
This document addresses the selection and preparation of antifungal agents; implementation and interpretation of test procedures; and quality control requirements for susceptibility testing of yeasts that cause invasive fungal infections.

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

Clinical and Laboratory Standards Institute document M27-A3—Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition describes a method for testing the susceptibility of antifungal agents to yeast that cause invasive fungal infections, including Candida spp. (and Candida glabrata), and Cryptococcus neoformans. Selection and preparation of antifungal agents, implementation and interpretation of test procedures, and the purpose and implementation of quality control procedures are discussed. A careful examination of the responsibilities of the manufacturer and the user in quality control is also presented.

## Contents

Abstract............................................................................................................................................i
Committee Membership................................................................................................................iii
Foreword........................................................................................................................................vii
Updated Information in This Edition...............................................................................................viii

1 Scope.............................................................................................................................................1
2 Introduction....................................................................................................................................1
3 Standard Precautions....................................................................................................................1
4 Definitions ......................................................................................................................................1
5 Indications for Performing Susceptibility Tests..........................................................................2
6 Antifungal Agents............................................................................................................................3
   6.1 Source .......................................................................................................................................3
   6.2 Weighing Antifungal Powders.................................................................................................3
   6.3 Preparing Stock Solutions.......................................................................................................4
   6.4 Number of Concentrations Tested ........................................................................................5
   6.5 Selection of Antifungal Agents for Routine Testing and Reporting ......................................5
7 Test Procedures..............................................................................................................................6
   7.1 Broth Medium.........................................................................................................................6
   7.2 Preparing Diluted Antifungal Agents......................................................................................6
   7.3 Inoculum Preparation..............................................................................................................7
   7.4 Inoculating RPMI-1640 Medium ............................................................................................8
   7.5 Incubation ...............................................................................................................................8
   7.6 Reading Results......................................................................................................................8
   7.7 Interpretation of Results.........................................................................................................8
   7.8 Broth Microdilution Modifications........................................................................................10
   7.9 Trailing Growth and the Impact of Time of Reading ..............................................................12
   7.10 Other Modifications.............................................................................................................12
8 Quality Control .............................................................................................................................12
   8.1 Purpose ....................................................................................................................................12
   8.2 QC Responsibilities ................................................................................................................12
   8.3 Selecting Reference Strains ..................................................................................................13
   8.4 Storing Reference Strains .....................................................................................................13
   8.5 Routine Use of Reference Strains ........................................................................................14
   8.6 Batch of Medium and Lot of Plasticware Control .................................................................15
   8.7 QC Frequency ......................................................................................................................15
   8.8 Other Control Procedures ..................................................................................................16
   8.9 QC Strains ............................................................................................................................16

References..........................................................................................................................................17

Appendix A. RPMI 1640 Medium.....................................................................................................19
Appendix B. McFarland 0.5 Barium Sulfate Turbidity Standard ....................................................20
Summary of Delegate Comments and Committee Responses.......................................................21
The Quality Management System Approach..................................................................................24
Related CLSI Reference Materials ..................................................................................................25
Foreword

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

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

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

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

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

Key Words

antifungal, broth macrodilution, broth microdilution, susceptibility testing, yeasts
Updated Information in This Edition

Definitions (Section 4)

Modified definition:
Minimal inhibitory concentration (MIC)

Added definition:
Antimicrobial susceptibility test interpretive category
Quality control

Additional Section

Indications for performing susceptibility tests (Section 5)
Time of reading (Section 7.8.1)

Data Inclusion/Exclusion

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

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

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

Tables

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

Table 1: Interpretive Guidelines for In Vitro Susceptibility Testing of Candida spp.
Added new column on “nonsusceptible (NS)” criteria for interpretive guidelines.

Added breakpoints criteria for the following antifungal agents:
- Anidulafungin
- Caspofungin
- Micafungin
- Voriconazole (first added in M27-S2, published February 2006)

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

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

Added solvents and diluents recommendations for the following antifungal agents:
- Anidulafungin
- Caspofungin
- Micafungin

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

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

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

Added the following antifungal agents:
- Anidulafungin (first added in M27-S2, published February 2006)
- Caspofungin (first added in M27-S2, published February 2006)
- Micafungin

1 Scope

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

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

2 Introduction

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

M27-A3 is a “reference” standard developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of yeasts to antifungal agents. An important use of a reference method is to provide a standard basis from which other methods can be developed, which also will result in interlaboratory agreement within specified ranges. For example, broth microdilution methods, described in this document, have been configured to produce results paralleling those obtained by the broth macrodilution reference method. Such methods might have particular advantages, such as ease of performance, economy, or more rapid results; therefore, their development could be highly desirable. To the extent that any method produces concordant results with this reference method, it would be considered to be in conformity with M27-A3.

3 Standard Precautions

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

4 Definitions

**antibiogram** — overall profile of antimicrobial susceptibility results of a microbial species to a battery of antimicrobial agents.
Related CLSI Reference Materials*


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

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

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

M29-A3 Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005). Based on U.S. regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

M38-A Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard (2002). This document addresses the selection of antifungal agents; preparation of antifungal stock solutions and dilutions for testing; implementation and interpretation of test procedures; and quality control requirements for susceptibility testing of filamentous fungi (moulds) that cause invasive fungal infections.

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