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

CLSI-EUCAST joint working group on antimicrobial susceptibility testing criteria and quality control

Contact information is available in the end of this document

Abbreviations, definitions and glossary

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AST</td>
<td>Antimicrobial Susceptibility Testing</td>
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<td>BMD</td>
<td>Broth Microdilution</td>
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<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<td>Non-wild type isolates</td>
<td>Isolates with phenotypically detectable acquired resistance mechanisms for test agent</td>
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Citation of this document

Kommentar [MEMkmV1]: To be added when the document is finalized
1. Introduction
The gold standard for antimicrobial susceptibility testing (AST) is MIC determination using broth microdilution (BMD) according to ISO standard 20776-1 [1], except for a few agents and/or organisms for which BMD does not provide reliable results. Both CLSI and EUCAST have developed standardized disk diffusion methods [2-3] calibrated to match the results of reference MIC methodology [1, 4-5] based in part on a method originally described by Bauer et al. [6]. The selection of the optimal disk content (potency) 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 10-14 mm range (see examples in Appendix D). Populations without and with resistance mechanisms that are clearly distinguishable by MIC should be clearly distinguishable by inhibition zone diameters. 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, 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. Since 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) for disk diffusion testing. These are described in this document and are endorsed by both CLSI and EUCAST. Pharmaceutical companies interested in having disk diffusion breakpoints in CLSI and/or EUCAST tables should follow the procedure when developing disks for disk diffusion testing.
2. Procedure for establishing the optimal disk content (potency)
This procedure describes the technical steps required to establish optimal disk content for single agents without the addition of enhancing or inhibiting substances. See section “Selection of the optimal disk content (potency) for combinations of agents” at the end of this document for guidance in establishing disk content for a combination of agents.

Selection Criteria
Studies are performed to achieve, when feasible:

- Reproducible inhibition zone diameters (within ± 2 mm) when testing QC strains and clinical isolates.
- A disk content (potency) that reproducibly distinguishes between isolates with different MIC values.
- A single disk content (potency) that can be used for all species. Multiple disk contents (potencies) should only be considered when this is absolutely necessary to achieve reproducibility in tests for all target species.
- An increase in zone diameters of 2-3 mm with each log₂ decrease in MIC for non-wild type isolates.
- Inhibition zone diameters between 15 and 35 mm for wild-type isolates of relevant species (target organisms) with the smallest zone diameter close to 15 mm for the least susceptible species.
- Optimal separation between wild-type and non-wild type isolates (when clinical breakpoints are not yet defined), if non-wild type isolates exist.
- Optimal separation between non-wild type isolates with various resistance mechanisms and MICs.

Establishing the disk content (potency) should be performed according to the procedures described in Tier 1 and Tier 2 below.

Basic criteria for Tier 1 and Tier 2 studies

- MIC testing is performed according to the reference method and MIC QC performance data should be available.
- Options for obtaining reference MIC values
  - MIC testing can be performed in parallel with disk diffusion testing.
  - Isolates with previously known MIC values can be selected.
- Isolates where 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) must be retested with both reference MIC methodology and disk diffusion using a single inoculum.
- Disk diffusion must be performed using a Mueller-Hinton medium that meets the requirements in ISO/TS 16782:2016 [7] and the QC criteria published by CLSI and EUCAST for standard QC strains [8-9]. To establish acceptable quality of the medium, results must be in range when testing QC strains and agents from similar and different antimicrobial classes.
- 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 3 separate days. The variation in zone diameter measurements from separate days should be no greater than ± 2 mm.
An appropriate control agent (preferably an antimicrobial agent belonging to the same or similar class as the agent being evaluated) with CLSI and/or EUCAST published QC ranges [8-9] must be included with disk diffusion testing of all isolates (clinical isolates and QC strains).

**Tier 1**

2.1. Initial screening of a series of disk contents (potencies) to select 2-4 contents (potencies) for further investigation.

Disks with at least 10 different contents (potencies) varying from very low (e.g. 0.1 µg) to very high (e.g. 50 µg) are produced in small batches and tested according to standardized disk diffusion methodology against relevant species. For some agents, it might be necessary to test disk contents (potencies) beyond the 0.1 to 50 µg range.

- A disk content (potency) previously used for the antimicrobial class of the agent being evaluated (e.g. 5 µg for fluoroquinolones, 30 µg for 3rd generation cephalosporins) can be included but not considered the optimal content (potency) by default.
- A minimum of two isolates per relevant target species are included, one wild-type isolate (a susceptible QC strain can be used) and one isolate with an MIC 2-3 two-fold dilutions above the wild-type distribution, if such isolates are available.
- Testing can be performed using one disk lot per content (potency) on Mueller-Hinton media from one manufacturer. These disks can be obtained from small-scale production by the pharmaceutical company or a contract laboratory. A description on how to prepare disks in house is available in Appendix A.
- The results are analysed according to the example in Figure 1 by preparing a graph correlating the disk content (potency) on the x-axis to the inhibition zone diameter on the y-axis for each tested isolate.

The aim of the Tier 1 initial screening is to identify 2-4 disk contents (potencies) for which the discriminatory power (defined as the part of the curve where the zone diameter increment between MIC values is the greatest, i.e. the steepest part of the curve) is greatest for all target species (see example in Figure 1). The selected disk contents (potencies) will be used for Tier 2 investigation that includes a larger number of isolates.

Figure 1. The relationship between disk content (potency) and inhibition zone diameters for several *Staphylococcus aureus* type culture collection strains with varying MIC values and hypothetical drug x.

**Tier 2**

2.2. Further investigation of the 2-4 selected disk contents (potencies)

A larger study is conducted with all relevant target species for the agent in question using the 2-4 disk contents that demonstrated the most discriminating power in Tier 1 studies.

- At least 30 isolates per species, or 60 isolates per group of organisms, should be included, of which at least 50%, and preferably not more than 80%, should be wild-type isolates. A larger number of isolates may be required for antimicrobial agents with a broad spectrum of activity (e.g. active against a variety of Gram-positive and Gram-negative genera).

- The non-wild type isolates should, when possible, represent a variety of MICs and resistance mechanisms and should include isolates with MICs 1-2 dilutions above the highest MIC in the wild-type population. When resistant isolates are not available, the minimum criterion is to define wild-type population of relevant species. If mutants with higher MICs can be provided by the pharmaceutical company, these should be included provided that growth characteristics are similar to those of wild-type isolates in the testing media used. It is also possible to include resistant isolates of non-target species if no resistant isolates are available for target species.

- Testing should preferably be performed using commercially produced disks (one disk lot per disk content (potency)) or using disks from small-scale production by the pharmaceutical company or a contract laboratory (two disk lots per disk content (potency)).

- Testing must be performed on media from at least two manufacturers in parallel.

- Inhibition zone diameters are correlated to the corresponding MIC values and presented in two different formats: i) as species-specific scattergrams (Figure 2a) and...
For CLSI agenda book and EUCAST consultation.

- ii) as inhibition zone diameter histograms with corresponding MIC values as coloured bars (Figure 2b). Examples of histograms and scattergrams for three different disk contents (potencies) vs. several species are shown in Appendix B.

**Figure 2a.** Zone diameter scattergram with zone diameters plotted against MIC values. Figures 2a and 2b represent the same data set.

**Figure 2b.** Zone diameter histogram with MIC values as coloured bars. Green colours correspond to wild-type isolates. Yellow, orange and red colours correspond to different MICs for non-wild type isolates. Figures 2a and 2b represent the same data set.

### 2.3. Selection of the optimal disk content (potency)

- The optimal disk content (potency) is determined using the Selection Criteria listed above following visual review of the raw data and data displayed in scattergrams and histograms.
- Populations without and with resistance mechanisms, clearly distinguishable by MIC, should be clearly distinguishable by inhibition zone diameters.
Tier 3

2.4. Establishment of QC criteria and zone diameter breakpoints

For establishment of QC criteria and zone diameter breakpoints, it is necessary to obtain commercially produced disks from at least two manufacturers.

Selection of the optimal disk content (potency) for combinations of agents

- 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 is taken forward.

- For combinations of an agent and an inhibitor without antimicrobial activity where an agreed 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 this agent must be part of the process.

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

Contact information

CLSI
www.clsi.org

EUCAST
www.EUCAST.org

References


Appendix A. Manual preparation of antimicrobial disks for Tier 1 Testing

Determining the optimal disk content (potency) for testing a novel antimicrobial agent using the disk diffusion method might require the in-house preparation of susceptibility disks impregnated with varying contents (potencies) of the antimicrobial agent.

To prepare the disks:

1. Prepare a stock solution at 50X the final highest desired disk content (potency) using solvents, diluents, and the procedure recommended in CLSI M100. If testing multiple contents (potencies), prepare dilutions from the 50X stock solution for the lower content (potency) disks.

2. Distribute sterile blank 6-mm paper disks (available from several manufacturers) in sterile plastic Petri dishes that have been appropriately labeled with the antimicrobial agent and potency. Make certain disks are not touching each other and can be easily accessed for pipetting. If static electricity is noted in the Petri dish, taping a small square of an anti-static sheet to the Petri dish lid will keep static interference to a minimum. Optionally, a sterile fine wire mesh can be placed 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 of the appropriate antimicrobial solution 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/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 (e.g. a 50 ml conical or glass tube) with desiccant until use. Wrap the lid of the storage container in parafilm and store at the appropriate storage temperature (2-8°C or -20°C or -80°C depending on the agent).

Notes:

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

2. As soon as possible after production, disks should be tested using relevant QC strains to obtain data which 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.

3. If a solvent/diluent other than sterile distilled water (e.g., DMSO, ethanol, etc.) is used to prepare stock solutions, a control disk impregnated with only the solvent/diluent at the appropriate concentration must be tested to ensure that there is no zone of inhibition for the solvent/diluent tested alone. Should a solvent produce an inhibition zone, different solvents should be tested.
4. When preparing a disk with two agents (antimicrobial plus inhibitor or two antimicrobials), the stock solutions (100X each) for each compound should be prepared separately and mixed together immediately before pipetting onto the disks, unless there is a known reason to pipette them separately.

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

6. Two disks (e.g., two lots) made from independently prepared stock solutions are tested to evaluate the reproducibility of the disk preparation.

Example for preparation of single agent disks (drug x) at 10 µg and 5 µg contents:

Prepare stock solution at 500 µg/ml (50 x 10 µg) = 500 µg/ml

For 10 µg disk:

Add 20 µl (0.02 ml, 1:50 dilution of 500 µg/ml) to each disk (final content = 10 µg)

For 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 of 250 µg/ml) to each disk (final content = 5 µg)

* In the United States, the standard paper is 740-E and should be 30 ± 4mg/cm²

References
Appendix B. Examples of histograms and scattergrams for 5, 10 and 30 µg disk vs. several species.

The 5 µg disk provides the best separation for all species.

Agent Y 30 μg vs MIC
*E. coli*, 30 isolates

Agent Y 5 μg vs MIC
*S. aureus*, 30 isolates
Agent Y 10 µg vs MIC
*S. aureus*, 30 isolates

Agent Y 30 µg vs MIC
*S. aureus*, 30 isolates


Agent Y 5 µg vs MIC
Enterococcus spp., 30 isolates

Agent Y 10 µg vs MIC
Enterococcus spp., 30 isolates

Agent Y 30 µg vs MIC
*Enterococcus* spp., 30 isolates

- MIC mg/L
- 4
- 2
- 1
- 0.5
- 0.25
- 0.125
- 0.06

Inhibition zone diameters (mm)

No of observations

Agent Y 5 µg vs MIC
*S. pneumoniae*, 30 isolates

- MIC mg/L
- 0.5
- 0.25
- 0.125
- 0.06

Inhibition zone diameters (mm)

No of observations
Appendix C – Required Documentation

1. Source, lot numbers and expiration dates of materials used in Tier 1 and Tier 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)

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

3. Bacterial isolates to include:
   - Source / storage
   - Characterization for resistance mechanisms, if appropriate

4. Quality Control to include:
   - Results obtained from each run for new agent and control agent (include Source, lot numbers and expiration dates)

5. Test results to include:
   - Dates of testing
   - Technician performing testing
   - All results (zone measurements and MIC values) from initial and any repeat testing
   - Description of appearance of inhibition zones (e.g., clear, slight haze, etc.) and any criteria recommended for measuring zone (e.g., read inner zone, 90% inhibition, etc).

6. 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.
Appendix D. Examples of zone diameter distributions with a defined wild-type distribution (black bars).

On-scale zone diameter distributions (± 2×SD) of wild-type organisms normally span 10-14 mm.