M38

Reference Method for Broth Dilution Antifungal Susceptibility Testing for Filamentous Fungi

This standard includes 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.
Clinical and Laboratory Standards Institute

Setting the standard for quality in clinical laboratory testing around the world.

The Clinical and Laboratory Standards Institute (CLSI) is a not-for-profit membership organization that brings together the varied perspectives and expertise of the worldwide laboratory community for the advancement of a common cause: to foster excellence in laboratory medicine by developing and implementing clinical laboratory standards and guidelines that help laboratories fulfill their responsibilities with efficiency, effectiveness, and global applicability.

Consensus Process

Consensus—the substantial agreement by materially affected, competent, and interested parties—is core to the development of all CLSI documents. It does not always connote unanimous agreement, but does mean that the participants in the development of a consensus document have considered and resolved all relevant objections and accept the resulting agreement.

Commenting on Documents

CLSI documents undergo periodic evaluation and modification to keep pace with advancements in technologies, procedures, methods, and protocols affecting the laboratory or health care.

CLSI’s consensus process depends on experts who volunteer to serve as contributing authors and/or as participants in the reviewing and commenting process. At the end of each comment period, the committee that developed the document is obligated to review all comments, respond in writing to all substantive comments, and revise the draft document as appropriate.

Comments on published CLSI documents are equally essential, and may be submitted by anyone, at any time, on any document. All comments are addressed according to the consensus process by a committee of experts.

Appeals Process

If it is believed that an objection has not been adequately addressed, the process for appeals is documented in the CLSI Standards Development Policies and Process document.

All comments and responses submitted on draft and published documents are retained on file at CLSI and are available upon request.

Get Involved—Volunteer!

Do you use CLSI documents in your workplace? Do you see room for improvement? Would you like to get involved in the revision process? Or maybe you see a need to develop a new document for an emerging technology? CLSI wants to hear from you. We are always looking for volunteers. By donating your time and talents to improve the standards that affect your own work, you will play an active role in improving public health across the globe.

For further information on committee participation or to submit comments, contact CLSI.

Clinical and Laboratory Standards Institute
950 West Valley Road, Suite 2500
Wayne, PA 19087 USA
P: 610.688.0100
F: 610.688.0700
www.clsi.org
standard@clsi.org
Reference Method for Broth Dilution Antifungal Susceptibility Testing for Filamentous Fungi

Barbara D. Alexander, MD, MHS
Gary W. Procop, MD
Sharon K. Cullen, BS, RAC
Philippe Dufresne, PhD (RMCCM)
Jeff Fuller, PhD, FCCM, ABMM
Mahmoud A. Ghannoum, MSc, PhD
Kimberly E. Hanson, MD, MHS
Shawn R. Lockhart, PhD, D(ABMM)
Luis Ostrosky-Zeichner, MD, FACP
David S. Perlin, PhD
Dee Shortridge, PhD
Nathan P Wiederhold, PharmD
Nancy L. Wengenack, PhD, D(ABMM)

Abstract

Clinical and Laboratory Standards Institute document M38—Reference Method for Broth Dilution Antifungal Susceptibility Testing for Filamentous Fungi describes a method for testing the susceptibility to antifungal agents of filamentous fungi (moulds) and dermatophytes that cause invasive and cutaneous fungal infections. 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 manufacturer and the user responsibilities in quality control is also presented. In addition, a brief discussion regarding newly defined epidemiological cutoff values for certain Aspergillus spp and species complexes has been introduced.


The Clinical and Laboratory Standards Institute consensus process, which is the mechanism for moving a document through two or more levels of review by the health care community, is an ongoing process. Users should expect revised editions of any given document. Because rapid changes in technology may affect the procedures, methods, and protocols in a standard or guideline, users should replace outdated editions with the current editions of CLSI documents. Current editions are listed in the CLSI catalog and posted on our website at www.clsi.org. If you or your organization is not a member and would like to become one, and to request a copy of the catalog, contact us at: Telephone: 610.688.0100; Fax: 610.688.0700; E-Mail: customerservice@clsi.org; Website: www.clsi.org.
Committee Membership

Consensus Council

Subcommittee on Antifungal Susceptibility Tests

Barbara D. Alexander, MD, MHS
Chairholder
Duke University Medical Center
USA

Gary W. Procop, MD
Cleveland Clinic
USA

Sharon K. Cullen, BS, RAC
Siemens Healthcare Diagnostics Inc.
USA

Philippe Dufresne, PhD (RMCCM)
Institut national de santé, publique du Québec
Canada

Jeff Fuller, PhD, FCCM, ABMM
Alberta Health Services Capital Health
Canada

Mahmoud A. Ghannoum, MSc, PhD
Case Western Reserve University
USA

Kimberly E. Hanson, MD, MHS
University of Utah and ARUP Laboratories
USA

Shawn R. Lockhart, PhD, D(ABMM)
Centers for Disease Control and Prevention
USA

Luis Ostrosky-Zeichner, MD, FACP
University of Texas Medical School at Houston
USA

David S. Perlin, PhD
New Jersey Medical Center-UMDNJ
USA

Dee Shortridge, PhD
bioMérieux, Inc.
USA

Nancy L. Wengenack, PhD, D(ABMM)
Mayo Clinic
USA

Nathan P. Wiederhold, PharmD
University of Texas, Health Science Center
USA

Staff

Marcy Hackenbrack, MCM, M(ASCP)
Project Manager

Megan L. Tertel, MA
Editorial Manager

Joanne P. Christopher, MA
Editor

Alexander B. Phucas
Editor
Acknowledgment

CLSI, the Consensus Committee on Microbiology, and the Subcommittee on Antifungal Susceptibility Tests gratefully acknowledge the following volunteers for their important contributions to the development of this document:

Ana Espinel-Ingroff, MS, PhD
Virginia Commonwealth University
USA

Phillippe Dufresne, PhD (RMCCM)
Institut national de santé publique du Québec,
Laboratoire de santé publique du Québec
Canada
Appendix E. Preparing RPMI Dilution Susceptibility Tests
Abbreviation: ........................................................................................................ 34
Appendix F. Oatmeal Agar.................................................................................................. 35
Appendix G. Minimal Effective Concentrations of Caspofungin and Anidulafungin............. 36
Appendix G. McFarland 0.5 Barium Sulfate Turbidity Standard........................................ 38
The Quality Management System Approach........................................................................ 40
Related CLSI Reference Materials .................................................................................. 41
Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory methods guiding the selection of antifungal therapy have gained greater attention. The CLSI Consensus Committee on Microbiology formed the Subcommittee on Antifungal Susceptibility Tests and concluded that a reproducible reference procedure for the antifungal susceptibility testing of filamentous fungi (moulds) would be useful. Accordingly, several studies were conducted to refine the methodology for performing nondermatophyte mould susceptibility testing.\(^1\)\(^2\)\(^3\)\(^4\)\(^5\) The resulting consensus method was published in 2002 as CLSI document M38 and the revised version in 2008.\(^1\)\(^2\)

In CLSI document M38-A2, supplemental material (QC data for mould isolates as well as echinocandin testing guidelines) was incorporated and guidelines for testing dermatophyte moulds were provided.\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) Since then, and in the absence of breakpoints for mould testing, epidemiological cutoff values (ECVs) for distinguishing wild-type and non-wild-type (isolates with intrinsic or acquired known resistance mechanisms or gene mutations) have been defined for some species and species complexes of *Aspergillus* (see CLSI documents M57 and M57S).\(^11\)\(^12\)\(^13\)\(^14\)\(^15\) Although a discussion regarding breakpoints was introduced in the previous edition of M38, breakpoints have not been established by CLSI for mould testing, ECV data and recommendations for the development and their use are incorporated into the newly developed CLSI documents M57\(^16\) and M57S\(^17\). QC data for testing mould isolates, as well as other testing guidelines, have been omitted from this edition of M38 and incorporated into the newly created CLSI document M38/M51\(^18\) Informational Supplement which combines supplemental material for both CLSI documents M38 [M38] and M51[M51].\(^18\)

**NOTE:** Current fungal taxonomy is under revision. Many genera have both a teleomorph (sexual state) and an anamorph (asexual state) name. In this document, the traditional anamorph names are used to provide continuity to both past procedures and associated documents such as the current edition of M27-S1.

Overview of Changes

**General**

Document formatting and organization was revised to reflect the new CLSI quality system essential/path-of-workflow document template.

References to the information supplement were updated to reflect the new informational supplement for broth dilution and disk diffusion testing of moulds.

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

Fungal taxonomy has undergone major changes in recent years with the dual (asexual and sexual stages) nomenclature having been abolished and the constant reclassification/renameing of species that results from improved molecular characterization of fungi. Species names listed in M38 were revised to reflect the most recent taxonomic changes based on classification according to DNA barcoding and internal transcribed spacer ribosomal DNA sequencing. For more information regarding updated reclassification and fungal species refer to publically available information.

For clarity, a list which includes previous and current taxonomical names for species used in the different collaborative studies (including QC studies) where each non-dermatophyte species was evaluated for testing guidelines of M38 methodology is also provided in Appendix A.
Subchapter 1.4.2 (Definitions)

Revised the interpretive breakpoint definitions and criteria for consistency with other CLSI antimicrobial susceptibility testing documents.

Added definitions for:
- Non-wild-type
- Wild-type

Chapter 2 (Indication for Performing Susceptibility Testing)

New indications added for testing of filamentous fungi with a discussion of environmental resistance of \textit{A. fumigatus}.

Chapter 3 (Testing Procedure)

An antifungal testing process flow-chart was added.

List of relevant drug concentrations to be tested for echinocandins expanded.

Procedural text converted to step/action tables.

Established guide for reading and interpretation of results for filamentous fungi including dermatophytes.

Modified text on reading results in Subchapter 3.4 to include new information on echinocandins and isavuconazole antifungal agents and minimal inhibitory concentration and minimal effective concentration comparison.

Appendixes (Original Tables)

The table providing the list of solvents has been updated, deleted from M38, and moved to the newly combined supplement, M38/M51S.

The table providing the recommended minimal inhibitory concentration or minimal effective concentration limits for QC and reference strains for broth dilution procedures have been deleted from M38 and moved to the combined supplement, M38/M51S.

A correction was made to Appendix E (Composition of RPMI-1640 Medium), to provide a single riboflavin concentration (0.0002 g /L) as found in CLSI document M27.

Dilution schemes for dermatophytes and non-dermatophytes isolates were harmonized with those of CLSI document M27 and revised to encompass the full dilution ranges recommended.

A table which lists previous and current taxonomical names of species found in the different collaborative studies is included in Appendix E.

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

Key Words

Antifungal agent, broth microdilution, dermatophytes, epidemiologic cutoff value, filamentous fungi, moulds, non-wild type, susceptibility testing, wild type
Reference Method for Broth Dilution Antifungal Susceptibility Testing for Filamentous Fungi

Chapter 1: Introduction

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

1.1 Scope

This document describes the standard broth microdilution testing method for antifungal susceptibility testing of filamentous fungi (moulds) that cause invasive and/or cutaneous fungal infections. This document also covers testing conditions including inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria end-point determination. QC reference ranges and specific epidemiologic cut off values (ECVs) are summarized in the current editions of CLSI documents M38/M51 and M57.

The intended audience includes diagnostic laboratory personnel, clinicians, and microbiologists who routinely perform antifungal susceptibility testing, use interpretive criteria to select suitable antifungal therapy, and those involved emerging resistance surveillance. The reference method is also useful for establishing ECVs and developing and validating alternate commercial methods for determining antifungal susceptibility of filamentous fungi. Therefore, the document is also of interest for both diagnostic and pharmaceutical companies and their regulatory counterparts.

This method has not been evaluated in studies of the yeast or mould forms of dimorphic fungi, such as Blastomyces dermatitidis, Coccidioides immitis/posadasii, Histoplasma capsulatum, or Talaromyces marneffei (Penicillium marneffei), and only has been evaluated for the mycelial form of Sporothrix schenckii spp. complex. This method also has not been used in studies of dermatophytes with the echinocandins nor nondermatophyte moulds with ciclopirox, griseofulvin, or terbinafine.

1.2 Background

The method described in this document is intended for testing common filamentous fungi including the dermatophytes, Trichophyton spp., Microsporum spp. and Epidermophyton spp.; more prevalent species of Aspergillus spp. and species complex, Fusarium spp. and species complex, Rhizopus spp., and other Mucorales (Zygomycetes); Scedosporium spp. (Pseudallescheria/Lomentospora), and the mycelial form of species included in the S. schenckii spp. complex, and dematiaceous (phacoid/black) moulds. Caution

Commented [MH7]: Sheryl, add reference to M575

Commented [MH8]: Sheryl, this should be a xref to reference 17

Commented [MH9]: Moulds don’t have interpretive criteria (only ECVs) so should this still be here?


should be used when interpreting the minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) results for any mould/drug combination.

This document discusses a “reference” method developed through a consensus process to facilitate agreement among laboratories in measuring mould susceptibility to antifungal agents. **NOTE:** The relationship between *in vitro* vs *in vivo* data has only been evaluated in animal models. An important use of a reference method is to provide a standard basis from which other methods can be developed and which will result in interlaboratory agreement within specified ranges. Such methods might have particular advantages, such as ease of performance, economy, or production of 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.

### 1.3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of all known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of bloodborne pathogens. The Centers for Disease Control and Prevention addresses this topic in published guidelines that address the daily operations of diagnostic medicine in humans and animals while encouraging a culture of safety in the laboratory. For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.

### 1.4 Terminology

#### 1.4.1 A Note on Terminology

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

**NOTE:** Although the breakpoints described below are not yet available for any antifungal agent/mould species at time of this document's publication, ECVs based solely on *in vitro* data have been established (see CLSI documents M57S and M38/51S). Currently, breakpoints only apply to some *Candida* spp. and antifungal agent combinations. Definitions for interpretive categories are provided below in the event that breakpoints are published for filamentous fungi in the future.

#### 1.4.2 Definitions

**Antimicrobial susceptibility test interpretive category** – a classification based on an *in vitro* response of an organism to an antimicrobial agent (including antifungal agents) at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent.
1) **susceptible (S)** – a category that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

2) **intermediate (I)** – a category that includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates; **NOTE:** The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher than normal dosage of a drug can be used. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

3) **resistant (R)** – a category that implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs that fall in the range in which specific microbial resistance mechanisms (eg, β-lactamas) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

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

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

For example, *Candida albicans* with an MIC of 0.5 µg/mL for fluconazole with the interpretive criteria of:

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>≤ 4</td>
</tr>
<tr>
<td>Intermediate</td>
<td>8–16</td>
</tr>
<tr>
<td>Resistant</td>
<td>≥ 32</td>
</tr>
</tbody>
</table>

The isolate would be categorized as susceptible (treatable):

“Susceptible breakpoint” is 24 µg/mL.

“Resistant breakpoint” is 8 µg/mL.

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

**epidemiological cut-off value (ECV) categorization criteria** – minimal inhibitory concentration (MIC)/minimal effective concentration values to indicate wild-type or non-wild-type.

For example, *A. fumigatus* with a MIC of 0.5 (µg/mL) for itraconazole with the ECV criteria of:

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>≤ 1</td>
</tr>
<tr>
<td>Non-wild-type</td>
<td>&gt; 1</td>
</tr>
</tbody>
</table>

The isolate would be categorized as wild-type (an MIC below the ECV of 1 µg/mL), which however does not indicate that the isolate susceptible.
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 prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

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

quality assurance (QA) – part of quality management focused on providing confidence that quality requirements will be fulfilled; NOTE: The practice that encompasses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. In the testing environment, this includes monitoring all the raw materials, supplies, instruments, procedures, sample collection/transport/storage/processing, recordkeeping, calibrating and maintaining of equipment, quality control, proficiency testing, training of personnel, and all else involved in the production of the data reported.

quality control (QC) – the operational techniques and activities that are used to fulfill requirements for quality; NOTE 1: In health care testing, the set of procedures designed to monitor the test method and the results to ensure test system performance; NOTE 2: QC includes testing control materials, charting the results and analyzing them to identify sources of error, and evaluating and documenting any remedial action taken as a result of this analysis.

wild-type (WT) – isolates without mechanisms of resistance.

1.4.3 Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC©</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MEC</td>
<td>minimal effective concentration</td>
</tr>
<tr>
<td>NWT</td>
<td>non-wild type</td>
</tr>
<tr>
<td>RPMI</td>
<td>Royal Park Memorial Institute (culture medium)</td>
</tr>
<tr>
<td>QA</td>
<td>quality assurance</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Chapter 2: Indications for Performing Susceptibility Tests

This chapter includes:

- References to information on the general indications for performing antifungal susceptibility tests

Although the general indications for performing susceptibility tests are provided in CLSI document M27-S4 (broth medium and all issues related to the antifungal agents), specific testing conditions for filamentous fungi that are different (eg, inoculum preparation and quantification, minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) readings and especially results interpretation) from those used for yeasts are described in the following chapters.

Antifungal resistance (other than intrinsic resistance of some species) is generally not widespread for prevalent fungal species and is frequently observed after exposure to antifungals and/or prolonged antifungal treatment. However, during the last ten years, an increase in triazole resistance among the complexes of Aspergillus, especially A. fumigatus spp. complex has been reported especially in Europe. Although it began as a single triazole resistance (mostly itraconazole), it is currently evident that cross resistance can be common in aspergillosis, especially in areas where environmental resistance can be as high as >10%. Resistant isolates have been recovered from patients not exposed to any azole with resistance being associated with the presence of gene mutations (cyp51A-gene). This is a concern given that the recommended therapy for aspergillosis is voriconazole and other triazoles and mutant isolates have been associated with treatment failure and high MICs. Although breakpoints are not currently available for any mould species as they have been for more prevalent Candida spp. and triazoles (only fluconazole and voriconazole) and echinocandins, it has been possible to calculate ECVs for Aspergillus spp. and triazoles (isavuconazole included).
Chapter 3: Antifungal Broth Dilution Susceptibility Testing Process for Filamentous Fungi

This chapter includes:

- Overview of antifungal broth dilution susceptibility testing process
- Selection of antifungal agents for routine testing and reporting
- Preparing the antifungal agents
- Testing procedures
- Reading and interpreting the results

Figure 1 provides an overview of the antifungal susceptibility testing process.
3.1 Selecting Antifungal Agents for Routine Testing and Reporting

Routine fungal susceptibility 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, diagnostic microbiology laboratory personnel, and the infection control committee.

3.1.1 Generic Names

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

3.1.2 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 principals apply.

3.1.3 Guidelines for Selective Testing

Testing may be warranted under certain selected circumstances including:

- As part of periodic batch surveys that establish antibiograms for collections of pathogenic isolates obtained within an institution
- 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 mould-drug combination remains uncertain. However, ECVs have been defined for the most common Aspergillus spp. vs amphotericin B, caspofungin, and the triazoles, including isavuconazole (see CLSI documents M57-16, M57S-17, M57S-18, M57S-19). Specimens for culture and other procedures should be obtained before initiating antifungal therapy.

3.2 Preparation of Antifungal Agents

3.2.1 Source

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

3.2.2 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. Therefore, a laboratory needs to standardize its antifungal solutions based on assays of the lots of antifungal powders used. Some agents may be affected by using treated plasticware. One such agent is caspofungin. Because of this, glass-ware or untreated plastic-ware should be used for weighing powders, preparing drug dilutions, and for the preparation of caspofungin microtiter trays.

Commented [MH17]: Weiderhold reference to be added when published
In some cases, a certificate of analysis with values for each of these components may be provided with antimicrobial agent powders. In this case, an overall value for potency may not be provided, but can be calculated from high performance liquid chromatography purity, water content, and, when applicable, the active fraction for drugs supplied as a salt (see CLSI document M07\(^2\) for examples).

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

\[
\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}} \quad (1)
\]

or

\[
\text{Volume (mL)} = \frac{\text{Weight (mg)} \times \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}} \quad (2)
\]

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 the required amount and to calculate the 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, the first formula should be used to establish the weight of powder needed:

\[
\text{Weight (mg)} = \frac{100 \text{ mL} \times 1280 \text{ µg/mL}}{750 \text{ µg/mg}} = 170.7 \text{ mg} \quad (3)
\]

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

\[
\text{Volume (mL)} = \frac{182.6 \text{ mg} \times 750 \text{ µg/mg}}{1280 \text{ µg/mL}} = 107.0 \text{ mL} \quad (4)
\]

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

### 3.2.3 Preparing Stock Solutions

Antifungal stock solutions should be prepared at concentrations of at least 1280 µg/mL or 10 times the highest concentration tested, whichever is greater. However, some antifungal agents of limited solubility need lower concentrations. In all cases, information provided by the drug manufacturer should be considered when determining solubility.
3.2.3.1 Using Solvents

Information on antifungal compound solubility should be included with the drug. Drugs should be dissolved at concentrations at least 100 times higher than the highest desired test concentration. The two most commonly used solvents are analytical grade dimethyl sulfoxide (DMSO) and water. Ethyl alcohol, polyethylene glycol, and carboxy methyl cellulose have also been used as solvents (see CLSI document M38/M51S). Because DMSO or other solvents should be used for all agents, 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 Appendixes A and B). This procedure avoids dilution artifacts resulting from precipitation of compounds with low solubility in aqueous media.

For example, to prepare for a broth microdilution test series containing a drug dissolved in DMSO, for which the highest desired test concentration is 16 µg/mL:

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Weigh 4.8 mg (assuming 100% potency) of the antifungal powder</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Dissolve the antifungal powder in 3.0 mL DMSO</td>
<td>This will provide a stock solution at 1600 µg/mL.</td>
</tr>
<tr>
<td>3.</td>
<td>Prepare further dilutions of this stock solution in DMSO.</td>
<td>See Appendixes A and B</td>
</tr>
<tr>
<td>4.</td>
<td>Dilute the solutions in DMSO 1:50 in the test medium</td>
<td>See Subchapter B.2. When inoculated, a further 2× (twofold) dilution will result (see Subchapter A.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.</td>
</tr>
</tbody>
</table>

The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations in Subchapter 3.2.2 should be applied.

3.2.3.2 Filtration

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

3.2.3.3 Storage

Small volumes of the sterile stock solutions should be dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at −60 °C or below, but never at a temperature greater than −20 °C). Vials should be removed as needed and used the same day. Any unused drug should be discarded at the end of the day. Most antifungal agent stock solutions can be stored at −60 °C or below for six months or more without significant loss of activity.29 In all cases, the instructions provided by the drug manufacturer need to be considered and supersede these general recommendations provided here. Any significant antifungal agent deterioration/loss of potency must be determined. This should be reflected in the susceptibility testing results using QC strains or reference strains such as those listed in CLSI document M38/M51S.28

28Clinical and Laboratory Standards Institute. All rights reserved. 9
3.2.4 Number of Concentrations Tested

3.2.4.1 Nondermatophyte Moulds

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**, itraconazole, ketoconazole, posaconazole, voriconazole, and isavuconazole: 0.0313 to 16 µg/mL.
- Flucytosine and fluconazole: 0.125 to 64 µg/mL.
- Echinocandins (anidulafungin, caspofungin, and micafungin): 0.008 to 4 µg/mL or lower, especially for anidulafungin and micafungin.

3.2.4.2 Dermatophyte Moulds

Suitable drug concentration ranges for testing dermatophytes are:

- Ciclopirox: 0.06 to 32 µg/mL.
- Fluconazole and griseofulvin: 0.125 to 64 µg/mL.
- Itraconazole, voriconazole, and terbinafine: 0.001 to 0.5 µg/mL.
- Posaconazole: 0.004 to 8 µg/mL.

3.3 Testing Procedures

3.3.1 Growth Medium

The completely synthetic medium, Royal Park Memorial Institute (culture medium) (RPMI-1640) (with glutamine, without bicarbonate, and with phenol red as a pH indicator), is satisfactory for testing filamentous fungi, and has been used to develop the reference method.\(^1\)\(^2\)\(^9\)

The formula for this medium is provided in Appendix D and directions for medium preparation from powder is provided in Appendix E.

3.3.2 Buffers

Media should be buffered to a pH of 7.0 ± 0.1 at 25 °C using 3-[N-morpholino] propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L at pH 7.0. The pH of each medium batch should be checked with a pH meter immediately after preparation. The pH should be between 6.9 and 7.1 at room temperature (25 °C). MIC performance characteristics of each batch of broth should be evaluated using a standard set of QC organisms (see Subchapter 4.3 and CLSI document M38/M51\(^18\)).

3.3.3 Preparing Diluted Antifungal Agents

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

- Sterile, plastic test tubes should be used to prepare drug dilutions and sterile, disposable, multiwell microdilution plates (96 U-shaped wells, untreated polystyrene) should be used to perform the tests (see Subchapter 3.2.2 for potential interlaboratory variability caused by plastic-ware and the use of untreated plastic ware to avoid this problem)\(^{15}\).

\(^{18}\)Clinical and Laboratory Standards Institute. All rights reserved.
• A growth control well containing RPMI-1640 medium without antifungal agents (but with nonaqueous solvent) should be used for each organism tested.

The total volume of each dilution prepared depends on the number of tests performed. Because 0.1 mL of each antifungal drug dilution is used for each test, 1.0 mL is adequate for approximately eight tests (one microdilution tray), allowing for pipetting. A single pipette should be used for measuring all diluents and then for adding the stock antifungal solution to the first tube. A separate pipette for each remaining dilution in that set should be used. 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.

An initial dilution series of the agent should be prepared at 100 times the final strength in an appropriate solvent (see Subchapter 3.2.3.1). 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 Subchapter 3.2.3.1 and Appendix A or B).

To prepare 5-mL volumes of diluted antifungal agent (sufficient for 45 tests):

1. Pipette 4.9-mL volumes of RPMI-1640 medium into each of 10 sterile test tubes.
2. 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.
3. 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 needed. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are twofold more concentrated than the final concentrations.

3.3.4 Broth Macrodilution Modifications

Published data show good concordance between results obtained using the broth microdilution methodology described above and a broth macrodilution adaptation. Some laboratories may choose to implement broth macrodilution rather than the broth microdilution method, primarily for safety issues. The steps and testing conditions relevant to the broth macrodilution test are provided below. 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 10× (10-fold) strength needed for the broth macrodilution test. The steps are listed below.
### 3.3.5 Preparing the Inoculum

When there is a risk of substantial spatter or aerosolization, the manipulation should be performed in a Class IIA or IIB biological safety cabinet. See CLSI document M29 for additional information and Subchapter 1.3.

#### 3.3.5.1 Nondermatophyte Moulds

Reliable nongerminated conidial or sporangiospore suspensions can be prepared by a spectrophotometric procedure. Concentrations of viable conidial or sporangiospore test inocula in a range of approximately $0.4 \times 10^4$ to $5 \times 10^4$ CFU/mL provide the most reproducible MIC data. The steps for preparing the inoculum are listed below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Induce conidium and sporangiospore formation.</td>
<td>Grow most fungi on sporulation media (e.g., potato dextrose agar, carrot juice agar, etc) 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 (e.g., Mucorales and Aspergillus spp. and species complex). <em>Fusarium</em> spp. and species complex may need to be incubated for 48 to 72 hours at 35 °C and then until day seven at 25 °C to 28 °C.</td>
</tr>
<tr>
<td>2.</td>
<td>Cover sporulating colonies with approximately 1 mL of sterile 0.85% saline.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Prepare a suspension by gently probing the colonies with the tip of a transfer pipette.</td>
<td>Adding one drop (approximately 0.01 mL) of polysorbate 20 will facilitate the preparation of <em>Aspergillus</em> spp. inocula.</td>
</tr>
<tr>
<td>4.</td>
<td>Withdraw and transfer the resulting mixture of conidia or sporangiospores and hyphal fragments to a sterile tube.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Allow heavy particles to settle for 3 to 5 minutes.</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Transfer the upper homogeneous suspension to a sterile tube and tighten the cap.</td>
<td></td>
</tr>
</tbody>
</table>
Mix by vortexing for 15 seconds.

CAUTION: Remove the cap carefully, as liquid adhering to the cap may produce aerosols when opened.

Read and adjust the densities of the conidial or sporangiospore suspensions to an OD at 530 nm.

0.09 to 0.13: Aspergillus spp. and species complex, Exophiala dermatitidis, Periconospora lilacinum, Paecilomyces variotii, S. schenckii and species complex.

0.15 to 0.17: Cladosiphialophora bantiana, Fusarium spp. and species complex, Ochroconis gallapova, Rhizopus spp. and Mucorales, Scedosporium (Lomentospora/Pseudallescheria)

0.25 to 0.30: Alternaria spp.*, Bipolaris spp.*

Dilute these suspensions 1:50 in the standard medium.

Inoculum suspensions of Scedosporium sp. (Lomentospora/Pseudallescheria), Bipolaris hawaiensis and spicifera, and Alternaria spp. and other less prevalent dematiaceous (black) moulds may require a lower (50%) dilution factor.

The 1:50 inoculum dilutions will be 2× (twofold) more concentrated than the density needed or approximately 0.4 × 10^4 to 5 × 10^4 CFU/mL.

Inoculate each well with 0.1 mL of the corresponding diluted inoculum suspension.

Prepare the test inoculum in a sufficient volume to inoculate all test wells.

*May require a lower (50%) dilution factor

Abbreviations: CFU, colony forming unit; OD, optical density

3.3.5.2 Dermatophyte moulds

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. Therefore, oatmeal agar (see Appendix F for preparation instructions) is recommended as the optimal growth medium for inducing conidium formation in T. rubrum isolates. The steps for preparing the inoculum are listed below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Inoculate dermatophytes onto potato dextrose or oatmeal agar (T. rubrum isolates only)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Incubate at 30 °C for 4 to 5 days or until good conidial growth is present.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Cover colonies with approximately 1 mL of sterile 0.85% saline</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Prepare a suspension by gently probing the colonies with the tip of a transfer pipette or sterile swab.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Let the resulting suspension settle for 5 to 10 minutes</td>
<td></td>
</tr>
</tbody>
</table>

© Clinical and Laboratory Standards Institute. All rights reserved.
6. Count the conidia using a hemacytometer. The final suspension should be made 2× more concentrated than the density needed for testing (1 × 10^3 to 3 × 10^3 CFU/mL).

Abbreviations: CFU, colony forming units

3.3.6 Quantifying the Inoculum

The final inoculum's accuracy may be verified as outlined in the following subchapters.

3.3.6.1 Quantifying the Inoculum 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 CFUs/mL. The plates should be incubated at 28 °C to 30 °C and observed daily for the presence of fungal colonies. Colonies should be counted as soon as possible after visible growth appears, especially for isolates Rhizopus spp. and other Mucorales. Incubation times will range from 24 hours or less (Rhizopus spp.) to five days (Scedosporium (Lomentospora/Pseudallescheria)).

3.3.6.2 Quantifying the Inoculum of Dermatophyte Moulds

This step can be performed by plating 0.01 mL of a 1:10 dilution of the adjusted inoculum on Sabouraud glucose agar to determine the viable number of CFU/mL. The plates should be incubated at 28 °C to 30 °C and observed daily for the presence of fungal colonies.

3.3.7 Inoculating the Wells

For microbroth dilution, each well should be inoculated on the day of the test with 0.1 mL of the 2× conidial or sporangiospore inoculum suspension. This step dilutes the drug concentrations, inoculum densities, and solvent, if used, to the final desired test concentrations. The growth control wells contain 0.1 mL of the corresponding diluted inoculum suspension and 0.1 mL of the drug diluent (2%) without antifungal agent (see Subchapter 3.3.3). QC and reference organisms should be tested in the same manner and included each time an isolate is tested.

For macrobroth dilution, each tube should be inoculated on the day of the test with 0.9 mL of the corresponding diluted inoculum suspension, bringing the drug dilutions and inoculum densities to the final concentrations listed for the microdilution method. The growth control receives 0.1 mL of 10× the drug diluent without antifungal agent and is inoculated with 0.9 mL of the corresponding diluted inoculum suspensions. QC organisms should be tested in the same manner and included each time an isolate is tested.

3.3.8 Incubating the Trays

All microdilution trays and macrobroth dilution tubes should be incubated at 35 °C without agitation and observed for the presence or absence of visible growth.

3.3.8.1 Non-dermatophyte Moulds

Some mould isolates may not grow at 35 °C and incubation at 30 °C is more suitable. See Table 2 for recommended incubation times for determining MICs/MECs.
Table 2. Recommended Incubation Times for Determining Minimal Inhibitory Concentrations or Minimal Effective Concentrations of Non-dermatophyte Moulds.

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Genus/species</th>
<th>Recommended Incubation (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All except echinocandins (amphotericin B, fluconazole, triazoles)</td>
<td>Rhizopus spp. Mucorales</td>
<td>21–26</td>
</tr>
<tr>
<td></td>
<td>Aspergillus spp. and species complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fusarium spp. and species complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. schenckii species complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. bantiana</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. dermatitidis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. lilacinum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. variotii</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scedosporium spp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L. prolificans</td>
<td>70–74</td>
</tr>
<tr>
<td>Echinocandins*</td>
<td>Aspergillus spp. and species complex</td>
<td>21–26</td>
</tr>
<tr>
<td></td>
<td>Paecilomyces variotti</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scedosporium spp.</td>
<td>46–72</td>
</tr>
<tr>
<td></td>
<td>(Lomentospora / Pseudallescheria)</td>
<td></td>
</tr>
</tbody>
</table>

*Alternative: Read on first day when sufficient growth (ie, confluent growth covering the bottom of the well) is present in the growth control well (ie, contained drug-free medium).

3.3.8.2 Dermatophyte Moulds

The dermatophyte mould isolate MICs should be determined after incubating for four days.9

3.4 Reading Minimal Inhibitory Concentration and Minimal Effective Concentration Results

3.4.1 General

The MIC is the lowest concentration of an antifungal agent that substantially inhibits organism growth, as detected visually when testing most antifungal agents. For the conventional microdilution procedure, the growth in each MIC well should be compared to the growth control with the aid of a reading mirror. For broth macrodilution, the tubes should be scored and the MICs determined as described for the broth microdilution procedure.

When testing echinocandin antifungal agents, the MEC evaluation provides more consistent and reproducible susceptibility data than the conventional MIC reading.6,7,8 The MEC is the lowest drug concentration 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 G). For evaluating the MEC, the growth in each well should be compared to the growth control (drug-free medium) using a reading mirror.

3.4.2 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.

3.4.3 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. Applying 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 (non-dermatophyte mould isolates) to 80% or more (dermatophyte isolates) reduction in growth compared to the control well growth (drug-free medium). When this turbidity persists, it is often identical for all drug concentrations above the MIC.

3.4.4 Itraconazole, Posaconazole, Voriconazole, and Isavuconazole

For non-dermatophyte mould isolates, 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 mould pathogens are not usually encountered. 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. 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).

3.4.5 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 improves reproducibility. The MEC is read as the lowest drug concentration 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 G).

3.4.6 Ciclopirox

For ciclopirox, end points are typically less well defined than that described for amphotericin B. Applying 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).

3.4.7 Griseofulvin

For griseofulvin, end points are typically less well defined than that described for amphotericin B. Applying 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).

3.4.8 Terbinafine

For terbinafine, end points are typically less well defined than that described for amphotericin B. Applying 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).

3.5 Interpreting Results

Although breakpoints for mould testing have not been established or approved by CLSI or any regulatory organization, ECVs have been defined for some *Aspergillus* spp. and species complex vs amphotericin B, caspofungin, and four triazoles (isavuconazole, itraconazole, posaconazole and voriconazole). These ECVs may help identify NWT *Aspergillus* spp. isolates (i.e., isolates for which the antifungal agent being evaluated appears to have reduced activity and may harbor resistance mechanisms). For information on ECVs, see CLSI documents M57P4 and M57S7.
For non-Aspergillus spp. and for analytical purposes, susceptibility endpoints were assigned by the error bounding method during the collaborative study evaluating agar disk diffusion method performance. MICs below 1 µg/mL are usually reported for S. apiospermum and Paracoccidioides brasilensis (Paecilomyces lilacinus) with posaconazole and voriconazole; for Alternaria spp. and Bipolaris spicifera with the triazoles. For some Mucorales with posaconazole and amphotericin B, isolates were grouped arbitrarily as “susceptible” (MIC or MEC < 1 µg/mL) and “resistant” (MIC or MEC > 4 µg/mL).

**Note:** Although these endpoints and ECVs would help identify certain mould isolates as potentially resistant to triazoles, amphotericin B, and/or caspofungin (see the subchapters below), these endpoints are based solely on *in vitro* data. Therefore, the clinical relevance of testing this group of fungal pathogens remains uncertain.

### 3.5.1 Amphotericin B

Experience using the procedures described in Subchapter 3.3 indicates that amphotericin B MICs for most nondermatophyte, opportunistic mould isolates are clustered between 0.5 and 2.0 µg/mL with modes (most frequent MIC) for Aspergillus spp. ranging between 0.5 and 1 µg/mL, except for *A. terreus* section complex (mode, 2 µg/mL). However, amphotericin B MICs for some other species (eg, species complexes of *A. flavus*, *A. nidulans* and *A. versicolor*) are above 2 µg/mL and as high as 16 µg/mL.

Although limited data are available regarding correlation between MIC and amphotericin B treatment outcomes for the filamentous fungi, MICs above 2 µg/mL (or above the ECV) have been associated with treatment failures. MICs below 2 µg/mL have been associated with clinical cure among 29 patients treated with amphotericin B for invasive aspergillosis caused by *A. fumigatus* species complex (eight cases), *A. terreus* species complex (nine cases) and *Aspergillus flavus* species complex (12 cases). Poor response to different formulations of amphotericin B was reported in patients with infections caused by *A. terreus* species complex (MICs > 1 µg/mL).

### 3.5.2 Flocculonitrolysis

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

### 3.5.3 Fluconazole

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

### 3.5.4 Ketoconazole

Experience using the procedures described in this document indicates that nondermatophyte mould MICs vary between 0.0313 and 16 µg/mL. However, data are not yet available to confirm a correlation between MIC and treatment outcome with ketoconazole. ECVs have not been defined for this agent.

### 3.5.5 Itraconazole, Posaconazole, Voriconazole, and Isavuconazole

The importance of proper drug dilution preparation for these water-insoluble compounds cannot be overemphasized (see CLSI document M27–3). Using incorrect solvents or deviating from the dilution scheme suggested in Appendix A and B can lead to substantial errors due to dilution artifacts.
3.5.5.1 Nondermatophyte Moulds.

Experience using the recommended procedures indicates that nondermatophyte mould MICs for triazoles vary between 0.0313 and 16 µg/mL (most Aspergillus spp. modes between 0.06 and 0.5 µg/mL). Reduced triazole susceptibility as well as cross phenotypic resistance has been documented for some members of the Aspergillus section Nigri, Aspergillus section Fumigati, and Fusarium species complex. Most ciclopirox MICs are yet to be determined. Although caspofungin MECs of 3.5.6 other moulds vary between 0.0313 and 16 µg/mL (most modes between 0.06 and 0.5 µg/mL), reduced echinocandin susceptibility (most FKS1 mutations) isolates of A. fumigatus species complex. A predictor of favorable treatment response was an MIC of <0.5 µg/mL with posaconazole and an MIC of 0.25 or 0.5 µg/mL with posaconazole. Voriconazole effectively prolonged survival and reduced the fungal load in a murine model of invasive infection caused by strains of A. terreus species complex for which the MICs were ≤ 1 µg/mL, ≤ the ECV for this species vs voriconazole. These results support the potential for using ECVs in the clinical setting for detecting triazole resistance (NWT MIC) due to cyp51A mutations, a resistance mechanism in some Aspergillus spp. Limited data are available to indicate a correlation between MIC and treatment outcome with these triazoles and other moulds. ECVs have only been defined for certain Aspergillus spp. and species complex (see CLSI documents M57 and M57S).

3.5.5.2 Dermatophyte Moulds

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

3.5.6 Echinocandins (anidulafungin, caspofungin, micafungin)

Work to date has focused principally on testing Aspergillus isolates and little information exists to guide work with other moulds; however, non-Aspergillus mould MECs are usually high (≥ 4 µg/mL). Caspofungin MECs for Aspergillus spp. vary between 0.016 and 16 µg/mL (most modes are between 0.06 and 0.25 µg/mL) with frequently lower values when testing anidulafungin and micafungin. The frequency of caspofungin MECs above the ECV for Aspergillus spp. vary according to the species and it can be high for the species complexes of A. nidulans, A. terreus and A. versicolor (6.7 to 14.1% MECs above ECVs of 0.25 to 0.5 µg/mL). ECVs have not been defined for the other two echinocandins or any other moulds. Correlation of high echinocandin MECs with clinical outcome and FKS1 gene mutations remains to be determined. Although caspofungin MECs of ≥1 µg/mL for A. fumigatus were obtained from breakthrough infections in patients receiving this agent, genetic information was not available.

3.5.7 Ciclopirox

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

3.5.8 Griseofulvin

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

Most terbinafine MICs are $\leq 0.25$ µg/mL for the dermatophytes, but MICs $\geq 0.5$ µg/mL have been reported for *T. rubrum.* Correlation of MIC with clinical outcome has yet to be determined.
Chapter 4: Quality System Essentials – Quality Control

This chapter includes:

- The purpose of quality control
- Responsibilities for quality control
- Selecting and storing quality control strains
- Routine uses for reference strains
- Controlling media batches and plasticware lots
- Quality control frequency
- Other control procedures

4.1 Purpose

The goals of a QC program are to monitor:

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

The goals are best accomplished by, but not limited to, using QC and reference strains selected for their genetic stability and usefulness in the particular method being controlled. 8,10,57,58,59,60

4.2 Quality Control Responsibilities

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

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

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

Manufacturers of commercial products should design and recommend a QC program that enables users to evaluate the 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.

Laboratories (user) is responsible for:

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

©Clinical and Laboratory Standards Institute. All rights reserved.
Laboratories should familiarize themselves with the regulatory and accreditation requirements for QC in their specific location.

### 4.3 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-log₂ series; however, strains with MICs between the third and seventh dilution are acceptable. Before a strain is accepted as a reference, it should be tested for as long as necessary to demonstrate that its antifungal susceptibility pattern is genetically stable. CLSI document M23 provides guidelines for selecting appropriate QC strains and determining acceptable MIC or MEC ranges. The QC strains listed in CLSI document M38/M51S were selected in accordance with the criteria in CLSI document M23 and can be used as controls for the antifungal susceptibility testing of moulds until mould isolates are selected. In addition, the reference mould isolates listed in CLSI document M38/M51S also can be used.

Also shown are additional strains that can be useful for conducting reference studies.

### 4.4 Storing Reference Strains

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

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

- There are two preferred methods for prolonged storage of reference strains. The fungal isolates may be grown on potato dextrose agar and then frozen at −70 °C. Alternatively, cells can be preserved in 10% to 15% glycerol solution in small vials and storing them at either −70 °C, in liquid nitrogen, or in the vapor of liquid nitrogen.

- 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 from frozen stock. 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 should be obtained.

#### 4.4.2 Sources for Reference Strains

Reference strains should be obtained from a source that can provide information on the culture's origin (e.g., 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.

#### 4.4.3 Preparing Strains for Storage

Use the following steps to prepare strains for storage.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Inoculate moulds onto an appropriate solid medium.</td>
<td>For most moulds, use potato dextrose agar. For dermatophytes, use Sabouraud dextrose agar or oatmeal agar (T. rubrum isolates).</td>
</tr>
<tr>
<td>2.</td>
<td>Incubate the plates for 7 days at 28-32 °C.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Select growth from several colonies and perform the appropriate susceptibility tests to</td>
<td>See CLSI document M38/M51S for expected MICs for QC and/or reference strains</td>
</tr>
</tbody>
</table>

Commented [MH41]: Sheryl, add a reference to M38/M57S
Commented [MH42]: Is this information in M38/M51 as well?
4. Demonstrate that they give the expected MIC or MEC results.

5. Subculture strains yielding expected results onto the same medium that was used for the primary culture.

6. Incubate the plates long enough for sufficient growth to occur. Usually 1–7 days

7. Examine the resulting growth carefully. Ensure the growth is a pure culture.

8. Distribute the turbid suspension in small volumes (1 or 2 drops/approximately 100 to 25 µL) into suitable sterile containers.

9. Store cultures at 2 to 8 °C.

Abbreviations: MEC, minimal effective concentration; MIC, minimal inhibitory concentration; QC, quality control

Stocks prepared using this procedure can be stored 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.

4.5 Routine Use of Reference Strains

Use the following steps to prepare reference strains for routine use.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Remove a container of the culture from the freezer or obtain a lyophilized vial.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Let the frozen mixture thaw or rehydrate the lyophilized culture.</td>
<td></td>
</tr>
</tbody>
</table>
| 3.   | For Candida spp.:  
- Transfer a portion of the mixture onto Sabouraud dextrose agar  
- Incubate at 35 °C for 24 hours.  
For moulds:  
- Subculture on potato dextrose agar or oatmeal agar (T. rubrum isolates only) | |
| 4.   | Incubate 4 to 5 days (dermatophyte isolates) or 7 days (nondermatophyte isolates) or until good conidial growth is present. Incubate at 35 °C | |
| 5.   | Remove 4 to 5 colonies and subculture them to medium for the appropriate susceptibility tests potato dextrose agar or Sabouraud dextrose agar | Incubate at 35 °C |
| 6.   | Store cultures at 2 to 8 °C. | |
| 7.   | Subculture from the slant to an agar plate.  
- Sabouraud dextrose for Candida spp.  
- Potato dextrose agar for filamentous fungi or oatmeal agar (for T. rubrum isolates only).  
Always perform susceptibility tests on colonies from:  
- Overnight plates (Candida spp.)  
- 7-day cultures (nondermatophyte isolates) | |
The agar slants may be used as working stock cultures. Agar slants should be replaced regularly with new slants prepared from the freezer supply at least every two weeks.

4.6 Controlling Media Batches and Plasticware Lots

Control batches and plasticware lots by using the following criteria.

- Each new batch of medium or lot of microdilution trays or macrodilution tubes should be tested with one of the QC strains listed in CLSI document M38/M51 to determine if MICs or MECs fall within the QC expected range. If they do not, the batch or lot should be rejected.

- At least one uninoculated tube from each batch should be incubated for the same amount of time as needed to complete the test to ensure the medium’s sterility.

- 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 Subchapter 6.1.2).

- The lot numbers of all materials and reagents used in these tests should be recorded.

4.7 Quality Control Frequency

4.7.1 Minimal Inhibitory Concentration or Minimal Effective Concentration Ranges

MIC or MEC accuracy ranges for a single control test are listed in CLSI document M38/M51. In general, 1 out of every 20 MIC values in a series of 20 consecutive tests might be out of control (ie, outside the stated range) due to random test variation. Two consecutive out-of-control results or any more than 2 out-of-control results in 20 consecutive control tests need corrective action. Any time corrective action is taken, the count of 20 begins again.

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

4.7.2 Frequency of Testing

The overall test system performance should be monitored by testing appropriate reference strains each day the test is performed. However, the 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:

- Documenting that all reference strains were tested for 30 consecutive test days.

- 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) were outside the accuracy ranges stated in CLSI document M38/M51.

NOTE: This procedure is only for establishing satisfactory performance of MIC or MEC tests for the purpose of performing weekly instead of daily QC tests. Do not confuse this procedure with the steps that must be taken for corrective action defined in Subchapter 4.7.1.
The overall test system performance evaluation (as outlined above) must 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.

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:

- Testing with appropriate reference strains for five consecutive test days should be performed.

- 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 the current edition of CLSI document M38/M51.18

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. To return to weekly testing, documenting satisfactory performance for another 30 consecutive test days as described in this Subchapter is necessary.

4.8 Other Control Procedures

4.8.1 Growth Control

To assess test organism viability, each broth microdilution or macrodilution series should include a growth control composed of RPMI-1640 medium without antifungal agent plus 1% of the solvent used (eg, DMSO). With 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 should be streaked onto a suitable agar medium and incubated until there is sufficient visible growth to detect mixed cultures and to provide freshly isolated colonies in the event retesting is needed.

4.8.3 Controlling End-point Interpretation

End-point interpretation should be monitored periodically to minimize variation in the interpretation of MIC or MEC end points among observers. All laboratory personnel performing these tests should independently read a selected set of dilution tests. The results should be recorded and compared to the results obtained by an experienced reader. Specific reference strains with predetermined MICs are particularly helpful for this purpose, especially with itraconazole and other triazoles.
Chapter 5: Conclusion

This document describes a broth dilution method to evaluate the susceptibility of filamentous fungi (including dermatophytes) to antifungal agents (e.g., amphotericin B, triazoles, echinocandins, etc). Detailed information regarding preparation of antifungal stock solutions, inoculum densities, and susceptibility endpoint interpretation (MICs and MECs) are provided. QC procedures are also provided.

With the proposal of species-specific ECVs for certain *Aspergillus* spp. vs the triazoles including isavuconazole, amphotericin B, and caspofungin, MICs/MECs obtained using this methodology should have some clinical relevance. The additional data establishing interpretive breakpoints for moulds is not available. ECVs could identify non-WT *Aspergillus* isolates with reduced susceptibility to the antifungal agent under consideration for clinical use. This is important since infections caused by *Aspergillus* spp. are the most common mould infections. For more information on developing and using ECVs, see CLSI documents [M57](#) and [M57S](#).

Chapter 6: Supplemental Information

This chapter includes:
- References
- Appendixes
- The Quality Management System Approach
- Related CLSI Reference Materials

Commented [MH43]: Sheryl, add reference to M57
Commented [MH44]: Sheryl, add a reference to M57S
References


16. M57

17. M57A


©Clinical and Laboratory Standards Institute. All rights reserved.


Additional References

Taxonomic References

**Aspergillus spp Nomenclature:**

**Talaromyces and Penicillium Nomenclature:**

**Changing Nomenclature:**

**Purpureocillium lilacinum Nomenclature:**

**Scedosporium Nomenclature:**

Appendix A. Scheme for Preparing Dilution Series of Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests for Nondermatophytes

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration (µg/mL)</th>
<th>Volume (mL)</th>
<th>Source</th>
<th>Volume (mL)</th>
<th>Solvent (eg, DMSO)</th>
<th>Intermediate Concentration (µg/mL)</th>
<th>Final Concentration at 1:100 (µg/mL)</th>
<th>Log</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1600</td>
<td>-</td>
<td>Stock</td>
<td>-</td>
<td>1600</td>
<td>1600</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>1600</td>
<td>0.5</td>
<td>Stock</td>
<td>0.5</td>
<td>800</td>
<td>800</td>
<td>8.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>1600</td>
<td>1.5</td>
<td>Stock</td>
<td>3.5</td>
<td>400</td>
<td>400</td>
<td>4.0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1600</td>
<td>3.5</td>
<td>Stock</td>
<td>7.5</td>
<td>200</td>
<td>200</td>
<td>2.0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>0.5</td>
<td>Step 4</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>1.5</td>
<td>Step 4</td>
<td>15</td>
<td>50</td>
<td>50</td>
<td>0.5</td>
<td>-1</td>
</tr>
<tr>
<td>7</td>
<td>200</td>
<td>3.5</td>
<td>Step 4</td>
<td>30</td>
<td>25</td>
<td>25</td>
<td>0.25</td>
<td>-2</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>0.5</td>
<td>Step 7</td>
<td>1</td>
<td>12.5</td>
<td>12.5</td>
<td>0.125</td>
<td>-3</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>1.5</td>
<td>Step 7</td>
<td>7.5</td>
<td>6.25</td>
<td>6.25</td>
<td>0.0625</td>
<td>-4</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>3.5</td>
<td>Step 7</td>
<td>22.5</td>
<td>3.13</td>
<td>3.13</td>
<td>0.0313</td>
<td>-5</td>
</tr>
<tr>
<td>11</td>
<td>3.13</td>
<td>0.5</td>
<td>Step 10</td>
<td>1.56</td>
<td>0.156</td>
<td>0.156</td>
<td>-6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3.13</td>
<td>1.5</td>
<td>Step 10</td>
<td>0.78</td>
<td>0.0078</td>
<td>0.0078</td>
<td>-7</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: DMSO, dimethyl sulfoxide

Commented [PD45]: Covers the full range of dilutions recommended. 1X final concentration rather that 2X. Harmonized with M27A4 draft

Commented [MH46]: Should this be water insoluble as in App B7?
Appendix B. Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests of Dermatophytes

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration (µg/mL)</th>
<th>Source</th>
<th>Volume (mL)</th>
<th>Solvent (mL)</th>
<th>Intermediate Concentration (µg/mL)</th>
<th>Final Concentration at 1:100 (µg/mL)</th>
<th>Log₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6400</td>
<td>Stock</td>
<td>-</td>
<td>-</td>
<td>6400</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>6400</td>
<td>Stock</td>
<td>0.5</td>
<td>0.5</td>
<td>3200</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>6400</td>
<td>Stock</td>
<td>0.5</td>
<td>1.5</td>
<td>1600</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>6400</td>
<td>Stock</td>
<td>0.5</td>
<td>3.5</td>
<td>800</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>800</td>
<td>Step 4</td>
<td>0.5</td>
<td>0.5</td>
<td>400</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>800</td>
<td>Step 4</td>
<td>0.5</td>
<td>1.5</td>
<td>200</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>800</td>
<td>Step 4</td>
<td>0.5</td>
<td>3.5</td>
<td>100</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>Step 7</td>
<td>0.5</td>
<td>0.5</td>
<td>50</td>
<td>0.5</td>
<td>−1</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>Step 7</td>
<td>0.5</td>
<td>1.5</td>
<td>25</td>
<td>0.25</td>
<td>−2</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>Step 7</td>
<td>0.5</td>
<td>3.5</td>
<td>12.5</td>
<td>0.125</td>
<td>−3</td>
</tr>
<tr>
<td>11</td>
<td>12.5</td>
<td>Step 10</td>
<td>0.5</td>
<td>0.5</td>
<td>6.25</td>
<td>0.0625</td>
<td>−4</td>
</tr>
<tr>
<td>12</td>
<td>12.5</td>
<td>Step 10</td>
<td>0.5</td>
<td>1.5</td>
<td>3.13</td>
<td>0.0313</td>
<td>−5</td>
</tr>
<tr>
<td>13</td>
<td>12.5</td>
<td>Step 10</td>
<td>0.5</td>
<td>3.5</td>
<td>1.56</td>
<td>0.0156</td>
<td>−6</td>
</tr>
<tr>
<td>14</td>
<td>1.56</td>
<td>Step 13</td>
<td>0.5</td>
<td>0.5</td>
<td>0.78</td>
<td>0.0078</td>
<td>−7</td>
</tr>
<tr>
<td>15</td>
<td>1.56</td>
<td>Step 13</td>
<td>0.5</td>
<td>1.5</td>
<td>0.39</td>
<td>0.0039</td>
<td>−8</td>
</tr>
<tr>
<td>16</td>
<td>1.56</td>
<td>Step 13</td>
<td>0.5</td>
<td>3.5</td>
<td>0.195</td>
<td>0.00195</td>
<td>−9</td>
</tr>
<tr>
<td>17</td>
<td>0.195</td>
<td>Step 16</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0975</td>
<td>0.000975</td>
<td>−10</td>
</tr>
</tbody>
</table>
### Appendix C. Scheme for Preparing Dilutions of Water-Soluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration (µg/mL)</th>
<th>Source</th>
<th>Volume (mL)</th>
<th>+ Medium (mL)</th>
<th>Intermediate Concentration (µg/mL)</th>
<th>Final Concentration at 1:10 (µg/mL)</th>
<th>Log2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5120</td>
<td>Stock</td>
<td>1.0</td>
<td>7.0</td>
<td>640</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>640</td>
<td>Step 1</td>
<td>1.0</td>
<td>1.0</td>
<td>320</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>640</td>
<td>Step 1</td>
<td>1.0</td>
<td>3.0</td>
<td>160</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>160</td>
<td>Step 3</td>
<td>1.0</td>
<td>1.0</td>
<td>80</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>160</td>
<td>Step 3</td>
<td>0.5</td>
<td>1.5</td>
<td>40</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>160</td>
<td>Step 3</td>
<td>0.5</td>
<td>3.5</td>
<td>20</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>Step 6</td>
<td>1.0</td>
<td>1.0</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>Step 6</td>
<td>0.5</td>
<td>1.5</td>
<td>5</td>
<td>0.5</td>
<td>−1</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>Step 6</td>
<td>0.5</td>
<td>3.5</td>
<td>2.5</td>
<td>0.25</td>
<td>−2</td>
</tr>
<tr>
<td>10</td>
<td>2.5</td>
<td>Step 9</td>
<td>1.0</td>
<td>1.0</td>
<td>7.25</td>
<td>0.125</td>
<td>−3</td>
</tr>
<tr>
<td>11</td>
<td>2.5</td>
<td>Step 9</td>
<td>0.5</td>
<td>1.5</td>
<td>0.625</td>
<td>0.0625</td>
<td>−4</td>
</tr>
<tr>
<td>12</td>
<td>2.5</td>
<td>Step 9</td>
<td>0.5</td>
<td>3.5</td>
<td>0.3125</td>
<td>0.03125</td>
<td>−5</td>
</tr>
</tbody>
</table>

Commented [MH47]: There is still no reference in the main body of the text to this appendix. Where should it be added.
### Appendix D. Composition of RPMI-1640 Medium

<table>
<thead>
<tr>
<th>Constituent</th>
<th>g/L Water</th>
<th>Constituent</th>
<th>g/L Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine (free base)</td>
<td>0.200</td>
<td>Biotin</td>
<td>0.0002</td>
</tr>
<tr>
<td>L-asparagine (anhydrous)</td>
<td>0.050</td>
<td>D-pantothenic</td>
<td>0.00025</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>0.020</td>
<td>Choline chloride</td>
<td>0.003</td>
</tr>
<tr>
<td>L-cystine • 2HCl</td>
<td>0.0652</td>
<td>Folic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>0.020</td>
<td>Myo-inositol</td>
<td>0.035</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.300</td>
<td>Niacinamide</td>
<td>0.001</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.010</td>
<td>PABA</td>
<td>0.001</td>
</tr>
<tr>
<td>L-histidine (free base)</td>
<td>0.015</td>
<td>Pyridoxine HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-hydroxyproline</td>
<td>0.020</td>
<td>Riboflavin</td>
<td>0.0002</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.050</td>
<td>Thiamine HCl</td>
<td>0.01</td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.050</td>
<td>Vitamin B₁₂</td>
<td>0.000005</td>
</tr>
<tr>
<td>L-lysine • HCl</td>
<td>0.040</td>
<td>Calcium nitrate • H₂O</td>
<td>0.10</td>
</tr>
<tr>
<td>L-methionine</td>
<td>0.015</td>
<td>Potassium chloride</td>
<td>0.40</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>0.015</td>
<td>Magnesium sulfate (anhydrous)</td>
<td>0.04884</td>
</tr>
<tr>
<td>L-proline</td>
<td>0.020</td>
<td>Sodium chloride</td>
<td>6.000</td>
</tr>
<tr>
<td>L-serine</td>
<td>0.030</td>
<td>Sodium phosphate, dibasic (anhydrous)</td>
<td>0.800</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.020</td>
<td>D-glucose</td>
<td>2.00</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>0.005</td>
<td>Glutathione, reduced</td>
<td>0.001</td>
</tr>
<tr>
<td>L-tyrosine • 2Na</td>
<td>0.02883</td>
<td>Phenol red, Na</td>
<td>0.0053</td>
</tr>
<tr>
<td>L-valine</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Commented [PD48]: Why 2 concentrations. Only 0.000005 in M27-S4*
Appendix E. Preparing RPMI-1640 Medium

RPMI-1640 medium buffered with 0.165 mol/L 3-[N-morpholino] propanesulfonic acid (MOPS), 1 L (see Appendix D)

To prepare 10.4 g powdered RPMI-1640 medium (with glutamine and phenol red, without bicarbonate) 34.53 g MOPS buffer, use the steps below.

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

Abbreviation:

Reference for Appendix E

Appendix F. Oatmeal Agar

To prepare oatmeal agar, use the steps below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>To 1 L of distilled water, add:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 100 g baby oatmeal cereal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 15 g granulated agar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 0.03 g gentamicin</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Mix well.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Dispense 500 mL into liter autoclavable beakers.</td>
<td>Note: The mixture tends to boil over.</td>
</tr>
<tr>
<td>4.</td>
<td>Autoclave at 121 °C for 20 minutes.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Immediately pour into petri plates and allow to cool</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Store at 4 °C to 6 °C.</td>
<td></td>
</tr>
</tbody>
</table>

QC:
Positive: *Trichophyton rubrum* – conidia formation
Negative: None
Sterility: No growth

Reference
Appendix G. Minimal Effective Concentrations of Caspofungin and Anidulafungin

G1. Minimal Effective Concentrations of Caspofungin

Figure G1. Example of Caspofungin MEC Results. 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). 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).

G2. Minimal Effective Concentrations of Anidulafungin

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MEC</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.13</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>Growth control</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. apiospermum</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Growth control</td>
</tr>
<tr>
<td>ATCC® MYA-3634</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. solani</td>
<td>&gt;32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Growth control</td>
</tr>
<tr>
<td>ATCC® MYA-3636</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>&lt;0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Growth control</td>
</tr>
<tr>
<td>ATCC® MYA-3727</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>&gt;0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Growth control</td>
</tr>
<tr>
<td>ATCC® MYA-3626</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure G2. Example of Anidulafungin MEC Results. 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. The *Scedosporium* isolate’s MEC might be read 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 G:


Commented [MHS2]: Are these references still relevant?
Appendix G. McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, use a BaSO₄ turbidity standard (0.5 McFarland standard).

The procedure for preparing this turbidity standard consists of the steps below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Comment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Add 0.5 mL of 0.048 mol/L BaCl₂ (1.175% w/v BaCl₂•2H₂O) to 99.5 mL of 0.18 mol/L (0.36 N) H₂SO₄ (1% v/v).</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Use a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance and verify the correct density of the turbidity standard.</td>
<td>The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.</td>
</tr>
<tr>
<td>3.</td>
<td>Distribute 4 to 6 mL into screw-cap tubes.</td>
<td>Tubes should be the same size as those used in growing or diluting the broth culture inoculum.</td>
</tr>
<tr>
<td>4.</td>
<td>Tightly seal the tubes.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Store the tubes in the dark at room temperature.</td>
<td>Vigorously agitate this turbidity standard on a mechanical vortex mixer just before use. Replace standards or recheck their densities monthly after preparation.</td>
</tr>
</tbody>
</table>

Commented [MH53]: There is no reference to this appendix in the text and no mention of a McFarland standard. If it is not discussed in M38, the appendix should be deleted.

Commented [MH54R53]: Can this be deleted?
“DRAFT DOCUMENT. This draft CLSI document is not to be reproduced or circulated for any purpose other than review and comment. It is not to be considered either final or published and may not be quoted or referenced. 28 December 2015.”
The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system (QMS) approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The QMS approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any health care service’s path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The QSEs are as follows:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M38 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.

Path of Workflow

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

M38 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.

<table>
<thead>
<tr>
<th>Preexamination</th>
<th>Examination</th>
<th>Postexamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examination ordering</td>
<td>Sample collection</td>
<td>Sample transport</td>
</tr>
<tr>
<td>M24</td>
<td>M7</td>
<td>M11</td>
</tr>
</tbody>
</table>
Related CLSI Reference Materials

EP23™ Laboratory Quality Control Based on Risk Management; Approved Guideline. 1st ed., 2011. This document provides guidance based on risk management for laboratories to develop quality control plans tailored to the particular combination of measuring system, laboratory setting, and clinical application of the test.


M23 Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters. 3rd ed., 2008. This document addresses the required and recommended data needed for the selection of appropriate interpretive criteria and quality control ranges for antimicrobial agents.

M27 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. 4th ed., 2016. 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 Protection of Laboratory Workers From Occupationally Acquired Infections. 4th ed., 2004. Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

M38/M51 Performance Standards for Antifungal Susceptibility Testing of Filamentous Fungi; Draft Informational Supplement. 1st ed., 2016. This supplement provides updated tables for the Clinical and Laboratory Standards Institute antifungal susceptibility testing documents M38-A3 and M51-A.

M51 Method for Antifungal Disk Diffusion Susceptibility Testing of Nondermatophyte Filamentous Fungi. 1st ed., 2010. This document describes the guidelines for antifungal susceptibility testing by the disk diffusion method of nondermatophyte filamentous fungi (moulds) that cause invasive disease.

M57 Principles and Procedures for the Development of Epidemiological Cutoff Values for Antifungal Susceptibility Testing; Draft Guideline. 1st ed., 2016. This document describes the criteria for development and use of epidemiological cutoff values for guiding clinical decisions for fungi/antifungal combinations for which there are no breakpoints.


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

©Clinical and Laboratory Standards Institute. All rights reserved.