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**Clinical laboratory testing — Criteria  
for acceptable lots of dehydrated  
Mueller-Hinton agar and broth for  
antimicrobial susceptibility testing**

*Détermination de la sensibilité aux antibiotiques — Critères  
d'acceptabilité pour les lots d'agar déshydraté et de bouillon Mueller-  
Hinton pour déterminer la sensibilité aux antibiotiques*

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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](http://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](http://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 on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

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## Introduction

Historically, although various media have been recommended for susceptibility testing, Mueller-Hinton broth (MHB) has been selected as the medium for the reference broth microdilution minimum inhibitory concentration (MIC) method (ISO 20776-1) and Mueller-Hinton agar (MHA) is most widely used for disc diffusion testing of rapidly growing bacteria. Mueller-Hinton medium provides satisfactory growth of most non-fastidious pathogens, acceptable batch-to-batch reproducibility, low sulfonamide, trimethoprim, and tetracycline inhibitors and a large amount of data has been collected from antimicrobial susceptibility tests with this medium over several decades. This International Standard is the result of an effort to establish a standard description and protocol by which manufacturers of dehydrated Mueller-Hinton agar (dMHA) and broth (dMHB) may determine its acceptable performance characteristics. This International Standard describes methods for evaluation of production lots of the dehydrated product. Performance characteristics of production lots of agar and broth prepared with the dehydrated product are determined by testing defined microorganism-antimicrobial agent combinations. The results of testing conform to defined quality control limit ranges for each combination of antimicrobial agent and quality control strains. Each production lot is tested at least against these combinations of antimicrobial agents and quality control strains. This International Standard does not address supplements (e.g. blood or blood products) that are added to the medium to support growth of fastidious bacteria.<sup>[1][2][3][4]</sup> Those additives are provided after the dehydrated medium is prepared in its liquid state as a final product and fall outside of the scope of this International Standard. Although dMHA can be used for determination of MICs using the agar dilution method<sup>[2][4]</sup> or the gradient diffusion method, this International Standard only includes performance testing of dMHA using disc diffusion methodology as described by the Clinical and Laboratory Standards Institute (CLSI)<sup>[3]</sup> and European Committee on Antimicrobial Susceptibility Testing (EUCAST).<sup>[1]</sup> Manufacturers may choose to test additional antimicrobial agents and strains, as well as Mueller-Hinton media supplemented for growth of fastidious strains. This is at the discretion of manufacturers but expected performance limits must be validated appropriately.

This International Standard has been developed in part based upon two previous Clinical and Laboratory Standards Institute documents, CLSI M6-A2 (protocols for evaluating dehydrated Mueller-Hinton agar) and CLSI M32-P (evaluation of lots of dehydrated Mueller-Hinton broth for antimicrobial susceptibility testing) with permission. This International Standard supersedes and replaces the previous CLSI publications.



# Clinical laboratory testing — Criteria for acceptable lots of dehydrated Mueller-Hinton agar and broth for antimicrobial susceptibility testing

## 1 Scope

This International Standard provides a standard description of the physical properties of dehydrated Mueller-Hinton broth (dMHB) and Mueller-Hinton agar (dMHA) and performance criteria by which manufacturers can assess the performance characteristics of their production lots of dMHA and dMHB. Production lots of broth or agar can then be utilized by all users, including *in vitro* susceptibility testing device manufacturers, as the test medium for performance of antimicrobial susceptibility testing.

## 2 Normative reference

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 20776-1:2006, *Clinical laboratory testing and in vitro diagnostic test systems — Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices — Part 1: Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases*

## 3 Terms and definitions

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

### 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.

[SOURCE: ISO 20776-1:2006, 2.1]

### 3.2

#### **antimicrobial disc**

small paper disc containing known amounts of antimicrobial agents used for *in vitro* susceptibility testing

### 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 = µg/ml but it is not recommended to use the unit µg/ml.

[SOURCE: ISO 20776-1:2006, 2.2.2]

### 3.4

#### **stock solution**

initial solution used for further dilutions

[SOURCE: ISO 20776-1:2006, 2.3]

### 3.5

#### **minimum inhibitory concentration**

##### **MIC**

lowest concentration of antimicrobial agent 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.

[SOURCE: ISO 20776-1:2006, 2.4, modified — “lowest concentration that” has been modified to “lowest concentration of antimicrobial agent that”.]

### 3.6

#### **reference strain**

catalogued, characterized microorganism with stable, defined antimicrobial susceptibility phenotype and/or genotype

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

[SOURCE: ISO 20776-1:2006, 2.7, modified — “characterized bacteria” has been modified to “characterized microorganism” and “culture collections” in Note 1 to entry has been modified to “recognized national culture collections”.]

### 3.7 Susceptibility testing method

#### 3.7.1

##### **broth dilution**

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

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

[SOURCE: ISO 20776-1:2006, 2.8.1, modified — “an antimicrobial solution, employing incrementally (usually two-fold) increasing concentrations of the antimicrobial agent and appropriate volumes of broth with” has been modified to “broth containing an antimicrobial agent in incrementally (usually two-fold) increasing concentrations and”.]

#### 3.7.2

##### **microdilution**

performance of broth dilution in microdilution trays with a capacity of 200 µl per well

[SOURCE: ISO 20776-1:2006, 2.8.2, modified — “a capacity of ≤200 µl per well” has been modified to “a capacity of 200 µl per well”.]

#### 3.7.3

##### **disc diffusion**

technique in which antimicrobial discs are applied to the surface of an agar medium that has been evenly inoculated with a defined inoculum and, following incubation under defined conditions, the resulting size of zones of growth inhibition of the microorganism corresponds to the susceptibility/resistance of the microorganism to the antimicrobial agent

#### 3.7.4

##### **zone diameter**

diameter (in mm) of the zone of growth inhibition around a paper disc containing an antimicrobial agent of specified amount used in a disc diffusion test



**3.8****broth**

liquid medium used for the *in vitro* growth of bacteria

[SOURCE: ISO 20776-1:2006, 2.9, modified — “fluid medium” has been modified to “liquid medium”.]

**3.9****inoculum**

number of viable 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).

[SOURCE: ISO 20776-1:2006, 2.10, modified — “number of bacteria” has been modified to “number of viable bacteria”.]

**3.10****dehydrated Mueller-Hinton broth****dMHB**

dried bacteriological medium which is used to prepare liquid medium for broth dilution antimicrobial susceptibility tests

**3.11****dehydrated Mueller-Hinton agar****dMHA**

dried bacteriological medium which is used to prepare antimicrobial susceptibility testing agar plates for disc diffusion, gradient diffusion MIC and agar dilution MIC methods

**4 Requirements for Mueller-Hinton broth****4.1 Components of Mueller-Hinton broth**

Historically, Mueller-Hinton broth medium for antimicrobial susceptibility testing contains approximately the following components per litre of purified water (adjustments may be needed to meet performance criteria):<sup>[5]</sup>

- dehydrated infusion from 300 g beef (i.e. 2 g of beef extract powder);
- acid digest of casein 17,5 g;
- starch 1,5 g.

**4.2 Physical and chemical characteristics****4.2.1 Dehydrated powder or granules**

Colour - beige to light beige

Uniform, free-flowing, homogeneous and free of extraneous material

**4.2.2 Prepared broth medium**

Once hydrated, the final pH measured after autoclaving shall be 7,2 to 7,4 at 25 °C.

The liquid is light straw coloured and clear with no visible precipitate.

**4.2.3 Cation supplementation and content for MHB**

The broth shall contain sufficient concentrations of cations to provide adequate growth and to permit the user to determine MIC values (e.g. aminoglycosides and quinolones) for quality control strains

within ranges identified in ISO 20776-1:2006, Table 4 (check the latest version of CLSI and EUCAST documents for QC ranges). New lots of MHB may require testing for acceptable cation content. For standard production lots of dMHB, the broth prepared from the dehydrated product shall contain no greater than 25 mg/l of total calcium and 12,5 mg/l of total magnesium. Manufacturers may choose to provide commercial lots of dMHB with required concentrations of cations or actual levels less than 20 mg/l of calcium and 10 mg/l of magnesium. In the latter case, the final label shall specify the actual amounts contained in the lot of broth. For final testing, the prepared MHB shall contain 20 mg/l to 25 mg/l of Ca<sup>2+</sup> and 10 mg/l to 12,5 mg/l of Mg<sup>2+</sup>.

While trace amounts of manganese are required for growth, the concentration shall be below 8 mg/l to avoid false resistant interpretations with glycylicyclines.<sup>[6]</sup> This shall be determined by an MIC value within the acceptable range obtained by testing *Escherichia coli* WDCM 00013 with tigecycline.

While trace amounts of zinc are required for growth, the concentration of zinc shall be below 3 mg/l to avoid false resistance interpretations with imipenem<sup>[7]</sup> and potentially with other carbapenems. This shall be determined by an MIC value within the acceptable range obtained by testing *Pseudomonas aeruginosa* WDCM 00025 with imipenem.

Cation concentrations of calcium, magnesium, manganese, and zinc shall be determined by inductively coupled plasma mass spectrometry (ICP-MS) or flame atomic absorption spectroscopy (FAAS).<sup>[8]</sup>

Although ion effects known to affect susceptibility test results for other antimicrobial agents are not included in this International Standard, they shall be considered for MHB dilution susceptibility tests by manufacturers at their discretion. Affected agents include daptomycin<sup>[9]</sup> and polymyxin.<sup>[10]</sup> When testing daptomycin, MHB shall be supplemented to a final concentration of 50 mg/l total Ca<sup>2+</sup>. Refer to ISO 20776-1 for appropriate instructions on preparation of media and antimicrobial susceptibility testing.

#### 4.2.4 Other medium components

The medium shall have a thymidine mass concentration of less than 0,03 mg/l as indicated by an MIC value of ≤0,5/9,5 mg/l obtained by testing *Enterococcus faecalis* WDCM 00087 with trimethoprim-sulfamethoxazole.<sup>[11]</sup>

#### 4.2.5 Specific adjustments required by the manufacturer

For antimicrobial agents included in [Table 1](#):

- a) incorporation of sodium chloride (2 % w/v NaCl) at a final concentration of 20 g/l in the broth is required for the detection of methicillin resistance in *Staphylococcus* spp. when testing with oxacillin;
- b) for broth microdilution testing of tigecycline, when MIC panels are prepared, the medium shall be prepared fresh on the day of use. The medium shall be no more than 12 h old at the time the panels are made; however, the panels may then be frozen for later use. For further details, refer to ISO 20776-1.

For organisms not included in [Table 1](#) (i.e. for extended testing at the discretion of the manufacturer):

- a) testing of fastidious organisms such as streptococci and *Haemophilus* spp. requires the addition of growth supplements (for example, blood or blood components). If a Mueller-Hinton agar or broth lot that is found to perform acceptably according to the criteria in this International Standard is to be used for testing fastidious organisms, the resulting MICs or zone diameters after addition of supplements shall fall within the acceptable quality control ranges published in 20776-1 for the specific medium and organism tested.

See [A.1](#) for a summary of specific effects on antimicrobial agents. Organisms/antimicrobial agents not specified may be tested by the manufacturer at their discretion.