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M23S

Procedure for Optimizing Disk Contents (Potencies) for Disk Diffusion Testing of Antimicrobial Agents Using Harmonized CLSI and EUCAST Criteria

This document describes the necessary technical steps for establishing the optimal disk content (potency) for single antimicrobial agents without the addition of enhancing or inhibiting substances.

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Procedure for Optimizing Disk Contents (Potencies) for Disk Diffusion Testing of Antimicrobial Agents Using Harmonized CLSI and EUCAST Criteria

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Abstract

Clinical and Laboratory Standards Institute document M23S—*Procedure for Optimizing Disk Contents (Potencies) for Disk Diffusion Testing of Antimicrobial Agents Using Harmonized CLSI and EUCAST Criteria* describes the necessary technical steps for establishing the optimal disk content (potency) for single antimicrobial agents without the addition of enhancing or inhibiting substances.

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Foreword

The disk diffusion antimicrobial susceptibility test has been widely used around the world for decades and was first standardized in 1966.[1] In the 1970s, CLSI (then the National Committee for Clinical Laboratory Standards) published additional guidance for disk diffusion testing. In Europe, different variants of the disk diffusion method were used in different countries until 2009, when the European Committee on Antimicrobial Susceptibility Testing (EUCAST) provided a standardized disk diffusion method calibrated to the harmonized European minimal inhibitory concentration breakpoints. The disk diffusion test is based on incorporating a standard amount of an antimicrobial agent into a filter paper disk. Because it is relatively easy to perform and uses standard microbiology laboratory equipment, the disk diffusion test is used in many types of laboratories, including those in low-resource settings.

The disk content (potency) recommended for new antimicrobial agents has sometimes varied among organizations that set criteria (eg, breakpoints) for interpreting results of disk diffusion testing. Subsequently, pharmaceutical manufacturers have performed testing with two different disk contents (potencies) for generating data to present to breakpoint-setting organizations. This burdensome situation was caused in part by a lack of harmonized recommendations for selecting optimal disk content (potencies). To correct this issue and improve efficiency for pharmaceutical manufacturers, disk manufacturers, researchers, and other organizations, CLSI and EUCAST initiated a joint venture to develop standardized recommendations for disk content selection. Their recommendations are presented in this document.

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

Key Words

Disk content, disk diffusion, disk potency

Procedure for Optimizing Disk Contents (Potencies) for Disk Diffusion Testing of Antimicrobial Agents Using Harmonized CLSI and EUCAST Criteria

Chapter 1: Introduction

This chapter includes:

- Document's scope and applicable exclusions
- Background information pertinent to the document's content
- Standard precautions information
- Terminology information, including:
  - Terms and definitions used in the document
  - Abbreviations and acronyms used in the document

1.1 Scope

This document is intended for pharmaceutical manufacturers involved in the development of antimicrobial agents and tests to support evaluation of antimicrobial agent activity. It is also intended for manufacturers of antimicrobial disks and any independent laboratory that supports the development of these disks. This document describes the process for selecting the optimal content (potency) of antimicrobial agent to be added to filter paper disks to obtain reliable results with the standardized disk diffusion test. It does not explain the steps needed to perform the standardized disk diffusion test, nor does it define the criteria (breakpoints) used to interpret zone diameters of inhibition into interpretive categories. These steps are described elsewhere (see CLSI documents M02[2] and M07[3]).[4,5] In some cases, the breakpoints defined by breakpoint-setting organizations for a single agent may differ even when the same disk content (potency) is used.

1.2 Background

The standard for antimicrobial susceptibility testing of rapidly growing aerobic bacteria is minimal inhibitory concentration (MIC) determination using broth microdilution according to international standards[6] or CLSI document M07,[3] except for a few agents and/or organisms for which broth microdilution does not provide reliable results. For fastidious organisms, the basic methodology is the same, but CLSI (see CLSI document M07[3]) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[7] recommend different media. Both CLSI (see CLSI document M02[2]) and EUCAST[4] have developed standardized disk diffusion methods calibrated to match the results of reference MIC methodology (see CLSI document M07[3])[7] based in part on a method originally described in 1966.[1] Optimal disk content (potency) selection for disk diffusion testing is critical for the development of an accurate and reproducible test. Disk contents (potencies) can only be developed once a reference MIC method has been established for the antimicrobial agent and organisms in question.

The CLSI and EUCAST disk diffusion methods are based on reproducible and reliable separation between isolates belonging to different interpretive categories as determined by reference MIC methodology. For each organism-agent combination, disk diffusion testing of clinical isolates should result in an on-scale

zone diameter distribution that spans a 10- to 14-mm range for wild-type (WT) organisms (see examples in Appendix A). Populations with and without resistance mechanisms that are clearly distinguishable by MIC should also be clearly distinguishable by inhibition zone diameter. Determining the optimal disk content (potency) is integral to achieving this goal.

The CLSI and EUCAST disk diffusion methods are based on the same basic methodology, ie, Mueller-Hinton agar and an inoculum size equivalent to a 0.5 McFarland standard. At present, there are differences between CLSI and EUCAST in supplements for media for fastidious organisms and in disk contents (potencies) for some antimicrobial agents. Because having common disk content (potency) for both CLSI and EUCAST disk diffusion testing is an advantage to users of the disk diffusion methods, pharmaceutical companies, and disk manufacturers, the CLSI-EUCAST joint working group formed in 2017 has agreed on common criteria for development of optimal disk contents (potencies) to be incorporated into 6-mm filter paper disks for disk diffusion testing. These disks are endorsed by both CLSI and EUCAST. Pharmaceutical companies interested in having disk diffusion breakpoints published in CLSI and/or EUCAST tables should follow the procedure when developing disks for disk diffusion testing.

1.3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of bloodborne pathogens. Published guidelines are available that discuss the daily operations of diagnostic medicine in humans and animals while encouraging a culture of safety in the laboratory.[8] For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.[9]

1.4 Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization whenever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in different countries and regions and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. CLSI recognizes its important role in these efforts, and its consensus process focuses on harmonization of terms to facilitate the global application of standards and guidelines. Table 1 is provided to clarify the intended interpretations of the following terms.

Table 1. Common Terms or Phrases With Intended Interpretations

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

1.4.1 Definitions

For purposes of this document, the terms and definitions listed below apply. Consult CLSI's Harmonized Terminology Database at http://htd.clsi.org for related terms and definitions.

disk content (potency) – the concentration of antimicrobial agent added to 6-mm filter paper disks to determine *in vitro* antimicrobial susceptibility testing results following a standardized disk diffusion method equivalent to disk load, disk mass, disk strength, and disk charge.

minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

non-wild-type isolates – isolates **with** phenotypically detectable acquired resistance mechanisms for the test agent.

wild-type isolates – isolates **without** phenotypically detectable acquired resistance mechanisms for the test agent.

1.4.2 Abbreviations and Acronyms

EUCAST	European Committee on Antimicrobial Susceptibility Testing
MIC	minimal inhibitory concentration
NWT	non-wild-type
QC	quality control
SOP	standard operating procedure
WT	wild-type

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Chapter 2: Procedure for Establishing the Optimal Disk Content (Potency)

This chapter includes:

- Selection criteria for disks containing a single antimicrobial agent
- Basic criteria for phase 1 and phase 2 disk content (potency) studies
- Considerations for selection of the optimal disk content (potency) for combinations of agents

This procedure describes the necessary technical steps for establishing the optimal disk content (potency) for single antimicrobial agents without the addition of enhancing or inhibiting substances. For guidance in establishing disk content for a combination of agents, see Subchapter 2.5. **NOTE:** The disk content (potency) must reflect the amount of active antimicrobial agent and not the salt form of the agent.

2.1 Selection Criteria for Disks Containing a Single Antimicrobial Agent

When feasible, studies are performed to achieve:

- Reproducible inhibition zone diameters when testing QC strains and clinical isolates
  - **NOTE:** The difference in zone diameter measurements obtained from testing a single QC strain or clinical isolate repetitively in one laboratory using the same lots of disks and media should not exceed 3 mm. This includes tests performed on a single day or over several days.

- A single disk content (potency) that can be used for all relevant species (target organisms)
  - **NOTE:** Multiple disk contents (potencies) should be considered only when absolutely necessary to meet the selection criteria for all target species.

- A general discriminatory power of 2- to 3-mm increase in zone diameters with each $\log_2$ decrease in MIC for non-wild-type (NWT) isolates

- Inhibition zone diameters between 15 and 35 mm (ideally not above 30 mm) for WT isolates of relevant species (target organisms)

- Optimal separation between WT and NWT isolates (when MIC clinical breakpoints are not yet defined), if NWT isolates exist

- Optimal separation between NWT isolates with different MICs, irrespective of resistance mechanisms

The disk content (potency) should be established according to the procedures described in phases 1 and 2 (see Subchapter 2.2).

2.2 Basic Criteria for Phase 1 and 2 Studies

The following basic criteria apply to phase 1 and phase 2 disk content (potency) studies:

- MIC testing is performed according to the reference method, and MIC QC performance data should be available.

- Options for obtaining reference MIC values for clinical isolates include:
  - Performing MIC testing in parallel with disk diffusion testing
  - Selecting isolates with previously established MIC values

- Isolates must be retested if the relationship between the MIC and zone diameter is not consistent with results from other similar isolates or not logical (ie, a low MIC and a small zone diameter or a high MIC and a large zone diameter). Retesting should be conducted using a single inoculum suspension for both reference MIC and disk diffusion methods in parallel. Three separate inoculum suspensions should be prepared to obtain triplicate results for each isolate.

- Disk diffusion must be performed using a Mueller-Hinton medium that meets the specifications in international standards[10] and the QC criteria published by CLSI (see CLSI document M100[11]) and EUCAST[12] for standard QC strains. To establish acceptable quality of the medium, results must be in range when testing QC strains and agents from similar and different antimicrobial classes. The numbers of QC strains and additional agents tested will vary depending on experience with particular lots of Mueller-Hinton medium used and the antimicrobial agent under investigation.

- For fastidious organisms, CLSI and EUCAST disk diffusion media must be tested in parallel.

- Testing can be performed on one or multiple days for clinical isolates.

- Relevant QC strains must be tested each day clinical isolates are tested and for a minimum of three separate days. The difference in zone diameter measurements obtained from testing a single QC strain or clinical isolate repetitively in one laboratory using the same lots of disks and media should not exceed 3 mm.

- An appropriate control agent (preferably an antimicrobial agent belonging to the same or similar class as the agent being evaluated) with CLSI (see CLSI document M100[11]) and/or EUCAST[12] published QC ranges must be included with disk diffusion testing of all isolates (clinical isolates and QC strains).

2.3 Phase 1: Initial Screening of a Series of Disk Contents (Potencies)

The aim of phase 1 is to screen up to 10 disks covering a wide range of contents (potencies) against a small number of isolates of the target species. From these results, the contents (potencies) of 2 to 4 disks will be selected for testing in phase 2 with a larger number of isolates.

Normally, 10 different disks ranging from very low to very high (eg, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 µg) content (potency) are produced in small batches and tested according to standardized disk diffusion methodology against relevant species. The most important target species should be included when evaluating organism groups, eg, *Escherichia coli* and *Klebsiella pneumoniae* for Enterobacterales. For agents with broad-spectrum activity against a variety of organism groups (eg, gram-positive and gram-negative genera), it might be necessary to test disk contents (potencies) beyond the 0.1- to 100-µg range. The contents (potencies) of fewer than 10 disks can be evaluated, but the risk of having to repeat the study if none of the disk contents (potencies) tested performs reliably is increased.

- A disk content (potency) previously used for the antimicrobial class of the agent being evaluated (eg, 5 µg for fluoroquinolones, 30 µg for third-generation cephalosporins) should be included but should not be considered the optimal content (potency) by default.

- A minimum of four isolates per relevant target species (as defined by the pharmaceutical company) are included: two WT isolates (a susceptible QC strain can be used) and two NWT isolates with different MICs, generally two to four twofold dilutions above the WT distribution. If no NWT isolates are available, testing is performed with three WT isolates with different MICs.

- Testing can be performed using one disk lot per content (potency) on Mueller-Hinton media from one manufacturer. These disks can be commercially produced or obtained from small-scale production by the pharmaceutical company or a contract laboratory. A procedure for manual preparation of disks is provided in Appendix B.

- The results should be summarized as presented in Tables 2A to 2C. For each target species, data are reviewed to identify:
  - The disk contents (potencies) that result in optimal zone sizes for WT isolates (15 to 35 mm and ideally not above 30 mm) (see Table 2A) and the disk contents (potencies) with the largest increase in zone sizes between two consecutive disk contents (potencies) for WT isolates (see Table 2B)
    - **NOTE:** Table 2C presents consolidated data from Tables 2A and 2B. Cells that are highlighted in both Tables 2A and 2B are highlighted in Table 2C. Overlapping or duplicated cells show the greatest difference between the zone sizes, as well as the optimal zone sizes.

  - The disk contents (potencies) that demonstrate the greatest difference between the smallest and largest zone size (highlighted in green), if NWT isolates are available (see Table 2C)

Tables 2A to 2C summarize phase 1 data for ceftazidime vs three target organisms. Disk contents (potencies) lower than 5 µg resulted in zones that are too small for WT isolates of *P. aeruginosa*. Overall optimal performance for the three species tested was therefore demonstrated with 5-, 10-, and 20-ug disks.

Table 2A. Zone Diameter Sizes (in mm) for Each Isolate and Disk Content (Potency)

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT ≤ 0.5 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
1	*E. coli*	0.06	WT	12	16	18	21	26	27	29	31	32	32
2	*E. coli*	0.25	WT	7	12	16	19	26	27	29	29	30	30
3	*E. coli*	8	NWT	6	6	6	9	16	19	21	23	24	23
4	*E. coli*	16	NWT	6	6	6	6	9	12	16	18	20	19

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT≤0.5 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
5	*K. pneumoniae*	0.25	WT	10	13	16	18	24	26	28	28	28	29
6	*K. pneumoniae*	0.5	WT	11	15	17	21	26	28	30	31	30	31
7	*K. pneumoniae*	32	NWT	6	6	6	6	8	10	13	15	15	16
8	*K. pneumoniae*	32	NWT	6	6	6	6	6	8	11	14	15	15

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT≤8 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
9	*P. aeruginosa*	2	WT	6	6	8	13	21	24	27	27	28	29
10	*P. aeruginosa*	4	WT	6	6	6	6	15	20	23	25	25	28
11	*P. aeruginosa*	>32	NWT	6	6	6	12	18	21	25	26	25	28
12	*P. aeruginosa*	>32	NWT	6	6	6	6	15	18	20	21	22	25

Abbreviations: ID, identification; MIC, minimal inhibitory concentration; NWT, non-wild-type; WT, wild-type.
[a] Optimal disk contents (potencies) and respective zone sizes are highlighted in yellow.

Table 2B. Zone Diameter Size Differences (in mm) Between the Contents (Potencies) of Two Consecutive Disks

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT ≤ 0.5 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
1	*E. coli*	0.06	WT	–	4	2	3	5	1	2	2	1	0
2	*E. coli*	0.25	WT	–	5	4	3	7	1	2	0	1	0
3	*E. coli*	8	NWT	–	0	0	3	7	3	2	2	1	−1
4	*E. coli*	16	NWT	–	0	0	0	3	3	4	2	2	−1

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT≤0.5 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
5	*K. pneumoniae*	0.25	WT	–	3	3	2	6	2	2	0	0	1
6	*K. pneumoniae*	0.5	WT	–	4	2	4	5	2	2	1	−1	1
7	*K. pneumoniae*	32	NWT	–	0	0	0	2	2	3	2	0	1
8	*K. pneumoniae*	32	NWT	–	0	0	0	0	2	3	3	1	0

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT≤8 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
9	*P. aeruginosa*	2	WT	–	0	2	5	8	3	3	0	1	1
10	*P. aeruginosa*	4	WT	–	0	0	0	9	5	3	2	0	3
11	*P. aeruginosa*	>32	NWT	–	0	0	6	6	3	4	1	−1	3
12	*P. aeruginosa*	>32	NWT	–	0	0	0	9	3	2	1	1	3

Abbreviations: ID, identification; MIC, minimal inhibitory concentration; NWT, non-wild-type; WT, wild-type.
[a] The disk contents (potencies) demonstrating the largest increase between two consecutive disks are highlighted in yellow.

Table 2C. Zone Diameter Sizes (in mm) and Zone Differences (last row) When Data From Tables 2A and 2B Are Consolidated

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT ≤ 0.5 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
1	*E. coli*	0.06	WT	12	16	18	21	26	27	29	31	32	32
2	*E. coli*	0.25	WT	7	12	16	19	26	27	29	29	30	30
3	*E. coli*	8	NWT	6	6	6	9	16	19	21	23	24	23
4	*E. coli*	16	NWT	6	6	6	6	9	12	16	18	20	19
Difference between largest and smallest zone				**6**	**10**	**12**	**15**	**17**	**15**	**13**	**13**	**12**	**13**

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT≤0.5 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
5	*K. pneumoniae*	0.25	WT	10	13	16	18	24	26	28	28	28	29
6	*K. pneumoniae*	0.5	WT	11	15	17	21	26	28	30	31	30	31
7	*K. pneumoniae*	32	NWT	6	6	6	6	8	10	13	15	15	16
8	*K. pneumoniae*	32	NWT	6	6	6	6	6	8	11	14	15	15
Difference between largest and smallest zone				**5**	**9**	**11**	**15**	**20**	**20**	**19**	**17**	**15**	**16**

Isolate ID	Species	MIC, µg/mL	WT/NWT (WT≤8 µg/mL)	Disk Content (Potency), µg[a]									
				0.1	0.2	0.4	1	5	10	20	30	40	50
9	*P. aeruginosa*	2	WT	6	6	8	13	21	24	27	27	28	29
10	*P. aeruginosa*	4	WT	6	6	6	6	15	20	23	24	25	28
11	*P. aeruginosa*	>32	NWT	6	6	6	12	18	21	25	26	25	28
12	*P. aeruginosa*	>32	NWT	6	6	6	6	15	18	20	21	22	25
Difference between largest and smallest zone				**0**	**0**	**2**	**7**	**6**	**6**	**7**	**6**	**6**	**4**

Abbreviations: ID, identification; MIC, minimal inhibitory concentration; NWT, non-wild-type; WT, wild-type.

[a] Disk contents (potencies) with both optimal zone sizes and the largest increase in zone sizes between two consecutive disk contents (potencies) for WT isolates are highlighted in yellow. The greatest difference (mm) between the largest and smallest zone within the optimal area is highlighted in green.

2.4 Phase 2: Disk Content (Potency) Study

A larger study is conducted with all relevant target species for the agent in question using the contents (potencies) of two to four disks that demonstrated the most discriminatory power in phase 1 studies. **NOTE:** The examples provided in this subchapter are not a continuation of those in phase 1. The examples were selected to optimally illustrate key points in each phase.

2.4.1 Additional Investigation of the Contents (Potencies) of Two to Four Selected Disks

At least 30 isolates per species or 60 isolates per group of organisms (eg, Enterobacterales, *Pseudomonas* spp., viridans group streptococci) should be included, of which at least 50% (preferably not more than 80%) should be WT isolates. A larger number of isolates may be needed for antimicrobial agents that are active against a variety of gram-positive and gram-negative genera.

The NWT isolates should, when possible, represent a variety of MICs and resistance mechanisms and include isolates with MICs one to two dilutions above the highest MIC in the WT population. When resistant isolates are not available, the minimum criterion is to define the WT population of relevant species. If the pharmaceutical company can provide variant isolates with higher MICs, these should be included, provided that growth characteristics are similar (ie, no reduction in growth) to those of WT isolates in the testing media used. It is also possible to include resistant isolates (including those with intrinsic resistance) of non-target species if no resistant isolates are available for target species.

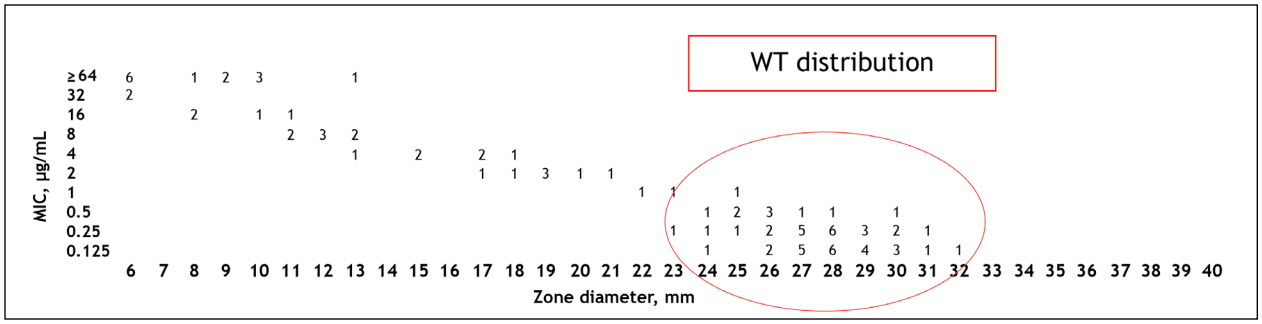
- Testing should be performed using commercially produced disks (one disk lot per disk content [potency]) or disks from small-scale production by the pharmaceutical company or a contract laboratory (two disk lots per disk content [potency]). A procedure for manual preparation of disks is provided in Appendix B.

- Testing must be performed on media from at least two manufacturers in parallel (ie, using the same inoculum suspension).

- If inhibition zones are difficult to read, zone diameters for a subset of isolates should be measured independently by two readers. Comments related to any peculiarities about zone measurement, including presence and size of colonies within the zone, should be provided.

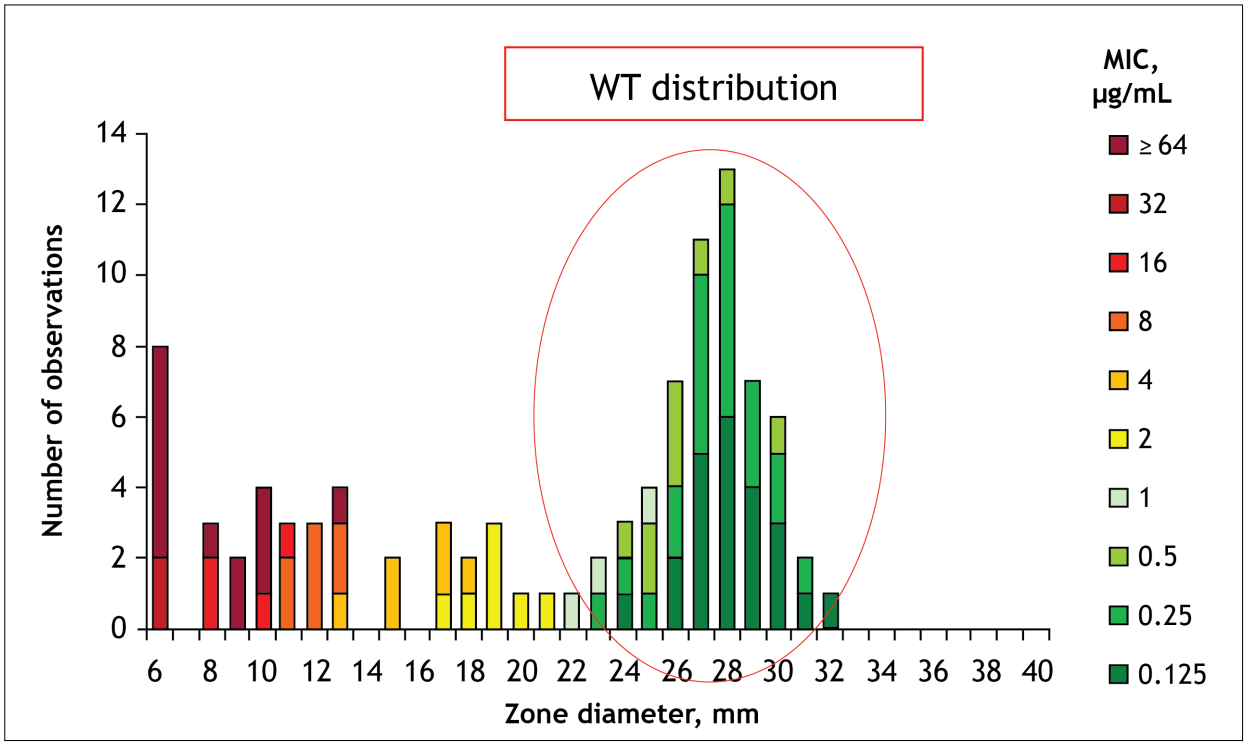
- Inhibition zone diameters are correlated to the corresponding MIC values and presented in two different graphical formats:
  - Species-specific scattergrams (see Figure 1A) and inhibition zone diameter histograms with corresponding MIC values as colored bars (see Figure 1B)
    - **NOTE:** Figures 1A and 1B represent the same dataset.

Examples of scattergrams and histograms for the contents (potencies) of three different disks vs several species are shown in Appendix C. Data should be analyzed and presented for all disk and media manufacturers combined and again for each disk and media manufacturer individually.



Abbreviations: MIC, minimal inhibitory concentration; WT, wild-type.

Figure 1A. Zone Diameter Scattergram With Zone Diameters Plotted Against Minimal Inhibitory Concentration Values. Figures 1A and 1B represent the same dataset.



Abbreviations: MIC, minimal inhibitory concentration; WT, wild-type.

Figure 1B. Zone Diameter Histogram With MIC Values Represented by Colored Bars. Green corresponds to WT isolates. Yellow, orange, and red correspond to different minimal inhibitory concentrations for non-wild-type isolates. Figures 1A and 1B represent the same dataset.

2.4.2 Selection of Optimal Disk Content (Potency)

Optimal disk content (potency) is determined using the selection criteria listed in Subchapter 2.1 following visual review of the raw data and data displayed in scattergrams and histograms. WT and NWT populations, clearly distinguishable by MIC, should also be clearly distinguishable by inhibition zone diameter.

2.5 Considerations for Selection of the Optimal Disk Content (Potency) for Combinations of Agents

For selection of the optimal disk content (potency) for combinations of agents, the following considerations apply:

- For disks consisting of combinations of agents (agent plus agent, agent plus inhibitor without antimicrobial activity, agent plus inhibitor with antimicrobial activity), a discussion with the joint working group is necessary before development moves forward.

- For combination agent tests, it is usually advisable to start with the standard potency for the active agent and vary the inhibitor component.

- For combinations of an agent and an inhibitor without antimicrobial activity for which an agreed-on disk for the parent agent exists, the amount of inhibitor is varied to obtain good correlation with the reference MIC values for the agent-inhibitor combination. If there is no disk for the parent agent alone, establishing the optimal content (potency) of the parent agent must be part of the process.

The necessary documentation for submitting data for optimizing disk contents (potencies) for disk diffusion testing of antimicrobial agents using harmonized CLSI and EUCAST criteria is presented in Appendix D.

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Chapter 3: Supplemental Information

This chapter includes:

- References
- Appendixes
- The Quality Management System Approach
- Related CLSI Reference Materials

References

1 Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*. 1966;45(4):493-496.

2 CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests.* 13th ed. CLSI standard M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

3 CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically.* 11th ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute; 2018

4 The European Committee on Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing: EUCAST disk diffusion method. Version 8.0; 2020. http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Accessed 12 June 2020.

5 The European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoints – breakpoints and guidance. https://www.eucast.org/clinical_breakpoints/. Accessed 6 July 2020.

6 ISO. *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*. ISO 20776-1:2019. Geneva, Switzerland: International Standards Organization; 2019.

7 The European Committee on Antimicrobial Susceptibility Testing. Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method. Version 6.0; 2020. http://www.eucast.org/ast_of_bacteria/media_preparation/. Accessed 12 June 2020.

8 Miller JM, Astles R, Baszler T, et al.; Biosafety Blue Ribbon Panel, Centers for Disease Control and Prevention (CDC). Guidelines for safe work practices in human and animal medical diagnostic laboratories. *MMWR Suppl.* 2012;61(1):1-102.

9 CLSI. *Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Fourth Edition.* CLSI document M29-A4. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.

10 ISO. *Criteria for acceptable lots of dehydrated Mueller-Hinton agar and broth for antimicrobial susceptibility testing*. ISO/TS 16782:2016. Geneva, Switzerland; International Standards Organization; 2016.

11 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing.* 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

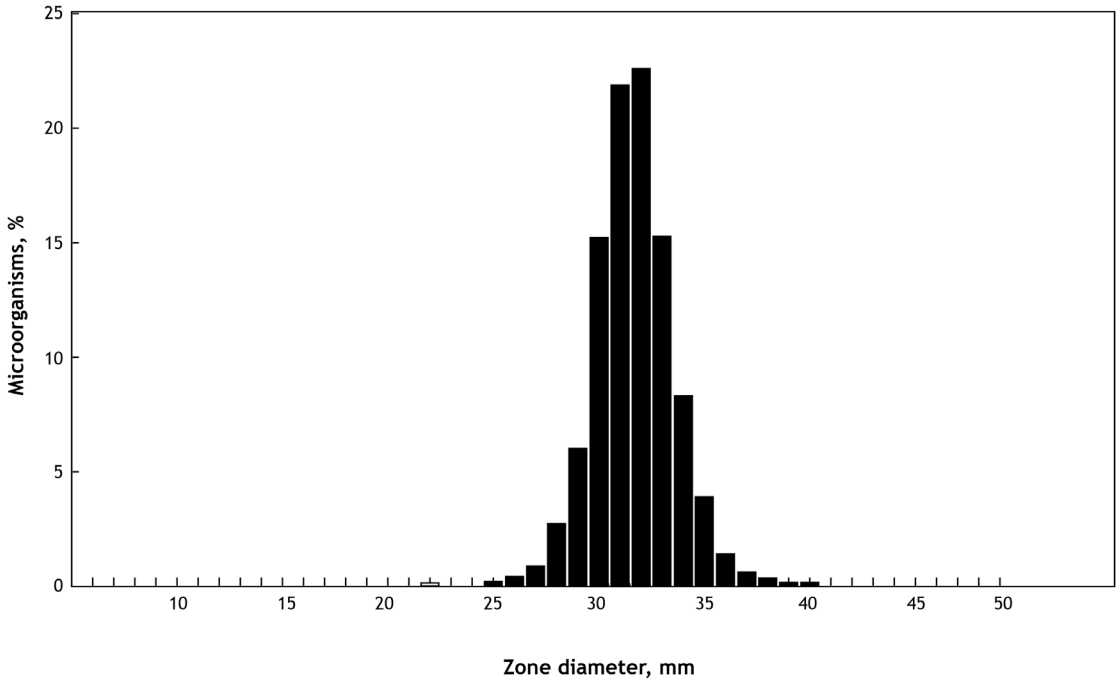
12 The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. Version 10.0; 2020. http://www.eucast.org/ast_of_bacteria/qc_tables/. Accessed 12 June 2020.

Appendix A. Examples of Zone Diameter Distributions With a Defined Wild-Type Distribution

Abbreviations for Appendix A

SD standard deviation
WT wild-type

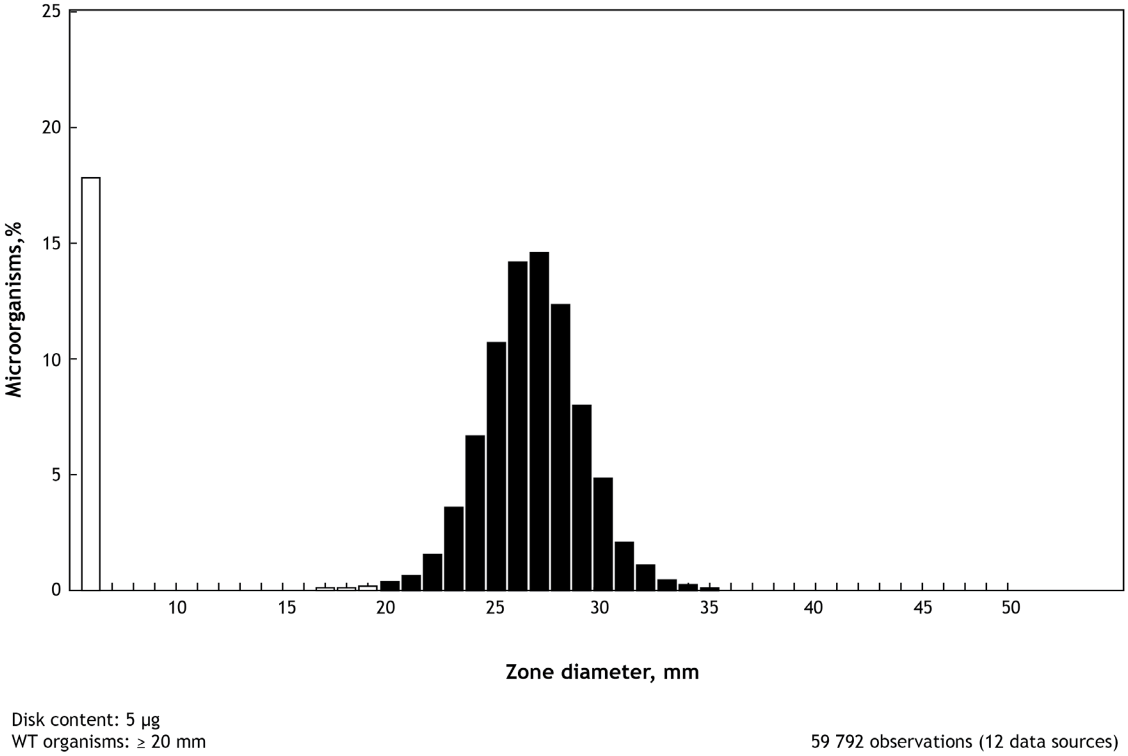
On-scale zone diameter distributions (± 2 SD) of wild-type (WT) organisms normally span 10 to 14 mm. **NOTE:** Zone diameter distributions with a defined WT distribution are represented by the black bars in Figures A1 through A4. Non-wild-type distributions are represented by the white bars in Figures A1 through A4.



Abbreviation: WT, wild-type.

Figure A1. Zone Diameter Distribution for Wild-Type *Escherichia coli* and Meropenem.[1] Distributions include collated data from multiple sources, geographical areas, and time periods and cannot be used to infer rates of resistance. (European Committee on Antimicrobial Susceptibility Testing. MIC and zone diameter distributions and ECOFFs. https://www.eucast.org/mic_distributions_and_ecoffs/. Accessed 17 March 2020.)

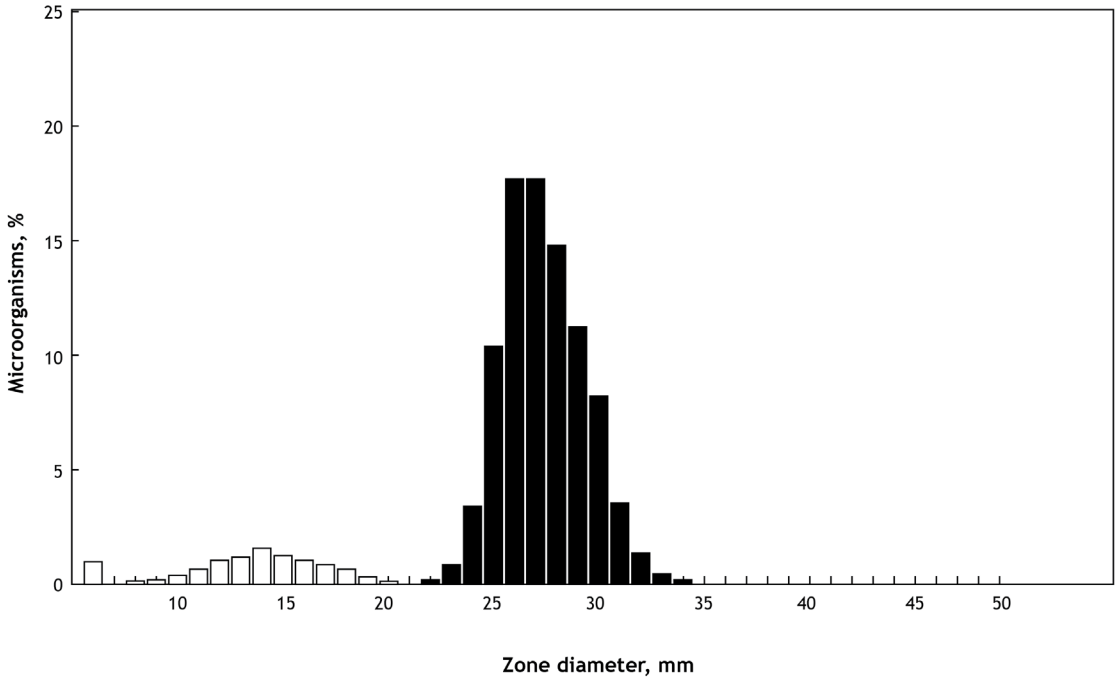
Appendix A. (Continued)



Abbreviation: WT, wild-type.

Figure A2. Zone Diameter Distribution for Wild-Type *E. coli* and Trimethoprim.[1] Distributions include collated data from multiple sources, geographical areas, and time periods and cannot be used to infer rates of resistance. (European Committee on Antimicrobial Susceptibility Testing. MIC and zone diameter distributions and ECOFFs. https://www.eucast.org/mic_distributions_and_ecoffs/. Accessed 17 March 2020.)

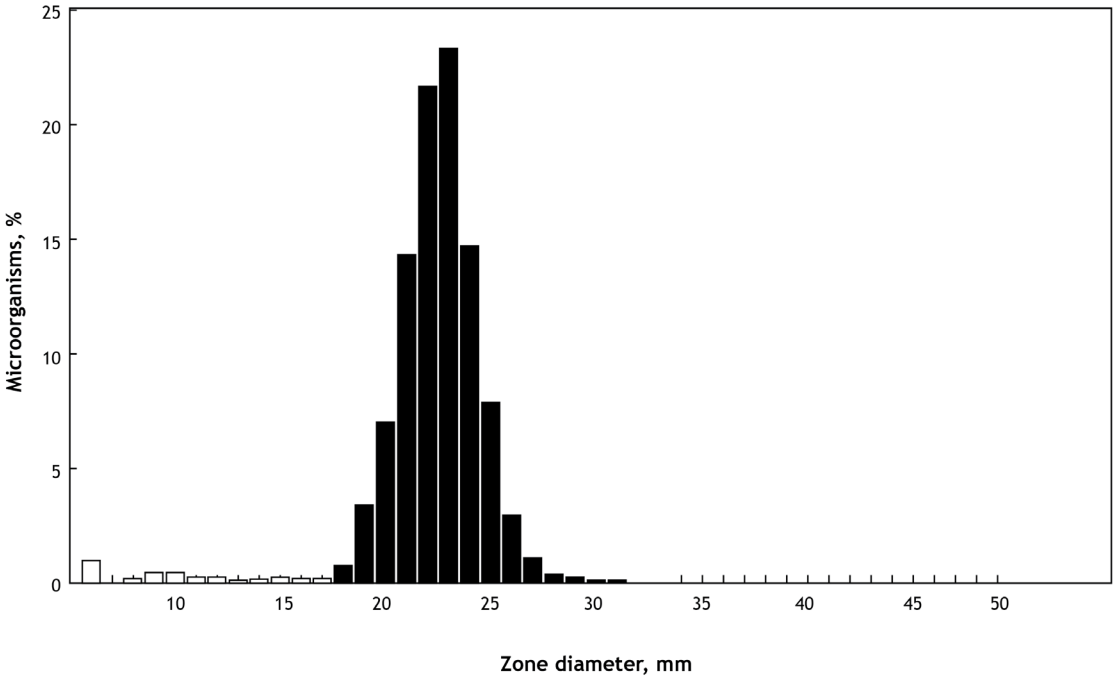
Appendix A. (Continued)



Abbreviation: WT, wild-type.

Figure A3. Zone Diameter Distribution for Wild-Type *Staphylococcus aureus* and Cefoxitin.[1] Distributions include collated data from multiple sources, geographical areas, and time periods and cannot be used to infer rates of resistance. (European Committee on Antimicrobial Susceptibility Testing. MIC and zone diameter distributions and ECOFFs. https://www.eucast.org/mic_distributions_and_ecoffs/. Accessed 17 March 2020.)

Appendix A. (Continued)



Abbreviation: WT, wild-type.

Figure A4. Zone Diameter Distribution for Wild-Type *S. aureus* and Gentamicin.[1] Distributions include collated data from multiple sources, geographical areas, and time periods and cannot be used to infer rates of resistance. (European Committee on Antimicrobial Susceptibility Testing. MIC and zone diameter distributions and ECOFFs. https://www.eucast.org/mic_distributions_and_ecoffs/. Accessed 17 March 2020.)

Reference for Appendix A

[1] European Committee on Antimicrobial Susceptibility Testing. MIC and zone diameter distributions and ECOFFs. https://www.eucast.org/mic_distributions_and_ecoffs/. Accessed 17 March 2020.

Appendix B. Manual Preparation of Antimicrobial Disks for Phase 1 Testing

Abbreviation for Appendix B

QC quality control

Determining the optimal disk content (potency) for testing a novel antimicrobial agent using the disk diffusion method (see CLSI document M02[1])[2] might necessitate in-house preparation of susceptibility disks impregnated with varying contents (potencies) of the antimicrobial agent. Presented here is a suggested method for in-house preparation of antimicrobial disks. The steps for preparing the disks are listed below.

Step	Action	Comment(s)
1	Prepare a stock solution at 50 times the final highest desired disk content (potency). If testing multiple contents (potencies), prepare dilutions from the 50 times stock solution for the lower-content (potency) disks.	• Use solvents and diluents as recommended by the pharmaceutical company and by the procedure recommended in CLSI document M100.[3,a] • The disk content (potency) must reflect the amount of active antimicrobial agent and not the salt form of the agent. If an organic solvent is used for preparation of stock solutions, the solvent alone must be incorporated as a control into a sampling of disks and tested against target organisms to ensure the solvent does not have an inhibitory effect against the organisms.
2	Distribute blank 6-mm paper disks in sterile plastic Petri dishes that have been appropriately labeled with the antimicrobial agent and potency.	• Blank disks are available from several manufacturers.[b] • Ensure certain disks are not touching each other and can be easily accessed for pipetting. • If static electricity in the Petri dish is observed, taping a small square of an antistatic sheet to the Petri dish lid will keep static interference to a minimum. Another option is to place a sterile fine wire mesh in the bottom of the Petri dish to create a surface on which the disks can be placed to aid the disk drying process.
3	Using an automatic pipettor, add 20 µL appropriate antimicrobial solution (or solvent control, if appropriate) to each of the disks in the Petri dish.	Do not touch the pipette tip to the disk as capillary action may result in absorption of extra solution onto the disk.
4	Allow the disks to air dry in a biological safety cabinet or laminar flow hood with the lids of the Petri dishes slightly ajar or completely removed.	• Reduce light exposure during the drying process by turning off the room light or covering the glass windows of the cabinet or hood with aluminum foil. • Drying time will vary according to the solvent used and may take up to 2 hours.
5	After drying, store the disks in a dry, clean sterile container (eg, a 50-mL conical or glass tube) with desiccant, with the disks separated from the desiccant until use.	Wrap the lid of the storage container in parafilm and store at the appropriate storage temperature (2 to 8°C or −20°C or −80°C depending on the agent).

[a] The safety data sheets should be consulted before any antimicrobial reference standard powder, solvent, or diluent is handled. Some of the compounds (eg, solvents such as DMSO or methanol) are more toxic than others and may necessitate handling in a chemical fume hood.

[b] In the United States, the standard paper is 740-E and should be 30 ± 4 mg/cm^2.

Appendix B. (Continued)

NOTE 1: In-house prepared disks should be used within two weeks of preparation, but certain agents might have a shorter shelf life.

NOTE 2: As soon as possible after production, disks should be tested using relevant QC strains to obtain data that can be used during subsequent testing to ascertain the potency and shelf life of the disk. During all subsequent testing, QC must be performed and results closely monitored.

NOTE 3: If a solvent or diluent other than sterile distilled water (eg, dimethyl sulfoxide, ethanol) is used to prepare stock solutions, a control disk impregnated with only the solvent or diluent at the appropriate concentration must be tested to ensure that there is no zone of inhibition for the solvent or diluent tested alone. If a solvent produces an inhibition zone, different solvents should be tested.

NOTE 4: When a disk with two agents (antimicrobial plus inhibitor or two antimicrobials) is prepared, the stock solutions for each compound should be prepared separately, usually at 100 times the final concentration. Equal volumes of each solution are mixed together immediately before pipetting onto the disks, unless there is a known reason to pipette them separately.

NOTE 5: Because the disks are unlabeled, the use of a testing map showing the position of each disk content is recommended to identify each antimicrobial agent and disk content. The testing map should be used under the agar plate when the disks are positioned, and the plate should be oriented to identify the correct mapping position.

NOTE 6: Two disks (eg, two lots) made from independently prepared stock solutions should be tested to evaluate the reproducibility of the disk preparation.

Below is an example for preparation of single-agent disks (agent X) at 10- and 5-µg contents:

1. Prepare stock solution at 500 µg/mL (50 • 10 µg) = 500 µg/mL.

2. For a 10-µg disk, add 20 µL (0.02 mL, 1:50 dilution 500 µg/mL) to each disk (final content = 10 µg).

3. For a 5-µg disk, dilute 500 µg/mL stock solution to 250 µg/mL (1:2 dilution). Add 20 µL (0.02 mL, 1:50 dilution 250 µg/mL) to each disk (final content = 5 µg).

References for Appendix B

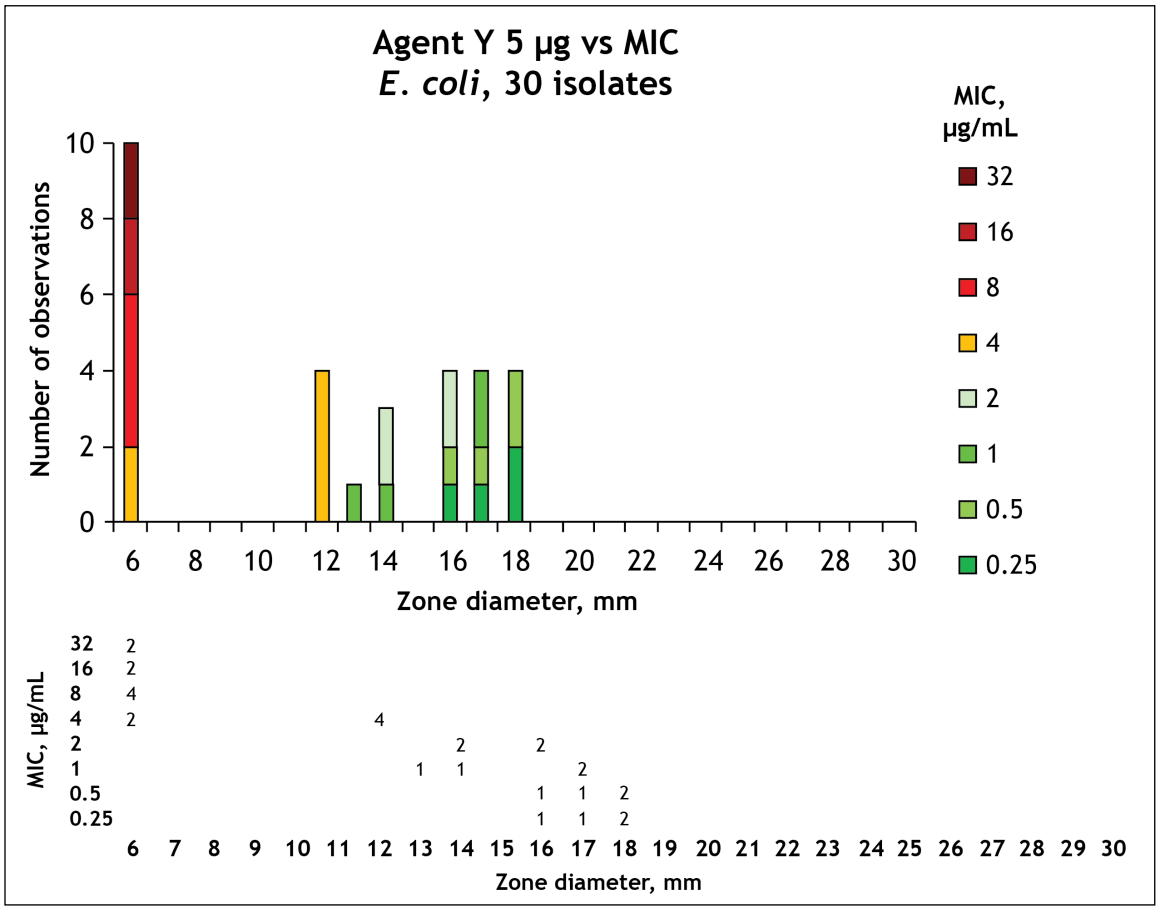
[1] CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests.* 13th ed. CLSI standard M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

[2] The European Committee on Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing: EUCAST disk diffusion method. Version 8.0; 2020. http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Accessed 12 June 2020.

[3] CLSI. *Performance Standards for Antimicrobial Susceptibility Testing.* 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

Appendix C. Examples of Histograms and Scattergrams for 5-, 10-, and 30-µg Disks vs Several Species During Phase 2 Testing

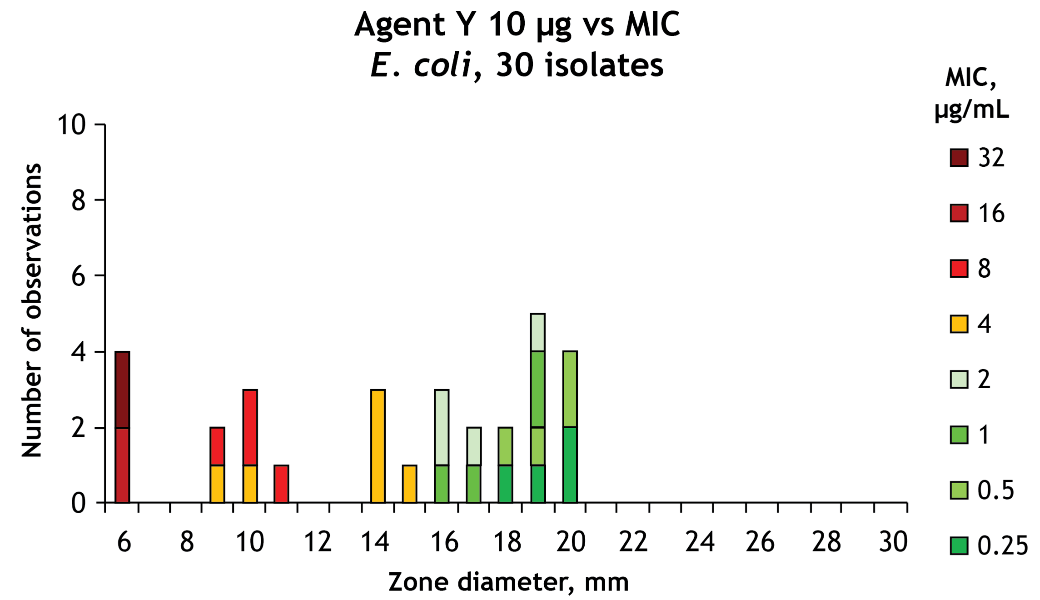
The 5-µg disk generally provides the best separation between wild-type and non-wild-type isolates for all species, compared with 10- and 30-µg disks. Examples of histograms and scattergrams from phase 2 testing with various disk contents (potencies) vs several species are shown in Figures C1 through C4.



Abbreviation: MIC, minimal inhibitory concentration.

Figure C1A. *Escherichia coli* Against Agent Y Using 5-µg Disks

Appendix C. (Continued)



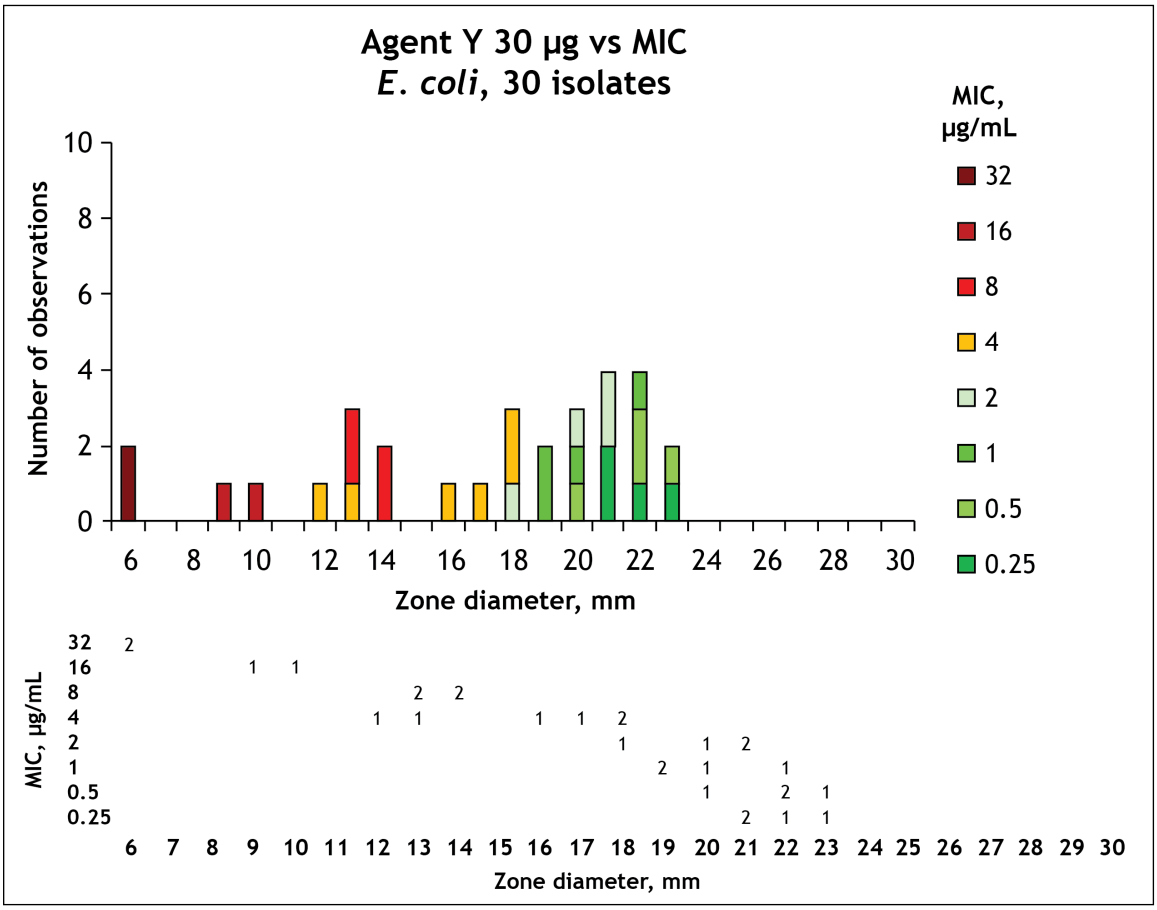
MIC, µg/mL	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
32	2																								
16	2																								
8				1	2	1																			
4				1	1				3	1															
2											2	1		1											
1											1	1		2											
0.5													1	1	2										
0.25													1	1	2										

Zone diameter, mm

Abbreviation: MIC, minimal inhibitory concentration.

Figure C1B. *Escherichia coli* Against Agent Y Using 10-µg Disks

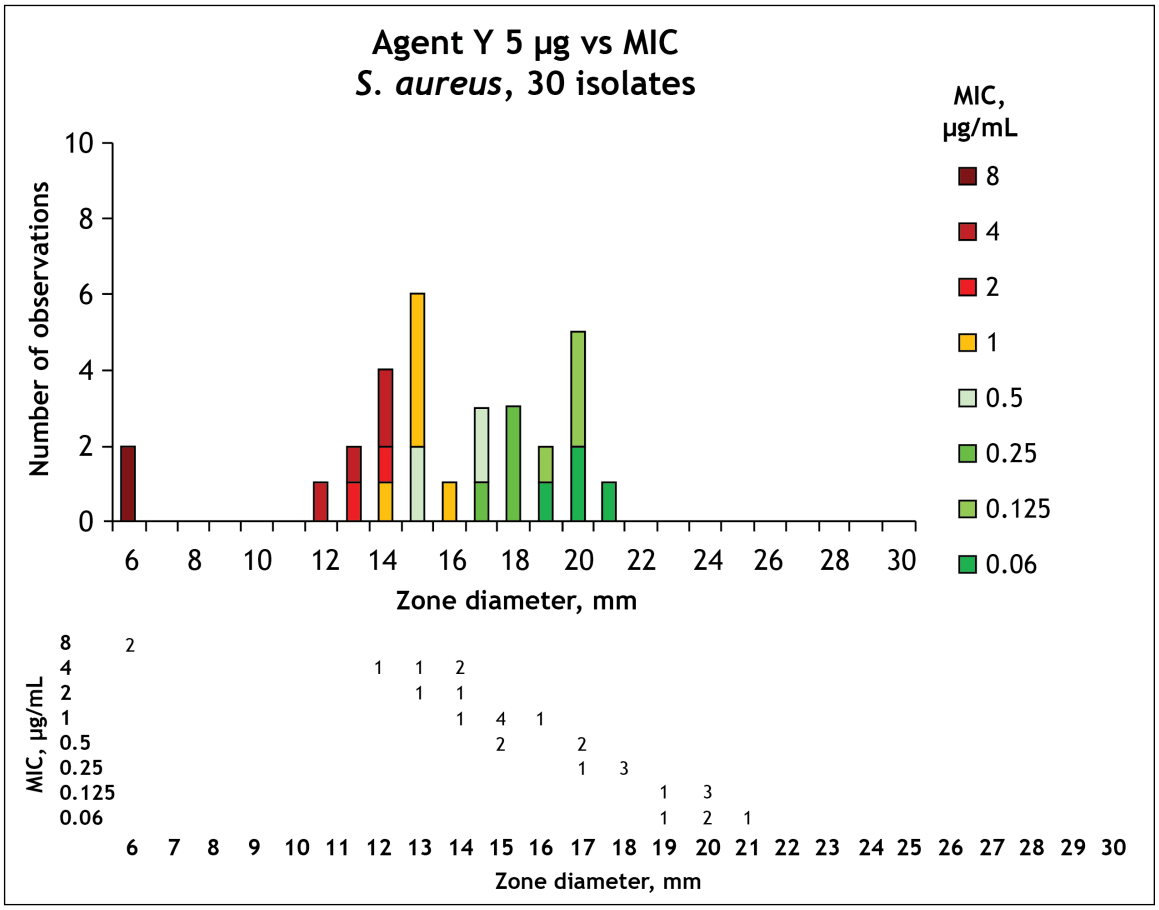
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C1C. *Escherichia coli* Against Agent Y Using 30-µg Disks

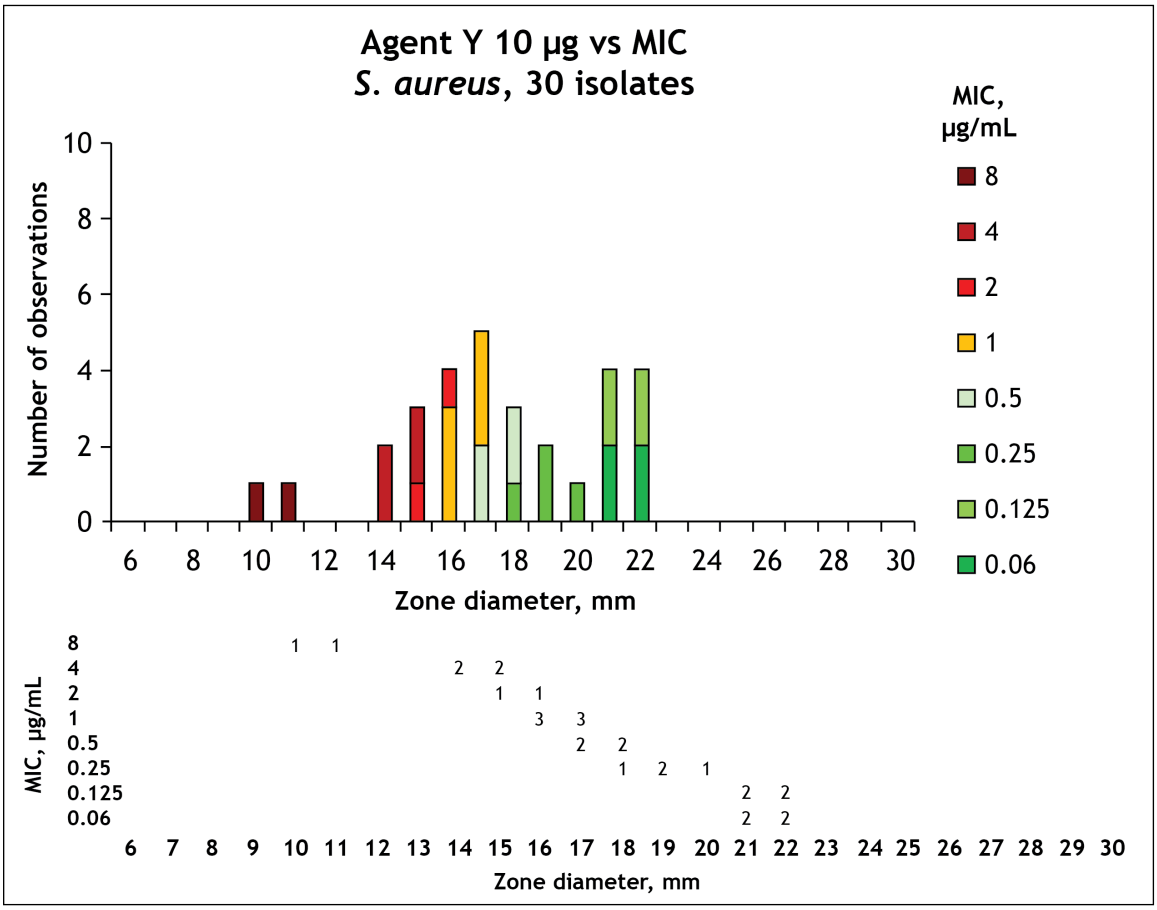
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C2A. *Staphylococcus aureus* Against Agent Y Using 5-µg Disks

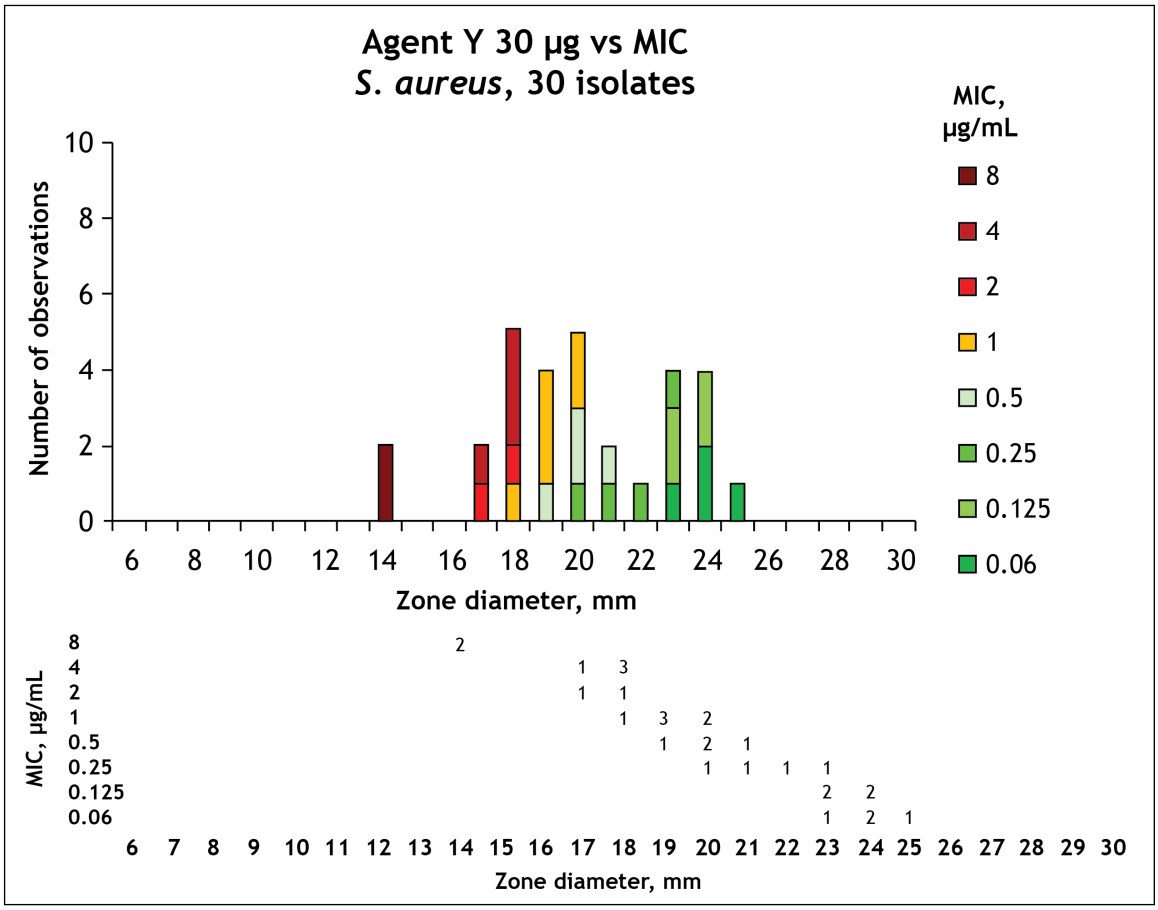
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C2B. *Staphylococcus aureus* Against Agent Y Using 10-µg Disks

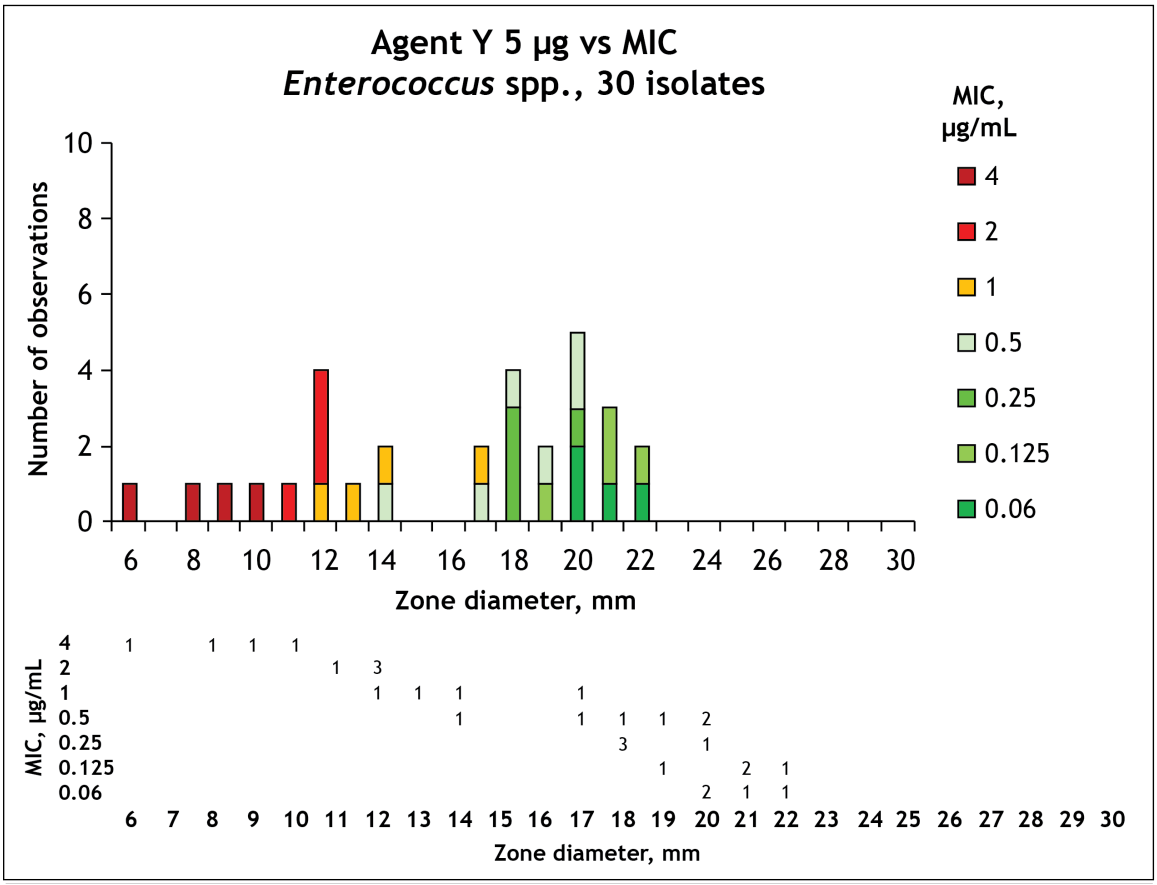
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C2C. *Staphylococcus aureus* Against Agent Y Using 30-µg Disks

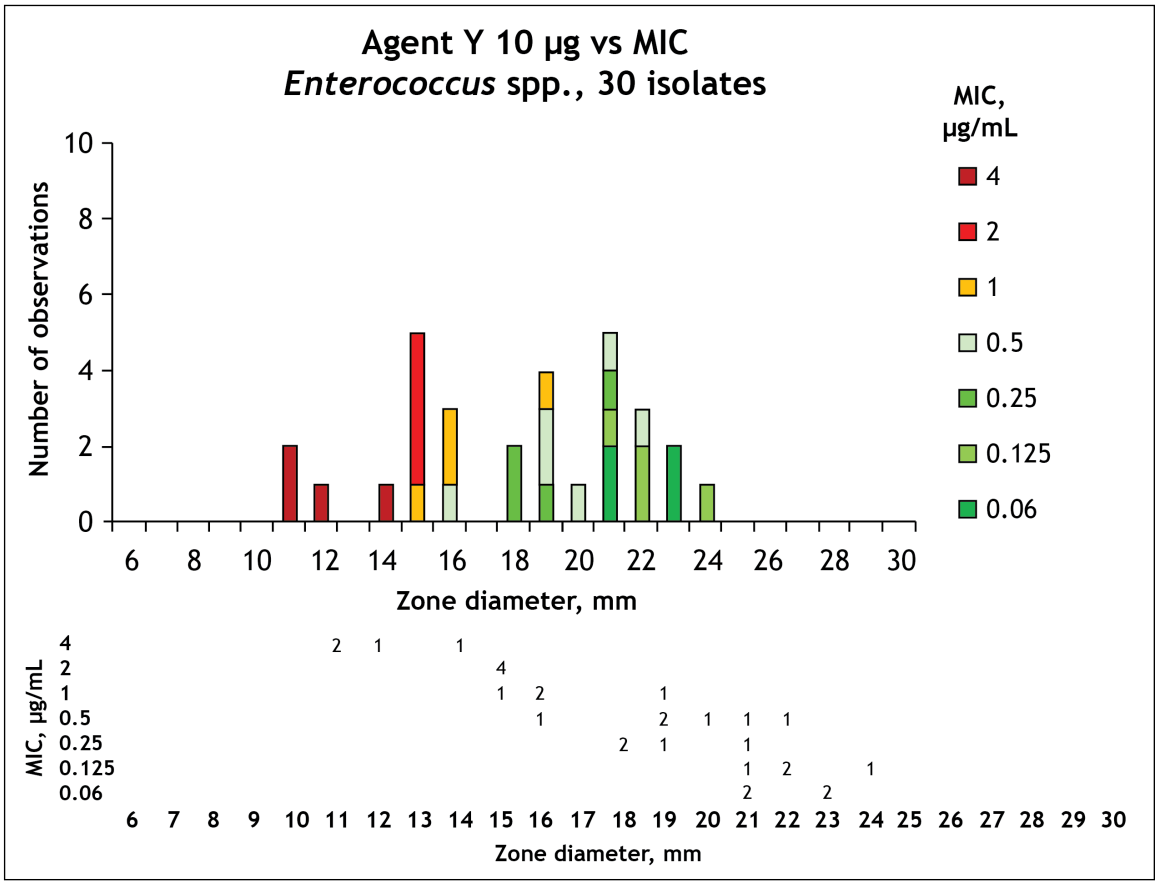
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C3A. *Enterococcus* spp. Against Agent Y Using 5-µg Disks

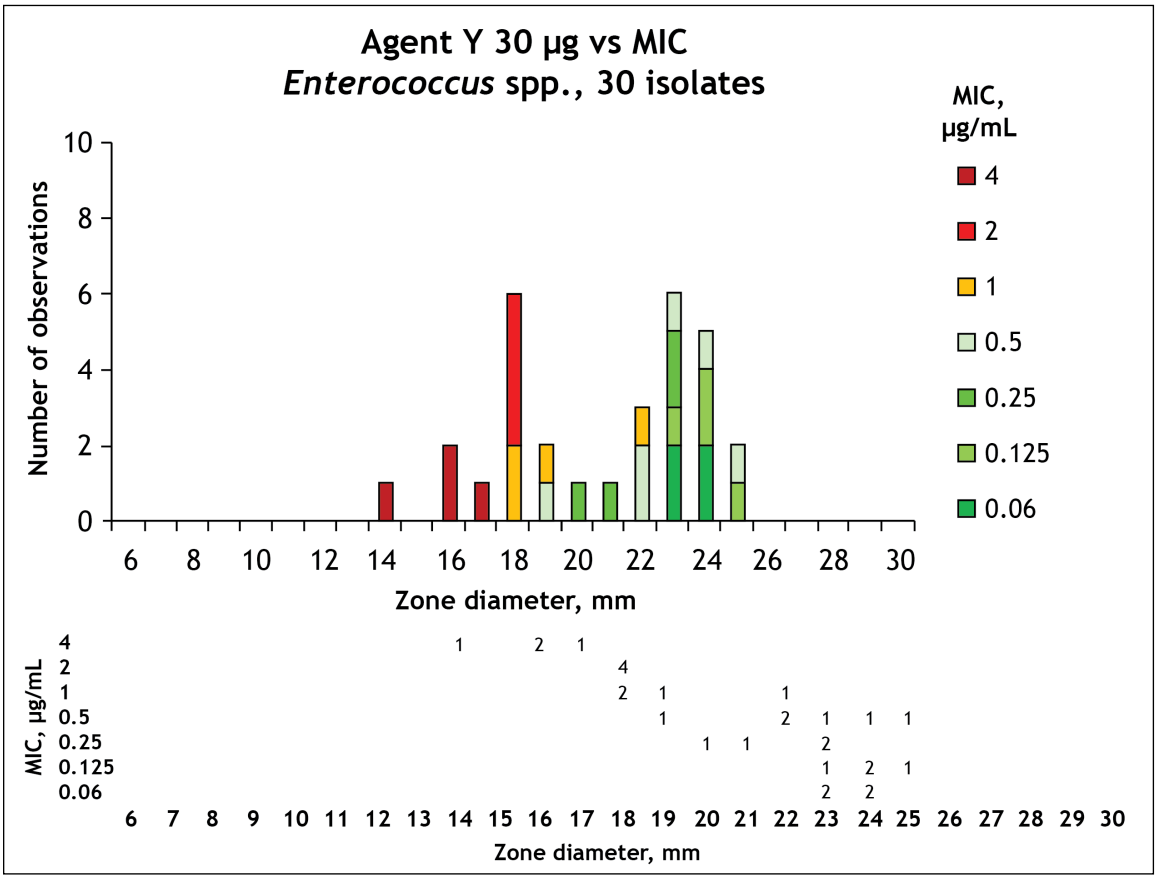
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C3B. *Enterococcus* spp. Against Agent Y Using 10-µg Disks

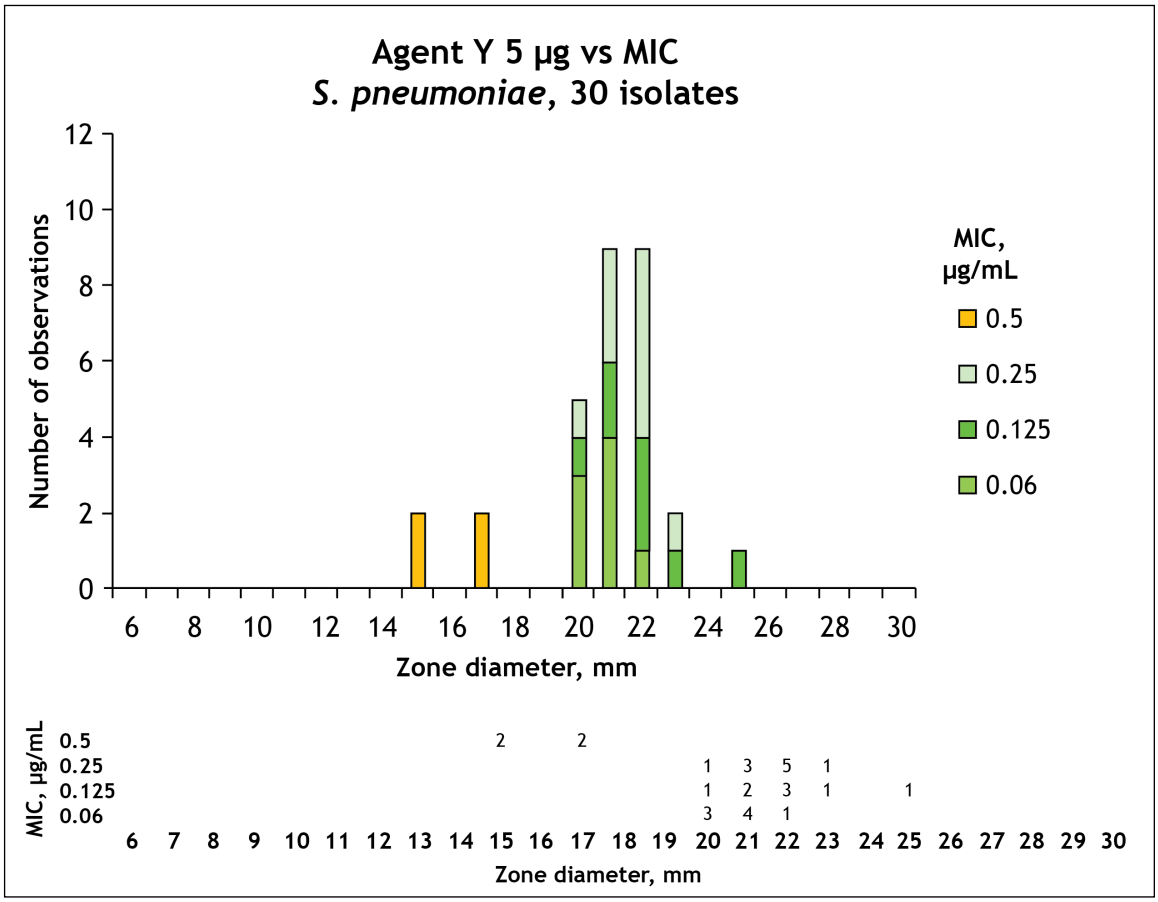
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C3C. *Enterococcus* spp. Against Agent Y Using 30-µg Disks

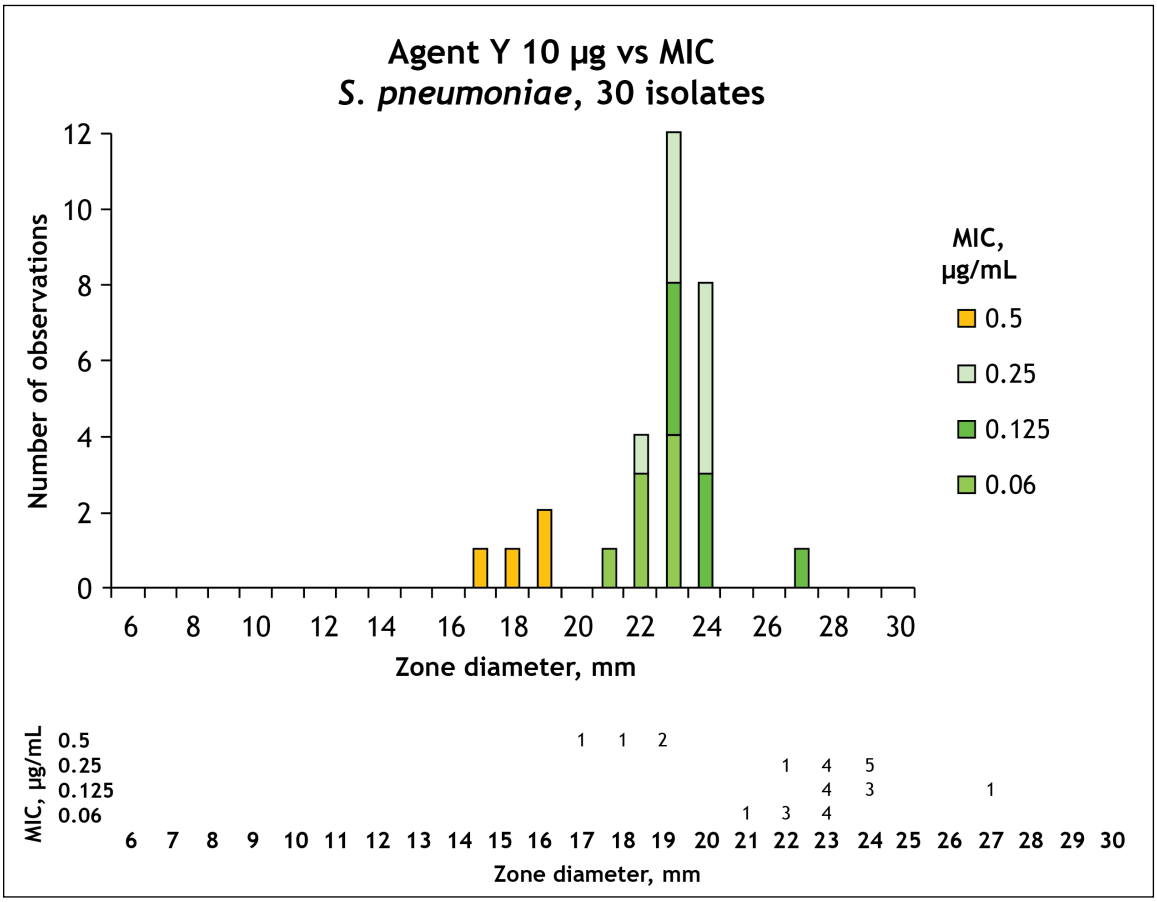
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C4A. *Streptococcus pneumoniae* Against Agent Y Using 5-µg Disks

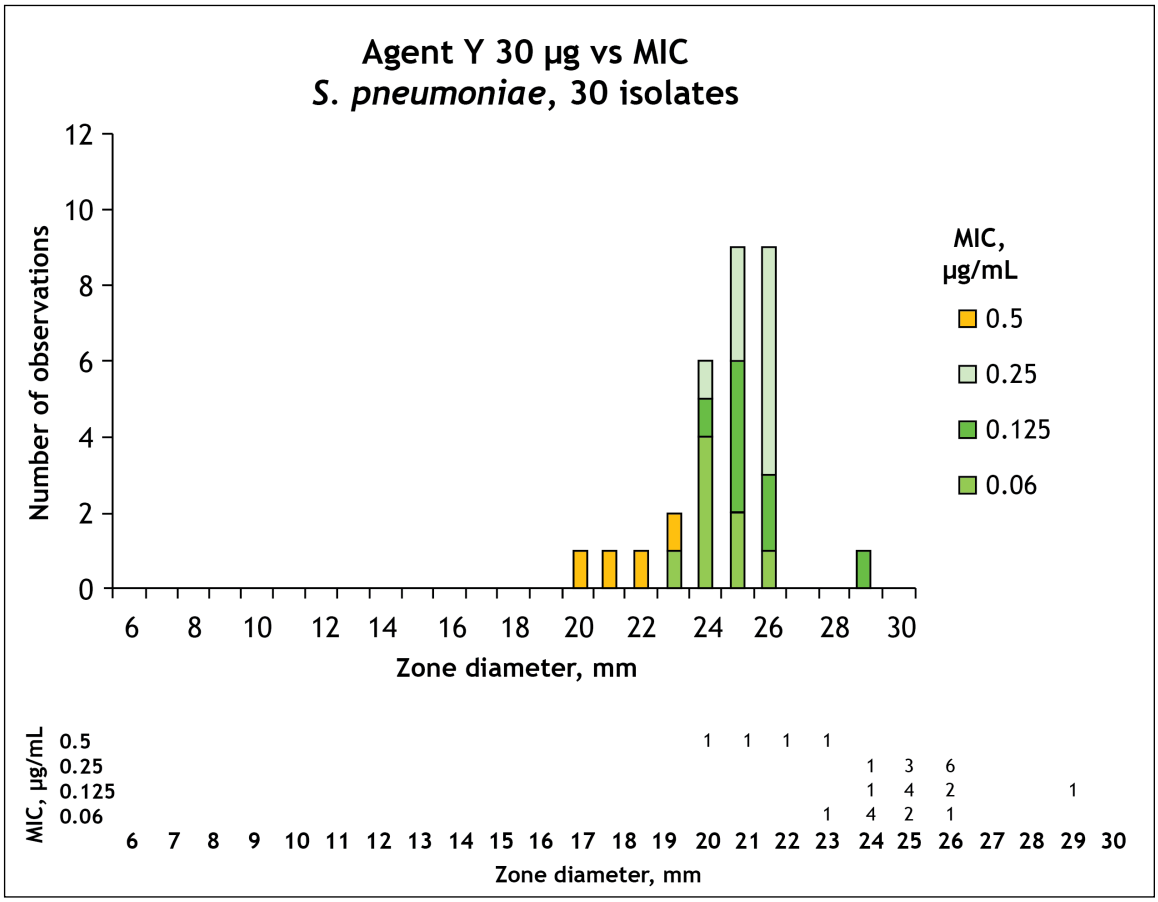
Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C4B. *Streptococcus pneumoniae* Against Agent Y Using 10-µg Disks

Appendix C. (Continued)



Abbreviation: MIC, minimal inhibitory concentration.

Figure C4C. *Streptococcus pneumoniae* Against Agent Y Using 30-µg Disks

Appendix D. Required Documentation

Abbreviation for Appendix D

QC quality control

The documentation listed below is required for submitting data for optimizing disk contents (potencies) for disk diffusion testing of antimicrobial agents using harmonized CLSI and EUCAST criteria.

- Source, lot numbers, and expiration dates of materials used in phase 1 and 2 studies to include:
  - Antimicrobial powders used to prepare stock solutions for incorporation into disks
  - Blank filter paper disks
  - Mueller-Hinton agar (with or without supplements)

- Additional procedures to include:
  - Preparation of stock solutions of antimicrobial agent(s)
  - Method for disk preparation (if different from that described in Appendix B)

- Bacterial isolates information to include:
  - Source and storage
  - Characterization for resistance mechanisms, if appropriate

- QC information to include:
  - Results obtained from each run for new agent and control agent (include source, lot numbers, and expiration dates)

- Test results to include:
  - Dates of testing
  - Technical staff performing testing
  - All results (zone measurements and minimal inhibitory concentration values) from initial and any repeat testing
  - Description of appearance of inhibition zones (eg, clear, slight haze) and any criteria recommended for measuring zone (eg, read inner zone, 90% inhibition)

- Brief summary to include:
  - Discussion of any differences noted between different disk or media sources, any discordant results or problems encountered during testing.
  - Discussion of overall experiences noted during testing to provide insight into the performance of disk diffusion testing of the agent under evaluation.

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The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system (QMS) approach in the development of standards and guidelines that facilitates project management, defines a document structure using a template, and provides a process to identify needed documents. The QMS approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any health care service's path of workflow (ie, operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The QSEs are:

• Organization and Leadership • Customer Focus • Facilities and Safety Management • Personnel Management	• Supplier and Inventory Management • Equipment Management • Process Management • Documents and Records Management	• Information Management • Nonconforming Event Management • Assessments • Continual Improvement

M23S covers the QSE indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section.

Organization and Leadership	Customer Focus	Facilities and Safety Management	Personnel Management	Supplier and Inventory Management	Equipment Management	Process Management	Documents and Records Management	Information Management	Nonconforming Event Management	Assessments	Continual Improvement
		M29				X M02 M07 M100					

Path of Workflow

A path of workflow is the description of the necessary processes to deliver the particular product or service that the organization or entity provides. A laboratory path of workflow consists of the sequential processes: preexamination, examination, and postexamination and their respective sequential subprocesses. All laboratories follow these processes to deliver their services, namely quality laboratory information.

M23S does not cover any of the medical laboratory path of workflow processes. For a description of the documents listed in the grid, please refer to the Related CLSI Reference Materials section.

Preexamination				**Examination**				**Postexamination**		
Examination ordering	Specimen collection	Specimen transport	Specimen receipt, accessioning, and processing	Examination method selection	Examination performance	Results review and follow-up	Laboratory results interpretation	Communication of alert values and issuance of preliminary reports	Release of final reports	Specimen management
					M02 M07	M02 M07 M100	M02 M07 M100		M02 M07 M100	

Related CLSI Reference Materials*

M02 **Performance Standards for Antimicrobial Disk Susceptibility Tests. 13th ed., 2018.** This standard covers the current recommended methods for disk susceptibility testing and criteria for quality control testing.

M07 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 11th ed., 2018.** This standard covers reference methods for determining minimal inhibitory concentrations of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

M29 **Protection of Laboratory Workers From Occupationally Acquired Infections. 4th ed., 2014.** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

M100 **Performance Standards for Antimicrobial Susceptibility Testing. 30th ed., 2020.** This document includes updated tables for the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M02, M07, and M11.

* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

NOTES

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