CLINICAL AND LABORATORY STANDARDS INSTITUTE®

3rd Edition

M44

Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts

This guideline provides an established methodology for disk diffusion testing of *Candida* spp., along with recommendations for results interpretation and quality control testing.

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

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Replaces M44-A2

Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts

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Abstract

Clinical and Laboratory Standards Institute guideline M44—*Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts* provides an approved method for determining zone diameter breakpoints for select *Candida* spp. with antifungal agents after 24 hours incubation, as well as quality control parameters for the same agents. This guideline fulfills the need for an alternative to the broth microdilution testing procedure (see CLSI document M27[1]) and describes a simple, rapid, and cost-effective approach for determining fungal organisms' susceptibility to various classes of antifungal agents. It also makes antifungal susceptibility testing more readily available to the medical microbiology laboratory and encourages the development of disk diffusion testing for newly discovered antifungal agents.

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Foreword

Because of the increased incidence of systemic fungal infections and increasing number of antifungal agents available for systemic administration, antifungal susceptibility testing has become more common. Today, antifungal susceptibility testing is an important tool for guiding physicians in selecting antifungal therapy. Broth macrodilution and microdilution reference methods are now available for yeast (see CLSI document M27[1]) and mould (see CLSI document M38[2]) susceptibility testing. For antifungal susceptibility testing to become more readily available to medical microbiology laboratories, simple, rapid, and cost-effective alternative approaches are needed. The disk diffusion method used for antibacterial testing (see CLSI document M02[3]) has provided the basis for a simple method for susceptibility testing of yeasts. Therefore, the CLSI Subcommittee on Antifungal Susceptibility Tests has developed a disk diffusion method for testing susceptibility of yeasts to antifungal agents.

Currently, this method is validated only for select *Candida* spp. tested vs various azoles and echinocandins. This method provides qualitative results after 24 hours incubation. Using supplemented Mueller-Hinton agar in lieu of RPMI 1640 medium makes antifungal susceptibility testing more readily available and less costly to some medical laboratories. Zone diameter breakpoints for caspofungin, micafungin, fluconazole, and voriconazole and quality control parameters for caspofungin, micafungin, fluconazole, posaconazole, and voriconazole have been established according to standard CLSI procedures. CLSI expects that this guideline will encourage disk diffusion testing development for other antifungal agents and fungal genera.

Overview of Changes

This guideline replaces the previous edition of the approved guideline, M44-A2, published in 2009. Several changes were made in this edition, including:

- Reorganized to fit the CLSI quality management system and path of workflow format
- Revised definitions for interpretive categories to align with other CLSI susceptibility testing documents
- Added disk diffusion standards for micafungin
- Described the specific *Candida* spp. for which there are zone diameter breakpoints and interpretive categories
- Updated yeast nomenclature
- Updated references to the previous informational supplement (M44-S3) to reflect CLSI document M60,[4] the new supplement for broth dilution and disk diffusion yeast susceptibility testing

NOTE: The content of this guideline is supported by the CLSI consensus process and does not necessarily reflect the views of any single individual or organization.

Key Words

Antifungal agent, antimicrobial agent, *Candida* spp., disk, disk diffusion, Kirby-Bauer method, susceptibility testing, yeast

Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts

Chapter 1: Introduction

This chapter includes:

- Guideline's scope and applicable exclusions
- 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 guideline
- Abbreviations and acronyms used in the guideline

1.1 Scope

This guideline provides an established methodology for disk diffusion testing of select *Candida* spp. For zone diameter breakpoints, interpretive categories, and recommended QC ranges for caspofungin, micafungin, fluconazole, and voriconazole, refer to CLSI document M60.[4]

The method described is intended for testing select *Candida* spp. This method is not currently applicable to any other genera and has not been used in studies of the yeast form of dimorphic fungi (eg, *Blastomyces dermatitidis* or *Histoplasma capsulatum*). Moreover, testing of filamentous fungi (ie, moulds) is not covered in the current procedure.

The method described in this guideline must be followed exactly to obtain reproducible results. When new problems are recognized or improvements in these criteria are developed, changes will be incorporated into future editions of M44 and new breakpoint information will be distributed in periodic informational supplements (see CLSI document M60[4]).

This guideline is intended for use by, among others, health care, academic, government, industry, or independent research organizations that perform antifungal susceptibility testing of yeasts.

1.2 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. Published guidelines are available that discuss the daily operations of diagnostic medicine in humans and animals while encouraging a culture of safety in the laboratory.[5] 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.[6]

1.3 Terminology

1.3.1 A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization whenever 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 different countries and regions and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. CLSI recognizes its important role in these efforts, and its consensus process focuses on harmonization of terms to facilitate the global application of standards and guidelines.

NOTE: Current fungal taxonomy is being revised. Many genera have both a teleomorph (sexual state) and an anamorph (asexual state) name. In this guideline, the traditional *Candida* spp. names are used to provide continuity both with past procedures and associated documents such as CLSI document M60.[4]

Table 1 is provided to clarify the intended interpretations of the following terms.

Table 1. Common Terms or Phrases With Intended Interpretations

Term or Phrase	Intended Interpretation
"Needs to" or "must"	Explains an action directly related to fulfilling a regulatory and/or accreditation requirement or is indicative of a necessary step to ensure patient safety or proper fulfillment of a procedure
"Require"	Represents a statement that directly reflects a regulatory, accreditation, performance, product, or organizational requirement or a requirement or specification identified in an approved documentary standard
"Should"	Describes a recommendation provided in laboratory literature, a statement of good laboratory practice, or a suggestion for how to meet a requirement

1.3.2 Definitions

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

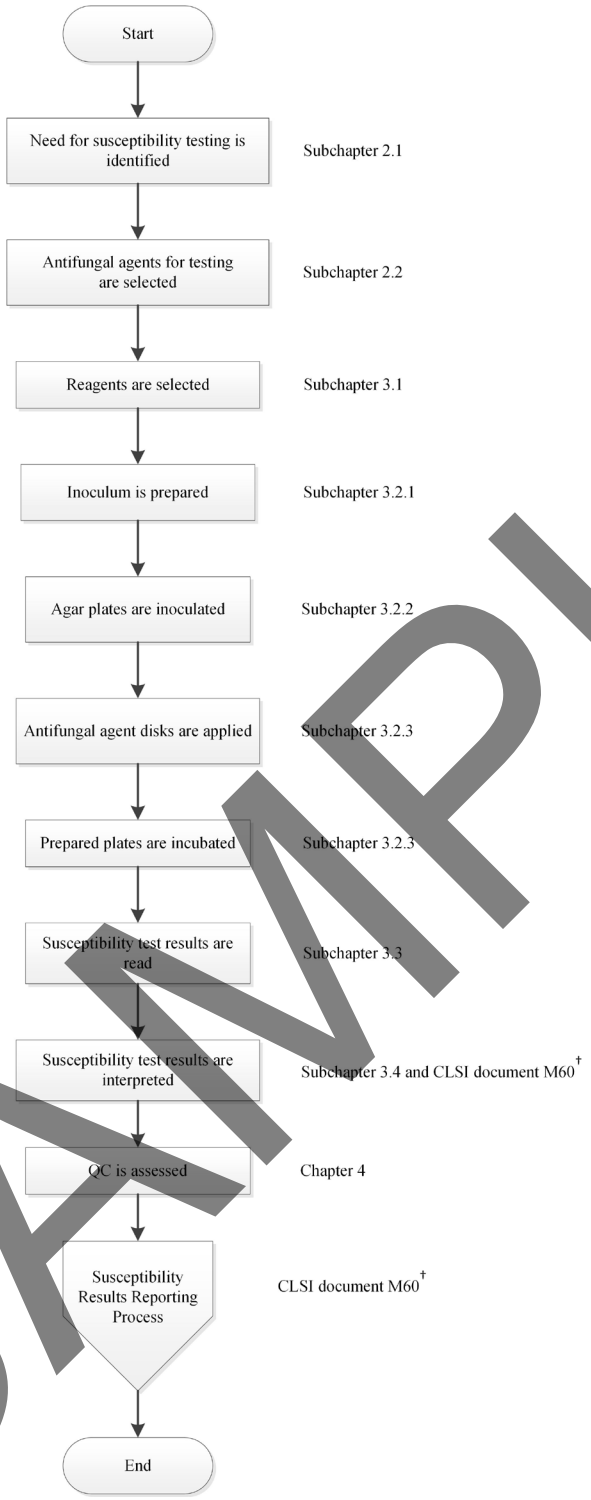
breakpoint – minimal inhibitory concentration (MIC) or zone diameter value used to categorize an organism as susceptible, susceptible-dose dependent, intermediate, or resistant; **NOTE 1:** MIC or zone diameter values generated by a susceptibility test can be interpreted based upon established breakpoints; **NOTE 2:** See **interpretive category.**

interpretive category – category derived from microbiological characteristics, pharmacokinetic-pharmacodynamic parameters, and clinical outcome data, when available; **NOTE 1:** Minimal inhibitory concentration (MIC) or zone diameter values generated by a susceptibility test can be interpreted based upon established breakpoints; **NOTE 2:** See **breakpoint.**

EXAMPLE:

Interpretive Category	Breakpoints*	
	MIC, µg/mL	Zone Diameter, mm
Susceptible	≤4	≥20
Susceptible-dose dependent	8–16	15–19
Intermediate	8–16	15–19
Resistant	≥32	≤14

* Formerly "interpretive criteria."



* Five basic symbols are used in process flow charts: oval (signifies the beginning or end of a process), arrow (connects process activities), box (designates process activities), diamond (includes a question with alternative "Yes" and "No" responses), pentagon (signifies another process).
† See CLSI document M60.[4]
Abbreviation: QC, quality control.

Figure 1. Antifungal Disk Diffusion Susceptibility Testing Process for Yeasts*

4.3 Selecting Reference Strains

Reference strains should be obtained from a source that can provide information on the culture's origin (eg, from commercial sources with documented culture history or from reference institutions with demonstrated ability to store and use organisms consistently with minimal contamination). A new stock culture should be obtained whenever a significant deviation from the expected result is observed. Recommended QC strains for antifungal susceptibility testing include:

- *C. albicans* ATCC® 90028
- *Candida parapsilosis* ATCC® 22019
- *Candida tropicalis* ATCC® 750
- *Candida krusei* ATCC® 6258

QC strains should be tested by the standard disk diffusion test procedure described in Chapter 3, using the same materials and methods used to test patient isolates.

4.4 Storing Reference Strains

4.4.1 Methods for Prolonged and Short-Term Storage

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

There are two preferred methods for prolonged reference strain storage. Yeasts may be grown on Sabouraud dextrose agar or potato dextrose agar and then frozen at −70°C on slants or plates.[20] Alternatively, reference strains can be preserved by suspending fungal cells in 15% glycerol solution in small vials and freezing and storing them at −70°C.[21]

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

4.4.2 Preparing Fungal Suspensions for Storage

The steps for preparing fungal suspensions for storage are listed below.

Step	Action	Comment
1.	Grow the organisms overnight on Sabouraud dextrose agar or potato dextrose agar plates.	
2.	Select growth from several colonies and perform the appropriate susceptibility tests to demonstrate that they produce the expected zone diameter results.	See CLSI document M60[4] for expected zone diameter of some reference strains.
3.	Subculture strains yielding expected results onto the same medium as the primary culture.	
4.	Incubate subcultures long enough for sufficient growth to occur.	Usually from 1–3 days.
5.	Examine the resulting growth carefully to ensure the culture is pure.	
6.	Suspend growth from the plate in 15% glycerol to make a heavy suspension if storing fungal suspensions.	If lyophilizing, suspend the growth in the appropriate medium.

