

Welcome to the NordicAST Basic AST webinar

Today's program:

- Welcome
- EUCAST Disk diffusion method (Erika Matuschek)
- Discussions/mentimeter (Barbara Holzkecht)
- 15 minutes' break
- Reading of zones (Erika Matuschek)
- Discussions/mentimeter (Ingvild Haugan)
- Conclusions and evaluation

For mentimeter questions: Use your browser and go to [menti.com](https://www.menti.com) or install the App "Mentimeter": Code: 76 40 64

Please stay muted.

The webinar will be recorded, link shared on the NordicAST homepage for later review.

The EUCAST disk diffusion method

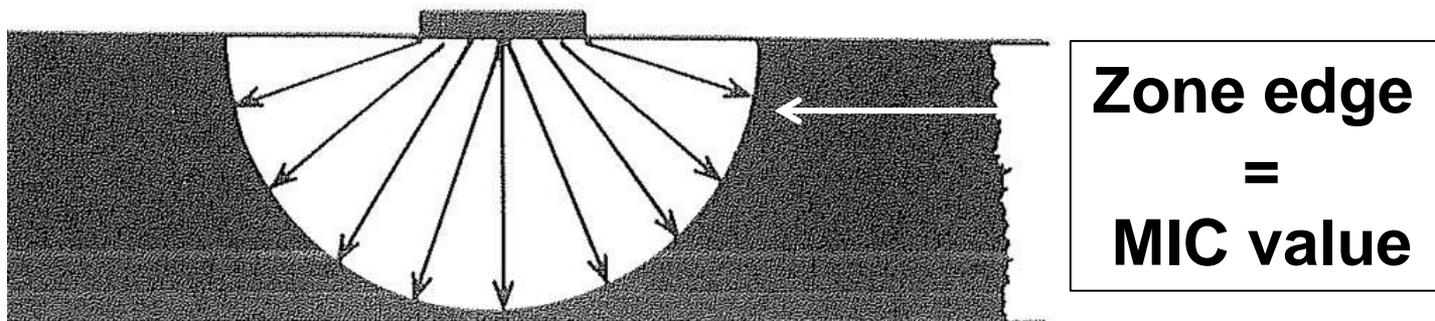
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Växjö, Sweden

Standardisation of AST

- Results change with changed parameters.
 - It is crucial to follow the methodology to get reproducible and reliable results!
- Standardisation of:
 - Disk potency
 - Media
 - Type of media, supplements, pH, agar depth etc.
 - Inoculum
 - Incubation
 - Time, temperature and atmosphere
 - Reading of results

General principle of disk diffusion

- Diffusion of the antibiotic from a disk to form a concentration gradient in the agar
- Must be calibrated to MIC values



Summary of the EUCAST disk diffusion method

Organism	Inoculum	Media	Incubation
Non-fastidious organisms	McF 0.5	Un-supplemented MH agar	Air 35°C, 16-20 h
Fastidious organisms	McF 0.5	MH-F	5% CO ₂ 35°C, 16-20 h
Campylobacter	McF 0.5	MH-F	Microaerobic 41°C, 24 h
Anaerobic bacteria	McF 1.0	FAA-HB	Anaerobic 35-37°C, 16-20 h

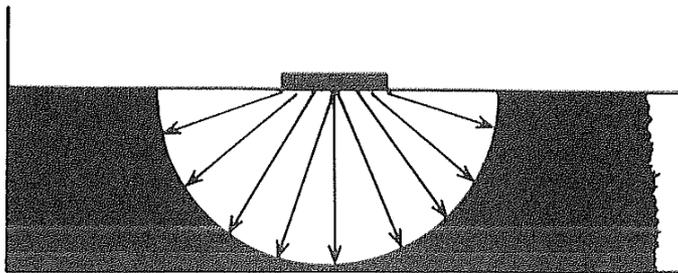
MH = Un-supplemented Mueller Hinton agar

MH-F = Mueller Hinton agar + 5% defibrinated horse blood and 20 mg/L β -NAD

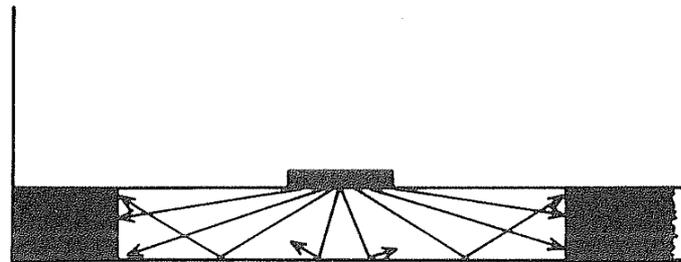
FAA-HB = Fastidious Anaerobe Agar + 5% defibrinated horse blood

Media: Agar depth

- The agar depth affects the diffusion of the antibiotic and thereby the zone sizes.
- The agar depth should be 4.0 ± 0.5 mm.
 - Agar depth < 4 mm: Larger zone diameters
 - Agar depth > 4 mm : Smaller zone diameters



Correct agar depth



Agar depth too low

Media: Storage and handling

- Store plates according to the manufacturer's instructions.
 - In-house prepared plates should be stored at 4-8°C.
- Make sure that agar plates are at room temperature prior to inoculation.
- The surface of the agar should be dry before use.
 - Excess moisture may cause fuzzy zone edges and/or haze within zones.

Media: Excess humidity

- Problems related to excess humidity in agar plates are most often seen on MH-F and FAA-HB plates.
- No drops of water should be visible on the surface of the agar or inside the lid. This is often seen with plates stored in plastic bags or sealed containers.
- If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min.

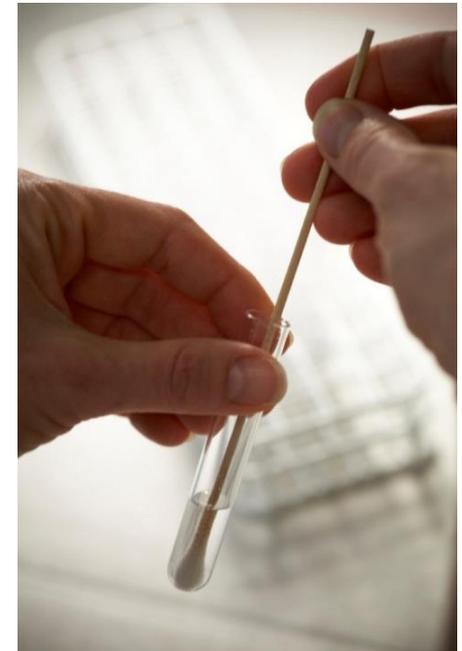


Excess humidity and fuzzy zone edges Example with MH-F agar plates



Inoculum preparation

- Use an overnight culture on non-selective media.
- Pick several morphologically similar colonies to avoid selecting an atypical variant.
- Suspend the colonies in saline and mix to an even turbidity.

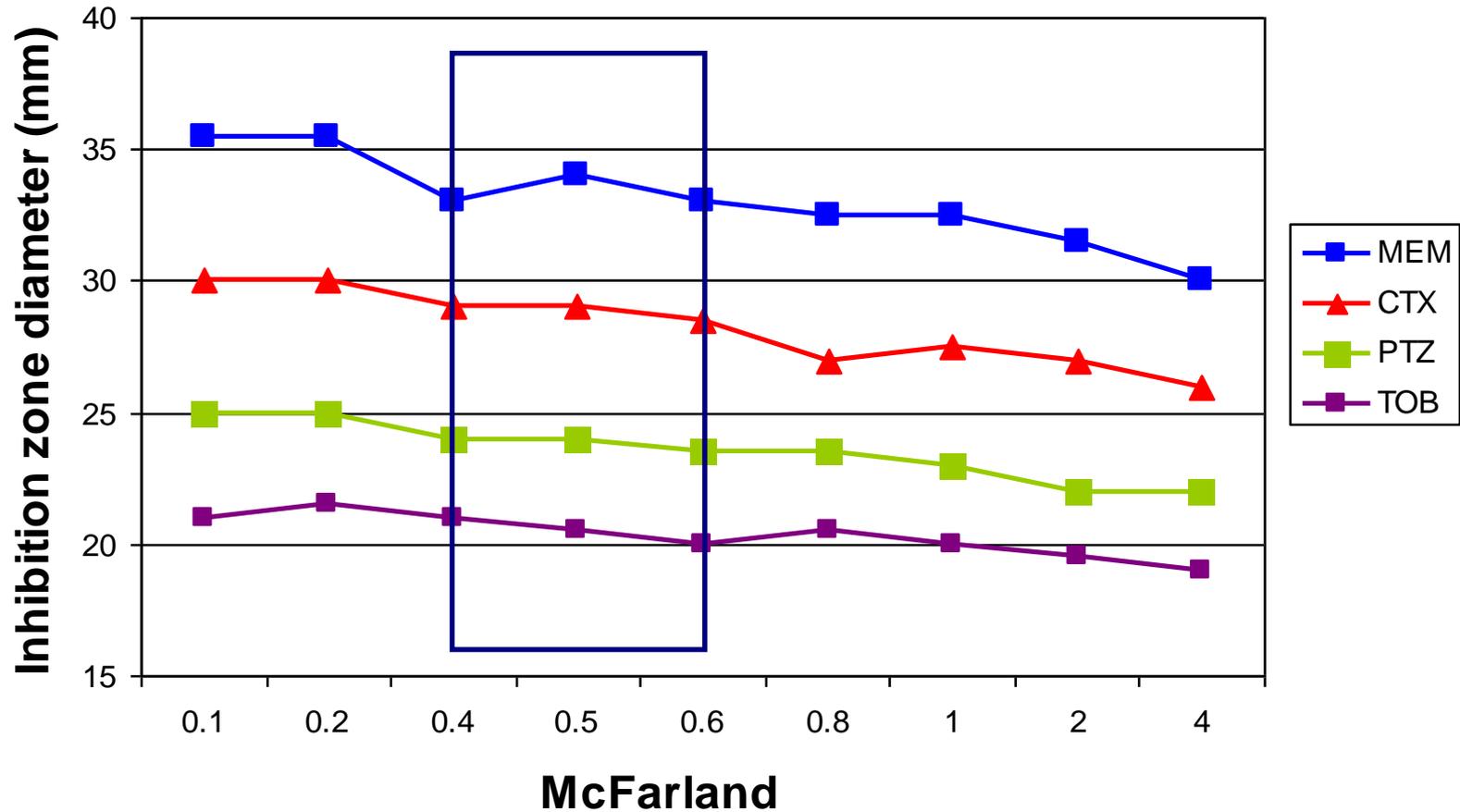


Inoculum preparation

- The inoculum should be equivalent to a 0.5 McFarland standard.
 - If the density is too low, add more bacteria.
 - If the density is too high, add more saline.
- It is recommended that the turbidity is measured with a photometric device.
- If *Streptococcus pneumoniae* is suspended from a chocolate agar plate and not a blood agar plate, the inoculum must be equivalent to a McFarland 1 turbidity standard.

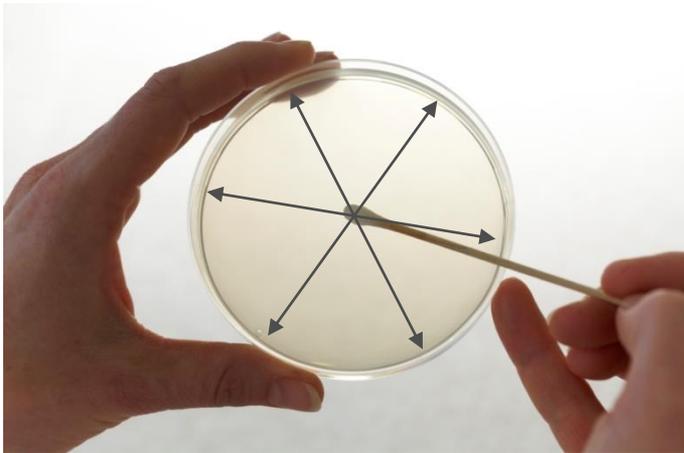
Effect of inoculum size on zone sizes

E. coli ATCC 25922



Inoculation of plates

- Dip a sterile cotton swab into the suspension.
- Spread the inoculum evenly over the entire surface by swabbing in three directions or by using a plate rotator.
- When inoculating several agar plates with the same inoculum, dip the cotton swab into the suspension for each agar plate.



Inoculation of plates

- For Gram-negative bacteria, remove excess fluid by pressing and turning the swab against the inside of the tube to avoid over-inoculation.
- For Gram-positive bacteria, do not press the swab against the inside of the tube and take particular care to ensure that there are no gaps between streaks.

The more carefully the plates are streaked, the easier it is to read the zone diameters!

Application of disks

- Apply disks within 15 min of inoculation.
- Disks must be in close and firm contact with the agar surface and must not be moved once they have been applied.
- The number of disks per plate should be limited:
 - To avoid overlapping of zones and interference between agents.
 - To allow sensitive organisms to grow.



Number of disks on a plate

- 6 disks are the maximum possible number on a 90-mm circular plate, but for many organisms, 4-5 disks per plate is appropriate.
- For bacteria that are very sensitive, the appropriate number of disks might be as low as 2 disks on a 90-mm plate.
 - Examples: *Corynebacterium* spp., *Aerococcus sanguinicola* and *urinae* and *Cutibacterium acnes*

Storage and handling of disks

- Store disks according to the manufacturers' instructions.
 - Some agents are more labile than others and may have specific recommendations (e.g. amoxicillin-clavulanic acid and carbapenems).
- Store disks in current use in sealed containers with a moisture-indicating desiccant and protected from light.
- Allow disks to reach room temperature before opening cartridges or containers used for disk storage. This is to prevent condensation, leading to rapid degradation of some agents.
 - It is better to keep disks at room temperature during the day than transfer repeatedly to and from cold storage.
- Do not use disks beyond the manufacturer's expiry date.

Incubation of plates

- Invert plates and make sure disks do not fall off the agar surface.
- Keep stacks of plates as small as possible to avoid uneven heating.
 - For most incubators, a maximum of five plates per stack is appropriate.
- Incubation time is 16-20 hours for all organisms unless otherwise stated in the EUCAST disk diffusion manual.

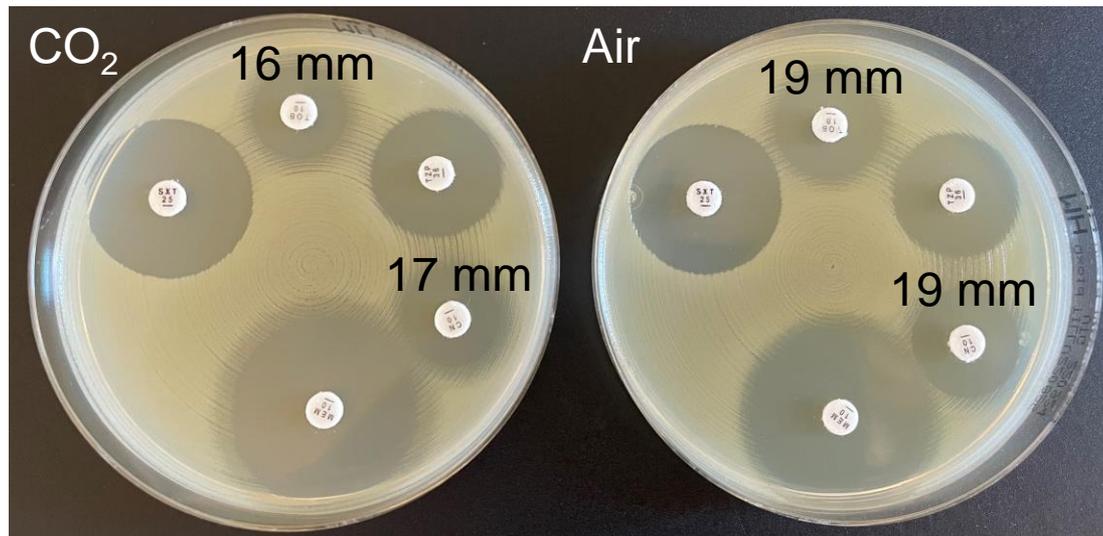
Incubation of plates

Organism	Media	Incubation	Exceptions
Non-fastidious organisms	Un-supplemented MH agar	Air 35°C, 16-20 h	24 h for enterococci and glycopeptides
Fastidious organisms	MH-F	5% CO ₂ 35°C, 16-20 h	See note
Campylobacter	MH-F	Microaerobic 41°C, 24 h	40-48 h if growth is non-sufficient after 24 h
Anaerobic bacteria	FAA-HB	Anaerobic 35-37°C, 16-20 h	

Note: Prolonged incubation (40-44 h) is allowed for *Corynebacterium* spp., *Aerococcus sanguinicola* and *urinae*, and *Kingella kingae* if growth is non-sufficient after 16-20 h incubation.

Incubation atmosphere

- The incubation atmosphere affects the activity of the antimicrobial agents.
 - Incubation in CO₂ changes the pH in the media, which affects some agents.



Always follow the recommendations for the organism tested!

The 15-15-15 minute rule

Follow these instructions for disk diffusion:

- Use the inoculum suspension optimally within **15 minutes** of preparation, and always within 60 minutes.
- Apply disks within **15 minutes** of inoculation.
- Incubate plates within **15 minutes** of disk application.

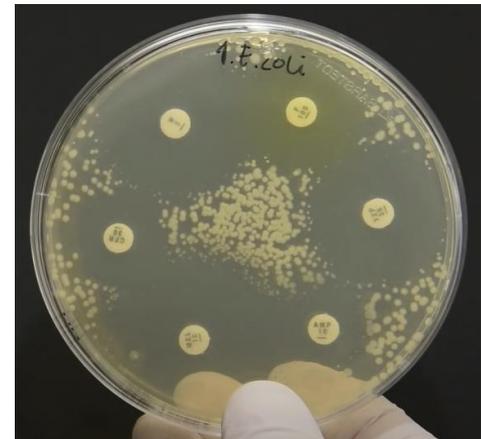
Why the 15-15-15 minute rule?

- Leaving inoculum suspensions at room temperature before inoculation:
 - Increased inoculum for fast-growing bacteria
 - Decreased inoculum for fastidious organisms
- Leaving inoculated plates at room temperature before the disks are applied:
 - The organism may begin to grow, resulting in erroneously small zones.
- Leaving the plates at room temperature after disks have been applied:
 - Pre-diffusion may result in erroneously large zones.

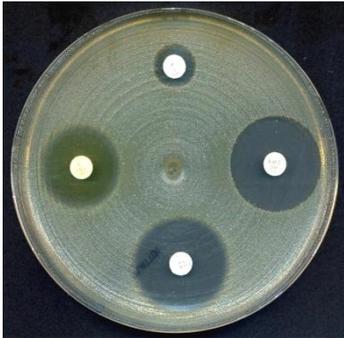
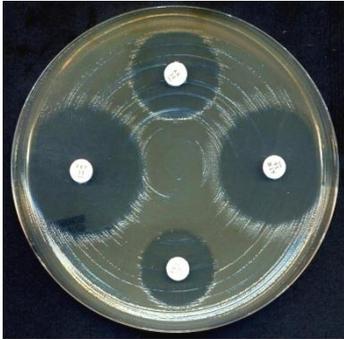
Examination of plates after incubation

- A correct inoculum and satisfactorily streaked plates should result in a confluent lawn of growth.
- The growth should be evenly distributed over the agar surface to achieve uniformly circular (non-jagged) inhibition zones.
- If individual colonies can be seen, the inoculum is too light and the test must be repeated.

Example of a plate with too light growth



The growth should be confluent and evenly spread over the plate



Plates should look like this..

..and NOT like this!

Reading of zones and interpretation of results

- Measure zone diameters to the nearest millimetre with a ruler or a or a calliper.

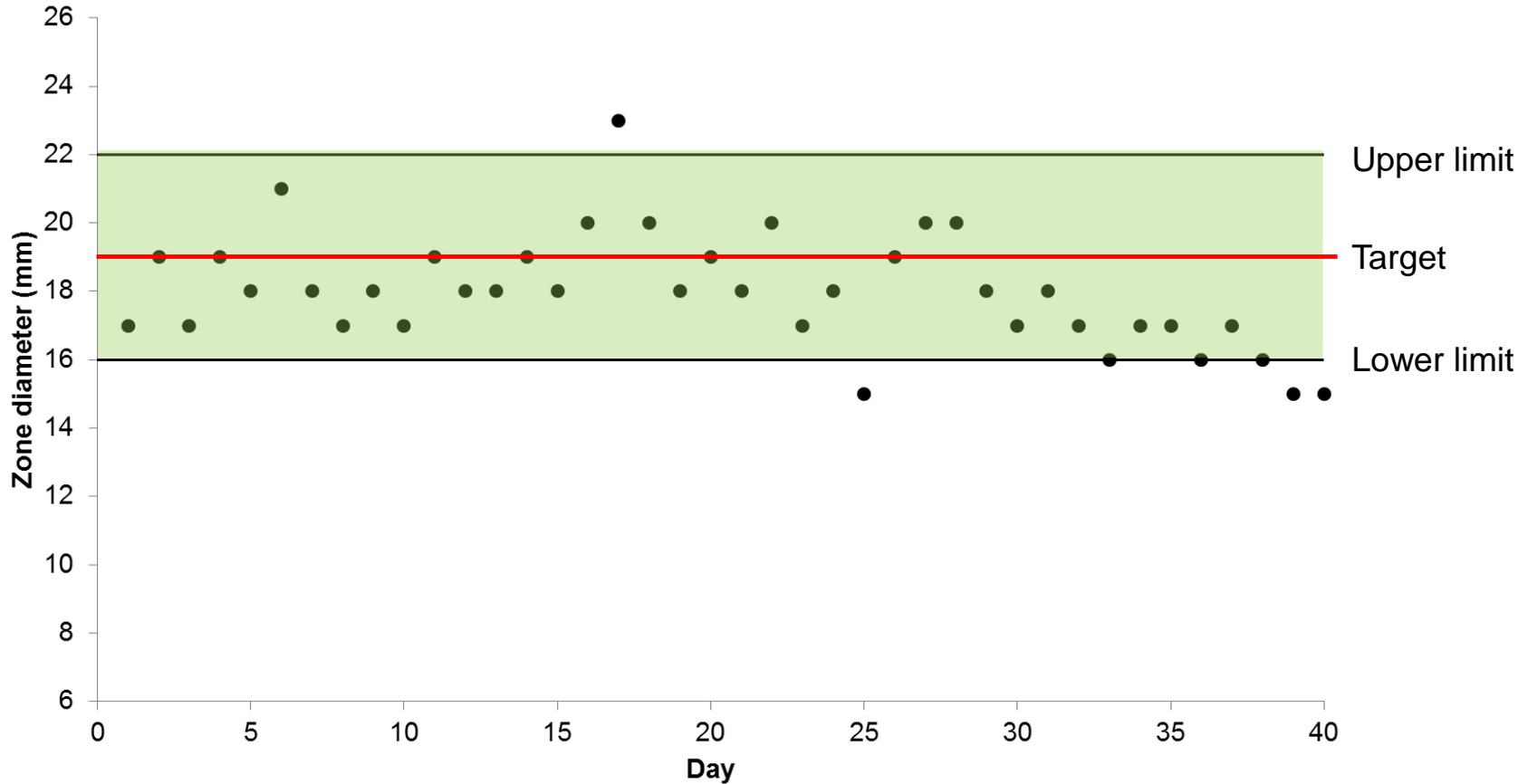


- Measured zone diameters are interpreted into S, I and R according to EUCAST breakpoints
 - Susceptible, standard dosing regimen (S)
 - Susceptible, increased exposure (I)
 - Resistant (R)

Quality control

- Use the recommended quality control (QC) strains to monitor the performance of the test:
 - Materials and equipment
 - Procedures (e.g. streaking of plates, reading of zones)
- Use an overnight culture of the QC strain and follow the same testing procedure as for clinical isolates.
- Include antimicrobial agents which are part of routine panels.
- Perform quality control daily, or at least four times a week.

Monitoring QC results



Factors affecting the zone size

- Medium (agar depth, pH, supplements)
- Size of inoculum (McF 0.5)
- Age of inoculum (overnight culture)
- Streaking of plates (technique, speed, removing excess fluid)
- Antimicrobial disks (correct potency, storage and handling)
- Incubation environment (air/CO₂/microaerobic/anaerobic)
- Incubation temperature and time
- ...and the reported zone diameter also by reading!

Reliable disk diffusion results

- Follow the described disk diffusion methodology!
- Use media and disks of good quality!
- Perform frequent quality control!



AST of bacteria

- Organization
- Consultations
- EUCAST News
- New definitions of S, I and R
- Clinical breakpoints and dosing
- Rapid AST in blood cultures
- Expert rules and expected phenotypes
- Resistance mechanisms
- Guidance documents
- SOP
- MIC and zone distributions and ECOFFs

- AST of bacteria**
 - Media preparation
 - MIC determination
 - Disk diffusion methodology**
 - Disk diffusion implementation
 - Breakpoint tables
 - Quality Control
 - Strains with defined susceptibility
 - Calibration and validation
 - Warnings!

EUCAST Disk Diffusion Test Methodology

The EUCAST disk diffusion test is based on MH media and disks of a good quality. It is calibrated to EUCAST clinical breakpoints using broth microdilution for MIC determination. Updates are published regularly.

See also [EUCAST instruction videos](#).

- [Disk diffusion - Manual v 10.0](#) (1 January, 2022)
- [Disk diffusion - Slide show v 10.0](#) (1 January, 2022)
- [Disk diffusion - Reading guide v 9.0](#) (1 January 2022)
- [Anaerobic bacteria - disk diffusion methodology v 1.0](#) (1 January 2022) including QC recommendations (the difficulties related to ordering the QC strain *C. perfringens* DSM 25589 from Germany have been solved - it can now be ordered from DSM and CCUG). Disk diffusion breakpoints for anaerobic bacteria are valid for FAA with 5% mechanically defibrinated horse blood as the only additive.
- [Anaerobic bacteria - disk diffusion reading guide v 1.0](#) (1 January 2022)
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For translations to other languages - contact National AST committees (NAC).
For previous versions of documents - see [➔ Previous versions](#).

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Disk diffusion methodology

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Videos and online seminars

[Organization](#)[Consultations](#)[EUCAST News](#)[New definitions of S, I and R](#)[Clinical breakpoints and dosing](#)[Rapid AST in blood cultures](#)[Expert rules and expected phenotypes](#)[Resistance mechanisms](#)[Guidance documents](#)[SOP](#)[MIC and zone distributions and ECOFFs](#)[AST of bacteria](#)[AST of mycobacteria](#)[AST of fungi](#)[AST of veterinary pathogens](#)[Frequently Asked Questions \(FAQ\)](#)[Meetings](#)[Publications and documents](#)[Presentations and statistics](#)[Videos and online seminars](#)[Online seminars](#)[Seminar links](#)[English](#)[Chinese](#)

Videos and Online seminars from EUCAST

Online seminars

- future seminars and recordings of previous seminars

[Online seminars and presentations.](#)

Instruction videos

In collaboration with the World Health Organisation (WHO), EUCAST publishes instruction videos on how to perform antimicrobial susceptibility testing (AST) using EUCAST recommended methods and interpretation.

The videos are published on Youtube™ and have an English speaker voice and English subtitles. Since not all countries may access Youtube™ videos in some languages are made available directly on the EUCAST web page.

The following topics are covered:

1. Preparation of inoculum (English).
2. Inoculation of agar plates for disk diffusion (English).
3. Application of antibiotic disks and incubation of plates (English).
4. Reading of inhibition zone diameters (English).
5. Guidance on the use of the breakpoint table (English).
6. Storage and handling of media and disks (English) - subtitles in other languages pending.
7. Quality control of AST in clinical microbiology (English) - subtitles in other languages pending.

Instruction videos on EUCAST susceptibility testing with subtitles in other languages than English:

[Instruction videos - English subtitles.](#)

- Alternative access to [instruction videos in English with English subtitles.](#)

[Instruction videos - German subtitles.](#)

[Instruction videos - Russian subtitles.](#)

Videos and online seminars



www.youtube.com



EUCAST disk diffusion antimicrobial susceptibility testing method

Preparation of inoculum

0:02 / 2:33

EUCAST videos (English)

Gunnar Kahlmeter • 1/5 videoer

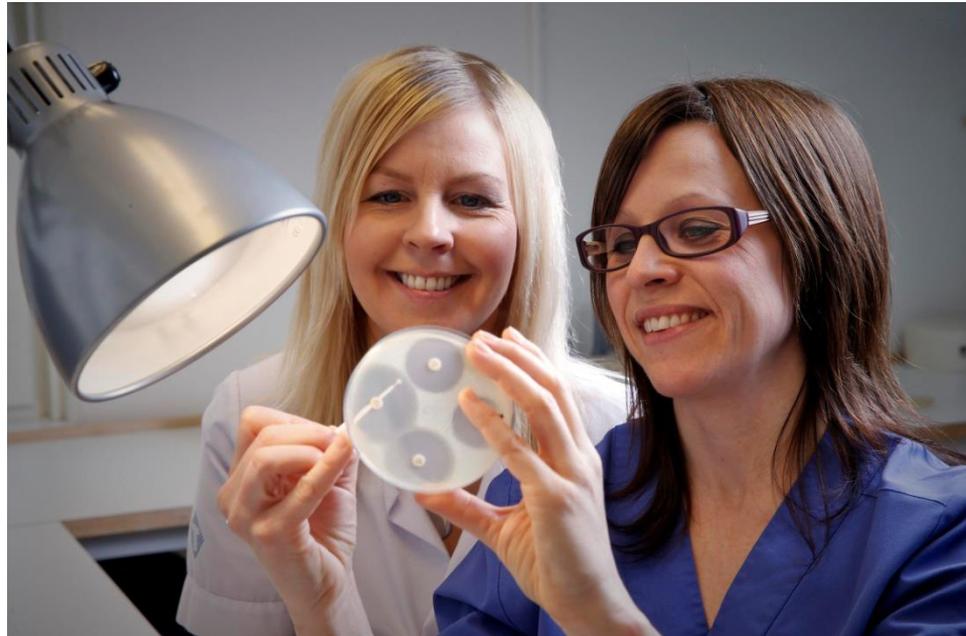


-  **1 Preparation of inoculum (english)**
Gunnar Kahlmeter
-  **2 Inoculation english**
Gunnar Kahlmeter
-  **3 Application of disks and incubation english**
Gunnar Kahlmeter
-  **4 Reading of zones english**
Gunnar Kahlmeter
-  **5 Guidance on breakpoint table english**
Gunnar Kahlmeter

Questions and/or problems?

Contact us!

erika.matuschek@eucast.org



EUCAST

EUROPEAN COMMITTEE
ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases