.

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

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Pathology and Cytology Laboratories, Inc. (KY)
Pathology Associates Medical Laboratories (WA)
Penn State Hershey Medical Center (PA)
Pennsylvania Hospital (PA)
Penrose St. Francis Health Services (CO)
The Permanente Medical Group (CA)
Perry County Memorial Hospital (IN)
Peterborough Regional Health Centre (Canada)
Piedmont Hospital (GA)
Pitt County Memorial Hospital (NC)
Prairie Lakes Hospital (SD)
Presbyterian Hospital of Dallas (TX)
Presbyterian/St. Luke's Medical Center (CO)
Prince County Hospital
Princess Margaret Hospital (Hong Kong)
Providence Alaska Medical Center (AK)
Providence Health Care (Canada)
Providence Medford Medical Center (OR)
Provincial Health Services Authority (Vancouver, BC, Canada)
Provincial Laboratory for Public Health (Edmonton, AB, Canada)
Queen Elizabeth Hospital (Canada)
Queensland Health Pathology Services (Australia)
Quest Diagnostics, Inc
Quest Diagnostics, Inc (San Juan Capistrano, CA)
Quest Diagnostics JV (IN, OH, PA)
Quest Diagnostics Laboratories (WA)
Quincy Hospital (MA)
Rady Children's Hospital San Diego (CA)
Redington-Fairview General Hospital (ME)
Regional Health Authority Four (RHA4) (Canada)
Regions Hospital (MN)
Reid Hospital & Health Care Services (IN)
Renown Regional Medical Center (NV)
Research Medical Center (MO)
Riverside Regional Medical Center (VA)
Riyadh Armed Forces Hospital, Sulaymainia
Robert Wood Johnson University Hospital (NJ)
Roxborough Memorial Hospital (PA)
Royal Victoria Hospital (Canada)
Rush North Shore Medical Center (IL)
SAAD Specialist Hospital (Saudi Arabia)
Sacred Heart Hospital (FL)
Sacred Heart Hospital (WI)
Sahlgrenska Universitetssjukhuset (Sweden)
Saint Elizabeth Regional Medical Center (NE)
Saint Francis Hospital & Medical Center (CT)
Saint Mary's Regional Medical Center (NV)
Saints Memorial Medical Center (MA)
St. Agnes Healthcare (MD)
St. Anthony Hospital (OK)
St. Anthony Hospital Central Laboratory (CO)
St. Anthony's Hospital (FL)
St. Barnabas Medical Center (NJ)
St. Christopher's Hospital for Children (PA)
St. Elizabeth Community Hospital (CA)
St. Francis Hospital (SC)
St. Francis Medical Center (MN)
St. John Hospital and Medical Center (MI)
St. John's Hospital (IL)
St. John's Hospital & Health Ctr. (CA)
St. John's Mercy Medical Center (MO)
St. John's Regional Health Center (MO)
St. Joseph Medical Center (MD)
St. Joseph Mercy – Oakland (MI)
St. Joseph Mercy Hospital (MI)
St. Joseph's Hospital (FL)
St. Joseph's Hospital & Health Center (ND)
St. Joseph's Medical Center (CA)
St. Joseph's Regional Medical Center (NJ)
St. Jude Children's Research Hospital (TN)
St. Louis University Hospital (MO)
St. Luke's Hospital (FL)
St. Luke's Hospital (IA)
St. Luke's Hospital (PA)
St. Margaret Memorial Hospital (PA)
St. Martha's Regional Hospital (Canada)
St. Mary Medical Center (CA)
St. Mary's Health Center (MO)
St Mary's Healthcare (SD)
St. Mary's Medical Center (IN)
St. Michael's Hospital Diagnostic Laboratories & Pathology (Canada)
St. Tammany Parish Hospital (LA)
St. Thomas More Hospital (CO)
Sampson Regional Medical Center (NC)
Samsung Medical Center (Korea)
San Francisco General Hospital-University of California San Francisco (CA)
Sanford USP Medical Center (SD)
SARL Laboratoire Carron (France)
Saudi Aramco Medical (Saudi Arabia)
Scott Air Force Base (IL)
Scott & White Memorial Hospital (TX)
Seoul National University Hospital (Korea)
Seton Medical Center (CA)
Shamokin Area Community Hospital (PA)
Sheik Kalifa Medical City (UAE)
Shore Memorial Hospital (NJ)
Shriners Hospitals for Children (SC)
Singapore General Hospital (Singapore)
SJRMC Plymouth Laboratory (IN)
Sky Lakes Medical Center (OR)
South Bend Medical Foundation (IN)
South County Hospital (RI)
South Dakota State Health Laboratory (SD)
South Miami Hospital (FL)
Southern Health Care Network (Australia)
Southern Maine Medical Center (ME)
Southwest Nova District Health Authority (Canada)
Speare Memorial Hospital (NH)
Spectrum Health - Blodgett Campus (MI)
Stanford Hospital and Clinics (CA)
State of Connecticut Department of Public Health (CT)
State of Hawaii Department of Health (HI)
State of Washington-Public Health Labs (WA)
Steele Memorial Hospital (ID)
Stillwater Medical Center (OK)
Stony Brook University Hospital (NY)
Stormont-Vail Regional Medical Center (KS)
Sudbury Regional Hospital (Canada)
Suncoast Medical Clinic (FL)
Sunnybrook Health Science Center (ON, Canada)
Sunrise Hospital and Medical Center (NV)
Swedish Medical Center (CO)
Sydney South West Pathology Service (Australia)
T.J. Samson Community Hospital (KY)
Taipei Veterans General Hospital (Taiwan)
Taiwan Society of Laboratory Medicine
Tallaght Hospital
Tartu University Clinics (Tartu)
Temple Univ. Hospital - Parkinson Pav. (PA)
Texas Children's Hospital (TX)
Texas Department of State Health Services
Thomason Hospital (TX)
Timmins and District Hospital (Canada)
The Toledo Hospital (OH)
Touro Infirmary (LA)
Tri-Cities Laboratory (WA)
Trident Medical Center (SC)
Trinity Medical Center (AL)
Tripler Army Medical Center (HI)
Tufts New England Medical Center Hospital (MA)
Tulane Medical Center Hospital & Clinic (LA)
Turku University Central Hospital
UCI Medical Center (CA)
UCLA Medical Center Clinical Laboratories (CA)
UCSD Medical Center (CA)
UCSF Medical Center China Basin (CA)
UMass Memorial Medical Center (MA)
UMC of Southern Nevada (NV)
UNC Hospitals (NC)
Union Clinical Laboratory (Taiwan)
United Christian Hospital (Hong Kong)
United Clinical Laboratories (IA)
Unity HealthCare (IA)
Universita Campus Bio-Medico (Italy)
Universitair Ziekenhuis Antwerpen (Belgium)
University College Hospital (Ireland)
University Medical Center at Princeton (NJ)
University of Alabama-Birmingham Hospital (AL)
University of Arkansas for Medical Sci. (AR)
University of Chicago Hospitals (IL)
University of Colorado Health Sciences Center (CO)
University of Colorado Hospital
University of Iowa Hospitals and Clinics (IA)
University of Kentucky Med. Ctr. (KY)
University of Maryland Medical System (MD)
University of Medicine & Dentistry, NJ University Hosp. (NJ)
University of Miami (FL)
University of Missouri Hospital (MO)
University of MN Medical Center - Fairview
University of MS Medical Center (MS)
University of So. Alabama Children's and Women's Hospital (AL)
University of Texas Health Center (TX)
The University of Texas Medical Branch (TX)
University of the Ryukyus (Japan)
University of Virginia Medical Center
University of Washington
UPMC Bedford Memorial (PA)
U.S.A. Meddac (Pathology Division) (MO)
UW Hospital (WI)
UZ-KUL Medical Center (Belgium)
VA (Asheville) Medical Center (NC)
VA (Bay Pines) Medical Center (FL)
VA (Chillicothe) Medical Center (OH)
VA (Cincinnati) Medical Center (OH)
VA (Dallas) Medical Center (TX)
VA (Dayton) Medical Center (OH)
VA (Decatur) Medical Center (GA)
VA (Hines) Medical Center (IL)
VA (Indianapolis) Medical Center (IN)
VA (Iowa City) Medical Center (IA)
VA (Long Beach) Medical Center (CA)
VA (Miami) Medical Center (FL)
VA New Jersey Health Care System (NJ)
VA Outpatient Clinic (OH)
VA (Phoenix) Medical Center (AZ)
VA (San Diego) Medical Center (CA)
VA (Seattle) Medical Center (WA)
VA (Sheridan) Medical Center (WY)
VA (Tucson) Medical Center (AZ)
Valley Health (VA)
Vancouver Hospital and Health Sciences Center (BC, Canada)
Vancouver Island Health Authority (Canada)
Vanderbilt University Medical Center (TN)
Via Christi Regional Medical Center (KS)
Virga Jessezieukenhuis (Belgium)
ViroMed Laboratories (LabCorp) (MN)
Virtua - West Jersey Hospital (NJ)
WakeMed (NC)
Walter Reed Army Medical Center (DC)
Warren Hospital (NJ)
Washington Hospital Center (DC)
Waterbury Hospital (CT)
Waterford Regional Hospital (Ireland)
Wayne Memorial Hospital (NC)
Weirton Medical Center (WV)
Wellstar Douglas Hospital Laboratory (GA)
Wellstar Paulding Hospital (GA)
Wellstar Windy Hill Hospital Laboratory (GA)
West China Second University Hospital, Sichuan University (P.R. China)
West Valley Medical Center Laboratory (ID)
Westchester Medical Center (NY)
Western Baptist Hospital (KY)
Western Healthcare Corporation (Canada)
Wheaton Franciscan & Midwest Clinical Laboratories (WI)
Wheeling Hospital (WV)
Whitehorse General Hospital (Canada)
William Beaumont Army Medical Center (TX)
William Beaumont Hospital (MI)
William Osler Health Centre (Canada)
Winchester Hospital (MA)
Winn Army Community Hospital (GA)
Wisconsin State Laboratory of Hygiene (WI)
Wishard Health Sciences (IN)
Womack Army Medical Center (NC)
Woodlawn Hospital (IN)
York Hospital (PA)

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