EUCAST THE EUCAST DEVELOPMENT LABORATORY

European Society of Clinical Microbiology and Infectious Diseases

EUCAST Disk Diffusion Method (Part 1)

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ICARS - ILRI Webinar Series
20 April 2022

Presentation Outline

EUCAST Disk Diffusion Method (1) (20 April 2022)

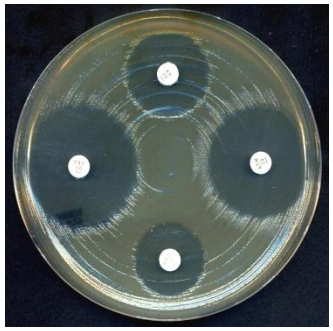
- Preparation and storage of media
- Disk diffusion methodology (inoculum preparation, inoculation of plates, application of disks, incubation of plates)
- Measurement of inhibition zones (general rules, specific instructions and exceptions)

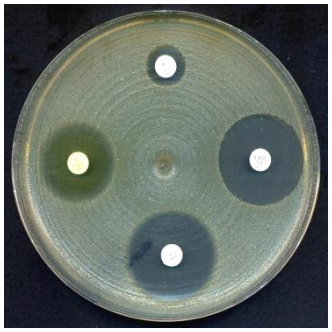
EUCAST Disk Diffusion Method (2) (4 May 2022)

- Interpretation of disk diffusion test results
- How to use EUCAST clinical breakpoint tables
- Caveats for disk diffusion method
- The importance of using a complete system

EUCAST disk diffusion test

- Based on a well-known technique (Kirby-Bauer)
- Calibrated to EUCAST MIC breakpoints
- Methodology, breakpoints and QC criteria are freely available on the EUCAST website






Antimicrobial susceptibility testing

Antimicrobial susceptibility testing is performed with phenotypic or genotypic methods. The basis of phenotypic methods is the minimum inhibitory concentration (MIC). Clinical MIC breakpoints determine whether the organism is categorised as susceptible, intermediate or resistant to the agent in question. Other methods should be calibrated to reference MIC methods.

Users of EUCAST breakpoints should use the EUCAST disk diffusion method or other susceptibility testing systems calibrated to EUCAST breakpoints and terminology in accordance with EUCAST breakpoint tables.

For videos on how to perform disk diffusion testing according to EUCAST - CLICK here!
For more information - CLICK here.

- **Media preparation**
  On how to prepare media for MIC and disk testing
- **MIC determination** of nonfastidious and fastidious organisms
  Broth microdilution methodology according to ISO and EUCAST
- **Disk diffusion methodology**
  Detailed description of the EUCAST disk diffusion test
- **Disk diffusion implementation**
  Guidance documents on how to implement the disk diffusion test
- **Breakpoint tables**
  Current MIC and zone diameter breakpoint tables
- **Quality Control and QC tables**
  Current tables of MIC and zone diameter ranges for quality control strains
- **Strains with well defined susceptibility**
  Strains with resistance mechanisms where MIC-values (and zone diameters) have been well defined using several different broth microdilution panels before and after freeze drying.
- **Calibration and validation**
  Presentation of raw data used in the development and calibration of EUCAST disk diffusion breakpoints against broth microdilution MIC-values.
- **Warnings!**
  EUCAST alerts on malfunctioning susceptibility testing material and procedures.
- **Guidance documents**
  Guidance notes on specific susceptibility testing issues
- **MIC determination services provided by the EUCAST Development Laboratory.**
- **Previous breakpoints and QC tables**
  Earlier versions of breakpoint and QC tables

... Disk diffusion methodology

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.
  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)
  Disk diffusion breakpoints for anaerobic bacteria are valid for FAA with 5% mechanically defibrinated horse blood as the only additive.

For translations to other languages - contact National AST committees (NAC).

For previous versions of documents - see → Previous versions.

EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Antimicrobial susceptibility testing

EUCAST disk diffusion method

Version 10.0

January 2022

Contents **Page**

Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories

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Abstract

With the support of ESCMID and European countries, EUCAST has developed a disk diffusion test with zone diameter breakpoints correlated with the EUCAST clinical MIC breakpoints. The development of the EUCAST disk diffusion method and quality control criteria are described, together with guidance on quality control and implementation of the method in clinical microbiology laboratories. The method includes the use of Mueller–Hinton agar without supplements for non-fastidious organisms and with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD for fastidious organisms, a standardized inoculum resulting in confluent growth, an incubation time of 16–20 h, a reading guide on how to read zone diameters on individual species-agent combinations and zone diameter breakpoints calibrated to the EUCAST clinical MIC breakpoints. EUCAST recommendations are described in detail and updated regularly on the EUCAST website (http://www.eucast.org).

Keywords: Antimicrobial susceptibility testing, disk diffusion, European Committee on Antimicrobial Susceptibility Testing, MIC, Mueller–Hinton agar, zone diameter breakpoints

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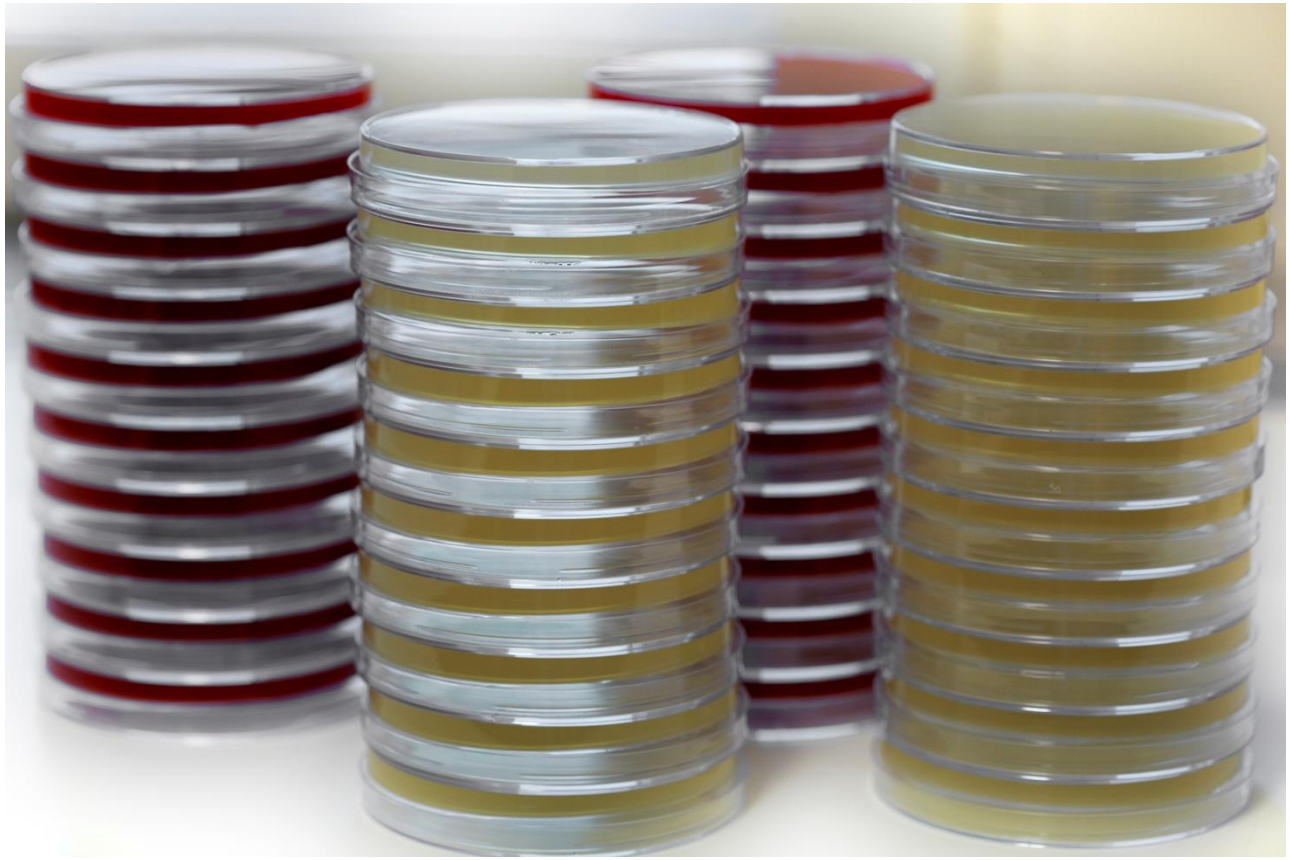
EUCAST disk diffusion method for antimicrobial susceptibility testing

Version 10.0
January 2022

Susceptibility testing media



EUCAST 2022 Version 10.0

Susceptibility testing media

- Un-supplemented Mueller-Hinton (MH) agar is used for non-fastidious organisms.
- MH with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD (MH-F, **M**ueller-**H**inton **F**astidious) is used for fastidious organisms.
- Use β-NAD with a purity of ≥ 98%.

Development of MH-F

- Good growth for *H. influenzae* and streptococci on MH with 5% horse blood and 20 mg/L β-NAD
- MH with 5% sheep blood required > 100 mg/L β-NAD for *H. influenzae* to grow –Expensive plate!
- We recommend β-NAD with a purity of ≥98%

Media for non-fastidious organisms

Organisms	Medium
Enterobacterales *Pseudomonas* spp. *Stenotrophomonas maltophilia* *Acinetobacter* spp. *Staphylococcus* spp. *Enterococcus* spp. *Aeromonas* spp. *Achromobacter xylosoxidans* *Vibrio* spp. *Bacillus* spp. *Burkholderia pseudomallei*	Mueller-Hinton agar

Media for fastidious organisms

Organisms	Medium
Streptococcus pneumoniae Streptococcus groups A, B, C and G Viridans group streptococci *Haemophilus influenzae* *Moraxella catarrhalis* *Listeria monocytogenes* *Pasteurella multocida* *Campylobacter jejuni* and *coli* *Corynebacterium* spp. *Aerococcus sanguinicola* and *urinae* *Kingella kingae*	Mueller-Hinton agar + 5% mechanically defibrinated horse blood + 20 mg/L β-NAD (MH-F)

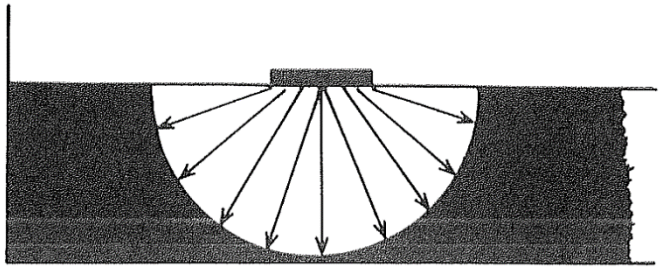
In-house preparation of media

- Prepare media according to the manufacturer's instructions.
- For MH-F, do not add blood or β-NAD until the medium has cooled to 42-45°C and mix well after the supplements have been added to the cooled medium.
- Pour plates on a level surface to give a uniform depth of 4.0 ± 0.5 mm. Adjust the volume if the agar depth is within the acceptable range but repeatedly above or below 4 mm.

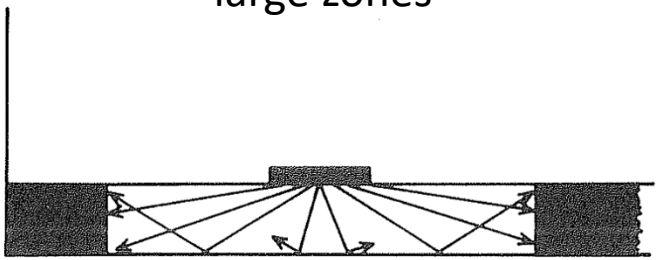
  Approximate volume for 90 mm circular plate: 25 mL, 100 mm circular plate: 31 mL, 150 mm circular plate: 71 mL, 100 mm square plate: 40 mL. Plate dimensions may differ between manufacturers. Ascertain that a correct volume, based on the true dimensions of the Petri dish in use, is calculated.

Agar depth

Correct agar depth



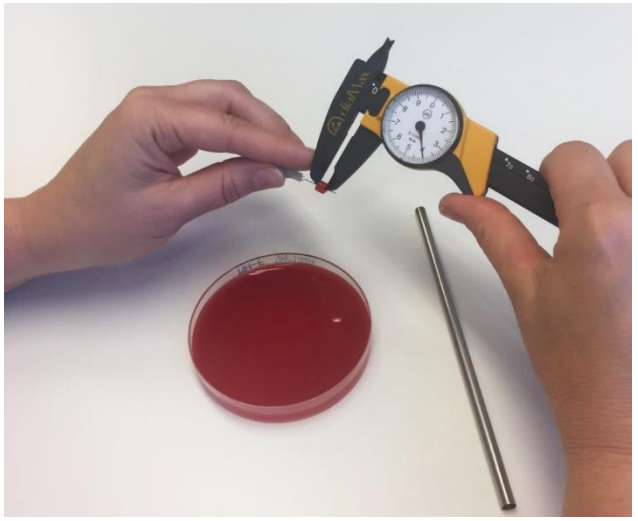
Thin layer of agar
= large zones



***P. aeruginosa* ATCC 27853** **Mean of 3 measurements (mm)**						
Volum	**22 mL**	**25 mL**	**28 mL**	**30 mL**	**Target**	**Range**
Agar depth	3.2 mm	3.7 mm	4.2 mm	4.6 mm		
Ceftazidime 10 µg	25.3	24.0	23.3	22.3	24	21-27
Piperacillin-tazobactam 30-6 µg	27.0	27.0	26.0	24.3	26	23-29

EM

Measuring agar depth



Adjust the volume if the agar depth is repeatedly above or below 4.0 mm!

Agar plates may be prepared in-house from dehydrated media or purchased ready-poured from commercial sources. Dehydrated Mueller-Hinton media should meet the requirements in the ISO Technical specification, ISO/TS 16782, 2016 and the quality control criteria published by EUCAST.

MH and MH-F agar plates are prepared as follows:

1. Reagents	
1.1	MH agar powder from commercial source.
1.2	Mechanically defibrinated horse blood.
1.3	β-Nicotinamide adenine dinucleotide (β-NAD), purity ≥98%.

2. Preparation of β-NAD stock solution	
2.1	Dissolve β-NAD in sterile deionized water to a concentration of 20 mg/mL.
2.2	Sterilize the solution through a 0.2 µm membrane filter.
2.3	The stock solution may be stored at -20°C in aliquots and defrosted as required. Do not refreeze unused solution.

MH and MH-F agar plates

3. Preparation of agar plates	
3.1	Prepare and autoclave MH agar according to the manufacturer's instructions.
3.2	Cool medium to 42-45°C.
3.3	For MH-F, aseptically add 50 mL mechanically defibrinated horse blood and 1 mL β-NAD stock solution per litre medium. Mix well and dispense immediately.
3.4	Dispense medium into sterile Petri dishes to give a level depth of 4 ± 0.5 mm (approximately 25 mL in a 90 mm circular plate, 31 mL in a 100 mm circular plate, 71 mL in a 150 mm circular plate, 40 mL in a 100 mm square plate). Ascertain that a correct volume, based on the true dimensions of the Petri dish in use, is calculated. Plate dimensions may differ between manufacturers.
3.5	Allow the agar to set before moving the plates.
3.6	The surface of the agar should be dry before use. No drops of water should be visible on the surface of the agar or inside the lid. If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min. Do not over-dry plates.

Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method
Version 7.0, January 2022 www.eucast.org

MH and MH-F agar plates

4. Storage of agar plates	
4.1	Store plates prepared in-house at 4-8°C.
4.2	For plates prepared in-house, plate drying, storage conditions and shelf life should be determined as part of the laboratory quality assurance programme.
4.3	Commercially prepared plates should be stored as recommended by the manufacturer and used within the labelled expiry date.
4.4	For agar plates (commercially or in-house prepared) stored in plastic bags or sealed containers, it may be necessary to dry the plates prior to use. This is to avoid excess moisture, which may result in problems with fuzzy zone edges and/or haze within zones.

Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method
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Drying and storage of agar plates

- Store plates at 4-8°C
- Make sure agar plates are at room temperature prior to inoculation.
- No drops of water should be visible on the surface of the agar or inside the lid.
- Excess moisture may cause

  fuzzy zone edges and/or

  haze within zones.

Drying and storage of agar plates

- In-house prepared plates:
  - Store at 4-8°C.
  - Plate drying, storage conditions and shelf life should be determined locally.
- Commercially prepared plates:
  - Store as recommended by the manufacturer.
  - Use within the labelled expiry date.

Drying and storage of agar plates

EM

MH-F agar plates
Excess humidity and fuzzy zone edges

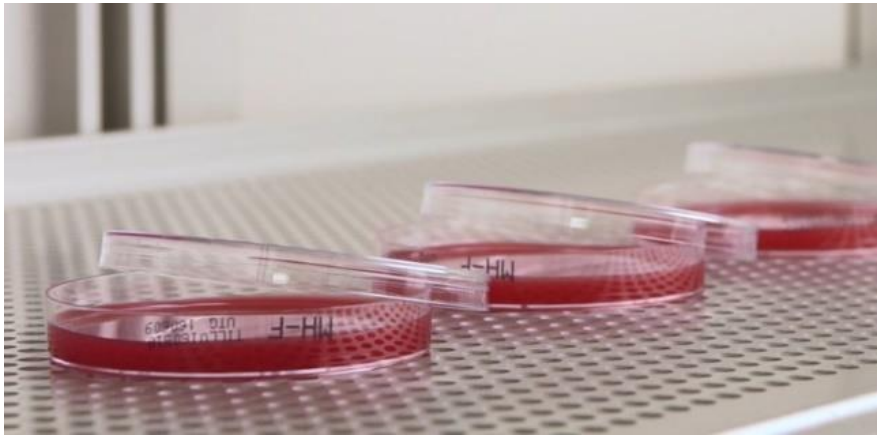
Drying and storage of agar plates

- 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.
  - 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.
- Do not over-dry plates.

Drying and storage of agar plates

- **In-house production:**
  - Dry newly produced plates in room temperature over night to remove excess humidity, before storing them in the fridge.
- **Plates stored in plastic bags or sealed containers:**
  - Unpack plates to be used within a short time and store in ventilated racks in the fridge.

- If necessary, dry plates
  - 20-25°C overnight or
  - 35°C for 15 min
    with the lid removed



MH and MH-F agar plates

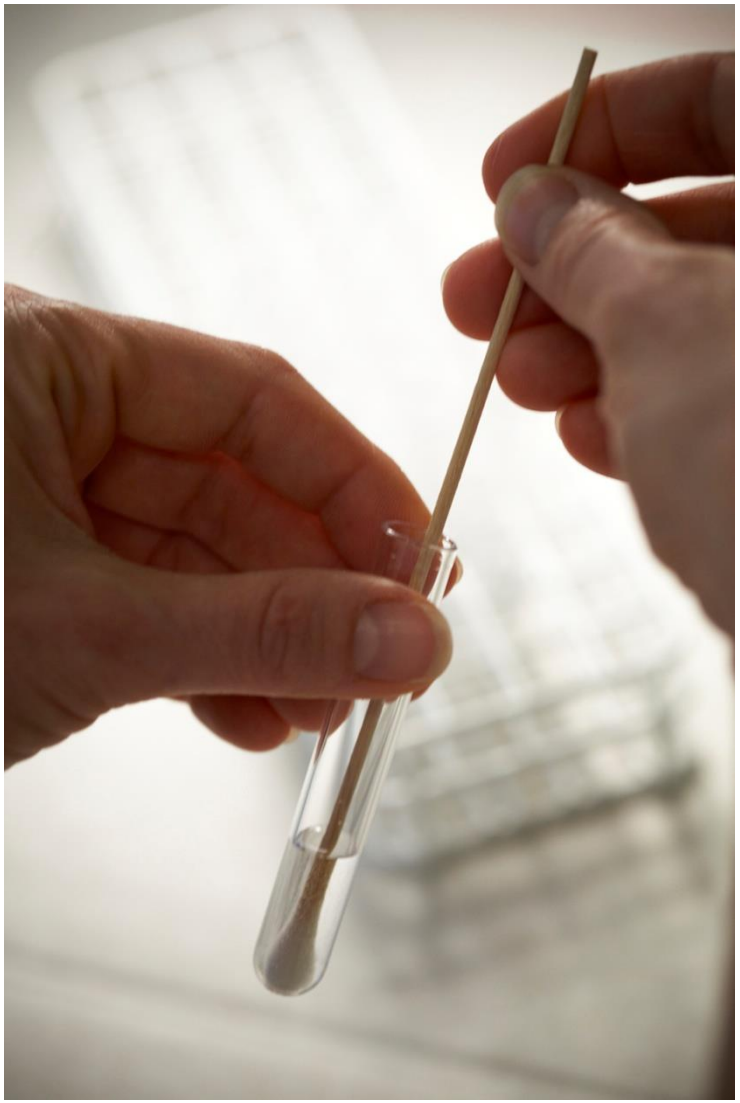
5. Quality control	
5.1	Use a surface pH electrode to check that the pH is within the range 7.2-7.4.
5.2	Check that the agar depth is 4 ± 0.5 mm.
5.3	Check that the medium supports good growth of control strain(s) of the intended test organisms.
5.4	Perform disk diffusion for quality control strains according to EUCAST recommendations and check that inhibition zones are within acceptable ranges for all bacteria-antimicrobial agent combinations used (EUCAST QC tables).

Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method
Version 7.0, January 2022 **www.eucast.org**

Inoculum

- The method requires an inoculum suspension with a turbidity equivalent to a 0.5 McFarland standard*.

* Approximately corresponding to 1-2 x10^8 CFU/mL for *E. coli.*



Select well-isolated colonies from overnight growth on non-selective medium

Inoculum preparation

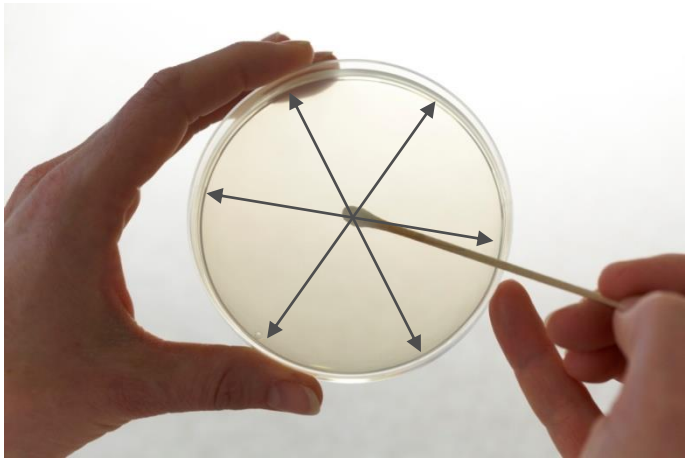
- Use a sterile loop or cotton swab to pick colonies from an overnight culture on non-selective media. If possible, use several morphologically similar colonies to avoid selecting an atypical variant.
- Suspend in saline and mix to an even turbidity.
- Adjust the density of the suspension to 0.5 McFarland by adding saline or more bacteria. Preferably use a photometric device to measure the turbidity.
  - Exception: *Streptococcus pneumoniae* is suspended to 0.5 McFarland from a blood agar plate, but to 1.0 McFarland from a chocolate agar plate.

Inoculation of plates

- Optimally, use the inoculum suspension within 15 minutes of preparation and always within 60 minutes.
- Make sure that agar plates are at room temperature prior to inoculation.
- Dip a sterile cotton swab into the suspension.
- 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 or turn the swab against the inside of the tube.

Inoculation of plates

- Spread the inoculum evenly over the entire surface by swabbing in three directions or by using a plate rotator.
- For Gram-positive bacteria, take particular care to ensure that there are no gaps between streaks.
- When inoculating several agar plates with the same inoculum, dip the cotton swab into the suspension for each agar plate.



Storage of antimicrobial disks

- Store stocks and working supplies of disks according to the manufacturers' instructions.
  - Some agents are more labile than others and may have specific recommendations.
- Store disks in current use in sealed containers with a moisture-indicating desiccant and protected from light.
- To prevent condensation, allow disks to reach room temperature before opening containers.
  - Rather 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.

Storage of antimicrobial disks

Storage of antimicrobial disks

Storage of antimicrobial disks

Storage of antimicrobial disks

TABLE 2. Differences in disk content between EUCAST and CLSI disk diffusion methods

Antimicrobial agent[a]	EUCAST disk content	CLSI disk content
Benzylpenicillin	1 unit	10 units
Ampicillin	2 and 10 μg[b]	10 μg
Amoxicillin-clavulanate	2–1 and 20–10 μg[c]	20–10 μg
Piperacillin	30 μg	100 μg
Piperacillin-tazobactam	30–6 μg	100–10 μg
Cefotaxime	5 μg	30 μg
Ceftaroline	5 μg	30 μg
Ceftazidime	10 μg	30 μg
Gentamicin (test for HLAR)	30 μg	120 μg
Vancomycin	5 μg	30 μg
Linezolid	10 μg	30 μg
Nitrofurantoin	100 μg	300 μg

HLAR, high-level aminoglycoside resistance.
[a]Ceftriaxone 30 μg and cefepime 30 μg are also under consideration for lower disk contents in the EUCAST disk diffusion test.
[b]2 μg for *Haemophilus influenzae*, *Pasteurella multocida*, *Listeria monocytogenes*, *Staphylococcus saprophyticus* and streptococci.
[c]2–1 μg for *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pasteurella multocida*.

Matuschek et al. *Clin Microbiol Infect* 2014;20:O255-O266
doi: 0.1111/1469-0691.12373

Application of antimicrobial disks

- Apply disks within 15 min of inoculation.
- Disks must be in close and even contact with the agar surface.
- The number of disks on a plate should be limited to avoid overlapping of zones and interference between agents. It is important that zone diameters can be reliably measured.

Incubation of plates

- Invert agar plates and make sure disks do not fall off the agar surface.
- Incubate plates within 15 min of disk application.
- Stacking plates in the incubator may affect results due to uneven heating. The efficiency of incubators varies, but for most incubators, a maximum of five plates per stack is appropriate.
- Incubate MH plates at 35±1°C in air.
- Incubate MH-F plates at 35±1°C in air with 4-6% CO_2 (except for Campylobacter).

Incubation of plates

Organism	Incubation conditions
Enterobacterales	35±1ºC in air for 18±2 h
Pseudomonas spp.	35±1ºC in air for 18±2 h
Stenotrophomonas maltophilia	35±1ºC in air for 18±2 h
Acinetobacter spp.	35±1ºC in air for 18±2 h
Staphylococcus spp.	35±1ºC in air for 18±2 h
Enterococcus spp.	35±1ºC in air for 18±2 h (24 h for glycopeptides)
Aeromonas spp.	35±1ºC in air for 18±2 h
Achromobacter xylosoxidans	35±1ºC in air for 18±2 h
Vibrio spp.	35±1ºC in air for 18±2 h
Bacillus spp.	35±1ºC in air for 18±2 h
Burkholderia pseudomallei	35±1ºC in air for 18±2 h

Incubation of plates

Organism	Incubation conditions
Streptococcus groups A, B, C and G	35±1ºC in air with 4-6% CO_2 for 18±2 h
Viridans group streptococci	35±1ºC in air with 4-6% CO_2 for 18±2 h
Streptococcus pneumoniae	35±1ºC in air with 4-6% CO_2 for 18±2 h
Haemophilus influenzae	35±1ºC in air with 4-6% CO_2 for 18±2 h
Moraxella catarrhalis	35±1ºC in air with 4-6% CO_2 for 18±2 h
Listeria monocytogenes	35±1ºC in air with 4-6% CO_2 for 18±2 h
Pasteurella multocida	35±1ºC in air with 4-6% CO_2 for 18±2 h
Campylobacter jejuni and *coli*	41±1ºC in microaerobic environment for 24 h (40-48 h)
Corynebacterium spp.	35±1ºC in air with 4-6% CO_2 for 18±2 h (40-44 h)
Aerococcus sanguinicola and *urinae*	35±1ºC in air with 4-6% CO_2 for 18±2 h (40-44 h)
Kingella kingae	35±1ºC in air with 4-6% CO_2 for 18±2 h (40-44 h)

The 15-15-15 minute rule

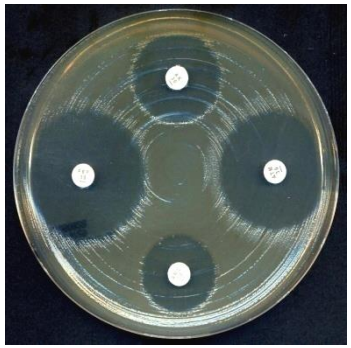
Follow these instructions for disk diffusion:

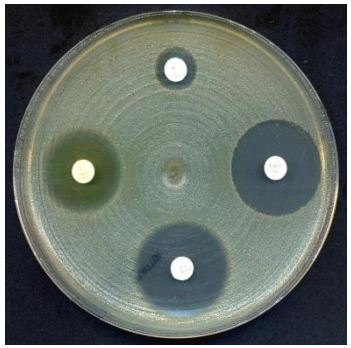
- Use the inoculum suspension optimally within **15 minutes** of preparation, and always within 60 minutes.
- Apply disks within **15 minutes** of inoculation.
- Incubate plates within **15 minutes** of disk application.

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 (see next slide).
- If individual colonies can be seen, the inoculum is too light and the test must be repeated.

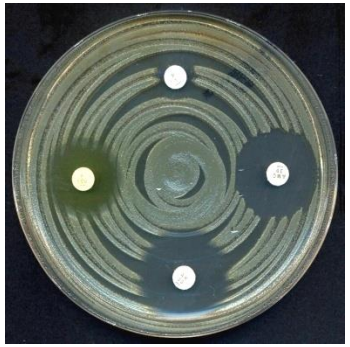
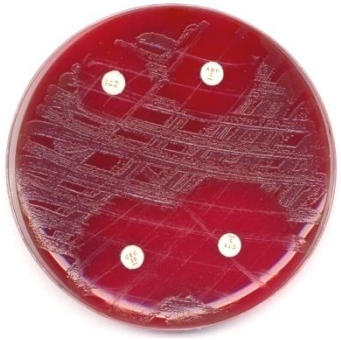
The growth should be confluent and evenly spread over the plate






Plates should look like this..



..and NOT like this!

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European Society of Clinical Microbiology and Infectious Diseases

Reading guide

EUCAST disk diffusion method for antimicrobial susceptibility testing

Version 9.0

January 2022

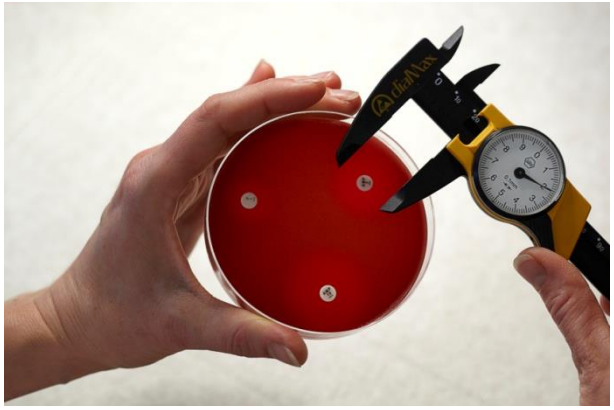
Reading zones

- The following instructions for reading inhibition zone diameters are part of the EUCAST disk diffusion method.
- Zone edges should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye (for exceptions and specific reading instructions, see slides 15-29).
- Holding the plate at a 45-degree angle to the work bench may facilitate reading when zone edges are difficult to define.
- Measure zone diameters to the nearest millimetre with a ruler or a calliper. If an automated zone reader is used, it must be calibrated to manual reading.

Reading zones

- Read **MH** plates from the back against a dark background illuminated with reflected light.

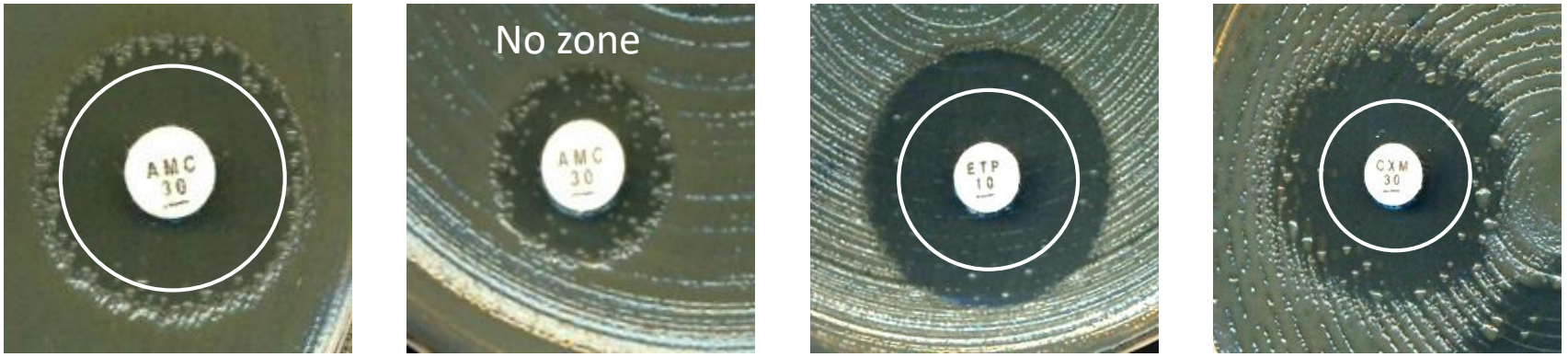
- Read **MH-F** plates from the front with the lid removed illuminated with reflected light.



Colonies within zone

- In case of distinct colonies within zones, check for purity and repeat the test if necessary.

- If cultures are pure, colonies within zones should be taken into account when measuring the diameter.



Reading of zones with colonies within the zone.

Colonies within zone

- In case of distinct colonies within zones, check for purity and repeat the test if necessary.

- If cultures are pure, colonies within zones should be taken into account when measuring the diameter.

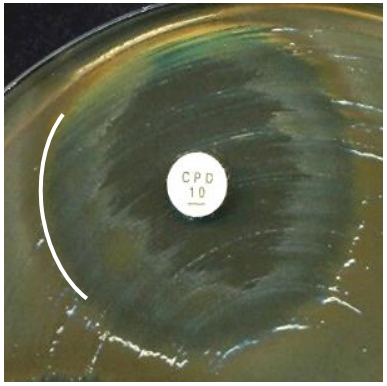
E. coli with ESBL

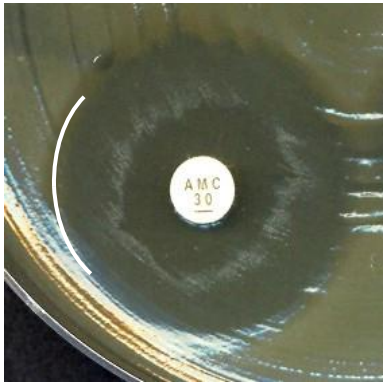
H. influenzae with PBP mutations

Reading of zones with colonies within the zone.

Swarming

- For *Proteus* spp., ignore swarming and read inhibition of growth.

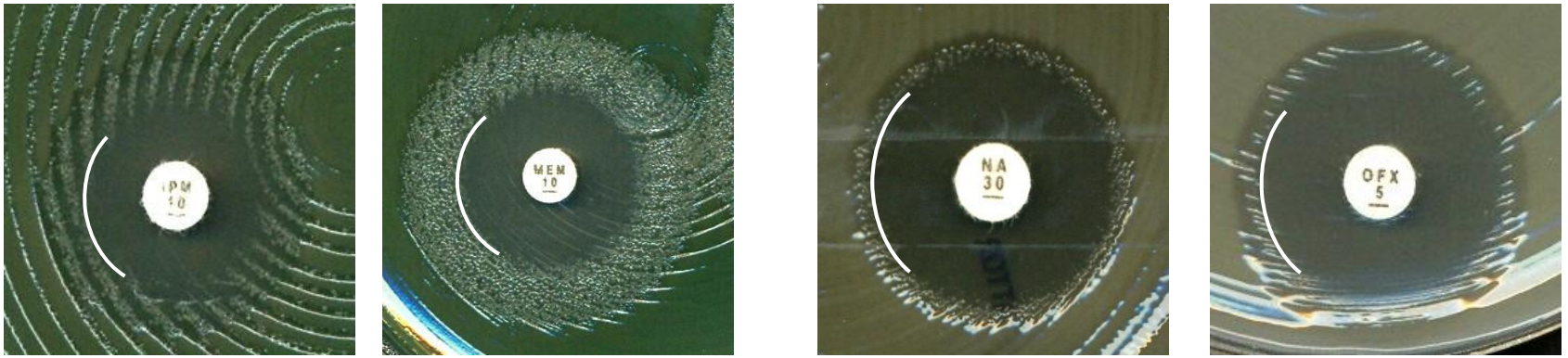




Double zones

- In case of double zones, check for purity and repeat the test if necessary.

- If cultures are pure, read the inner zone.



Reading of double zones.

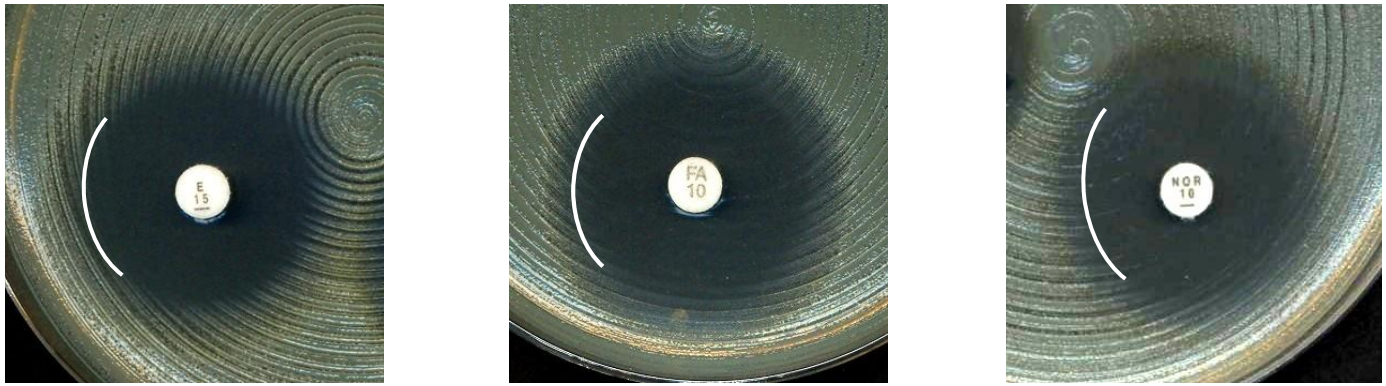
Fuzzy zone edges
Enterobacterales

- Hold the plate against a dark background about 30 cm from the naked eye and estimate where the zone edge is. Do not hold the plate up to light (transmitted light) or use a magnifying glass.

Reading of zones with fuzzy zone edges for Enterobacterales.

Fuzzy zone edges
Staphylococci

- Hold the plate against a dark background about 30 cm from the naked eye and estimate where the zone edge is. Do not hold the plate up to light (transmitted light) or use a magnifying glass.



Reading of zones with fuzzy zone edges for staphylococci.

Fuzzy zone edges
S. pneumoniae

- Small colonies that are visible when the plate is held about 30 cm from the naked eye at a 45-degree angle to the work bench should be taken into account when reading zones.
- The presence of small colonies close to the zone edge may be related to excess humidity in the MH-F media, and may be reduced by drying the plates prior to use.

Reading of zones with fuzzy zone edges for *S. pneumoniae*.

Growth or haemolysis?

- Read inhibition of growth and not inhibition of haemolysis.

- It is sometimes difficult to distinguish between haemolysis and growth.
  - β-Haemolysins diffuse in agar. β-haemolysis is therefore usually free from growth.
  - α-Haemolysins do not diffuse. There is often growth within areas of α-haemolysis.
  - Zone edges accompanied with α-haemolysis is most common with *S. pneumoniae* and β-lactam antibiotics.

β-haemolysis

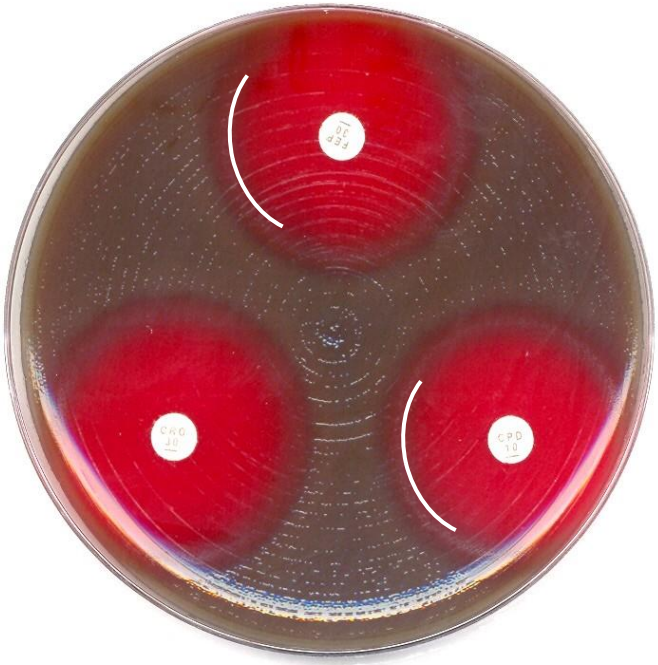
- Tilt the plate back and forth to better differentiate between haemolysis and growth.
- β-haemolysis is usually free from growth.

S. pyogenes

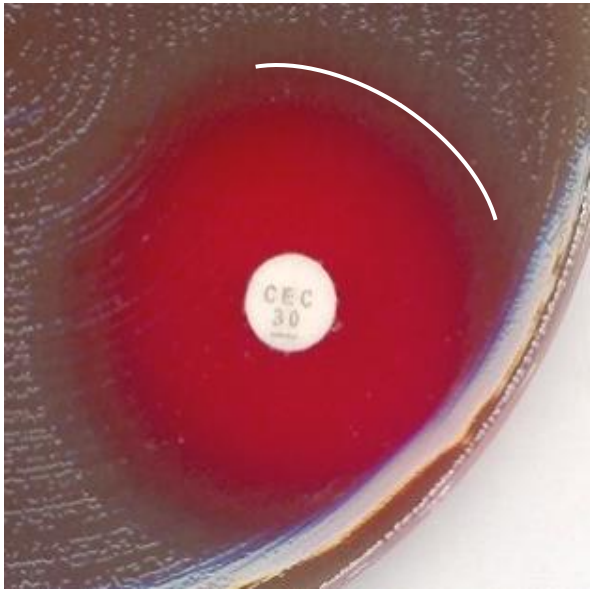
Streptococcus group C

α-haemolysis

- Tilt the plate back and forth to better differentiate between haemolysis and growth.



There is usually growth in the whole area of α-haemolysis.



For some organisms, there is additional α-haemolysis without growth. Tilt the plate to differentiate between haemolysis and growth.

Reading zones – exceptions (1)

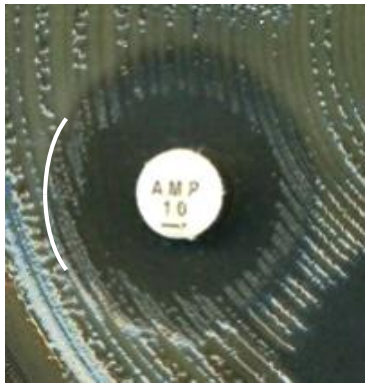
Organism	Antimicrobial agent	Reading inhibition zones
Enterobacterales	Ampicillin Ampicillin-sulbactam Amoxicillin-clavulanic acid	Ignore fine growth that may appear as an inner zone on some batches of MH agar.
Enterobacterales	Temocillin	Ignore isolated colonies within the inhibition zone.
Enterobacterales	Mecillinam	Ignore isolated colonies within the inhibition zone.
E. coli	Fosfomycin	Ignore isolated colonies within the inhibition zone and read the outer zone edge.
Proteus spp.	Any	Ignore swarming.
S. maltophilia, *A. xylosoxidans* and *B. pseudomallei*	Trimethoprim-sulfamethoxazole	Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
S. aureus	Benzylpenicillin	Examine zone edge from the front of the plate with transmitted light (plate held up to light).

Reading zones – exceptions (2)

Organism	Antimicrobial agent	Reading inhibition zones
Staphylococci	Cefoxitin	Examine zones carefully to detect colonies within the inhibition zone.
Enterococcus spp.	Vancomycin	Examine zone edge from the front of the plate with transmitted light (plate held up to light).
Streptococcus spp.	Any	Read inhibition of growth and not the inhibition of haemolysis.
H. influenzae	Beta-lactam agents	Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk.
Aeromonas spp.	Trimethoprim-sulfamethoxazole	Read the obvious zone edge and disregard haze or growth within the inhibition zone
Any	Trimethoprim Trimethoprim-sulfamethoxazole	Ignore faint growth up to the disk and measure at the more obvious zone edge.

Enterobacterales with ampicillin, ampicillin-sulbactam and amoxicillin-clavulanic acid

- Ignore growth that may appear as a thin inner zone on some batches of Mueller-Hinton agars. The inner zone is not seen with some batches of agar and when the outer zone is read there is no difference between batches.

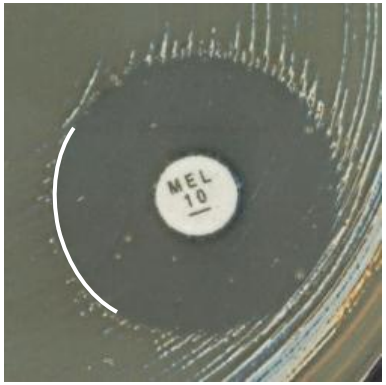


Enterobacterales and temocillin

- Ignore isolated colonies within the inhibition zone and read the outer zone edge.

Enterobacterales and mecillinam

- Ignore isolated colonies within the inhibition zone and read the outer zone edge.



E. coli and fosfomycin

- Ignore isolated colonies within the inhibition zone and read the outer zone edge.

Trimethoprim and trimethoprim-sulfamethoxazole

- Follow the instructions for reading and read the inner zone when double zones appear (see examples below).
- Ignore haze or faint growth up to the disk within a zone with otherwise clear zone edge.

E. coli

CoNS

Moraxella

Haemophilus

S. maltophilia with trimethoprim-sulfamethoxazole

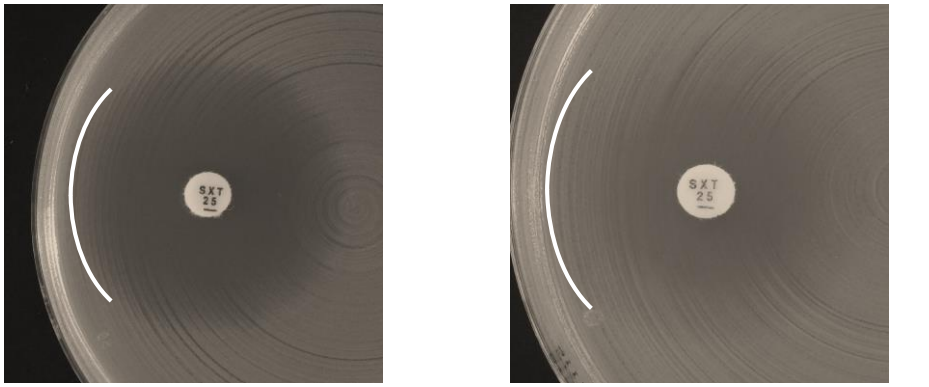
- Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
  - Read the outer zone edge and interpret according to the breakpoints.
- If there is growth up to the disk and no sign of inhibition zone, report resistant.

An outer zone can be seen

Growth up to the disk

A. xylosoxidans with trimethoprim-sulfamethoxazole

- Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
  - Read the outer zone edge and interpret according to the breakpoints.
- If there is growth up to the disk and no sign of inhibition zone, report resistant.

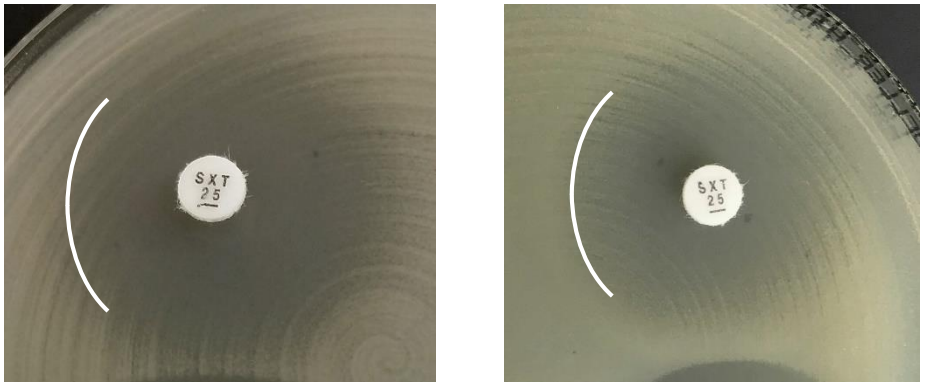


An outer zone can be seen

Growth up to the disk

B. pseudomallei with trimethoprim-sulfamethoxazole

- Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
  - Read the outer zone edge and interpret according to the breakpoints.
- If there is growth up to the disk and no sign of inhibition zone, report resistant.

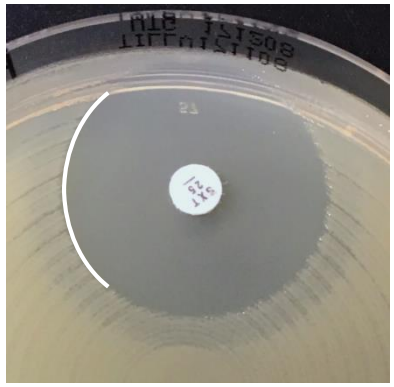


An outer zone can be seen

Growth up to the disk

Aeromonas spp. and trimethoprim-sulfamethoxazole

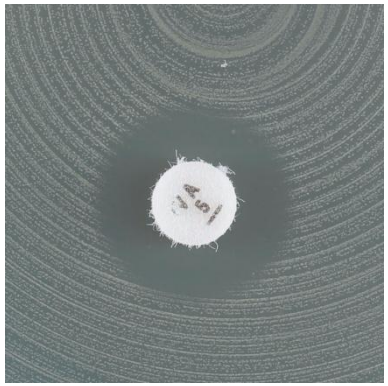
- Read the obvious zone edge and disregard haze or growth within the inhibition zone.
- If there is an obvious inner zone edge, read the inhibition zone as the inner zone.



Enterococci and vancomycin

- Examine zone edge from the front of the plate with transmitted light (plate held up to light).
  - If the zone edge is sharp, report susceptible.
  - If the zone edge is fuzzy, colonies grow within the zone or if you are uncertain, suspect VRE and perform confirmatory testing, even if the zone diameter is ≥ 12 mm.
  - Isolates must not be reported susceptible before 24 h incubation.

non-VRE



VRE

S. aureus and benzylpenicillin

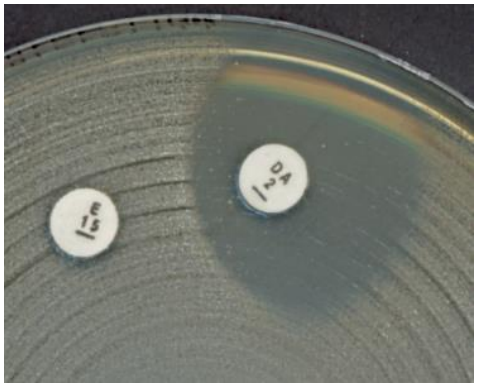
- Examine zone edge from the front of the plate with transmitted light (plate held up to light).
  - To detect penicillinase production, read the zone diameter AND examine the zone edge closely.
  - If the zone is ≥ 26 mm and the zone edge is sharp (no reduction of growth towards zone edge, like a “cliff”), the isolate is a pencillinase producer, report resistant.
  - If the zone is ≥ 26 mm and the zone edge is fuzzy (reduction of growth towards zone edge, like a “beach”), report susceptible.

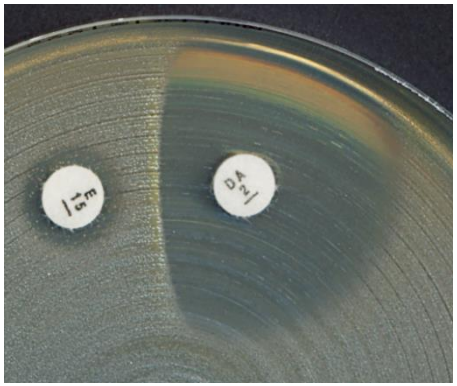
Zone ≥ 26 mm and
sharp zone edge= Resistant

Zone ≥ 26 mm and
fuzzy zone edge = Susceptible

Detection of inducible clindamycin resistance in staphylococci

- Inducible clindamycin resistance can be detected by antagonism of clindamycin activity and a macrolide agent.
- Place the erythromycin and clindamycin disks **12-20 mm apart** (edge to edge) and look for antagonism (the D phenomenon).

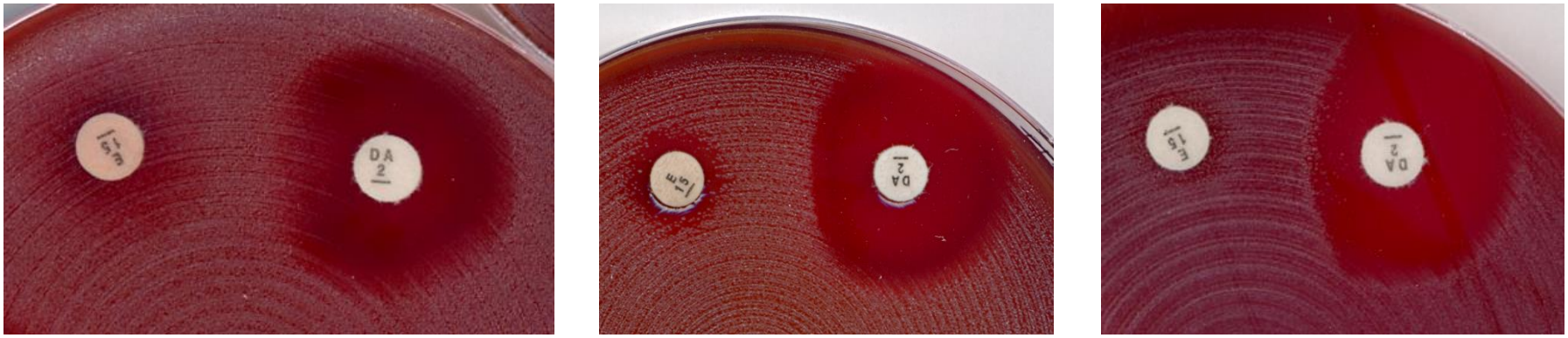




Examples of D phenomenon for staphylococci.

Detection of inducible clindamycin resistance in streptococci

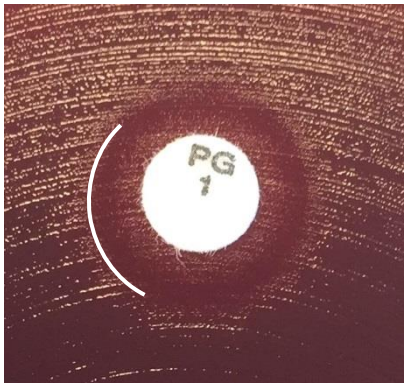
- Inducible clindamycin resistance can be detected by antagonism of clindamycin activity and a macrolide agent.
- Place the erythromycin and clindamycin disks **12-16 mm apart** (edge to edge) and look for antagonism (the D phenomenon).



Examples of D phenomenon for streptococci.

H. influenzae and beta-lactam agents

- Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk.



Standardisation of AST

- Results change with changed parameters.
  - It is crucial to adhere to the methodology to get reproducible and reliable results!

- Standardisation of:
  - Disk potency
  - Media
    - Type of media, supplements, pH, agar depth etc.
  - Inoculum
  - Incubation
  - Reading of results

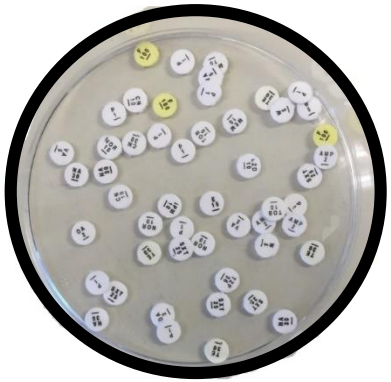
All antimicrobial susceptibility testing must be standardised!

Problems? Please check:

- Inoculum
  - Too light, too heavy or uneven?
- Incubation
  - Always 16-20 h and 35°C!
- Reading
  - Sharp/fuzzy zone egdes, Growth/haemolysis?
- Agar depth
  - 4.0 mm ± 0.5 mm (occasional deviations)
- QC strains
- Disks

Potential sources of error

- Antimicrobial disks
  - Decreased disk potency
    - Loss of potency during handling and storage
    - Disks passed expiry date
  - Wrong agent
  - Wrong disk potency
  - Poor disk quality



EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Frequently Asked Questions (FAQ)

Frequently Asked Questions (FAQ)

The EUCAST secretariat receives many questions on subjects ranging from how we determine breakpoints, the MIC-distribution website, and the disk diffusion methodology. We try to answer each question individually but also publish frequently asked questions and answers in a classical FAQ document.

The file is updated at regular intervals.

Frequently Asked Questions - valid from 2021-06-04

Frequently asked questions concerning EUCAST RAST (Rapid AST directly from blood culture bottles).

Previous versions of FAQ:

- **Frequently Asked Questions** - valid from 2019-08-12 - 2021-04-03
- **Frequently Asked Questions** (and an **update** on question 8) - valid from 2018-02-18 - 2019-08-12
- **Frequently Asked Questions** - valid from 2016-03-29
- **Frequently Asked Questions** - valid 2015-03-23 - 2016-03-29
- **Frequently Asked Questions** - valid 2014-02-26 - 2015-03-23
- **Frequently Asked Questions** - valid 2013-04-24 - 2014-02-26

EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING
European Society of Clinical Microbiology and Infectious Diseases

Questions? Please contact
erika.matuschek@escmid.org

EUCAST Frequently Asked Questions

1. EUCAST Disk Diffusion Test - Medium

1. Which manufacturer of Mueller-Hinton agar does EUCAST recommend?
2. What is the difference between Mueller-Hinton agar and Mueller-Hinton II agar?
3. Do we need to quality control each new batch of Mueller-Hinton agar?
4. Can we use sheep blood instead of horse blood for the MH-F medium?
5. Which β-NAD should we use?
6. Can MH-F be used as medium for gradient tests?
7. It is stated in the EUCAST disk diffusion manual that the agar depth should be 4.0 ± 0.5 mm. Does this mean that it is acceptable to use plates with an agar depth of 3.5-3.7 mm?
8. We have problems with haze within the inhibition zones and growth of colonies close to the zone edge, particularly on the MH-F media. Can we do something to improve this?

2. EUCAST Disk Diffusion Test - Disks

1. Are EUCAST disk contents all the same as CLSI?

3. EUCAST Disk Diffusion Test - Inoculum preparation

1. Do we have to measure the McFarland value on all suspensions?
2. Can we pick colonies from selective media?
3. Should we pick more than one colony to be sure that we do not miss hetero-resistance?
4. Can we use water or buffer instead of saline for inoculum preparation?
5. In the EUCAST disk diffusion manual it is stated that we have to adjust the inoculum to a density of a McFarland 0.5 turbidity standard. What is the range we can use?
6. Can flooding be used to inoculate plates for antimicrobial susceptibility testing?

4. EUCAST Disk Diffusion Test - Reading zones of inhibition

1. Do we have to measure all inhibition zones?
2. Should inhibition zones on both MH and MH-F be read against a black background?
3. Are all bactericidal and bacteriostatic agents read according to the same recommendations?
4. Why is there sometimes growth within zones of beta-lactams for *Haemophilus influenzae* ATCC 49766?
5. Are isolated colonies within mecillinam inhibition zones significant?
6. Why are there sometimes colonies within the inhibition zones of carbapenems and *Pseudomonas aeruginosa* ATCC 27853?

Videos and online seminars from EUCAST

Instruction videos with subtitles in several languages (below)

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.

Instruction videos - Turkish subtitles.

Instruction videos - French subtitles.

Instruction videos - Spanish subtitles.

Instruction videos - Portuguese subtitles.

Instruction videos - Arabic subtitles.

Instruction videos - Czech subtitles.

Instruction videos - Chinese subtitles.
- Alternative access to instruction videos in english with **chinese subtitles.**

Instruction videos - Romanian subtitles.

- Check the EUCAST website regularly for updates on methodology, QC ranges and breakpoints.

www.eucast.org

- Please send any questions, comments and suggestions to

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