
**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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Contents

	Page
Foreword	iv
Introduction	v
1 Scope	1
2 Normative reference	1
3 Terms and definitions	1
4 Requirements for Mueller-Hinton broth	3
4.1 Components of Mueller-Hinton broth.....	3
4.2 Physical and chemical characteristics.....	3
4.2.1 Dehydrated powder or granules.....	3
4.2.2 Prepared broth medium.....	4
4.2.3 Cation supplementation and content for MHB.....	4
4.2.4 Other medium components.....	4
4.2.5 Specific adjustments required by the manufacturer.....	4
4.3 Manufacturers protocol for testing production lots of dehydrated Mueller-Hinton broth.....	5
4.4 Interpreting the results.....	5
4.5 Evaluating the results.....	6
5 Requirements for Muller-Hinton agar	6
5.1 Components of Mueller-Hinton agar.....	6
5.2 Physical and chemical characteristics.....	6
5.2.1 Dehydrated powder or granules.....	6
5.2.2 Prepared agar medium.....	7
5.2.3 Cation supplementation and content for MHA.....	7
5.2.4 Other medium components.....	7
5.2.5 Specific adjustments required by the manufacturer.....	7
5.3 Manufacturer's protocol for testing production lots of dehydrated Mueller-Hinton agar.....	8
5.4 Interpreting the results.....	8
5.5 Evaluating the results.....	10
6 Testing new antimicrobial agents with production lots of dehydrated Mueller-Hinton broth or agar	11
Annex A (informative) Mueller-Hinton medium	12
Annex B (informative) Preparing control cultures	14
Annex C (informative) Suggested data sheet for testing of production lots	16
Annex D (informative) Label statement	19
Bibliography	20

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing Technical Specifications 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).

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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*.

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.

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 Technical Specification has been developed in part based upon two Clinical and Laboratory Standards Institute (CLSI) documents, CLSI M6-A2^[1] (protocols for evaluating dehydrated Mueller-Hinton agar) and CLSI M32-P^[2] (evaluation of lots of dehydrated Mueller-Hinton broth for antimicrobial susceptibility testing) with permission. Upon publication of ISO 16782, CLSI documents M6-A2^[1] and M32-P^[2] will no longer be available. Manufacturers can follow ISO 16782 to assess the performance characteristics of their production lots of dMHA and dMHB.

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

1 Scope

This Technical Specification 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.

This Technical Specification does not address supplements (e.g. blood or blood products) that are added to the medium to support growth of fastidious bacteria^{[3][4][5][6]}. 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 Technical Specification. Although dMHA can be used for determination of MICs using the agar dilution method^{[4][6]} or the gradient diffusion method, this Technical Specification only includes performance testing of dMHA using disc diffusion methodology as described by the Clinical and Laboratory Standards Institute (CLSI)^[5] and European Committee on Antimicrobial Susceptibility Testing (EUCAST)^[3].

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*

CLSI M100, *Performance Standards for Antimicrobial Susceptibility Testing; Informational Supplement*

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)[7]:

- 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 Ca²⁺ and 10 mg/l to 12,5 mg/l of Mg²⁺.

While trace amounts of manganese are required for growth, the concentration shall be below 8 mg/l to avoid false resistant interpretations with glycylicyclines^[8]. 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^[9] 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)^[10].

Although ion effects known to affect susceptibility test results for other antimicrobial agents are not included in this Technical Specification, they shall be considered for MHB dilution susceptibility tests by manufacturers at their discretion. Affected agents include daptomycin^[11] and polymyxin^[12]. When testing daptomycin, MHB shall be supplemented to a final concentration of 50 mg/l total Ca²⁺. 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^[13].

4.2.5 Specific adjustments required by the manufacturer

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

- a) incorporation of sodium chloride (2 % *m/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.

Manufacturers may choose to test additional antimicrobial agents and strains, as well as Mueller-Hinton media supplemented for growth of fastidious strains. The expected performance limits shall be validated.

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

- c) 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 Technical Specification 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.

4.3 Manufacturers protocol for testing production lots of dehydrated Mueller-Hinton broth

Procedures for preparing microdilution trays and performing the test are described in ISO 20776-1. Those procedures shall be followed with restrictions noted below.

- a) The minimum and maximum concentration of each antimicrobial agent on each tray shall bracket the quality control limit range by at least two doubling dilutions beyond each limit.
- b) As a minimum, test a single microbial inoculum in three separate trays for each of the microorganism-antimicrobial combinations listed in [4.4](#). This list of microorganism-antimicrobial agent combinations represents the minimum requirements for testing and includes agents likely to detect particular problems with the medium. Other antimicrobial agents may be tested at the manufacturer's discretion as needed to ensure consistent performance of the medium. The medium shall be appropriate for the antimicrobial agents tested.
- c) See ISO 20776-1, CLSI^[6] or EUCAST^[4] for specific details of quality control strain maintenance. At least two days before testing, thaw a vial of each of the control cultures that will be needed (see [4.4](#)). Inoculate each culture onto a plate of non-selective nutritive agar medium and incubate it for 18 h to 24 h at 34 °C to 37 °C in ambient air as described in ISO 20776-1. After incubation, check for purity. The day before the inoculation of the test plates, subculture again to provide fresh colonies for inoculum preparation. All microorganisms shall be subcultured at least twice from the frozen state before being used for testing.
- d) If frozen trays are used, they shall be allowed to thaw completely at ambient room temperature (usually takes 1 h to 2 h) before use. Trays shall be used on the same day that they are thawed.
- e) Tests shall be set up as described in ISO 20776-1. A single inoculum for each quality control strain shall be prepared using the colony suspension method. Inoculated microdilution trays should be incubated for 16 h to 20 h (24 h for oxacillin with *Staphylococcus aureus*) and read within one hour of removal from the incubator.
- f) Results shall be recorded and maintained according to the manufacturer's policies for record retention. A suggested data sheet for this purpose is shown in [Annex C](#).

4.4 Interpreting the results

The acceptable MIC ranges in [Table 1](#) were obtained with permission from CLSI^[14] and EUCAST [http://www.eucast.org/ast_of_bacteria/qc_tables/]^[15].

The acceptable ranges are subject to revision. Therefore the latest version of Reference [\[14\]](#) or the EUCAST Tables shall be checked for possible updates.

See [Annex B](#) for alternative numbers for the same control microorganism from different culture collections.

Table 1 — MIC ranges (mg/l) for control strains

Quality control strain	Antimicrobial agent	Acceptable range mg/l
<i>Pseudomonas aeruginosa</i> WDCM 00025	Ciprofloxacin	0,25–1
	Gentamicin	0,5–2
	Imipenem	1–4
	Piperacillin-tazobactam	1/4–8/4
<i>Escherichia coli</i> WDCM 00013	Ampicillin	2–8
	Cefotaxime	0,03–0,12
	Tigecycline	0,03–0,25
<i>Staphylococcus aureus</i> WDCM 00131	Clindamycin	0,06–0,25
	Erythromycin	0,25–1
	Oxacillin	0,12–0,5
	Tetracycline	0,12–1
	Vancomycin	0,5–2
<i>Enterococcus faecalis</i> WDCM 00087	Ampicillin	0,5–2
	Trimethoprim- sulfamethoxazole	≤0,5/9,5 ^a
	Vancomycin	1–4
<i>Staphylococcus aureus</i> WDCM 00211	Oxacillin	4–32

^a CLSI or EUCAST has not yet established a control range for trimethoprim-sulfamethoxazole. The MIC results for trimethoprim-sulfamethoxazole shall be ≤0,5/9,5 mg/l.

4.5 Evaluating the results

If all performance criteria for all microorganism-antimicrobial agent combinations are within acceptable ranges listed in 4.4 and all physical and chemical characteristics are met (see 4.2), the manufacturer may apply the label statement given in Annex D. Manufacturers should attempt to achieve mean MIC values close to the midpoint of the control ranges. Data shall be maintained on file and results made available to anyone upon request.

5 Requirements for Muller-Hinton agar

5.1 Components of Mueller-Hinton agar

Historically, Mueller-Hinton agar medium for antimicrobial susceptibility testing contains approximately (adjustment may be needed to meet performance criteria) the following components per litre of purified water^[2]:

- 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;
- agar 17 g.

5.2 Physical and chemical characteristics

5.2.1 Dehydrated powder or granules

Colour: beige to light beige.

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

5.2.2 Prepared agar medium

The final pH measured after autoclaving and gelling shall be 7,2 to 7,4 at 25 °C.

The gelled medium is light straw coloured and slightly opalescent. The depth of medium in the plate shall be uniform and within the range of 3,5 mm to 5,0 mm [EUCAST specifies 4 mm $\pm$ 0,5 mm and CLSI either approximately 4 mm^[6] or 4 mm to 5 mm (CLSI document M6^[1])]. Plates from different sources might differ in diameter (measured internally at the base of the plate) and the agar volume required to provide the specified depth is calculated from the formula “3,143 $\times$ plate radius (cm) squared $\times$ depth (cm)”. Hence, the acceptable range of depth of the medium for 90 mm, 100 mm and 150 mm internal diameter round plates is achieved with volumes of 23 ml to 31 ml, 28 ml to 39 ml, and 62 ml to 88 ml, respectively. For other plate sizes, the volume of medium required shall be calculated.

5.2.3 Cation supplementation and content for MHA

The agar shall contain sufficient concentrations of cations to provide adequate growth and to permit the user to determine zone diameters for quality control strains within ranges identified in [5.4](#).

The medium shall have Ca²⁺ and Mg²⁺ cation concentrations such as to provide zone diameter values within expected range for *Pseudomonas aeruginosa* vs aminoglycoside class of antimicrobial agents as shown by zone diameter with gentamicin and *Pseudomonas aeruginosa* WDCM 00025 within the acceptable range.

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

To avoid false resistant interpretations when testing carbapenems, the medium shall have a zinc concentration below 3 mg/l, as shown by zone diameter with imipenem and *Pseudomonas aeruginosa* WDCM 00025 within the acceptable range. The effect of excess zinc concentration is known to be true for imipenem and may apply to other carbapenems.

5.2.4 Other medium components

The medium shall have a thymidine mass concentration of less than 0,03 mg/l as shown by the clarity and zone of inhibition with trimethoprim-sulfamethoxazole of ≥ 20 mm for *Enterococcus faecalis* WDCM 00210 or by a zone diameter within the acceptable range obtained by testing *Enterococcus faecalis* WDCM 00087.

Consistent and adequate gel strength is required for reproducible zone sizes that meet quality control specifications.

5.2.5 Specific adjustments required by the manufacturer

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 Technical Specification 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 CLSI^[5] or EUCAST^[3] documents for the specific medium and organism tested.

See [A.2](#) for a summary of specific effects on antimicrobial agents. Organisms/antimicrobial agents not specified may be tested by the manufacturer at their discretion.

5.3 Manufacturer's protocol for testing production lots of dehydrated Mueller-Hinton agar

The disc diffusion method shall be used to evaluate production lots of dMHA. CLSI^[5] and EUCAST^[3] describe the procedures for preparing plates, performing and reading the test, and strain maintenance. Those procedures shall be followed with the restrictions noted below.

- a) As a minimum, test a single inoculum on three separate plates for each microorganism-antimicrobial agent combinations listed in [5.4](#). This list of microorganism-antimicrobial agent combinations represents the minimum requirements for testing and includes agents likely to detect particular problems with the medium. Other antimicrobial agents may be tested at the manufacturer's discretion as needed to ensure consistent performance of the medium. The medium shall be appropriate for the antimicrobial agents to be tested.
- b) At least two days before the inoculation of the test plates, thaw a vial of each of the control cultures that will be needed (see below and [Annex C](#)). Inoculate each culture onto a plate of non-selective nutritive agar medium and incubate it for 18 h to 24 h at $35\text{ °C} \pm 2\text{ °C}$ (CLSI) or $35\text{ °C} \pm 1\text{ °C}$ (EUCAST) in ambient air. After incubation, check for purity. The day before the inoculation of the test plates, subculture should be repeated. All microorganisms shall be subcultured at least twice from the frozen state before being used for testing.
- c) A single inoculum for each quality control strain shall be prepared using the colony suspension method as described in the most recent version of CLSI or EUCAST documents.
- d) Inoculate the three replicate plates of each medium with the standardized inoculum of each of the control cultures. Inoculate plates within 15 min after adjusting the inoculum suspension.
- f) After incubation at the appropriate temperature [i.e. $35\text{ °C} \pm 2\text{ °C}$ (CLSI) or $35\text{ °C} \pm 1\text{ °C}$ (EUCAST)] and for the appropriate time (i.e. 16 h to 20 h), remove plates from the incubator and read within one hour. For enterococci with vancomycin, incubation time shall be increased to 24 h.
- g) Results shall be recorded and maintained according to the manufacturer's policies for record retention. A suggested data sheet for this purpose is shown in [Annex C](#).

5.4 Interpreting the results

See [Annex B](#) for alternative numbers for the same control microorganism from different culture collections.

Table 2 — Disc diffusion ranges (in mm) for control strains

Quality control strain	Disc content µg	Antimicrobial agent	Acceptable range mm
<i>Staphylococcus aureus</i> WDCM 00034 ^a	20/10	Amoxicillin-clavulanate	28–36
	10/10	Ampicillin-sulbactam	29–37
	30	Cefoxitin	23–29
	5	Ciprofloxacin	22–30
	15	Erythromycin	22–30
	10	Gentamicin	19–27
	30	Linezolid	25–32
	10 units	Penicillin	26–37
	30	Tetracycline	24–30
<i>Staphylococcus aureus</i> WDCM 00131 ^{a,b}	1 unit	Penicillin (benzylpenicillin)	12–18
	30	Cefoxitin	24–30
	5	Ciprofloxacin	21–27
	15	Erythromycin	23–29
	10	Gentamicin	19–25
	10	Linezolid	21–27
	30	Tetracycline	23–31
<i>Enterococcus faecalis</i> WDCM 00087 ^c	2	Ampicillin	15–21
	10	Imipenem	24–30
	10	Linezolid	19–25
	100	Nitrofurantoin	18–24
	5	Trimethoprim	24–32
	1,25–23,75	Trimethoprim-sulfamethoxazole	26–34
	5	Vancomycin	10–16

^a With *S. aureus* WDCM 00034, use linezolid 30 µg discs and penicillin 10 unit discs. With *S. aureus* WDCM 00131, use linezolid 10 µg discs and penicillin (benzylpenicillin) 1 unit discs.

^b Disc diffusion ranges from EUCAST control range tables^[3]. Check the latest version from EUCAST <http://www.eucast.org> for updated ranges as they are subject to periodic updates. *S. aureus* WDCM 00131 is an alternative quality control microorganism to *S. aureus* WDCM 00034 for disc diffusion testing for the antimicrobial agents in common. *S. aureus* WDCM 00034 shall be tested with antimicrobial agents for which no acceptable range for WDCM 00131 is available. Cefoxitin is replacing oxacillin as the surrogate for detection of methicillin-resistant *Staphylococcus aureus* (MRSA). Oxacillin disc diffusion testing is no longer recommended. Refer to CLSI^[5] or EUCAST^[3] documents.

^c The zone of inhibition for trimethoprim-sulfamethoxazole shall be ≥20 mm.

^d The zone of inhibition for cefoxitin (CLSI^[3]) shall be ≤21 mm.

Table 2 (continued)

Quality control strain	Disc content µg	Antimicrobial agent	Acceptable range mm
<i>Escherichia coli</i> WDCM 00013	20/10	Amoxicillin-clavulanate	18–24
	10	Ampicillin	15–22
	30 or 5	Cefotaxime	29–35 25–31
	30	Chloramphenicol	21–27
	5	Ciprofloxacin	30–40
	10	Gentamicin	19–26
	250 or 300	Sulfisoxazole	15–23
	30	Tetracycline	18–25
	15	Tigecycline	20–27
	1,25/23,75	Trimethoprim-sulfamethoxazole	23–29
<i>Pseudomonas aeruginosa</i> WDCM 00025	30	Aztreonam	23–29
	10 or 30	Ceftazidime	21–27 22–29
	5	Ciprofloxacin	25–33
	10	Gentamicin	17–23
	10	Imipenem	20–28
	100/10 or 30/6	Piperacillin-tazobactam	25–33 23–29
	10	Tobramycin	20–26
<i>Enterococcus faecalis</i> WDCM 00210	1,25/23,75	Trimethoprim-sulfamethoxazole	c
<i>Staphylococcus aureus</i> WDCM 00211	30	Cefoxitin	d
<i>Staphylococcus aureus</i> WDCM 00212	30	Cefoxitin	14–20

a With *S. aureus* WDCM 00034, use linezolid 30 µg discs and penicillin 10 unit discs. With *S. aureus* WDCM 00131, use linezolid 10 µg discs and penicillin (benzylpenicillin) 1 unit discs.

b Disc diffusion ranges from EUCAST control range tables^[3]. Check the latest version from EUCAST <http://www.eucast.org> for updated ranges as they are subject to periodic updates. *S. aureus* WDCM 00131 is an alternative quality control microorganism to *S. aureus* WDCM 00034 for disc diffusion testing for the antimicrobial agents in common. *S. aureus* WDCM 00034 shall be tested with antimicrobial agents for which no acceptable range for WDCM 00131 is available. Cefoxitin is replacing oxacillin as the surrogate for detection of methicillin-resistant *Staphylococcus aureus* (MRSA). Oxacillin disc diffusion testing is no longer recommended. Refer to CLSI^[5] or EUCAST^[3] documents.

c The zone of inhibition for trimethoprim-sulfamethoxazole shall be ≥20 mm.

d The zone of inhibition for cefoxitin (CLSI^[3]) shall be ≤21 mm.

5.5 Evaluating the results

If all performance criteria for all microorganism-antimicrobial agent combinations are within acceptable limits (see 5.4) and all physical and chemical characteristics are met (see 5.2), the manufacturer may apply the label statement specified in Annex D. Manufacturers should attempt to achieve mean zone diameter values close to the midpoint of the control ranges. Data shall be maintained on file and results made available to anyone upon request.

6 Testing new antimicrobial agents with production lots of dehydrated Mueller-Hinton broth or agar

When *in vitro* data on new antimicrobial agents are being developed, dMHB or dMHA production lots meeting the criteria in this Technical Specification shall be used to develop *in vitro* quality control parameters for these new antimicrobial agents. Testing of production lots of dMHB or dMHA with new antimicrobial agents shall follow the procedures outlined in this Technical Specification. For dMHB, ion content shall be examined to determine whether the new antimicrobial agent is affected by specific cations or anions or concentrations of ions in the medium that differ from the ranges suggested in this Technical Specification. Other medium components that could affect results of *in vitro* quality control susceptibility tests that come to light during these investigations shall be identified. Adjustments shall be made as necessary to achieve stable, reproducible test results, and this information shall be widely circulated to others involved in antimicrobial susceptibility testing and quality control including the CLSI Subcommittee on Antimicrobial Susceptibility Testing and EUCAST. This is the responsibility of the company manufacturing the new agent.

Annex A (informative)

Mueller-Hinton medium

A.1 Broth

Table A.1 — Effects of dehydrated Mueller-Hinton broth

Antimicrobial	Remarks
Aminoglycosides	Specified levels of calcium (20 mg/l to 25 mg/l) and magnesium (10 mg/l to 12,5 mg/l) are based on studies ^{[16][17]} comparing Mueller-Hinton agar and broth dilution for clinical strains of <i>P. aeruginosa</i> .
Carbapenems	While trace amounts of zinc are required for growth, the concentration of zinc shall be below 3 mg/l to avoid false resistance interpretations ^[9] . This is known to be true for imipenem and may apply to other carbapenems.
Daptomycin	Medium shall be supplemented to a final concentration of 50 mg/l total Ca ²⁺ .
Folate pathway inhibitors (eg. Sulfonamides and Trimethoprim)	The medium shall have a thymidine mass concentration of less than 0,03 mg/l as indicated by a value of <0,5/9,5 mg/l obtained by testing <i>Enterococcus faecalis</i> WDCM 00087 with trimethoprim-sulfamethoxazole.
Fosfomycin	Only agar dilution shall be used as the reference method because broth dilution is not reliable ^{[4][6]} .
Glycylcyclines (eg. Tigecycline)	Freshly prepared (<12 h) test medium shall be used. This may also apply to other antimicrobial agents in this drug class. While trace amounts of manganese are required for growth, the concentration shall be below 8 mg/l to avoid false resistant interpretations ^[8] . This shall be determined by a value within the acceptable MIC range obtained by testing <i>Escherichia coli</i> WDCM 00013 with tigecycline.
Lipoglycopeptides (eg. Dalbavancin and Oritavancin)	The broth shall be supplemented with 0,002 % v/v polysorbate-80. This may also apply to other members of this drug class.
Oxacillin	Incorporation of NaCl at a final concentration of 20 g/l in the broth is required for the detection of methicillin resistance in <i>Staphylococcus</i> spp. when testing with oxacillin.
Quinolones	Data from studies of quinolone activity in human urine suggest that decreased quinolone activity may occur when magnesium concentrations are 8 mm to 10 mm (100 mg/l to 150 mg/l) in Mueller-Hinton medium ^[18] . Other data indicate that 35 mg/l to 60 mg/l of magnesium cause an increase in MICs for several species of bacteria ^{[19][20]} .
Tetracyclines	Mueller-Hinton broth supplemented to contain 50 mg/l of calcium and 25 mg/l of magnesium has been shown to increase MICs for <i>E. coli</i> , <i>P. aeruginosa</i> , and other species of <i>Pseudomonas</i> by 2- to 32- fold ^[12] .
All	Testing of fastidious microorganisms such as streptococci and <i>Haemophilus</i> spp. requires the addition of growth supplements (for example, blood or blood components) ^{[4][6]} .

A.2 Agar

Table A.2 — Effects of dehydrated Mueller-Hinton agar

Antimicrobial	Remarks
Aminoglycosides	The medium shall have Ca^{2+} and Mg^{2+} concentrations such as to provide zone diameter values within expected range for <i>Pseudomonas aeruginosa</i> vs the aminoglycoside class of antimicrobial agents as shown by zone diameter with gentamicin and <i>Pseudomonas aeruginosa</i> WDCM 00025 within the acceptable range.
Carbapenems	While trace amounts of zinc are required for growth, the concentration of zinc shall be below 3 mg/l to avoid false resistance interpretations ^[9] . This is known to be true for imipenem and may apply to other carbapenems.
Daptomycin	Cannot be tested by disc diffusion
Folate pathway inhibitors (eg. Sulfonamides and Trimethoprim)	The medium shall have a thymidine mass concentration of less than 0,03 mg/l as shown by the clarity and zone of inhibition with trimethoprim-sulfamethoxazole of ≥ 20 mm for <i>E. faecalis</i> WDCM 00210 or by a zone diameter within the acceptable range obtained using <i>E. faecalis</i> WDCM 00087.
Fosfomycin	Only agar dilution shall be used as the reference method because broth dilution is not reliable ^{[4][6]} . The test agar shall be supplemented with 25 mg/l glucose-6-phosphate.
Glycylcyclines (eg. Tigecycline)	Freshly prepared (<12 h) test medium shall be used. This may also apply to other antimicrobial agents in this drug class. While trace amounts of manganese are required for growth, the concentration shall be below 8 mg/l to avoid false resistant interpretations ^[8] . This shall be determined by a value within the acceptable range obtained by testing <i>Escherichia coli</i> WDCM 00013 with tigecycline.
Quinolones	Data indicate that 35 mg/l to 60 mg/l of magnesium cause a reduction in zone diameters and an increase in MICs for several species of bacteria ^{[19][20]} .
Tetracyclines	The medium shall have calcium and magnesium concentrations such as to provide zone diameter values within expected range for <i>Pseudomonas aeruginosa</i> vs aminoglycoside class of antimicrobial agents as shown by zone diameter with gentamicin and <i>Pseudomonas aeruginosa</i> WDCM 00025 within the acceptable range.
All	Testing of fastidious microorganisms such as streptococci and <i>Haemophilus</i> spp. requires the addition of growth supplements (e.g. blood or blood components) ^{[3][5]} .

Annex B (informative)

Preparing control cultures

B.1 Stock cultures

Stock cultures are prepared from lyophilized cultures obtained from a recognized national culture collection. Cultures shall be reconstituted according to procedures defined by the culture collection and maintained in such a way that selection of genetic variants is minimized. Alternative numbers for the same microorganism from different culture collections are listed below when available. The following are the cultures required for the purposes of this protocol:

<i>Staphylococcus aureus</i>	WDCM ^a 00131; ATCC ^{®b} 29213; NCTC ^{®c} 12973; CIP ^d 103429; DSM ^e 2569; CCUG ^f 15915; CECT ^g 794
<i>Staphylococcus aureus</i>	WDCM ^a 00034; ATCC ^{®b} 25923; NCTC ^{®c} 12981; CIP ^d 76.25; DSM ^e 1104; CCUG ^f 17621; CECT ^g 435
<i>Staphylococcus aureus</i>	WDCM ^a 00211; ATCC ^{®b} 43300
or	
<i>Staphylococcus aureus</i>	WDCM ^a 00212; NCTC ^{®c} 12493
<i>Escherichia coli</i>	WDCM ^a 00013; ATCC ^{®b} 25922; NCTC ^{®c} 12241; CIP ^d 76.24; DSM ^e 1103; CCUG ^f 17620; CECT ^g 434
<i>Pseudomonas aeruginosa</i>	WDCM ^a 00025; ATCC ^{®b} 27853; NCTC ^{®c} 12903; CIP ^d 76.110; DSM ^e 1117; CCUG ^f 17619; CECT ^g 108
<i>Enterococcus faecalis</i>	WDCM ^a 00087; ATCC ^{®b} 29212; NCTC ^{®c} 12697; CIP ^d 103214; DSM ^e 2570; CCUG ^f 9997; CECT ^g 795
or	
<i>Enterococcus faecalis</i>	WDCM ^a 00210; ATCC ^{®b} 33186
^a WDCM, World Data Centre for Microorganisms, www.wdcm.org. ^b ATCC is the trademark of a product supplied by American Type Culture Collection, www.atcc.org. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO of the product named. Equivalent products can be used as listed above. ^c NCTC is the trademark of a product supplied by National Collection of Type Cultures, a culture collection of Public Health England, www.hpacultures.org.uk. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO of the product named. Equivalent products can be used as listed above. ^d CIP, Collection de l'Institut Pasteur, www.pasteur.fr. ^e DSMZ, Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, www.dsmz.de. ^f CCUG, Culture Collection, University of Göteborg, www.ccug.se. ^g CECT, Colección Española de Cultivos Tipo, www.cect.org.	

B.1.1 Preparing the initial stock cultures

Using a sterile loop or swab, inoculate one or more plates (depending on the number of frozen replicate vials that are to be prepared) of non-selective nutritive agar with each control microorganism listed in [B.1](#). Streak each plate to obtain isolated colonies. Incubate the plates for 18 h to 24 h as per ISO 20776-1 at 34 °C to 37 °C in ambient air.

B.1.2 Preparing frozen stock cultures

After incubation, check for purity and harvest the entire growth from each set of plates and emulsify it in soybean-casein digest broth [tryptic soy broth (TSB)] containing 15 % w/v glycerol to prepare a uniformly dense suspension. Store the vials at -60°C or a lower temperature. With this method, cultures shall be viable for at least one year. Other methods of preparing stock cultures may be used if they provide adequate viability and stability. Periodically renew stock cultures from fresh lyophilized cultures obtained from recognized culture collections.

NOTE To prepare the TSB with glycerol, add the manufacturer's recommended amount of TSB powder or granule for preparing one litre, then add 500 ml of deionized water and 150 ml of glycerol. Adjust the final total volume to 1 l with deionized water, heat to dissolve and mix well, and sterilize it at 121°C for 15 min. Dispense the suspension into small, sterile vials.

B.1.3 Preparing the stock culture test inoculum

Refer to [4.3](#) for preparing test inoculum.

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Annex C (informative)

Suggested data sheet for testing of production lots

C.1 Suggested data sheet for testing of production lots of dMHB

Date:

Production lot number:

Batch size:

Description of colour:

Clarity:

pH:

Antimicrobial agent	Acceptable range mg/l	MIC mg/l		
		1	2	3
<i>P. aeruginosa</i> WDCM 00025				
Ciprofloxacin	0,25-1			
Gentamicin	0,5-2			
Imipenem	1-4			
Piperacillin-tazobactam	1/4-8/4			
<i>E. coli</i> WDCM 00013				
Ampicillin	2-8			
Cefotaxime	0,03-0,12			
Tigecycline	0,03-0,25			
<i>S. aureus</i> WDCM 00131				
Clindamycin	0,06-0,25			
Erythromycin	0,25-1			
Oxacillin	0,12-0,5			
Tetracycline	0,12-1			
Vancomycin	0,5-2			
<i>E. faecalis</i> WDCM 00087				
Ampicillin	0,5-2			
Trimethoprim-sulfamethoxazole	≤0,5/9,5			
Vancomycin	1-4			
<i>S. aureus</i> WDCM 00211				
Oxacillin	4-32			

Ranges are subject to periodic updates. Check the latest version of M100 available from CLSI for updated ranges. CLSI, 950 West Valley Road, Suite 2500, Wayne, PA 19087, USA^[14] or check the latest version of EUCAST QC tables available from EUCAST at http://www.eucast.org/ast_of_bacteria/qc_tables/.