
Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices —

Part 1:

Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases

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Sensibilité in vitro des agents infectieux et évaluation des performances des dispositifs pour antibiogrammes —

Partie 1: Méthode de référence de microdilution en bouillon pour la détermination de la sensibilité in vitro aux agents antimicrobiens des bactéries aérobies à croissance rapide impliquées dans les maladies infectieuses



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Published in Switzerland

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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*.

This second edition cancels and replaces the first edition (ISO 20776-1:2006), which has been technically revised.

The main changes compared to the previous edition are as follows:

- revised to a broth micro-dilution only performance document;
- removal of S, I, R breakpoint definitions and information;
- moved embedded tables to annexes;
- removed quality control range table;
- updated table (now [Annex B](#)) on solvents and diluents for antimicrobial agents used globally;
- updated information on special culture media and method performance for specific currently used antimicrobial agents.

A list of all parts in the ISO 20776 series can be found on the ISO website.

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.

This corrected version of ISO 20776-1:2019 incorporates the following correction:

- Correction of the diluent pH value for ampicillin from 8,0 to 6,0 in [Annex B](#).

Introduction

In vitro antimicrobial susceptibility tests are performed on micro-organisms suspected of causing disease, particularly if the organism is thought to belong to a species that may exhibit 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 for antimicrobial susceptibility testing. MIC methods are used in resistance surveillance, defining identifying wild type phenotypes, comparative testing of new agents, to establish the susceptibility of organisms that give equivocal results in routine tests, for tests on organisms where routine tests may be unreliable and when a quantitative result is required for clinical management. In dilution tests, micro-organisms are tested for their ability to produce visible growth in broth (broth dilution) containing serial dilutions of the antimicrobial agent or on a series of agar plates (agar dilution).

The lowest concentration of an antimicrobial agent (in mg/l) that, under defined in vitro conditions, prevents the appearance of visible growth of a micro-organism 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 is required for intra- and inter-laboratory reproducibility of broth MIC tests. The MICs generally span two to three doubling dilutions with a dominant central value.

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

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

The method described in this document is intended for the testing of pure cultures of aerobic bacteria that are easily grown by overnight incubation on agar and grow well in standardized micro-dilution trays containing standardized Mueller-Hinton broth (volume of $\leq 200 \mu\text{l}$), which may need to be modified depending on the antimicrobial agent being tested.

The broth micro-dilution method described in this document is essentially the same as those used in many countries, and as the methods published by the Clinical and Laboratory Standards Institute (CLSI) [1] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [2]. These methods are based on those described by Ericsson and Sherris [3].

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WARNING — The use of this document may 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 to determine the applicability of any other restrictions prior to use.

1 Scope

This document describes one reference method, broth micro-dilution, for determination of MICs. The MIC can be a guide for the clinician, and reflects the activity of the drug under the described test conditions, by taking into account other factors, such as drug pharmacology, pharmacokinetics, or bacterial resistance mechanisms. This allows categorisation of bacteria as “susceptible” (S), “intermediate” (I), or “resistant” (R). In addition, MIC distributions can be used to define wild type or non-wild type bacterial populations. Although clinical interpretation of the MIC value is beyond the scope of this document, modifications of the basic method are required for certain antimicrobial agent - bacteria combinations to facilitate clinical interpretation. These modifications are included in a separate annex of this document. It is necessary to compare other susceptibility testing methods (e.g. disc diffusion or diagnostic test devices) with this reference method for validation, in order to ensure comparable and reliable results.

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 terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

antimicrobial agent

substance of biological, semi-synthetic or synthetic origin that inhibits the growth of or kills bacteria, 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

potency

measure of drug activity expressed in terms of the amount required to produce an effect of given intensity

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 (molar fraction) in mole per litre of ingredients in the test substance.

3.3

concentration

amount of an antimicrobial agent in a defined volume of liquid

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

Note 2 to entry: mg/l is the designated ISO unit.

3.4

stock solution

initial solution used for further dilutions

3.5

minimum inhibitory concentration

MIC

lowest concentration that, under defined in vitro conditions, prevents visible growth of bacteria within a defined period of time

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

3.6

breakpoint

BP

specific values of parameters, such as MICs, on the basis of which bacteria can be assigned to the clinical categories "susceptible", "intermediate" and "resistant"

Note 1 to entry: For current interpretive breakpoints, reference can be made to the latest publications of organisations employing this reference method (e.g. CLSI and EUCAST).

3.7

wild type

absence of known resistance mechanisms to the antimicrobial agent for a given strain

3.8

reference strain

catalogued, characterized bacteria with stable, defined antimicrobial 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.

3.9

broth dilution

technique in which containers are filled with appropriate volumes of an antimicrobial solution, employing incrementally (usually two-fold) increasing concentrations of the antimicrobial agent and appropriate volumes of broth with a defined inoculum

Note 1 to entry: The aim of this method is the determination of the MIC.

3.10

micro-dilution

performance of broth dilution in micro-dilution trays with a final test volume of $\leq 200 \mu\text{l}$ per well

3.11**broth**

fluid medium used for the in vitro growth of bacteria

Note 1 to entry: For the broth reference method the medium is standardised Mueller-Hinton broth (see [Annex A](#)).

3.12**inoculum**

number of bacteria 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).

3.13**inoculum effect**

change in MIC related to change in inoculum in CFU/ml

4 Test procedures**4.1 General**

The tests are performed in polystyrene micro-dilution trays. The method is based on the preparation of antimicrobial agent working solutions, either in 50 µl volumes per well (with the addition of an inoculum also in a volume of 50 µl), or in a volume of 100 µl per well (with the addition of a maximum of 10 µl inoculum volume).

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4.2 Medium

Mueller-Hinton broth shall be used (see [Annex A](#) for details and [Annex D](#) for special test situations).

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4.3 Antimicrobial agents

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4.3.1 General

Antimicrobial agents shall be obtained directly from the manufacturer or from reliable commercial sources; pharmaceutical preparations for clinical use are not acceptable. The antimicrobial agents shall be supplied as powders 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, with a desiccant at the recommended temperature of the supplier. Hygroscopic agents should be dispensed into aliquots, one of which is used on each test occasion.

To avoid condensation, allow containers to warm to room temperature before opening.

4.3.2 Preparation of stock solutions

The use of a calibrated analytical balance is required to weigh antimicrobial agents. Allowance for the potency of the powder shall be made by use of the following formula to obtain the amount of antimicrobial agent substance or the volume of diluent needed for a standard solution:

$$m = \frac{V \times \rho}{P} \quad (1)$$

$$V = \frac{m \times P}{\rho} \quad (2)$$

where

- ρ is the concentration of the stock solution, in mg/l;
- m is mass of the antimicrobial agent (powder), in g;
- P is the potency of the antimicrobial 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). Agents should be dissolved and diluted in sterile distilled water unless the manufacturer states otherwise. Some agents require alternative solvents (see [Annex B](#)).

NOTE For newer antimicrobial agents not identified in [Annex B](#) of this current document, consult manufacturer information on the most appropriate solvent and diluent for the specific agent. Sterilisation of solutions is not usually necessary. If required, sterilisation should be done by membrane filtration, and samples before and after sterilisation 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.

4.3.3 Preparation of working solutions

The range of concentrations selected for testing depends on the micro-organisms and antimicrobial agent. The chosen range shall allow full endpoint MIC determination for appropriate reference strains. A two-fold dilution series based on 1 mg/l is prepared in Mueller-Hinton broth. Dilutions should not be prepared by serial dilution steps, but according to the procedure outlined in [Annex C](#). Working solutions shall be used the same day unless information is available on stability of the solutions under specified storage conditions.

4.3.4 Preparation of micro-dilution trays

Working solutions are dispensed into micro-dilution trays at 50 μ l per well with double the desired final concentrations of antimicrobial agent, or at 100 μ l per well in the desired final concentrations.

At least one well, containing 50 μ l or 100 μ l of antimicrobial agent-free medium, should be included as a growth control for each strain tested. Likewise, a well containing 100 μ l of antimicrobial agent-free medium should be included as an un-inoculated negative control well for each micro-organism type tested.

4.3.5 Storage of micro-dilution trays

Filled trays may be used immediately or may be stored for up to three months or until documented quality control or other evidence indicates degradation of the antimicrobial agent. For storage the filled trays should be sealed in plastic bags and immediately placed in a freezer at ≤ -60 °C unless the antimicrobial agents are known to be stable at higher temperatures.

Trays shall not be stored in a self-defrosting freezer, and thawed antimicrobial solutions shall not be re-frozen, as repeated freeze-thaw cycles accelerate the degradation of some antimicrobial agents, particularly β -lactams.

Allow frozen plates to thaw for up to 2 h and inoculate by 4 h of removal from the freezer.

4.4 Preparation of inoculum

4.4.1 General

Standardization of the inoculum is essential for accurate and reproducible broth micro-dilution susceptibility tests. Therefore, purity checks and viable colony counts shall be performed on every isolate tested with this reference procedure

4.4.2 Broth culture method

The inoculum may be prepared by diluting a broth culture or by suspending several morphologically similar colonies (when possible) from an overnight culture on non-selective agar medium in broth with a sterile loop or swab. When suspending colonies, morphologically similar colonies should be picked to avoid contamination of other species or atypical variants of the same species.

The broth used shall not be antagonistic to the antimicrobial agent tested. The broth is incubated at $(35 \pm 1)^\circ\text{C}$ until the growth reaches a turbidity equal to or greater than that of a 0,5 McFarland standard^[4]. If needed, the culture is adjusted with saline or broth to give a turbidity equivalent to the 0,5 McFarland standard. This can be done by means of a photometric device (using 625 nm wavelength and a 1 cm path cuvette, the absorbance will be in the range of 0,08 to 0,13), or by employing a suitably calibrated nephelometer. Alternatively, this can be achieved visually by comparing the appearance of black lines through the inoculum and 0,5 McFarland standard suspensions (the inoculum and McFarland standard shall be in tubes of the same size) or any other method that gives reproducible CFU/ml. The final inoculum shall be 5×10^5 CFU/ml (target range, 2×10^5 CFU/ml to 8×10^5 CFU/ml).

NOTE A 0,5 McFarland standard can be produced by adding a 0,5 ml aliquot of 0,048 mol/l barium chloride (BaCl_2) (11,72 g/l $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) to 99,5 ml of 0,18 mol/l sulphuric acid (H_2SO_4), with constant stirring to maintain a suspension.

4.4.3 Direct colony suspension method

Several morphologically similar colonies from a non-selective nutritive agar medium, incubated at $35 \pm 1^\circ\text{C}$ for 18 h to 24 h, unless longer incubation is required, are touched with a sterile loop and the growth transferred to sterile broth or saline. The suspension is adjusted to give a turbidity equivalent to that of a 0,5 McFarland standard, as described in 4.4.2 for the broth culture method.

For all micro-organisms, the number of viable cells (in CFU/ml) in the final inoculum depends on the growth phase of the culture. This effect is most pronounced for fastidious organisms such as *Streptococcus pneumoniae*, where use of older cultures can significantly reduce the number of viable cells in the suspension.

A correctly adjusted suspension prepared by either method contains approximately 1×10^8 CFU/ml to 2×10^8 CFU/ml for the common relevant bacteria.

The adjusted inoculum prepared as above is diluted in broth to give a final cell number of 5×10^5 CFU/ml (target range 2×10^5 CFU/ml to 8×10^5 CFU/ml). The dilution required depends upon the bacterial species being tested and the method used for inoculum delivery. Transfer of 0,1 ml of standardized micro-organism suspension to a tube containing 9,9 ml (1:100 dilution) of broth results in a suspension of 1×10^6 CFU/ml which, when 50 μl is added to an equal volume (50 μl) of antimicrobial agent solution, results in a final inoculum of 5×10^5 CFU/ml with many Gram-negative bacteria (e.g. *Escherichia coli*). If the wells already contain 100 μl of antimicrobial agents in broth, an appropriate dilution to give the required final inoculum should be prepared prior to addition of up to 10 μl of the diluted suspension to each well. Different dilutions of the 0,5 McFarland suspension may be necessary, as determined by colony counts in preliminary tests^[5].

4.5 Inoculation of micro-dilution trays

The trays shall be inoculated within 30 min of standardizing the inoculum suspension, in order to maintain viable cell number in CFU/ml. To each well containing 50 μl of diluted antimicrobial agent in