INTERNATIONAL STANDARD

ISO 16256

Second edition
2021-10

Clinical laboratory testing and in vitro diagnostic test systems — Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

Laboratoires d'analyses de biologie médicale et systèmes de diagnostic in vitro — Méthode de référence de microdilution en milieu liquide pour soumettre à essai l'activité in vitro des agents antimicrobiens par rapport aux levures impliquées dans les maladies infectieuses

Reference number
ISO 16256:2021(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 140, *In vitro diagnostic medical devices,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 16256:2012), which has been technically revised.

The main changes are as follows:

— addition of “broth micro-dilution” to the title;

— removal of 48 h reading for *Candida* species by the visual reading method;

— removal of definitions for susceptibility and resistance that are beyond the scope of this test performance document;

— inclusion of considerations for antifungal testing of yeast species with micro-dilution trays “treated” by manufacturers of the trays prior to use in the tests;

— updating of viable count testing methods for visual and spectrophotometer test pathways.

— addition of new antifungals (isavuconazole, rezafungin) to the testing and quality control range tables;

— detailed characterization of the components of one formulation of RPMI-1640 known to provide reproducible results of antifungal susceptibility tests for *Candida* species and *Cryptococcus neoformans*;

— reassigning of annexes;

— update of bibliography to more relevant information about performance of antifungal susceptibility testing for yeast fungi.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

In vitro susceptibility tests are performed on microorganisms suspected of causing disease, particularly if the organism is thought to belong to a species that can exhibit acquired resistance to frequently used antimicrobial agents. The tests are also important in resistance surveillance, epidemiological studies of susceptibility and in comparisons of new and existing agents.

Dilution procedures are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and represent the reference method for antifungal susceptibility testing. MIC methods are used in resistance surveillance, comparative testing of new agents for research or registration purposes, to establish the susceptibility of organisms that give equivocal results in routine tests, for tests with organisms where routine tests can be unreliable and when a quantitative result is needed for clinical management. In dilution tests, microorganisms are tested for their ability to produce discernible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing serial dilutions of the antimicrobial agent.

The lowest concentration of an antimicrobial agent (in mg/l) that, under defined in vitro test conditions, reduces visible or optically measurable growth of a microorganism within a defined period of time is known as the MIC. The MIC is a guide for the clinician to the susceptibility of the organism to the antimicrobial agent and aids treatment decisions. Careful control and standardization are required for intra- and inter-laboratory reproducibility, as results can be influenced by the method used. It is generally accepted that broth MIC tests are reproducible to within one doubling dilution of the true end point (i.e. ±1 well or tube in a doubling dilution series).

Broth dilution is a technique in which containers holding identical volumes of broth with antimicrobial agent solutions in incrementally (usually two-fold) increasing concentrations are inoculated with a known number of microorganisms.

Broth micro-dilution denotes the performance of the broth dilution test in micro-dilution trays.

The reference methods described in this document are intended for the testing of pure cultures of yeast fungi. The broth micro-dilution methods described in this document are the same as those described by the Clinical and Laboratory Standards Institute (CLSI)[1][5] and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[2][10]. These methods were initially shown to provide MICs of fluconazole that were similar, if not identical up to 2 mg/l[3]. Further the methods have been shown to provide MICs for two quality control strains of licensed antifungal agents that are similar as described in this document although quality control results for the spectrophotometer can trend slightly lower than for the visual reading method. The laboratory that wishes to use this document for conducting studies of newer antifungal agents, or as a reference method for comparison to MICs generated by a diagnostic device, can select which of the procedure options to use based upon the choice of MIC reading determined by visual inspection (CLSI method)[5] or by use of a spectrophotometer (EUCAST method)[2][10]. In either case, the procedural details for that option should be followed explicitly. In the first edition of this document, i.e. ISO 16256:2012, the reported quality control tests were performed using broth micro-dilution trays that were not treated in some way by the manufacturers of the plastic trays for either the visual or spectrophotometer method.

In this document the following verbal forms are used:

— “shall” indicates a requirement;

— “should” indicates a recommendation;

— ”may” indicates a permission;

— “can” indicates a possibility or a capability.

Clinical laboratory testing and in vitro diagnostic test systems — Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

WARNING — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

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. The reference method described here has not been used in studies of the yeast forms of dimorphic fungi, such as *Blastomyces dermatitidis* and/or *Histoplasma capsulatum* variety capsulatum. Moreover, testing filamentous fungi (moulds) introduces several additional problems in standardization not addressed by the current procedure. Those methods are beyond the scope of this document.

This document describes the broth micro-dilution reference method, which can be implemented by either of two pathways. One pathway involves visual determination of MICs (CLSI method)[1][5]; the second pathway involves spectrophotometric determination of MICs (EUCAST method)[2][10]. The MIC reflects the activity of the drug under the described test conditions and can be interpreted for clinical management purposes by taking into account other factors, such as drug pharmacology or antifungal resistance mechanisms. In addition, MIC distributions can be used to define wild type or non-wild type fungal populations. Clinical interpretation of the MIC value is beyond the scope of this document; interpretive category breakpoints specific to the CLSI- and EUCAST-derived methods can be found by consulting the latest interpretive tables provided by the organizations[5][15]. Routine susceptibility testing methods or diagnostic test devices can be compared with this reference method in order to ensure comparable and reliable results for validation or registration purposes.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
antifungal agent
substance of biological, semi-synthetic or synthetic origin that inhibits the growth of or kills fungi, and is thus of potential use in the treatment of infections

Note 1 to entry: Disinfectants, antiseptics and preservatives are not included in this definition.

3.2 Antifungal agents — properties

3.2.1
potency
active fraction of a test substance, determined in a bioassay against a reference powder of the same substance

Note 1 to entry: The potency is expressed as mass fraction in milligrams per gram (mg/g), or as activity content in International Units (IU) per gram, or as a volume fraction or mass fraction in percent, or as an amount-of-substance concentration (mass fraction) in mole per litre of ingredients in the test substance.

3.2.2
concentration
amount of an *antifungal agent* (3.1) in a specified volume of liquid

Note 1 to entry: The concentration is expressed as mg/l.

Note 2 to entry: mg/l = µg/ml but use of the unit µg/ml is not recommended.

3.3
stock solution
initial solution used for further dilutions

3.4
minimum inhibitory concentration
MIC
lowest *concentration* (3.2.2) that, under specified in vitro test conditions, reduces growth by an agreed amount within a specified period of time

Note 1 to entry: The MIC is expressed in mg/l.

3.5
wild type
absence of phenotypically-detectable acquired resistance mechanisms to the *antifungal agent* (3.1) in a given fungal strain

3.6
reference strain
catalogued, well-characterized fungal strain with stable, specified antifungal susceptibility phenotypes and/or genotypes

Note 1 to entry: Reference strains are kept as stock cultures, from which working cultures are derived. They are obtainable from culture collections and used for quality control.

3.7 Susceptibility testing method

3.7.1
broth dilution
technique in which containers are filled with appropriate volumes of an antifungal solution, employing incrementally (usually two-fold) increasing *concentrations* (3.2.2) of the *antifungal agent* (3.1) and appropriate volumes of *broth* (3.8) with a specified *inoculum* (3.9)

Note 1 to entry: The aim of this method is the determination of the *minimum inhibitory concentration* (3.4).

3.7.2
broth micro-dilution
performance of *broth dilution* (3.7.1) in micro-dilution trays with a capacity of ≤300 µl per well

3.8
broth
fluid medium used for the in vitro growth of yeast fungi

3.9
inoculum
number of colony-forming units of yeast in a suspension, calculated with respect to the final volume

Note 1 to entry: The inoculum is expressed as colony-forming units per millilitre (CFU/ml).

4 Test procedures

4.1 General

4.1.1 Trays and method

The tests are performed in plastic disposable micro-dilution trays. The method is based on the preparation of double strength antifungal agent working solutions in 100 µl volumes per well with the addition of an inoculum also in a volume of 100 µl.

4.1.2 Conditions for use of disposable micro-dilution trays

The tests were originally performed in broth micro-dilution trays that have had no additional treatment by the manufacturer. Quality control data by manufacturers of untreated trays (and on which this document was originally based) have shown that quality control results are consistently in specification for all antifungal agents tested. In some jurisdictions there has been a suggestion that results can be more consistent using treatment of the plastic trays. Treatment of the plastic, either by coating or corona discharge to impart an electrical charge to the plastic, is used in tissue culture studies and allows the tissue cells to adhere to the plastic. It is unknown if this process has been standardized for all micro-dilution tray manufacturers. It is known that with some antifungal agents the treated trays can result in elevated MICs compared to untreated trays. Such treatment can affect the reporting of results for those agents[13]. Those laboratories that use "treated" micro-dilution trays and read by spectrophotometer should ensure that the treated trays being utilized in testing provide the same quality control results as those indicated in Table 5. Those quality control ranges were originally performed with untreated trays. The data indicates that for almost all antifungal agents, the quality control ranges for the two standard strains listed in this document (*Candida parapsilosis* ATCC®[1)] 22019 and *Candida krusei* ATCC® 6258) are the within one log2 dilution for both testing/reading methods. Comparative quality control ranges for those strains for the spectrophotometer method are the same as originally reported using untreated trays[10] and for treated trays[2], with the exception of caspofungin (see Table 5). Comparative MIC observations for clinical isolates provided by the visual reading method[5] and those spectrophotometer readings using treated plates[2]for both testing methods should be interpreted with caution.

4.2 Medium

4.2.1 General

RPMI-1640 broth shall be used (see Table A.1 and Table A.2 for details for preparation of the two complete product versions of RPMI-1640 glucose broth) for both reading methods.

4.2.2 Visual reading pathway

The RPMI-1640 medium should contain 0,2 % glucose. The RPMI-1640 broth is prepared and dispensed at single strength with double strength antifungal agent dilutions and the inoculum is delivered in equal volumes of RPMI-1640 broth containing the adjusted yeast inoculum suspension.

1) ATCC is the registered trademark of a product supplied by the American Type Culture Collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.2.3 Spectrophotometric reading pathway

The RPMI-1640 medium should contain 2,0 % glucose. The RPMI-1640 broth and antifungal agents are both prepared at double strength with the inoculum subsequently added in an equal volume of sterile distilled water.

4.3 Antifungal agents

4.3.1 General

Antifungal agents shall be obtained directly from the manufacturer or from reliable commercial sources; pharmaceutical preparations for clinical use are not acceptable. The antifungal agents shall be supplied with a lot number, potency, an expiry date and details of recommended storage conditions. Substances shall be stored in tightly closed containers in the dark, at −20 °C, with a desiccant unless otherwise recommended by the manufacturer. Hygroscopic agents should be dispensed into aliquots, one of which is used on each test occasion.

Allow containers to warm to room temperature before opening them in order to avoid condensation and loss of potency.

4.3.2 Preparation of stock solutions

The use of a calibrated analytical balance is required for weighing antifungal agents. Allowance for the potency of the powder shall be made by use of Formulae (1) and (2) to obtain the amount of antifungal agent substance or the volume of diluent needed for a standard solution:

$$m = \frac{V \times \rho}{P} \tag{1}$$

$$V = \frac{m \times P}{\rho} \tag{2}$$

where

ρ is the concentration of the stock solution, in mg/l;

m is mass of the antifungal agent (powder), in g;

P is the potency of the antifungal agent (powder), in mg/g;

V is the volume of diluent, in l.

Concentrations of stock solutions should be 1 000 mg/l or greater, although the solubility of some agents is a limiting factor. The actual concentrations of stock solutions depend on the method of preparing working solutions (serial dilutions). Some agents require alternative solvents (see Table 1). Sterilization of solutions is not usually necessary. If required, sterilization should be done by membrane filtration and samples before and after sterilization should be compared by assay to ensure that adsorption has not occurred.

Unless information is available on stability of stock solutions under specified storage conditions, they should be prepared fresh for each test batch.