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Standard Operating Procedure

Procedure for optimizing disk contents (potencies) for disk diffusion testing of antimicrobial agents using harmonized CLSI and EUCAST criteria

EUCAST SOP 11.1

21 January 2025

Procedure for optimizing disk contents (potencies) for disk diffusion testing of antimicrobial agents using harmonized CLSI and EUCAST criteria

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Foreword

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is organised by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID), the European Centre for Disease Prevention and Control (ECDC), and the active national antimicrobial breakpoint committees in Europe. EUCAST was established by ESCMID in 1997, was restructured in 2001-2002 and has been in operation in its current form since 2002.

The current remit of EUCAST is to harmonise clinical breakpoints for existing antimicrobial agents in Europe, to determine clinical breakpoints for new agents, to set epidemiological (microbiological) breakpoints, to revise breakpoints as required, to harmonise methodology for antimicrobial susceptibility testing, to develop a website with MIC and zone diameter distributions of antimicrobial agents for a wide range of organisms and to liaise with European governmental agencies and European networks involved with antimicrobial resistance and resistance surveillance.

Information on EUCAST, EUCAST breakpoints and all documents are freely available on the EUCAST website at www.eucast.org.

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Abbreviations	
CLSI	Clinical and Laboratory Standards Institute
EOR	Ease of reading
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESCMID	European Society for Clinical Microbiology and Infectious Diseases
ISO	International Organization for Standardisation
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
NWT	Non wild type
QC	Quality control
SOP	Standard Operating Procedure
WOG	Weight of growth
WT	Wild type

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 standardised disk diffusion method equivalent to disk load, disk mass, disk strength, and disk charge.
Minimum 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.

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1	Introduction
	<p>The disk diffusion antimicrobial susceptibility test has been widely used around the world for decades and was first standardised in 1966¹. 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 EUCAST (European Committee on Antimicrobial Susceptibility Testing) provided a standardised disk diffusion method calibrated to the harmonized European minimum 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.</p> <p>The disk content (potency) recommended for new antimicrobial agents has sometimes varied among organizations that set criteria (e.g. 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 harmonised 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 standardised recommendations for disk content (potency) selection. Their recommendations are presented in this document.</p>
1.1	Scope
	<p>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.</p> <p>This document is intended for pharmaceutical manufacturers involved in the development of antimicrobial agents and tests to support evaluation of antimicrobial agent activity for testing of bacteria. 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 standardised disk diffusion test. It does not explain the steps needed to perform the standardised 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.²⁻⁴ 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.</p> <p>The development of zone diameter breakpoints and QC criteria for EUCAST tables is described in “European Committee on Antimicrobial Susceptibility</p>

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	Testing. Procedure for establishing zone diameter breakpoints and quality control criteria for new antimicrobial agents, EUCAST SOP 9.3, 2022. http://www.eucast.org .”
1.2	<p>Background</p> <p>The standard for antimicrobial susceptibility testing of rapidly growing aerobic bacteria is minimum inhibitory concentration (MIC) determination using broth microdilution according to international standards^{3,5}, 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 CLSI³ and EUCAST⁶ recommend different media. Both CLSI² and EUCAST⁴ have developed standardised disk diffusion methods calibrated to match the results of reference MIC methodology^{3,5}, 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 be developed only once a reference MIC method has been established for the antimicrobial agent and organisms in question.</p> <p>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.</p> <p>The CLSI and EUCAST disk diffusion methods are based on the same basic methodology, i.e. 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 joint CLSI-EUCAST 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.</p> <p>Although CLSI and EUCAST strongly suggest that pharmaceutical companies approach the joint CLSI-EUCAST working group to develop disk content (potency) for a new drug or for a drug for which an alternative disk content (potency) might be needed, some companies may wish to proceed without collaborating with the joint CLSI-EUCAST working group. If the company subsequently seeks approval for disk QC ranges, breakpoints, or epidemiological</p>

cut-off values the company will be asked to provide the data supporting disk content (potency) selection to the joint CLSI-EUCAST working group. Additional studies may be requested if the data are deemed insufficient to approve the disk content (potency).

2 Procedure for Establishing the Optimal Disk Content (Potency)

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 (potency) 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 QC strains and clinical isolates are tested

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 log₂ 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

	<ul style="list-style-type: none"> • Optimal separation between NWT isolates with different MICs, irrespective of resistance mechanisms <p>The disk content (potency) should be established according to the procedures described in phases 1 and 2 (see Subchapter 2.2).</p>
2.2	<p>Basic Criteria for Phase 1 and 2 Studies</p> <p>The following basic criteria apply to phase 1 and phase 2 disk content (potency) studies:</p> <ul style="list-style-type: none"> • 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: <ul style="list-style-type: none"> – 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 (i.e. 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 standards⁷ and the QC criteria published by CLSI⁸ and EUCAST⁹ for standard QC strains. To establish acceptable quality of the medium, results must be in range when QC strains and agents from similar and different antimicrobial classes are tested. 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. See CLSI M23S3 (EUCAST SOP 13.0) for the necessary technical steps for confirming the acceptability of Mueller-Hinton agar sources for subsequent use in CLSI and/or EUCAST studies to establish disk diffusion QC ranges. • 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.

	<ul style="list-style-type: none"> • 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 CLSI⁸ and/or EUCAST⁹ published QC ranges must be included with disk diffusion testing of all isolates (clinical isolates and QC strains). • The quality of zones produced by the agent for each organism and Mueller-Hinton medium source should be assessed by documenting Ease of Reading (EOR) and Weight of Growth (WOG) (see Table 1). This assessment helps reviewers identify any potential challenges encountered during zone measurements. The CLSI¹⁰ and EUCAST¹¹ reading guides are resources for this evaluation. Additionally, photographs can be submitted with the test data. • If the appearances of zones (eg, sharp, clear zones) and growth (confluent) are unambiguous for all test isolates, a comment summarizing these two observations in the report summary is sufficient. • If zone measurements are difficult to determine or growth varies on different media sources (eg, for fastidious organisms), it is suggested that the following scores in Table 1 be recorded with readings for all or a representative sample of tests.
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Table 1. EOR and WOG Scores

EOR	EOR Score	WOG	WOG Score
Very fuzzy and/or double zone and/or edge difficult to read	1	Faint confluent growth	1
Moderate	2	Moderate confluent growth	2
Easy to read (sharp, clear zones edges) ^a	3	Good confluent growth ^a	3

Abbreviations: EOR, ease of reading; WOG, weight of growth.

^a If all readings from test isolates fall into both of these categories, it is not necessary to record scores for each isolate and a comment in the report is sufficient.

Submission of photographs to capture zone appearances is encouraged, as deemed appropriate.

2.3	<p>Phase 1: Initial Screening of a Series of Disk Contents (Potencies)</p> <p>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 two to four disks will be selected for testing in phase 2 with a larger number of isolates.</p> <p>Normally, 10 different disks ranging from very low to very high (e.g. 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 standardised disk diffusion methodology against relevant species. The most important target species should be included when organism groups are evaluated, e.g. <i>Escherichia coli</i> and <i>Klebsiella pneumoniae</i> for <i>Enterobacterales</i>. For agents with broad-spectrum activity against a variety of organism groups (e.g. 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.</p> <ul style="list-style-type: none">• A disk content (potency) previously used for the antimicrobial class of the agent being evaluated (e.g. 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:<ul style="list-style-type: none">– 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)
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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-µg 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	<i>E. coli</i>	0.06	WT	12	16	18	21	26	27	29	31	32	32
2	<i>E. coli</i>	0.25	WT	7	12	16	19	26	27	29	29	30	30
3	<i>E. coli</i>	8	NWT	6	6	6	9	16	19	21	23	24	23
4	<i>E. coli</i>	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	<i>K. pneumoniae</i>	0.25	WT	10	13	16	18	24	26	28	28	28	29
6	<i>K. pneumoniae</i>	0.5	WT	11	15	17	21	26	28	30	31	30	31
7	<i>K. pneumoniae</i>	32	NWT	6	6	6	6	8	10	13	15	15	16
8	<i>K. pneumoniae</i>	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	<i>P. aeruginosa</i>	2	WT	6	6	8	13	21	24	27	27	28	29
10	<i>P. aeruginosa</i>	4	WT	6	6	6	6	15	20	23	25	25	28
11	<i>P. aeruginosa</i>	>32	NWT	6	6	6	12	18	21	25	26	25	28
12	<i>P. aeruginosa</i>	>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	<i>E. coli</i>	0.06	WT	–	4	2	3	5	1	2	2	1	0
2	<i>E. coli</i>	0.25	WT	–	5	4	3	7	1	2	0	1	0
3	<i>E. coli</i>	8	NWT	–	0	0	3	7	3	2	2	1	–1
4	<i>E. coli</i>	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	<i>K. pneumoniae</i>	0.25	WT	–	3	3	2	6	2	2	0	0	1
6	<i>K. pneumoniae</i>	0.5	WT	–	4	2	4	5	2	2	1	–1	1
7	<i>K. pneumoniae</i>	32	NWT	–	0	0	0	2	2	3	2	0	1
8	<i>K. pneumoniae</i>	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	<i>P. aeruginosa</i>	2	WT	–	0	2	5	8	3	3	0	1	1
10	<i>P. aeruginosa</i>	4	WT	–	0	0	0	9	5	3	2	0	3
11	<i>P. aeruginosa</i>	>32	NWT	–	0	0	6	6	3	4	1	–1	3
12	<i>P. aeruginosa</i>	>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	<i>E. coli</i>	0.06	WT	12	16	18	21	26	27	29	31	32	32
2	<i>E. coli</i>	0.25	WT	7	12	16	19	26	27	29	29	30	30
3	<i>E. coli</i>	8	NWT	6	6	6	9	16	19	21	23	24	23
4	<i>E. coli</i>	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	<i>K. pneumoniae</i>	0.25	WT	10	13	16	18	24	26	28	28	28	29
6	<i>K. pneumoniae</i>	0.5	WT	11	15	17	21	26	28	30	31	30	31
7	<i>K. pneumoniae</i>	32	NWT	6	6	6	6	8	10	13	15	15	16
8	<i>K. pneumoniae</i>	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	<i>P. aeruginosa</i>	2	WT	6	6	8	13	21	24	27	27	28	29
10	<i>P. aeruginosa</i>	4	WT	6	6	6	6	15	20	23	24	25	28
11	<i>P. aeruginosa</i>	>32	NWT	6	6	6	12	18	21	25	26	25	28
12	<i>P. aeruginosa</i>	>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	<p>Phase 2 Disk Content (Potency) Study</p> <p>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.</p> <p>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.</p>
2.4.1	<p>Additional Investigation of the Contents (Potencies) of Two to Four Selected Disks</p> <p>At least 30 isolates per species or 60 isolates per group of organisms (e.g. <i>Enterobacteriales</i>, <i>Pseudomonas</i> 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.</p>

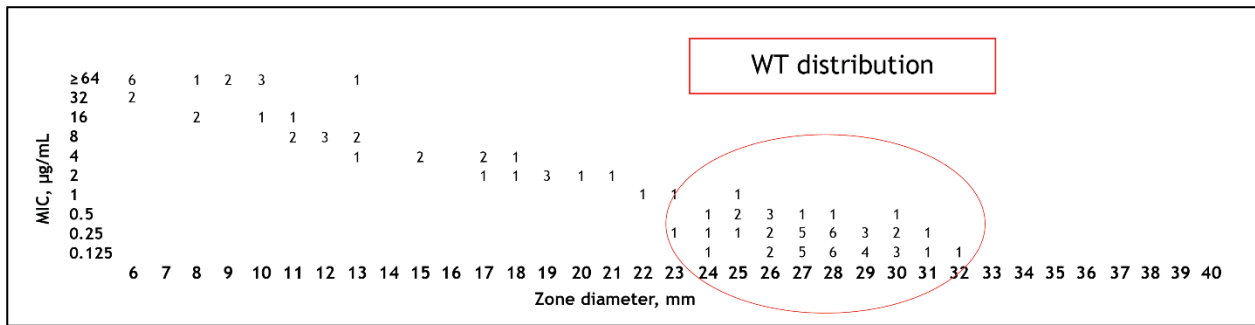
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The NWT isolates should, when possible, represent a variety of MICs and resistance mechanisms and include isolates with MICs one to two dilutions above the highest MIC in the WT population. When resistant isolates are not available, the minimum criterion 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 (i.e. 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 (i.e. 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 coloured bars (see Figure 1B)

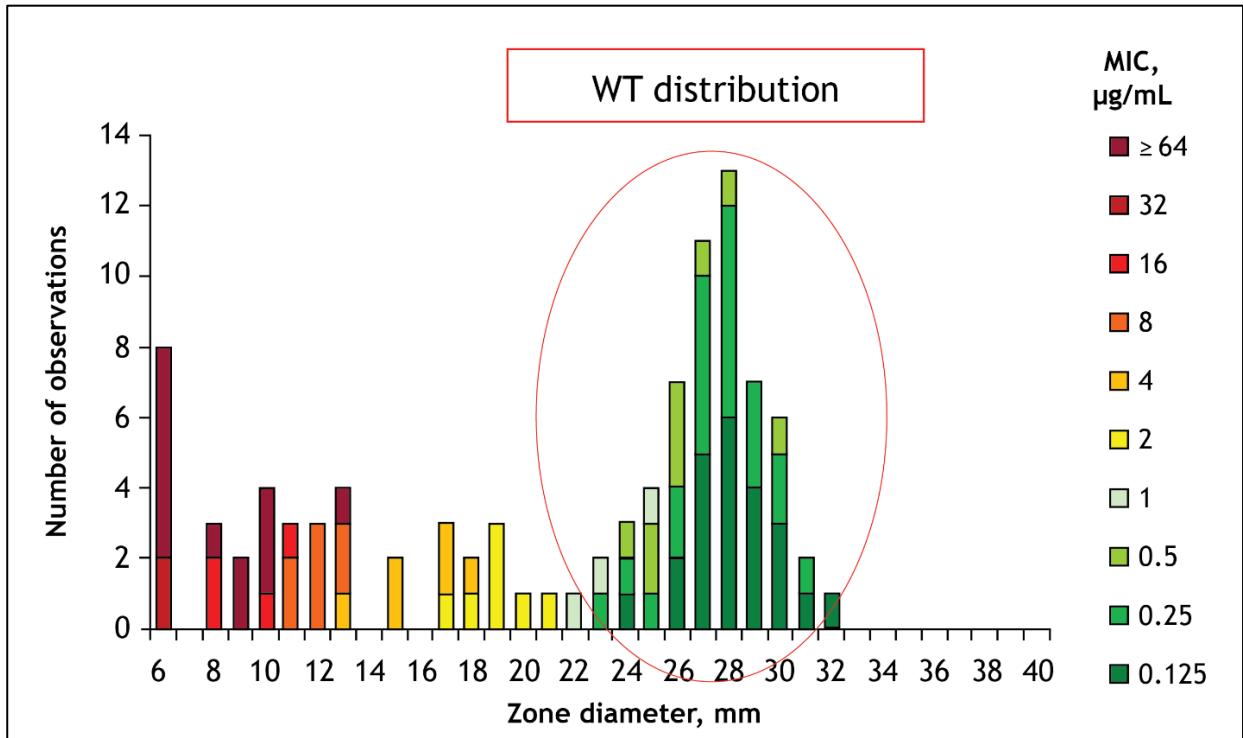
NOTE: Figures 1A and 1B represent the same dataset.

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



Abbreviations: MIC, minimum inhibitory concentration; WT, wild-type.
Figure 1A. Zone Diameter Scattergram With Zone Diameters Plotted Against MIC Values.

Figures 1A and 1B represent the same dataset.



Abbreviations: MIC, minimum inhibitory concentration; WT, wild-type; NWT, non-wild type.
Figure 1B. Zone Diameter Histogram with MIC Values Represented by Coloured Bars. Green corresponds to WT isolates. Yellow, orange, and red correspond to different MICs for NWT isolates.

Figures 1A and 1B represent the same dataset.

2.4.2	<p>Selection of Optimal Disk Content (Potency)</p> <p>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.</p>
2.5	<p>Considerations for Selection of the Optimal Disk Content (Potency) for Combinations of Agents</p> <p>The following considerations apply to the selection of the optimal disk content (potency) for combinations of agents:</p> <ul style="list-style-type: none"> • For disks consisting of combinations of agents (agent plus agent or agent plus inhibitor), a discussion with the joint CLSI-EUCAST working group is recommended before development moves forward. • It is usually best to initially consider the standard disk content (potency) for the active agent (referred to here as the parent compound) and vary the inhibitor component. If there is no agreed disk content (potency) for the parent compound alone, establishing the optimal disk content (potency) of the parent compound must be part of the process. This must also be considered when the existing disk content (potency) is suboptimal or when CLSI and EUCAST disk contents (potencies) differ. • At the outset of any studies with combination disks, there are important points that should be considered: <ul style="list-style-type: none"> – Does the inhibitor have any secondary antimicrobial activity unrelated to the target inhibition (eg, another form of antimicrobial activity or synergy with the parent compound) that may influence the combination disk results and require additional evaluation? – Do the zone diameters for parent compound plus an inhibitor (without secondary activity) coincide with those of the parent compound alone against isolates that are WT to the parent compound? If not, this may be indicative of an on-disk interaction between the parent compound and inhibitor. In these cases, an alternative disk content (potency) of the parent compound should be evaluated to ensure the zone diameters match. • When test isolates are selected for development of a combination disk, it is important to ensure that a sufficient number of isolates are

	<p>NWT to the parent compound alone (or resistant if a clinical breakpoint exists).</p> <p>NOTE: Isolates WT to the parent compound alone will not provide useful data for evaluation of the disk content (potency) for the inhibitor, but they are important to test to assess whether there is an on-disk interaction.</p> <ul style="list-style-type: none"> To select the optimal disk content (potency) for combination agents, a greater number of isolates than suggested for a single agent should be tested (see Table 3). <p>NOTE: For Enterobacterales, a variety of target species should be included, and differences in MIC values between species should be considered when the laboratory is determining the numbers of each species to evaluate.</p>
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Table 3. Selection of Isolates for Phase 1 and Phase 2 Testing of Combination Agents

Phenotype	Phase 1	Phase 2
WT to the parent compound	≥ 2	≥ 10
WT to the combination (NWT to parent compound)	≥ 2	≥ 30
NWT to the combination	≥ 2	≥ 30

Abbreviations: NWT, non-wild-type; WT, wild-type.

	<ul style="list-style-type: none"> It is advisable to include in the report information available on relevant resistance mechanisms in the test isolates, and the effect of the inhibitor on these resistance mechanisms. Results should be summarized in species-specific or organism-specific scattergrams (see Figure 1A) and inhibition zone diameter histograms with corresponding MIC values as coloured bars (see Figure 1B).
	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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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.* 14th ed. CLSI standard M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2024.
- 3 CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically.* 12th ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute; 2024
- 4 The European Committee on Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing: EUCAST disk diffusion method. Version 12.0; 2024. http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Accessed 19 September 2024.
- 5 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.
- 6 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 7.0; 2022. http://www.eucast.org/ast_of_bacteria/media_preparation/. Accessed 19 September 2024.
- 7 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.
- 8 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing.* 34th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2024.
- 9 The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. Version 14.0; 2024. http://www.eucast.org/ast_of_bacteria/qc_tables/. Accessed 19 September 2024.
- 10 CLSI. M02 Disk Diffusion Reading Guide. 2nd ed. CLSI quick guide M02QG. Clinical and Laboratory Standards Institute; 2024.
- 11 The European Committee on Antimicrobial Susceptibility Testing. EUCAST disk diffusion reading guide. Version 10.0; 2023. http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Accessed 19 September 2024.

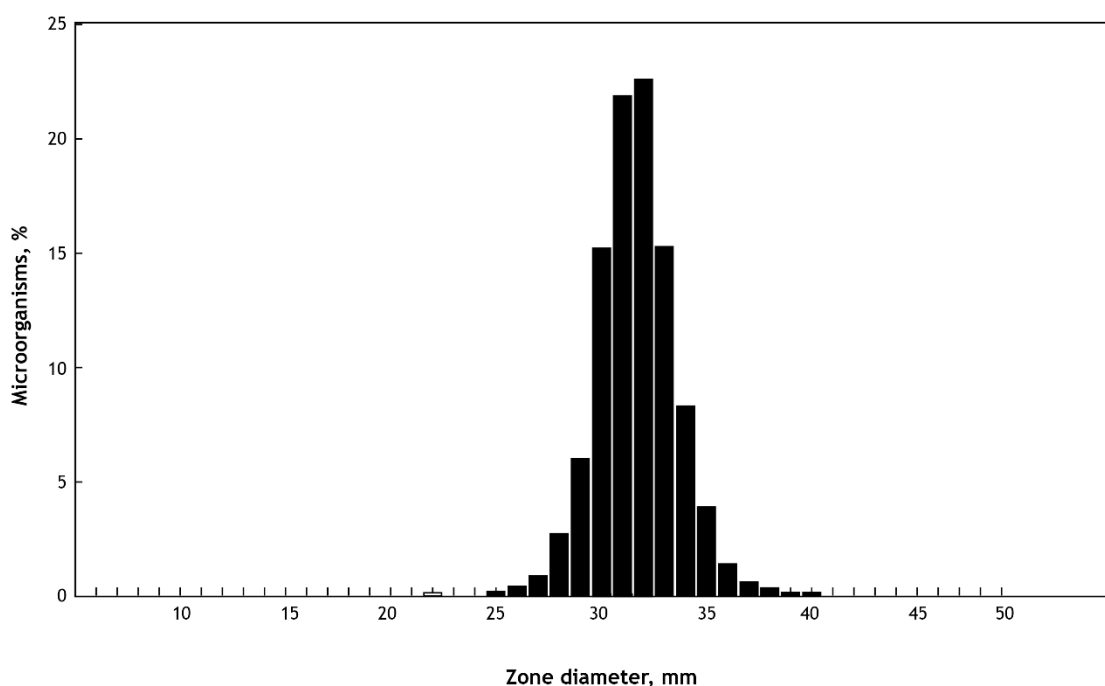
Appendix A. Examples of Zone Diameter Distributions with a Defined Wild-Type Distribution

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.



Disk content: 10 μ g
WT organisms: ≥ 25 mm

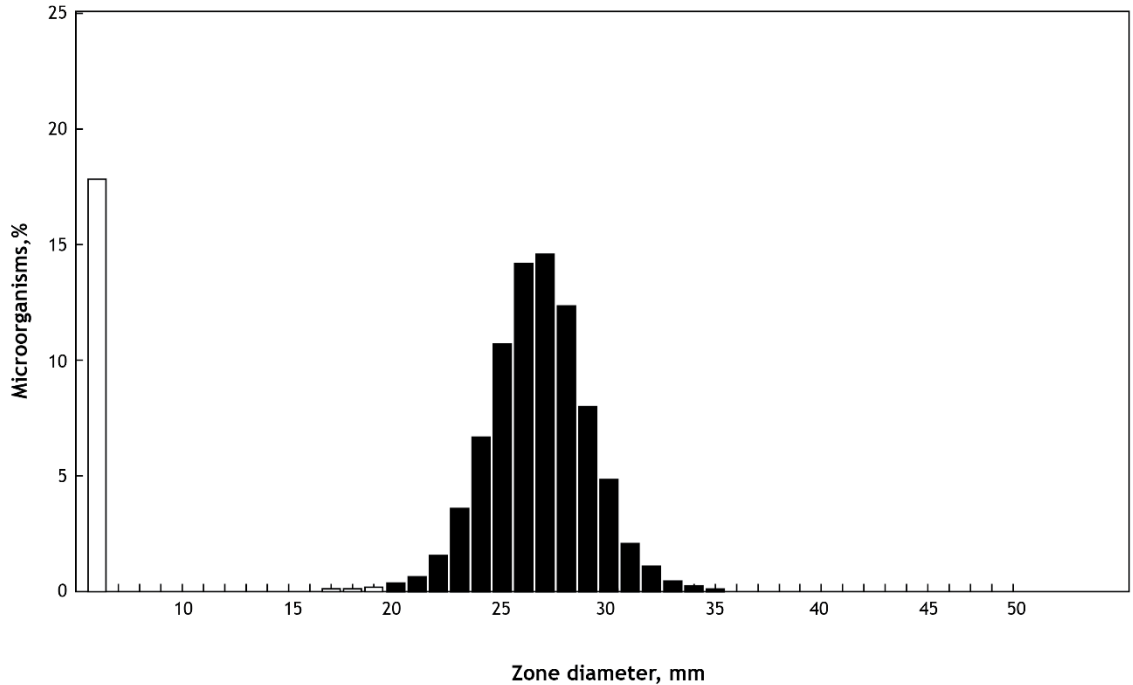
15 566 observations (17 data sources)

Abbreviation: WT, wild-type.

Figure A1. Zone Diameter Distribution for WT *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)



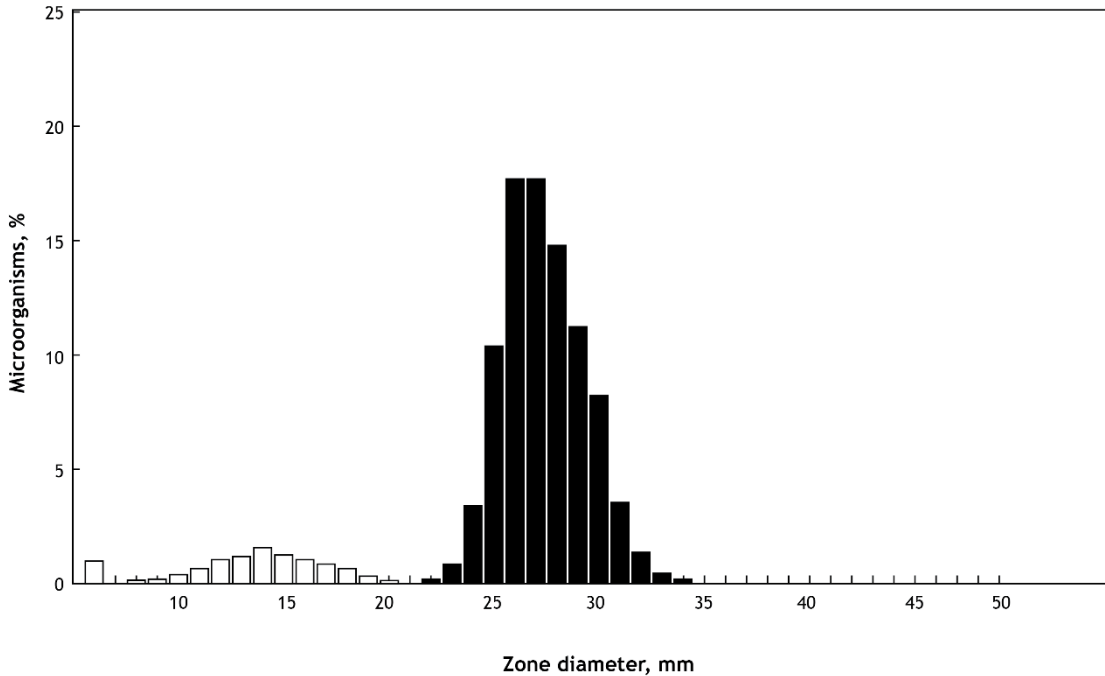
Disk content: 5 µg
 WT organisms: ≥ 20 mm

59 792 observations (12 data sources)

Abbreviation: WT, wild-type.

Figure A2. Zone Diameter Distribution for WT *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)



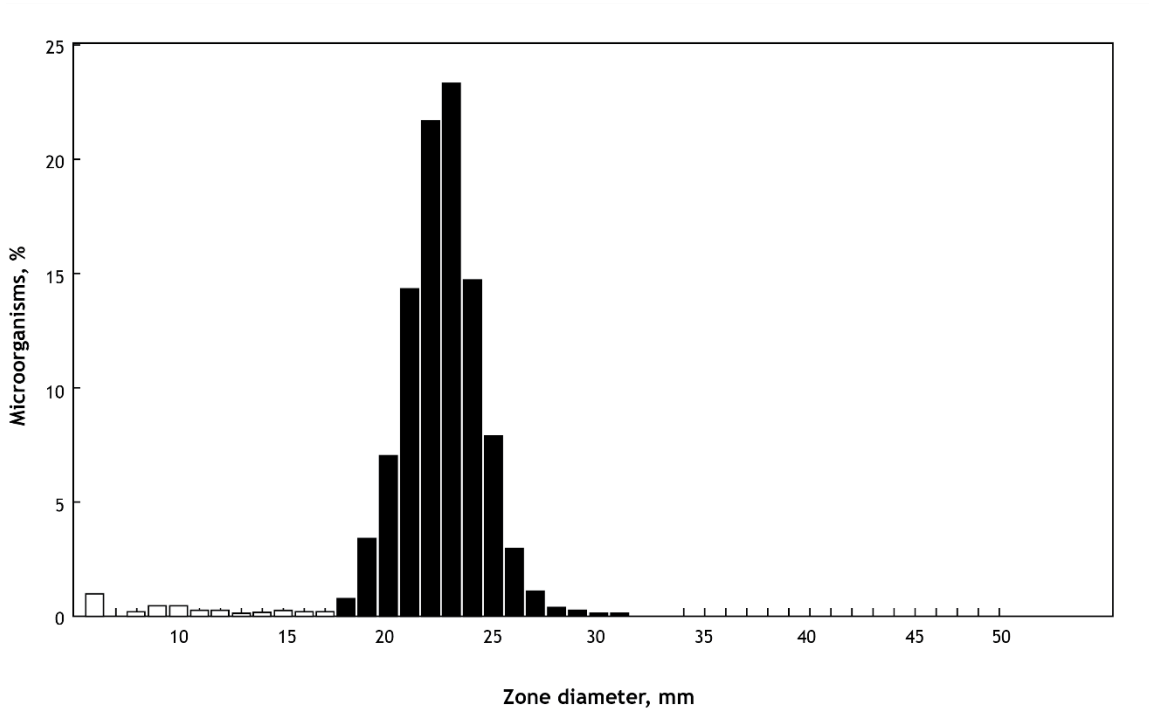
Disk content: 30 µg
 WT organisms: ≥ 22 mm

36 460 observations (15 data sources)

Abbreviation: WT, wild-type.

Figure A3. Zone Diameter Distribution for WT *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)



Disk content: 10 µg
 WT organisms: ≥ 18 mm

13 195 observations (15 data sources)

Abbreviation: WT, wild-type.

Figure A4. Zone Diameter Distribution for WT *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^{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	<p>Prepare a stock solution at 50 times the final highest desired disk content (potency).</p> <p>If multiple contents (potencies) are being tested, prepare dilutions from the 50 times stock solution for the lower-content (potency) disks.</p>	<ul style="list-style-type: none"> 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 labelled with the antimicrobial agent and disk content (potency).	<ul style="list-style-type: none"> 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.	<ul style="list-style-type: none"> Reduce light exposure during the drying process by turning off the room light or covering the glass windows of the cabinet or hood with aluminium 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 (e.g. 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 (e.g. 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/cm².

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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 disk content (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 (e.g. 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 agent plus inhibitor or two antimicrobial agents) 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 unlabelled, the use of a testing map showing the position of each disk content (potency) is recommended to identify each antimicrobial agent and disk content (potency). 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 (e.g. 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- μ g disk contents (potencies):

1. Prepare stock solution at 500 mg/L ($50 \cdot 10 \mu\text{g}$) = 500 mg/L.
2. For a 10- μ g disk, add 20 μ L (0.02 mL, 1:50 dilution 500 mg/L) to each disk (final content = 10 μ g).
3. For a 5- μ g disk, dilute 500 mg/L stock solution to 250 mg/L (1:2 dilution). Add 20 μ L (0.02 mL, 1:50 dilution 250 mg/L) to each disk (final content = 5 μ g).

References for Appendix B

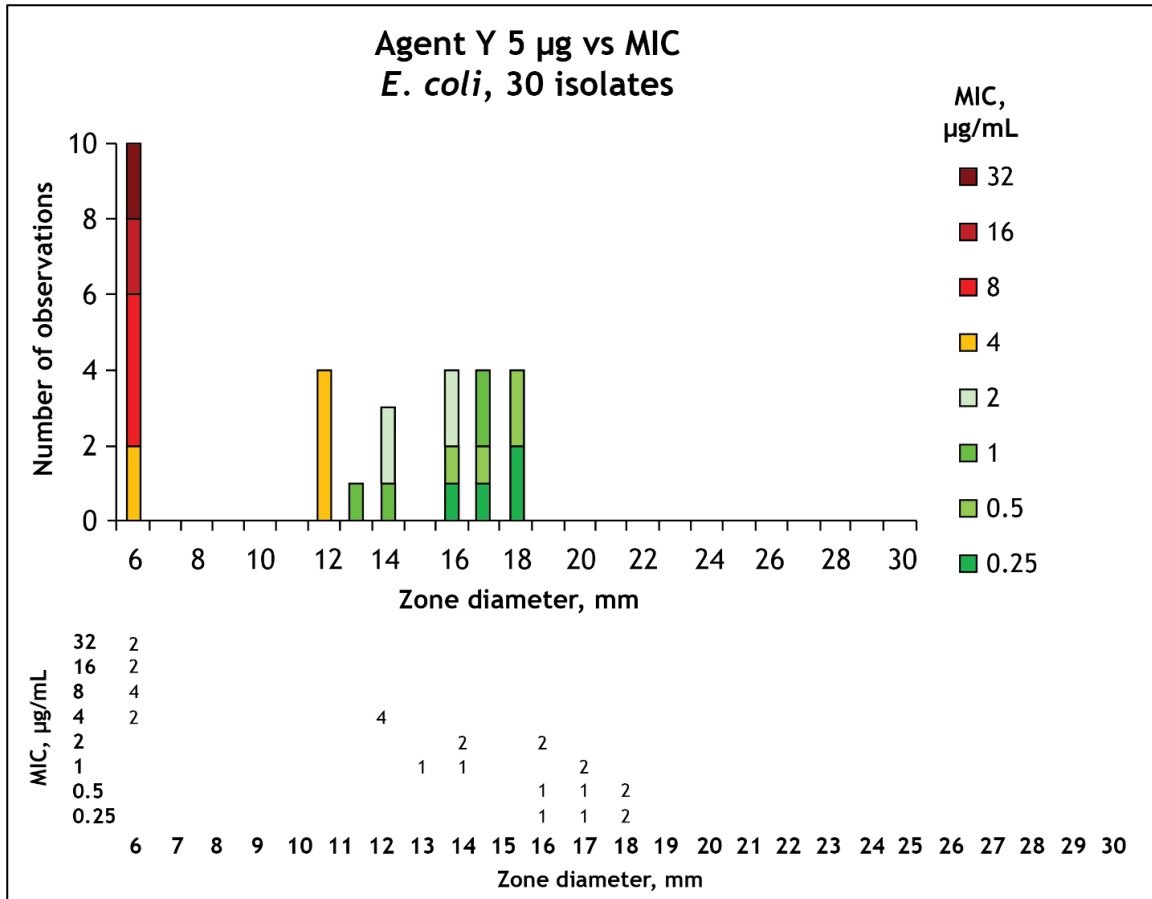
¹ CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests. 14th ed. CLSI standard M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2024.

² The European Committee on Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing: EUCAST disk diffusion method. Version 12.0; 2024.
http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Accessed 19 September 2024.

³ CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 34th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2024.

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

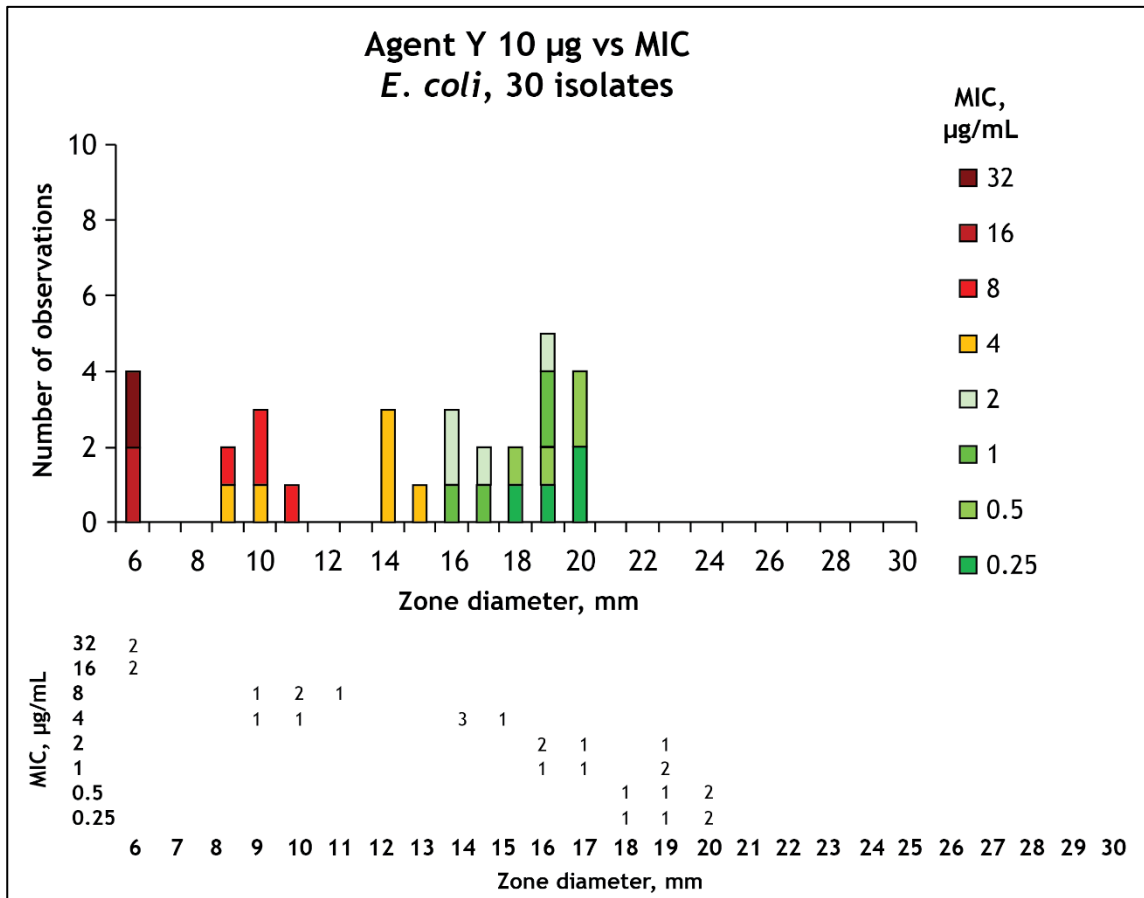
The 5-µg disk generally provides the best separation between wild-type and non-wild-type isolates for all species, compared with 10- and 30-µg disks. Examples of histograms and scattergrams from phase 2 testing with various disk contents (potencies) vs several species are shown in Figures C1 through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



Abbreviation: MIC, minimum inhibitory concentration.

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

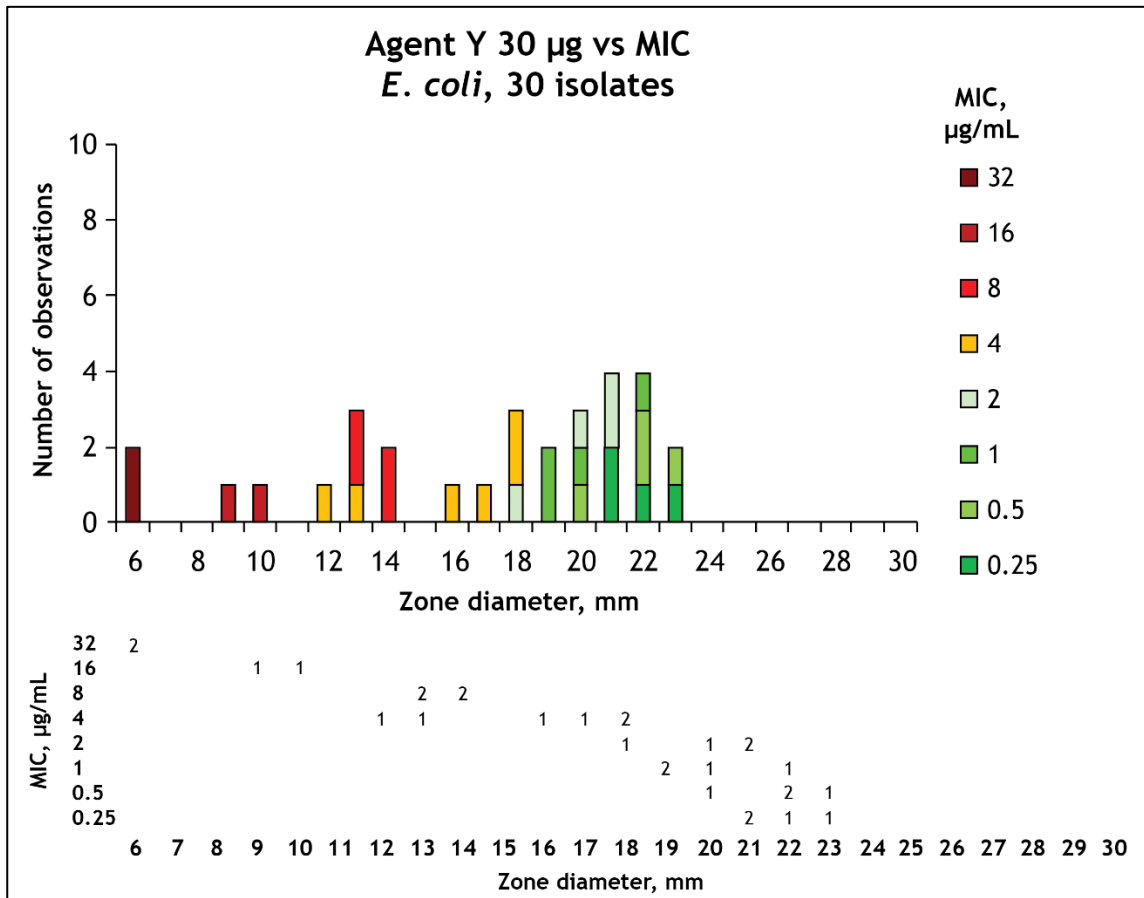
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

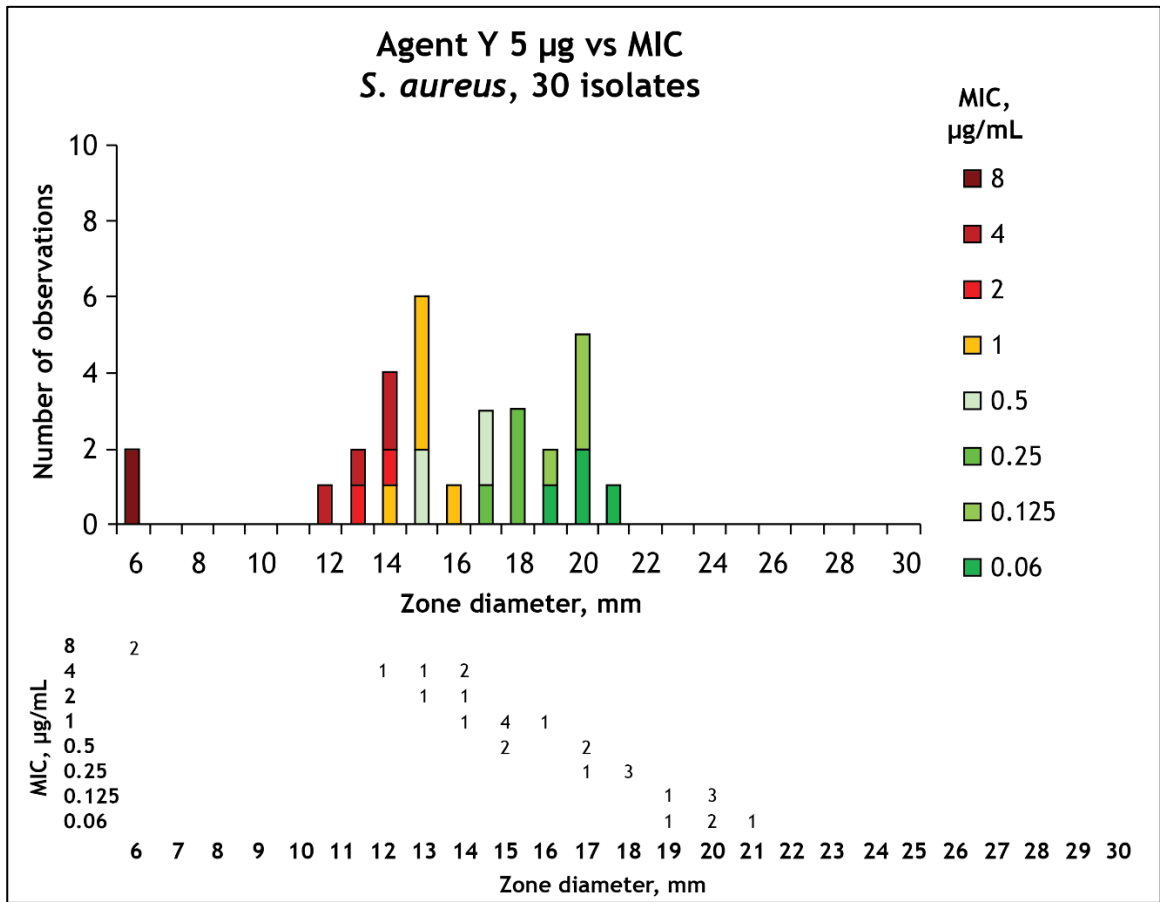
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

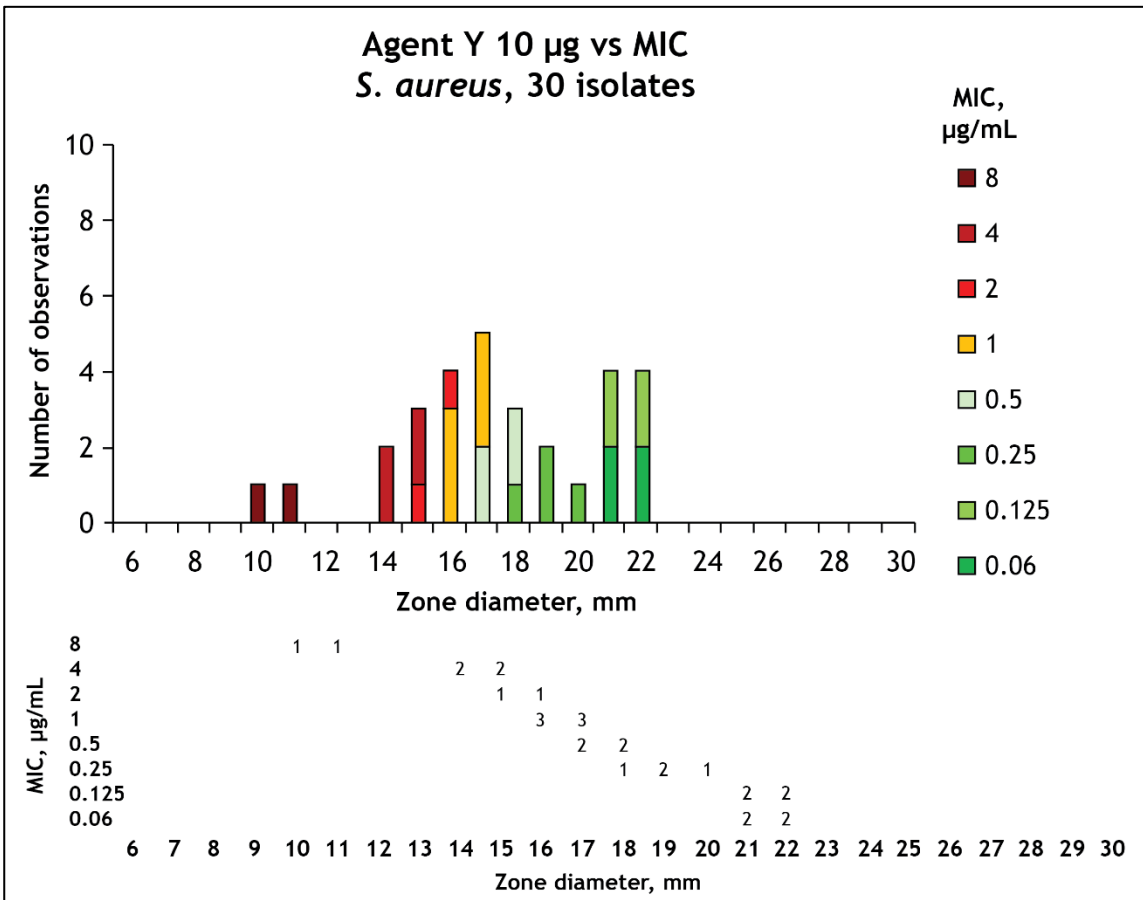
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

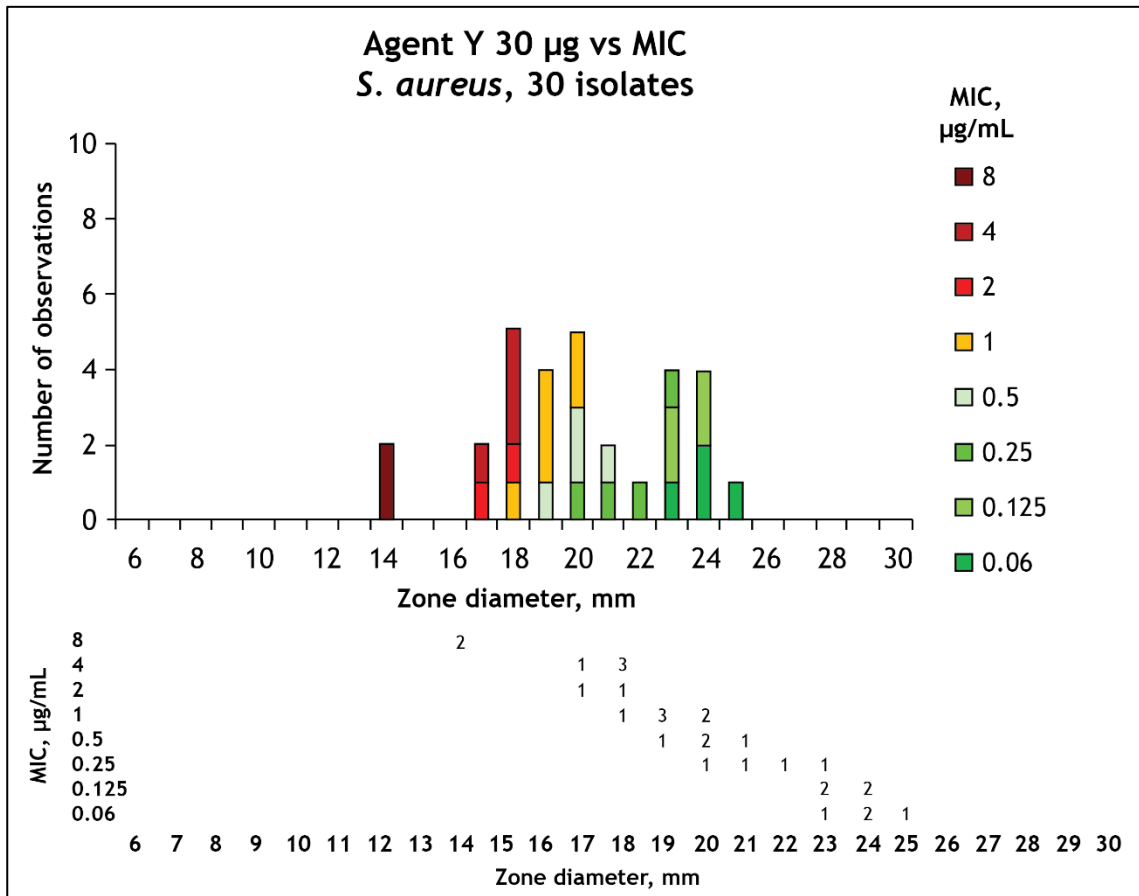
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

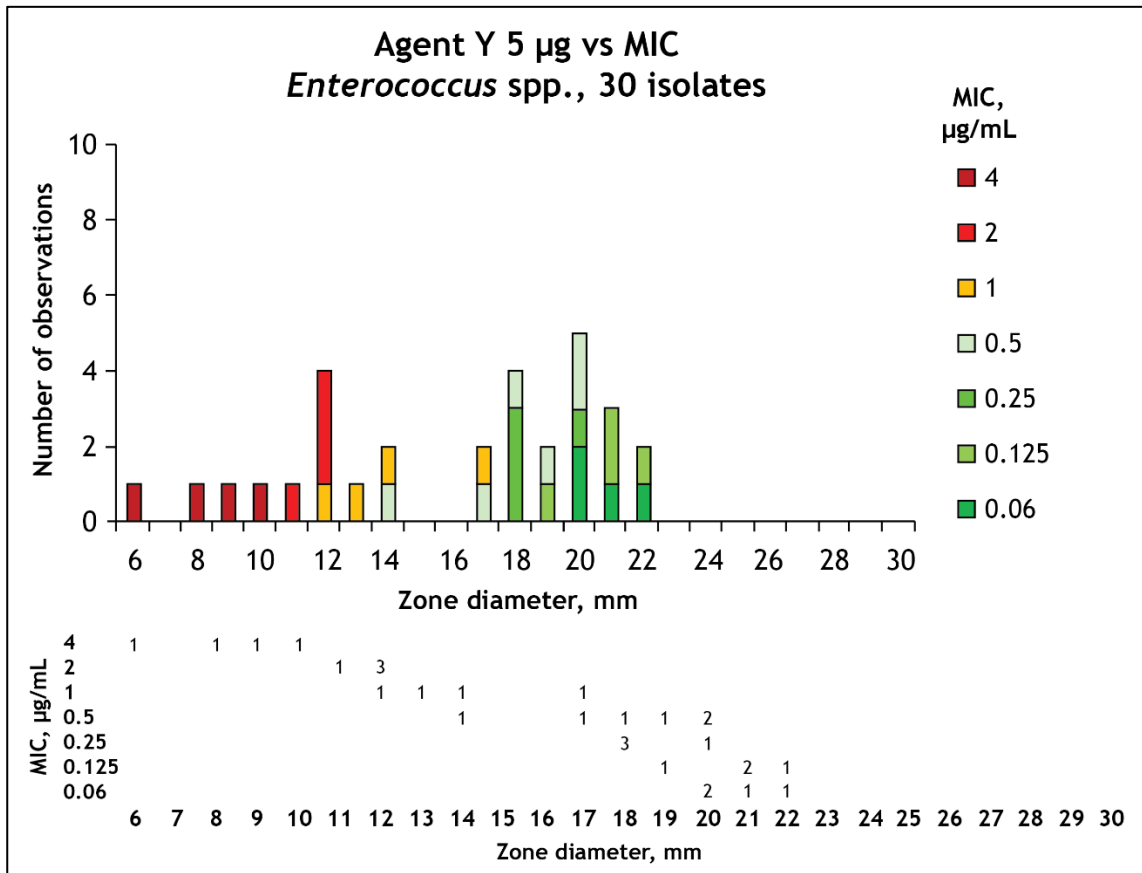
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

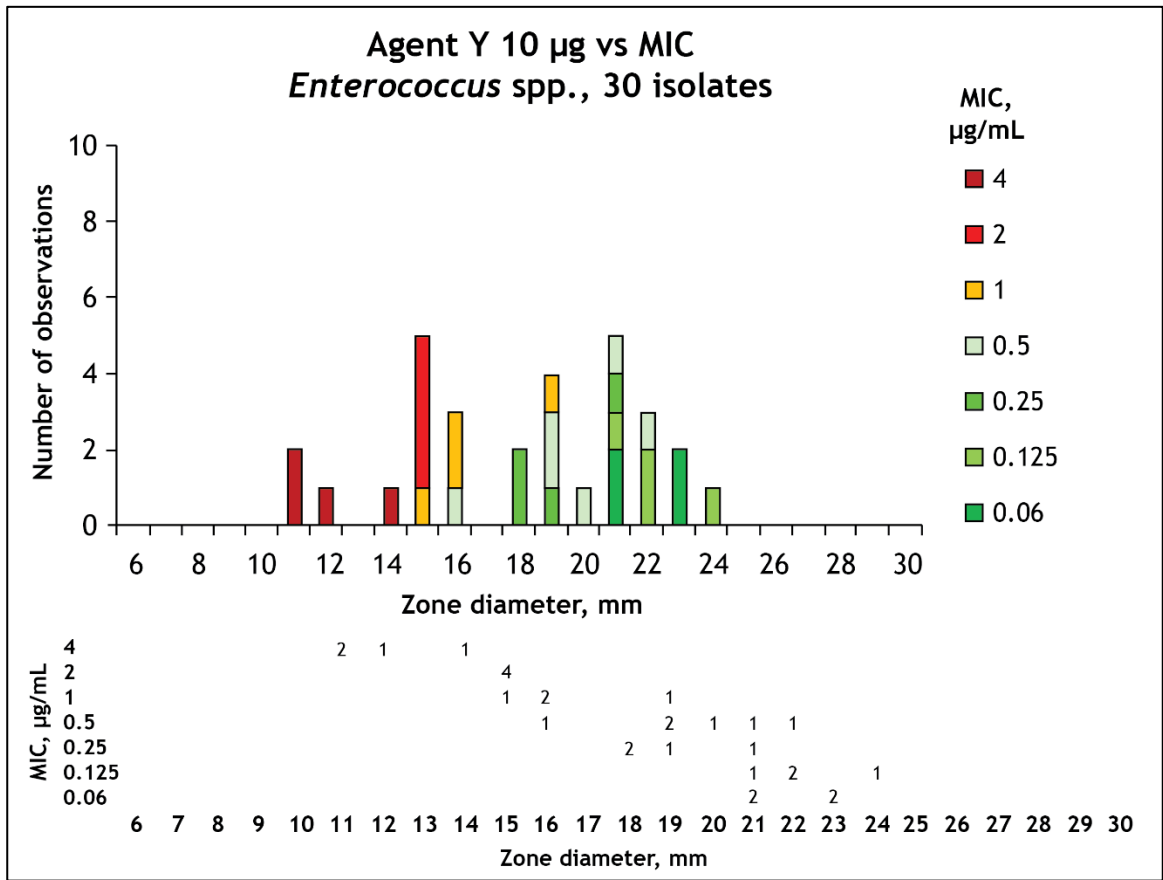
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

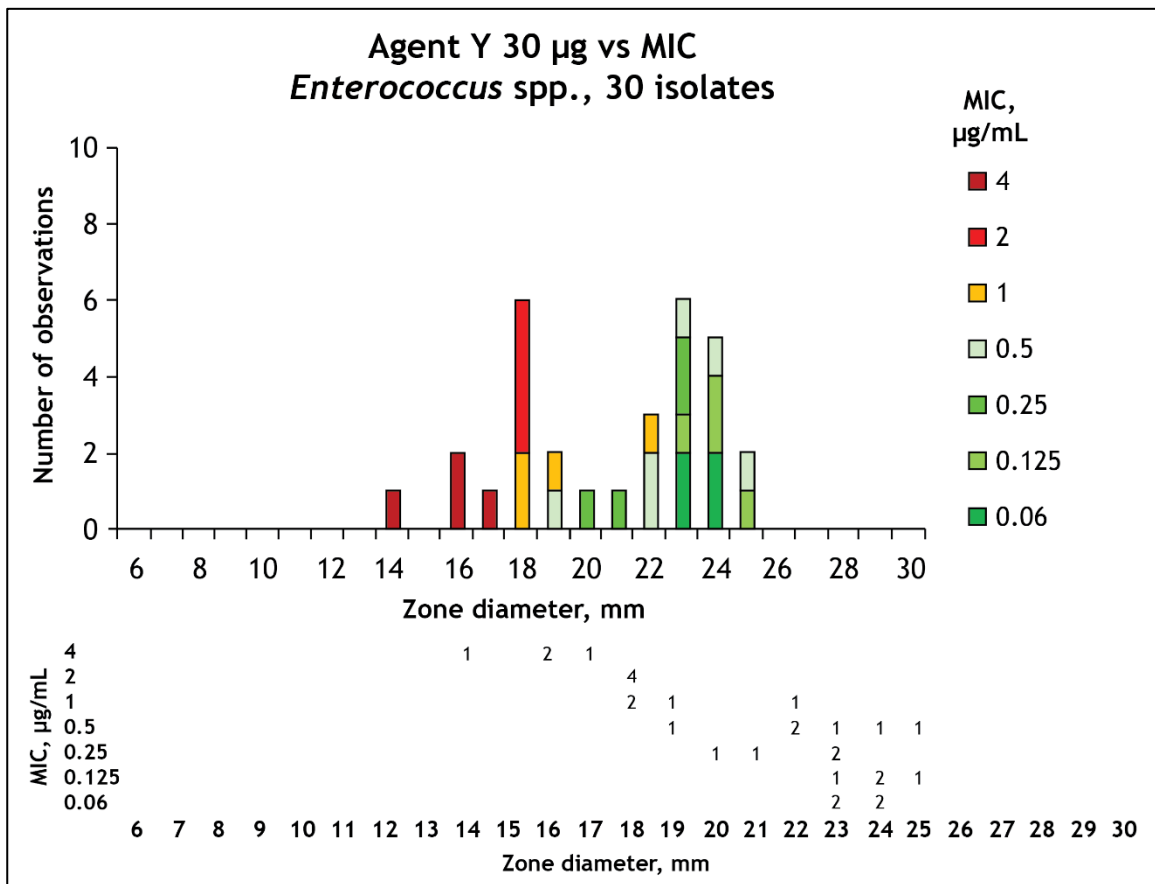
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

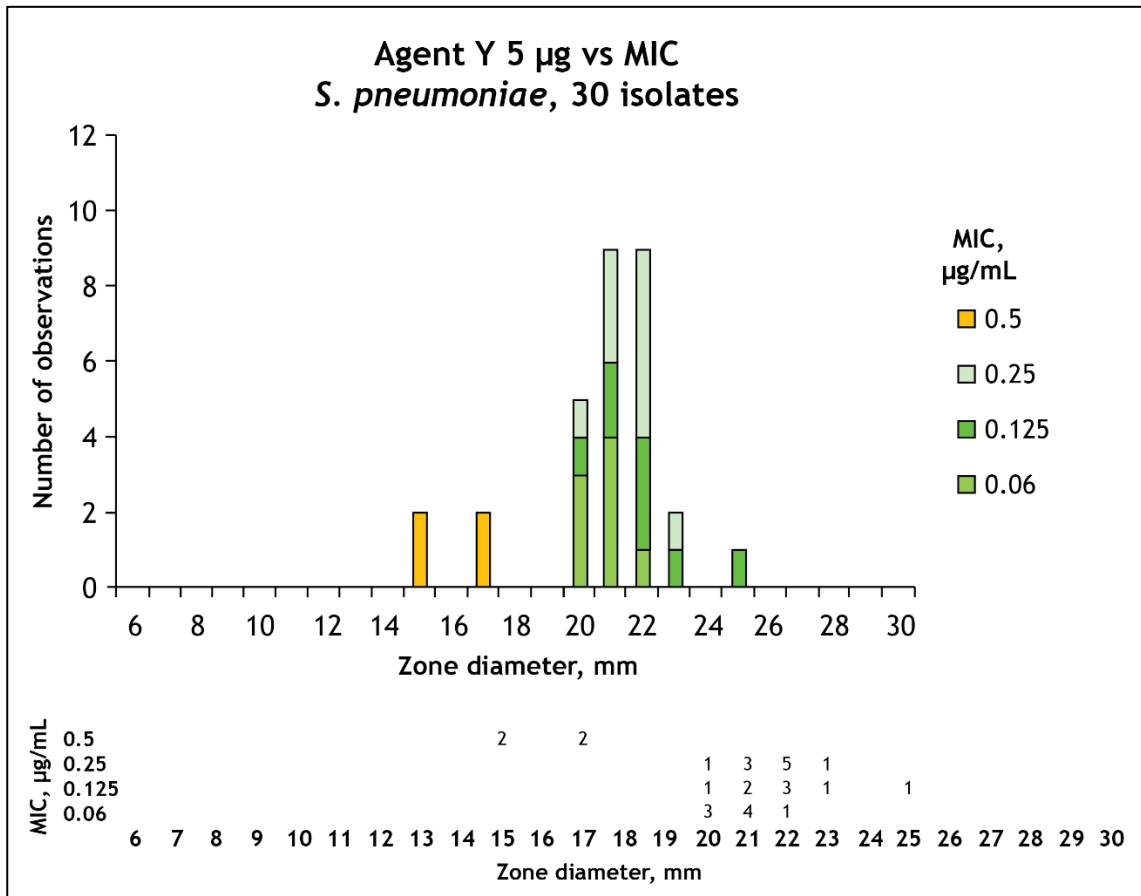
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

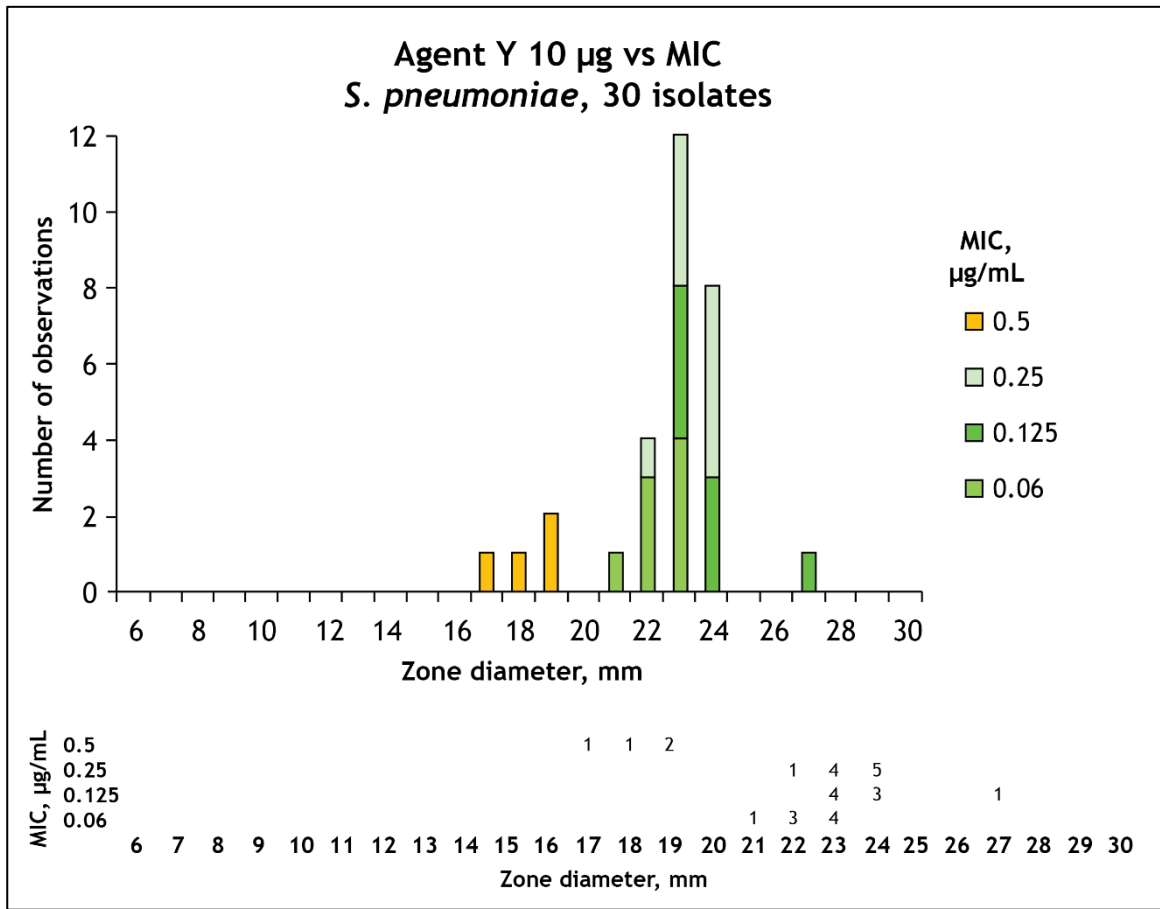
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

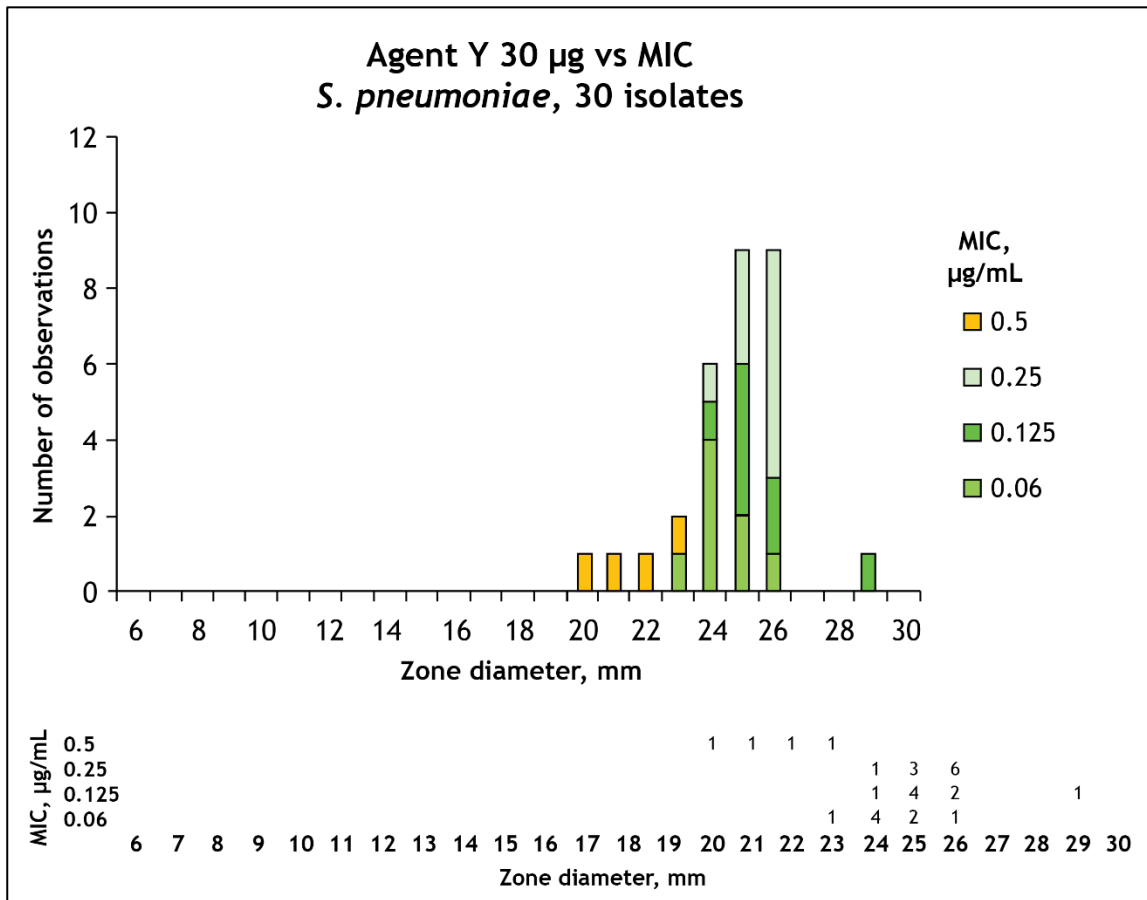
Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

Appendix C. (Continued)



Abbreviation: MIC, minimum inhibitory concentration.

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

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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 harmonised CLSI and EUCAST criteria.

- Source, lot numbers, and expiry 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 expiry dates)
- Test results to include:
 - Dates of testing
 - Technical staff performing testing
 - All results (zone measurements and minimum inhibitory concentration values) from initial and any repeat testing
 - Description of appearance of inhibition zones (e.g. clear, slight haze) and any criteria recommended for measuring zone (e.g. 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.