



Troubleshooting in antimicrobial susceptibility testing and Frequently Asked Questions (FAQ)

Erika Matuschek

Jenny Åhman

Gunnar Kahlmeter

EUCAST Development Laboratory (EDL)

NordicAST workshop 2017

Troubleshooting in disk diffusion

- Quality control (QC)
 - QC results out of range
 - Investigate if two consecutive tests are out of range or multiple disks out of range on one day.
 - Evaluation of QC data over time
 - Look for trends and zones falling consistently above or below the target.
- Zone distributions for clinical isolates not in agreement with reference distributions.

Routine quality control

- Materials and equipment
 - Agar plates
 - Antimicrobial disks
 - Incubator
 - Densitometer
- Performance
 - Inoculum
 - Inoculation
 - Reading
 - etc.

QC ranges and targets

Range

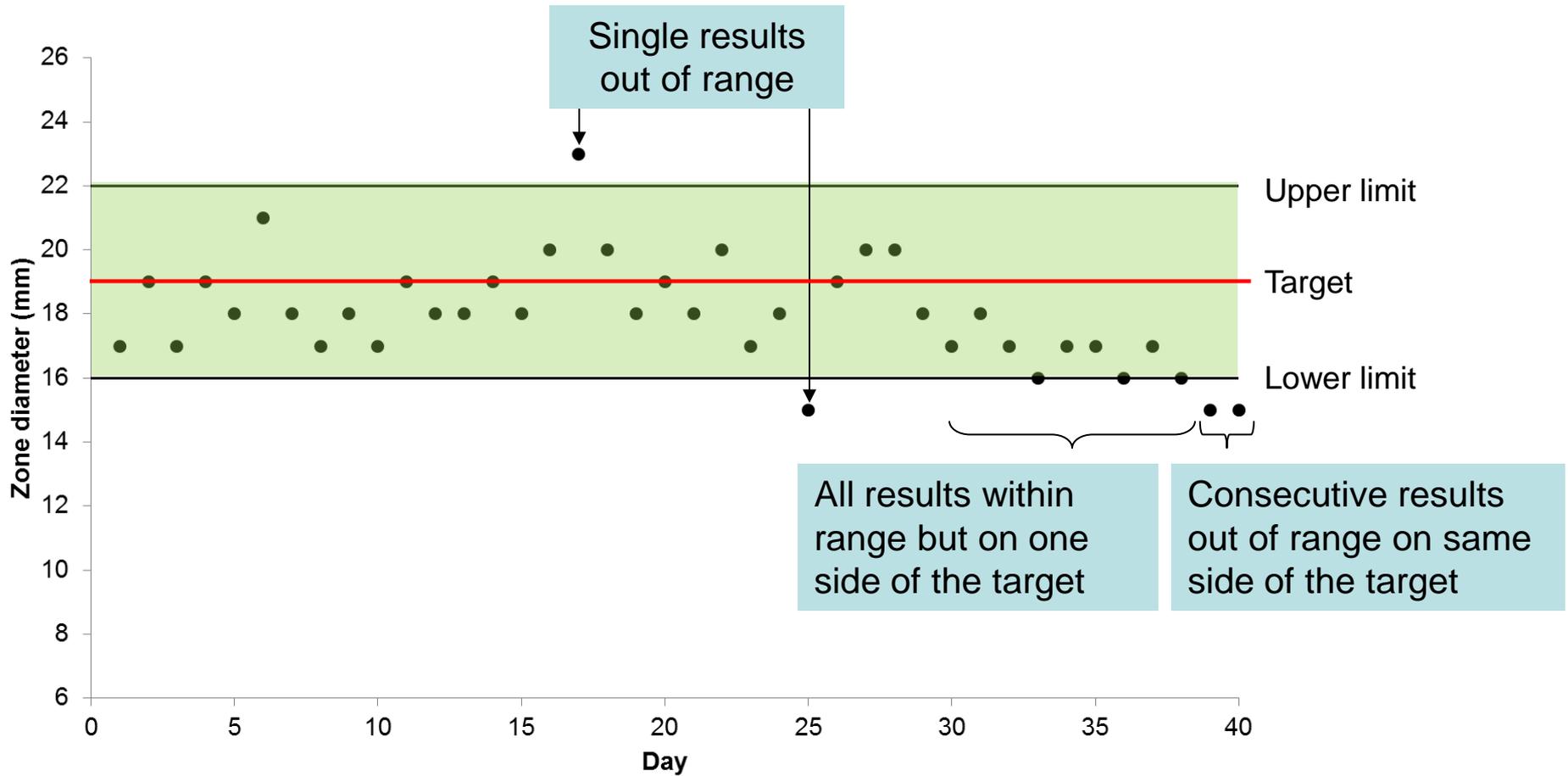
Used to allow occasional variation

Antimicrobial agent	Disk content (µg)	Inhibition zone diameter (mm)	
		Target	Range
Amikacin	30	23	19-26
Amoxicillin-clavulanic acid	20-10	21	18-24
Ampicillin	10	19	15-22

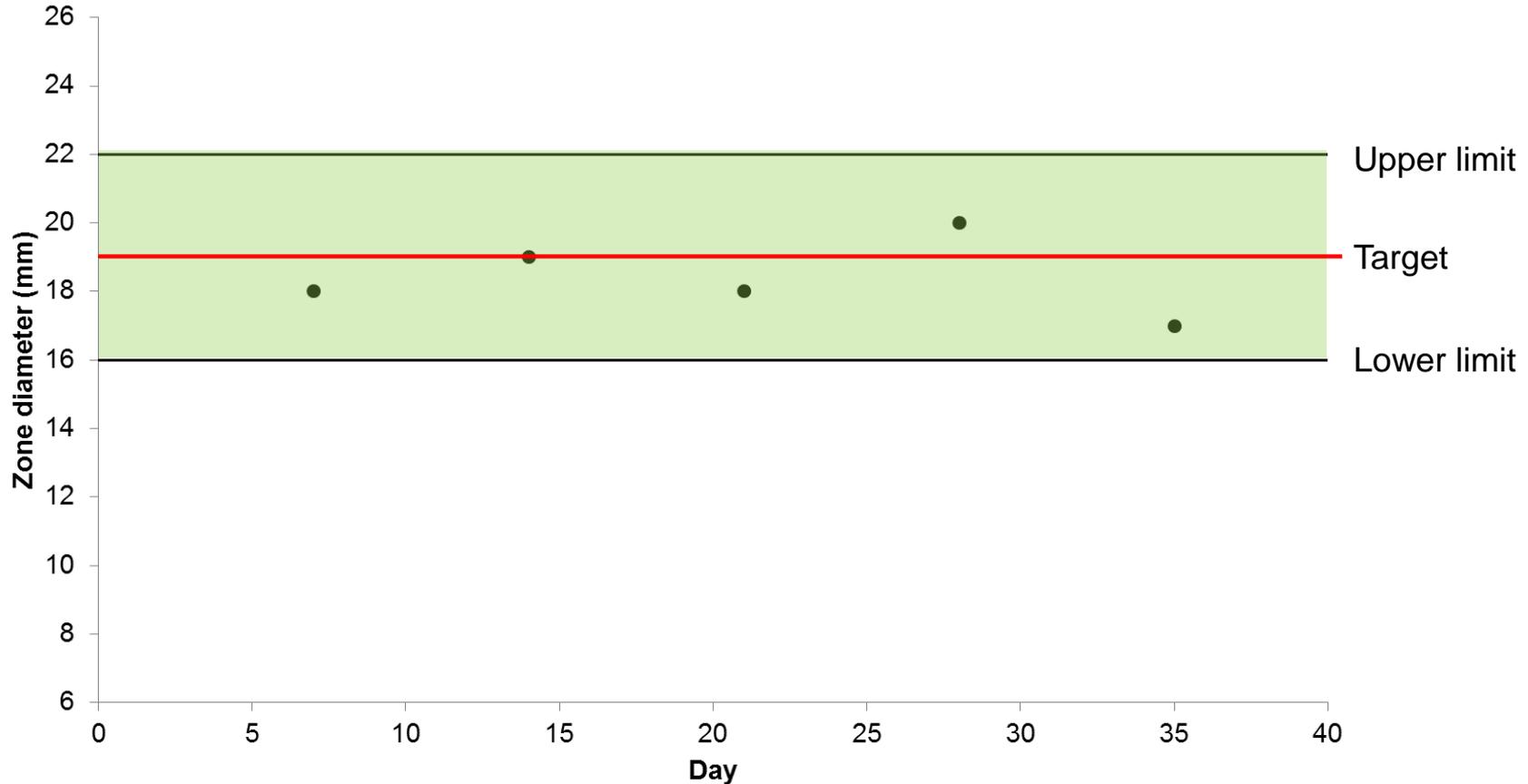
Target

Mean values from repeated measurements (≥ 10) should optimally be on target ± 1 mm

Monitoring of QC results over time



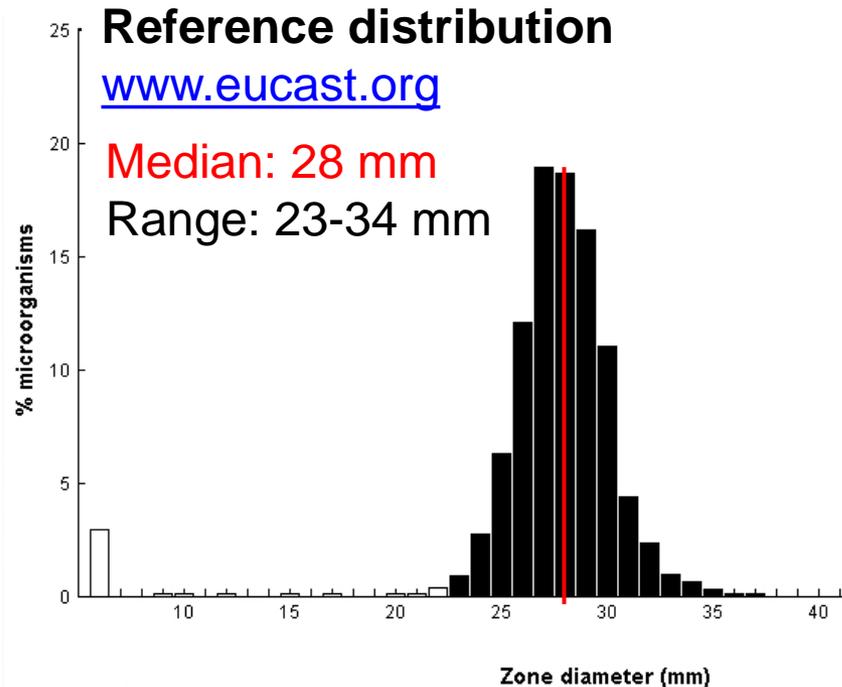
Monitoring of QC results over time



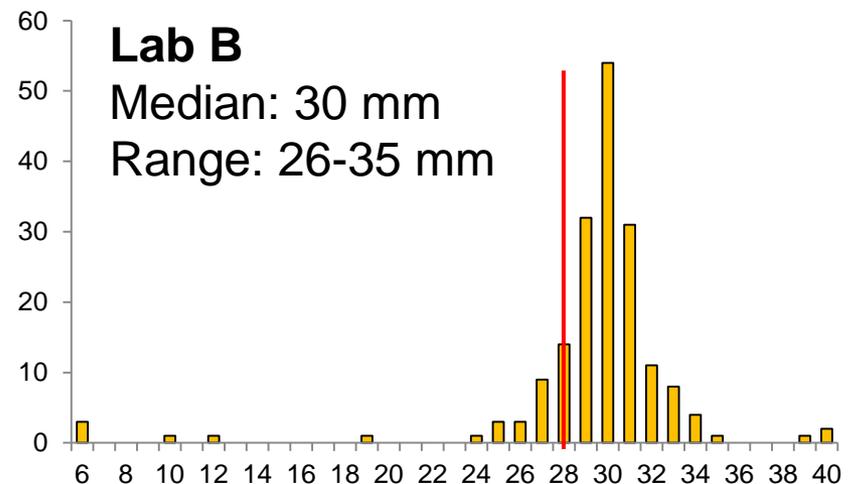
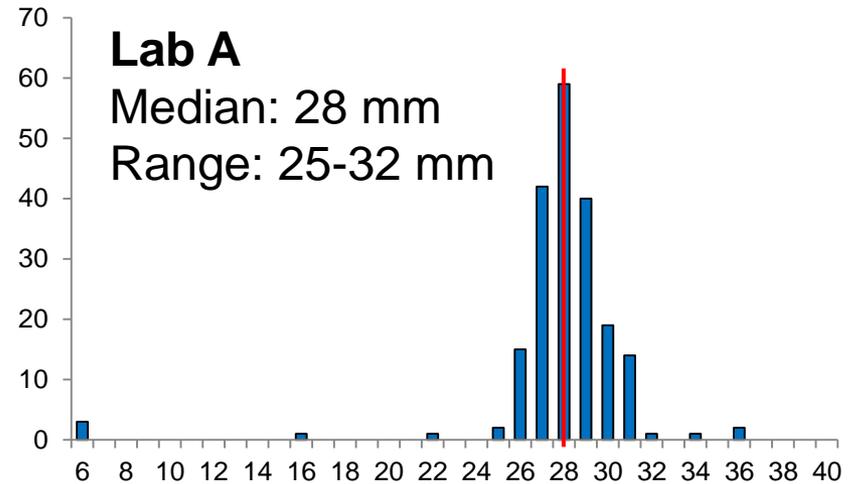
Daily QC versus weekly QC!

Routine quality control should be performed daily, or at least four times a week!

Comparison with reference distributions

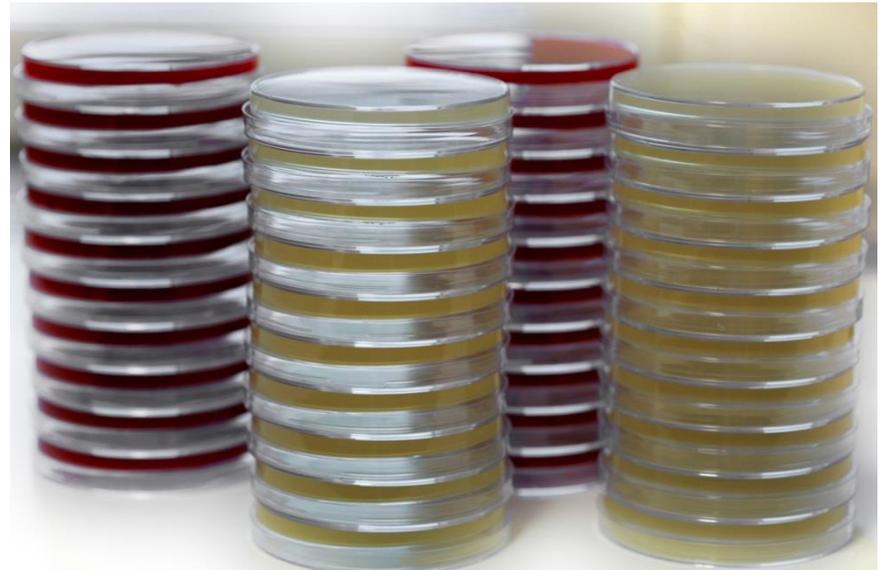


Median of wild-type distribution should be at median for reference ± 1 mm.



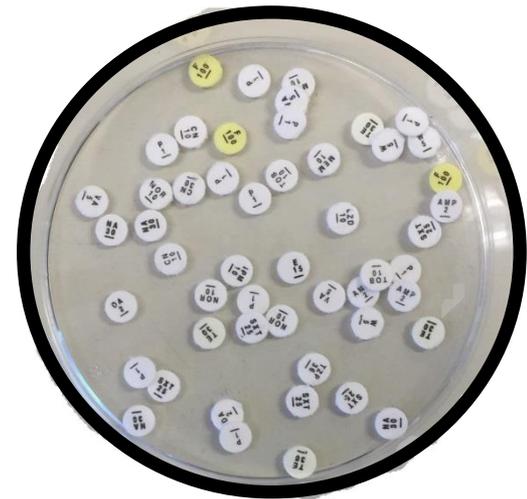
Potential sources of errors

1. Disks
2. Media
3. Not adhering to methodology
4. Equipment
5. QC strain



Example 1

- **Problem:** Amoxicillin-clavulanic acid zone diameters above QC range for *H. influenzae* ATCC 49766.
 - Correct disk potency?
 - No, 20-10 µg-disk used!
- **Solution:** Change to correct disk potency



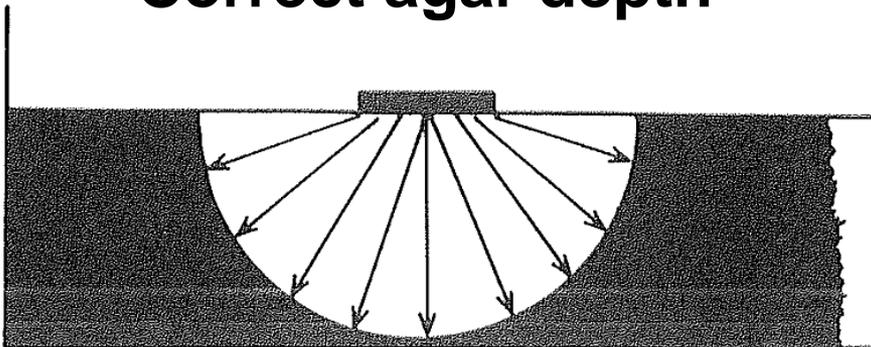
Example 2

- **Problem:** Amoxicillin-clavulanic acid zone diameters above QC range for *H. influenzae* ATCC 49766.
 - Correct disk potency?
 - Yes, 2-1 µg-disk used.
 - Other agents within range?
 - Yes, but some are in the upper part of the QC ranges.
 - Amoxicillin-clavulanic acid zones for *S. pneumoniae* ATCC 49619 within range?
 - Yes, but in the upper part of the QC range.

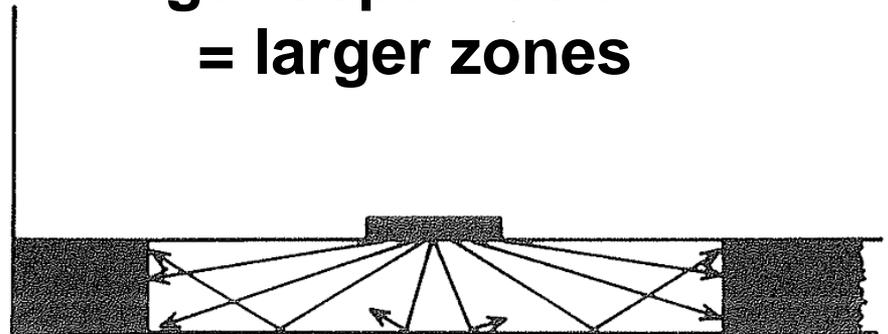
Example 2

- **Problem:** Amoxicillin-clavulanic acid zone diameters above QC range for *H. influenzae* ATCC 49766.
 - Correct agar depth?
 - Agar depth 3.0 mm
- **Solution:** Adjust volume of agar to achieve agar depth of 4.0 mm.

Correct agar depth

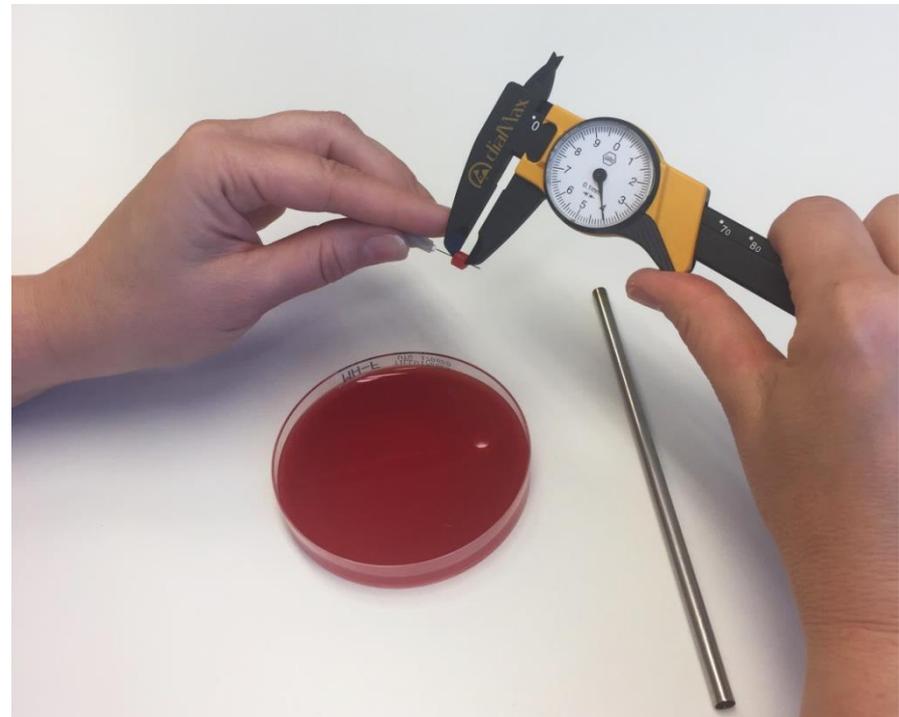


Agar depth too thin
= larger zones



Measuring agar depth

- Check the agar depth for all new lots of plates, both commercial and in-house produced.



Correct agar depth: 4.0 mm, with occasional variation between 3.5 and 4.5 mm!

Example 3

- **Problem:** Meropenem zone diameters below QC range for *H. influenzae* ATCC 49766.
 - Disks lost potency?
 - Disks within the expiry date?
 - Yes!
 - Active desiccant?
 - Yes!
 - Correct storage?
 - Yes!
 - Meropenem zones for *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. pneumoniae* ATCC 49619 within range?
 - Yes, but in the lower part of the QC range for *S. pneumoniae*.

Example 3

- **Problem:** Meropenem zone diameters below QC range for *H. influenzae* ATCC 49766.
 - MH-F plates within the expiry date?
 - Yes!
 - MH-F plates sufficiently dry?
 - No, condensation inside the lid!



- **Solution:** Check storage conditions and/or dry plates prior to inoculation.

Drying and storage of MH-F



No drops of water should be visible on the surface of the agar or inside the lid.

- Store plates unpacked in the fridge
- Dry plates before inoculation
 - 20-25°C overnight, or at 35°C, with the lid removed, for 15 min.

Drying and storage of MH-F

For agar plates 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.



Example 4

- **Problem:** Ciprofloxacin zone diameters below QC range for *E. coli* ATCC 25922.
 - Disks lost potency?
 - Disks within the expiry date?
 - Yes!
 - Correct storage of disks?
 - Yes!
 - Media within the expiry date?
 - Yes!
 - Ciprofloxacin zones within ranges for other QC strains?
 - Yes!

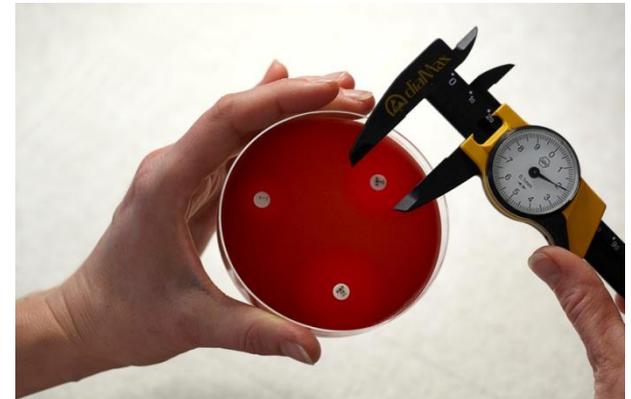
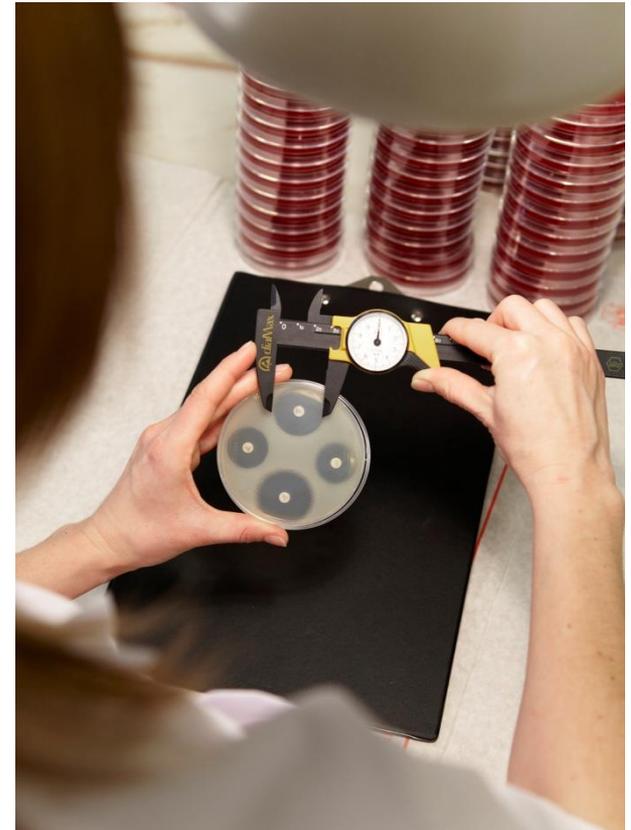
Example 4

- **Problem:** Ciprofloxacin zone diameters below QC range for *E. coli* ATCC 25922.
 - Agar plates correctly inoculated?
 - Yes!
 - Ciprofloxacin zones edges?
 - Fuzzy zone edges!
- **Solution:** Follow reading instructions, perform reading exercises.



Reading zones

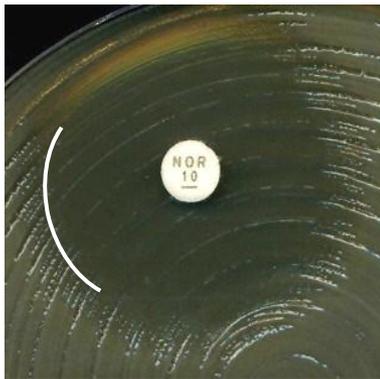
- Read **MH** plates from the back against a dark background illuminated with reflected light.
- Read **MH-F** plates from the front with the lid removed illuminated with reflected light.



Fuzzy zone edges

Enterobacteriaceae

- 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.



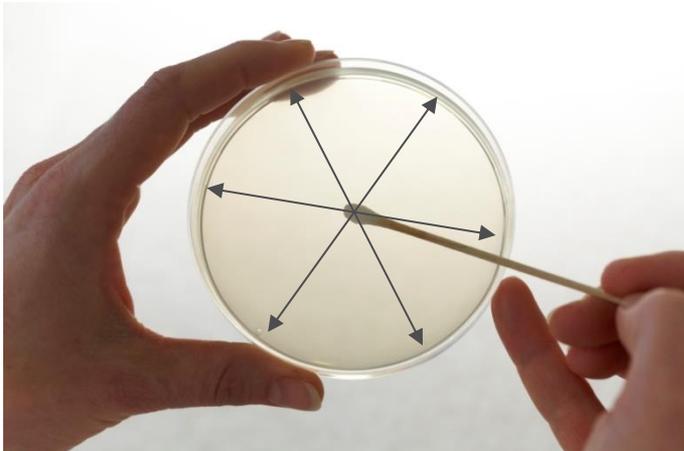
Reading of zones with fuzzy zone edges for Enterobacteriaceae.

Example 5

- **Problem:** Zone diameters for multiple agents below QC ranges for *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922.
 - Correct agar depth?
 - Yes!
 - Agar plates correctly inoculated?
 - No, growth too heavy due to over-inoculation!
- **Solution:** Make sure that plates are correctly inoculated.

Inoculation of agar plates

- Spread the inoculum evenly over the entire agar surface.
- **Gram-negative bacteria:** remove excess fluid by pressing and turning the swab against the inside of the tube to avoid over-inoculation.
- **Gram-positive bacteria:** do not press or turn the swab against the inside of the tube. Take particular care to ensure that there are no gaps between streaks.



Example 6

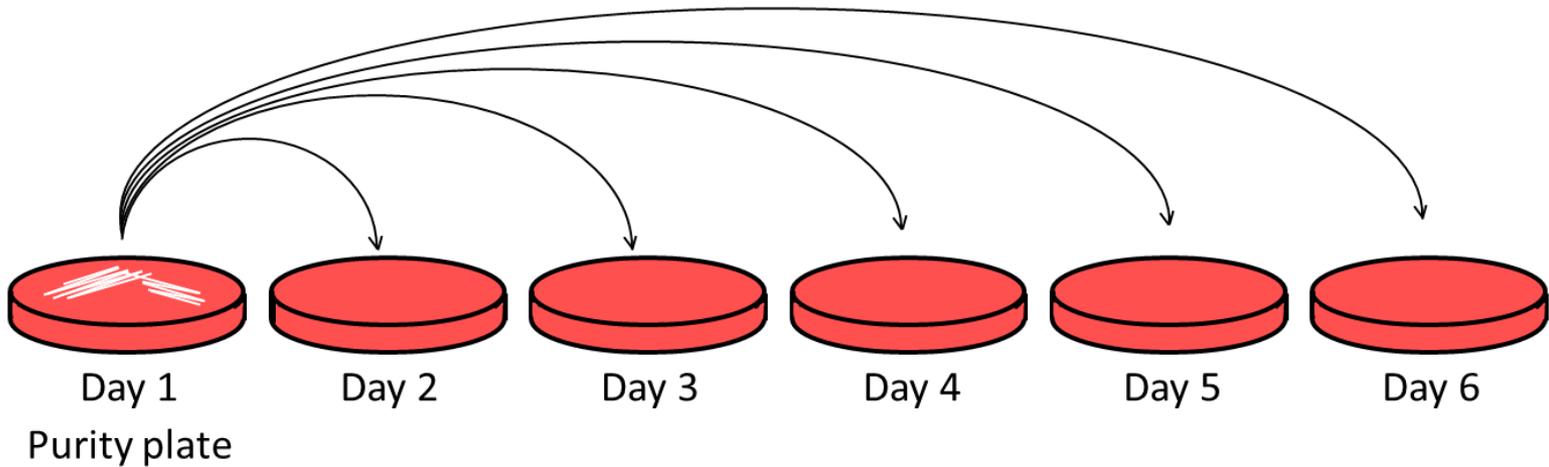
- **Problem:** Colonies within zones for *H. influenzae* ATCC 49766 and several agents.
 - Agar plates correctly inoculated?
 - Yes!
 - MH-F plates dry?
 - Yes!
 - 15-15-15 minute rule adhered to?
 - Yes!
 - Correct incubation time and temperature?
 - 35°C, but 20-22 hours incubation!
- **Solution:** Follow recommendations and incubate for 16-20 hours.

Example 7

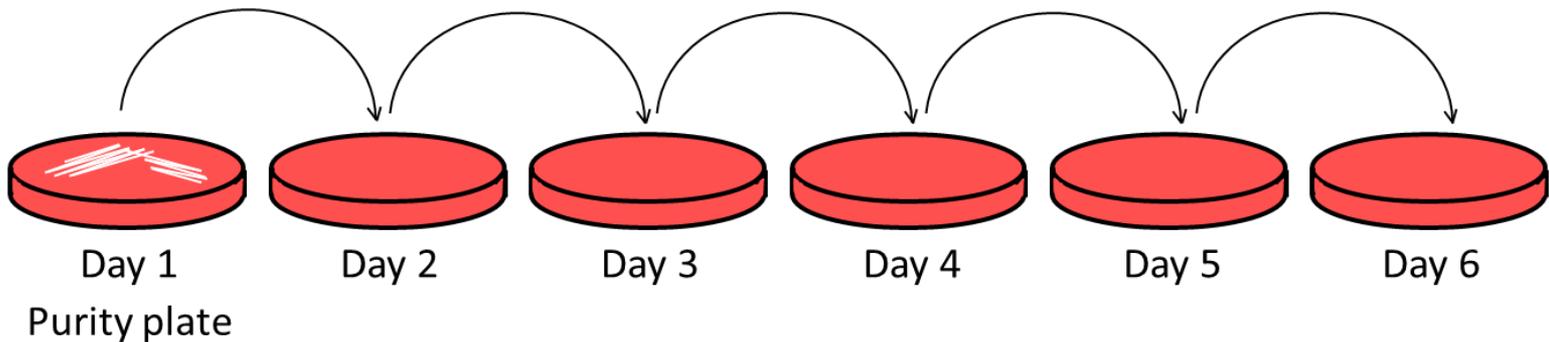
- **Problem:** Piperacillin-tazobactam zone diameters above QC range for *K. pneumoniae* ATCC 700603.
 - Pip-tazo zones within range for *E. coli* ATCC 25922?
 - Yes, piperacillin content ok!
 - Pip-tazo zones within range for *E. coli* ATCC 35218?
 - Yes, tazobactam content ok!
 - QC strain lost resistance?
- **Solution:** Prepare a new subculture of the QC strain from frozen stock.

Handling of QC strains

Non-fastidious QC strains



Fastidious QC strains



Example 8

- **Problem:** Discrepant SIR categorisation between disk diffusion and gradient test for clinical isolate of *P. aeruginosa* and meropenem.
 - QC for meropenem ok?
 - Disk diffusion: Yes, mean value on target ± 1 mm.
 - Gradient test: Yes, within range and on target.
- **Solution:** Use disk diffusion for SIR categorisation, perform BMD (or send to EDL) if an MIC is needed.

Gradient tests vs. BMD

Example *P. aeruginosa* and carbapenems

Gradient tests
may overestimate
MICs!

Meropenem

Isolate No	BMD MIC	Etest MIC
1	8	>32
2	8	>32
3	8	>32
4	8	>32
5	8	>32
6	8	>32
7	8	>32
8	8	>32
9	8	32
10	8	>32
11	8	>32
12	8	>32
13	4	>32
14	2	8
15	2	8
16	2	4
17	2	4
18	1	8
19	0,25	4

Meropenem QC

E. coli ATCC 25922

Range: 0.008-0.06 mg/L

Target: 0.016-0.032 mg/L

Results: 0.016-0.032 mg/L

QC on target!

Imipenem

Isolate No	BMD MIC	Etest MIC
1	8	>32
2	8	>32
3	8	>32
4	8	>32
5	4	>32
6	8	>32
7	2	4
8	2	4
9	0,25	2
10	0,5	4
11	1	1

Imipenem QC

E. coli ATCC 25922

Range: 0.06-0.25 mg/L

Target: 0.125 mg/L

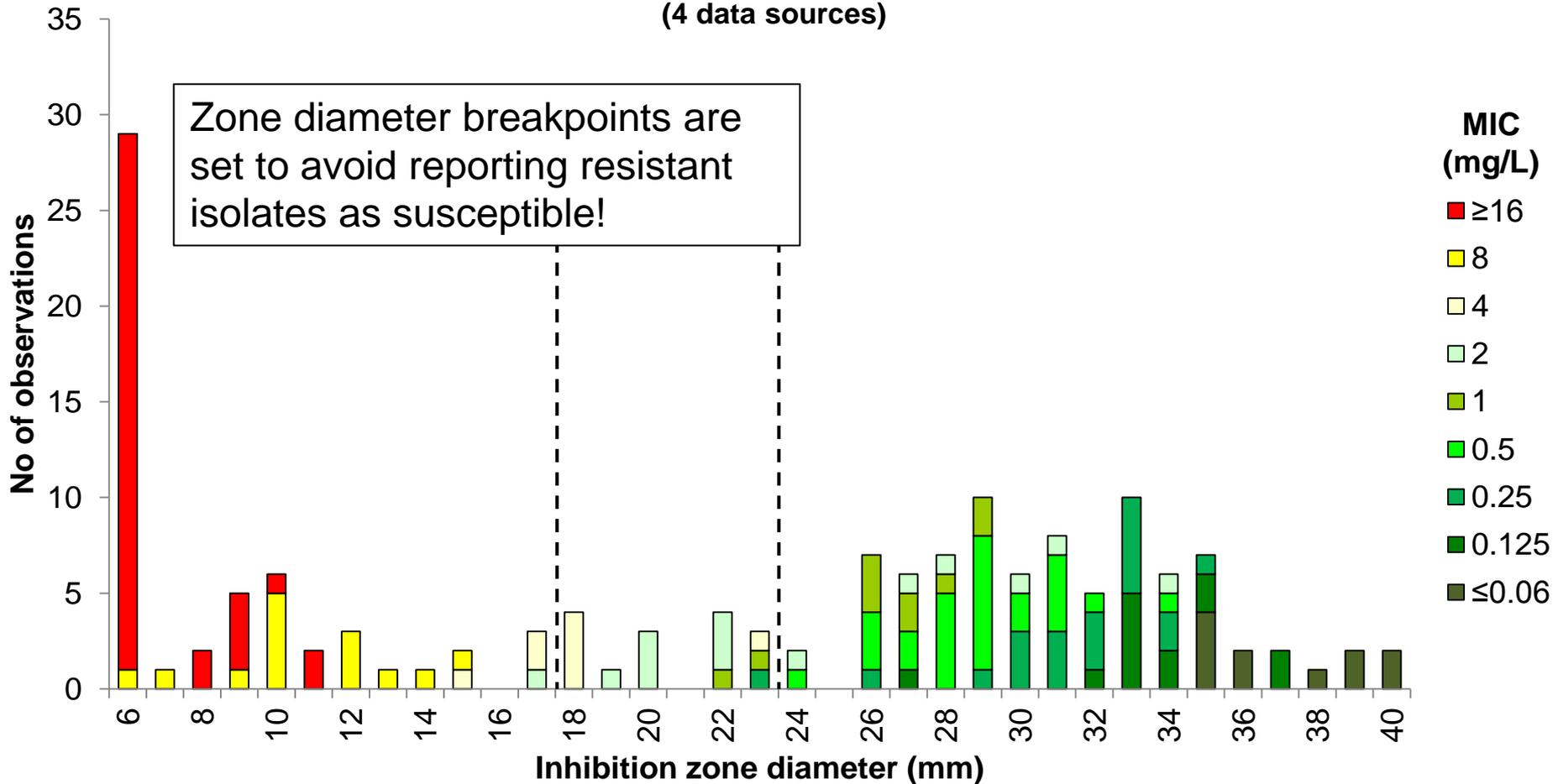
Results: 0.25-0.5 mg/L

Upper limit /above range!

Meropenem 10 µg vs. MIC

Pseudomonas aeruginosa, 153 isolates

(4 data sources)

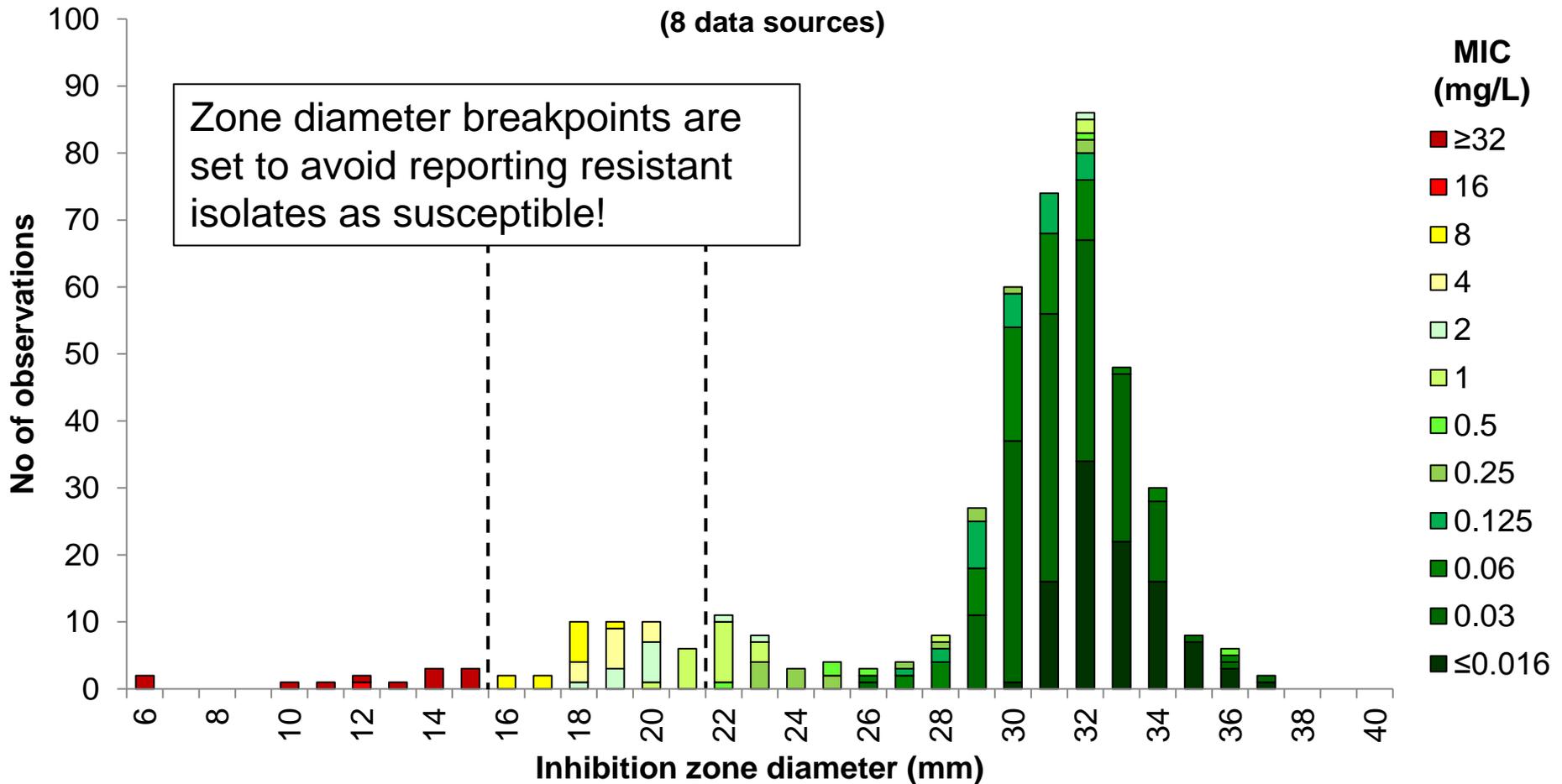


Breakpoints		ECOFF
MIC	S ≤ 2, R > 8 mg/L	2 mg/L
Zone diameter	S ≥ 24, R < 18 mm	

Meropenem 10 µg vs. MIC

Enterobacteriaceae, 378 isolates (435 correlates)

(8 data sources)



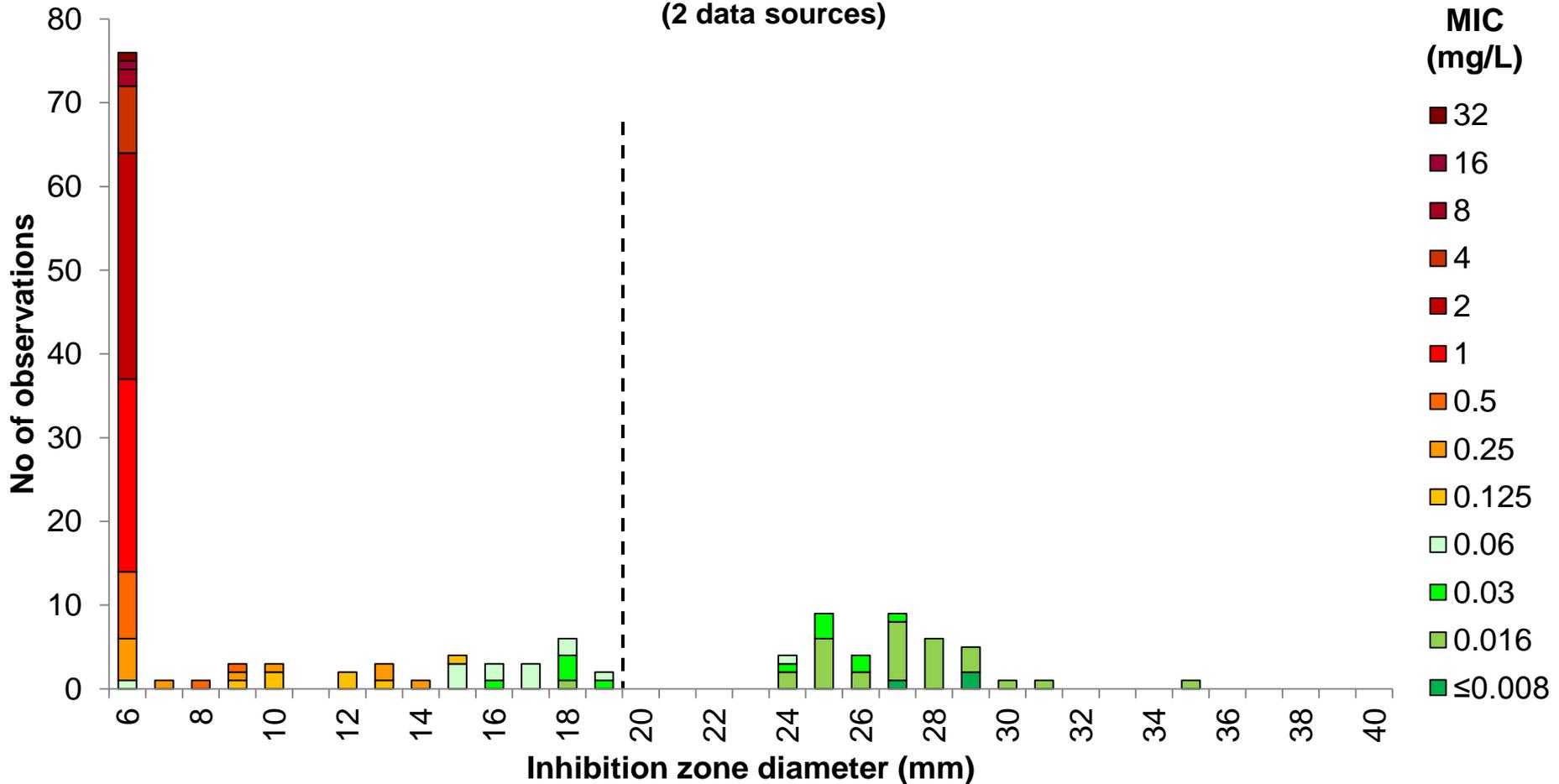
Breakpoints	
MIC	S ≤ 2, R > 8 mg/L
Zone diameter	S ≥ 22, R < 16 mm

Example 9

- **Problem:** Clinical isolate of *S. pneumoniae* with oxacillin zone 12 mm and PCG gradient test MIC 0.032 mg/L.
 - QC data ok?
 - Oxacillin disk diffusion: Yes, the mean value is on target ± 1 mm.
 - PCG gradient test: Within range, but in the lower part.
- **Solution:** Perform BMD (or send to EDL)!

Oxacillin 1 µg vs. Benzylpenicillin MIC *S. pneumoniae*, 148 isolates

(2 data sources)



Breakpoints

Benzylpenicillin (meningitis) MIC
Oxacillin zone diameter (screen)

$S \leq 0.06$, $R > 0.06$ mg/L
 $S \geq 20$ mm

ECOFF

0.06 mg/L

Evaluation of gradient tests for *S. pneumoniae* and β -lactam agents

	No of tests	Within ± 1 dilution (%)
Etest on BBL MH-F	162	88
Etest on Oxoid MH-F	162	79
MTS on BBL MH-F	162	97
MTS on Oxoid MH-F	162	93
M.I.C.E. on BBL MH-F	133	99
M.I.C.E. on Oxoid MH-F	133	95

Conclusion: Gradient tests systematically underestimates β -lactam MICs for *S. pneumoniae*.

AST of bacteria

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Expert rules and intrinsic resistance

Resistance mechanisms

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Zone distributions and ECOFFs

AST of bacteria

Media preparation

MIC determination

Disk diffusion methodology

Disk diffusion implementation

Compliance of manufacturers

Breakpoint tables

QC Tables



... Disk diffusion methodology

EUCAST Disk Diffusion Test Methodology

EUCAST has developed a disk diffusion test based on MH media and calibrated to EUCAST clinical breakpoints. Updates are published regularly.

[EUCAST Disk Diffusion - Manual v 6.0](#) (9 January, 2017)

[EUCAST Disk Diffusion - Slide Show](#) (9 January, 2017)

[EUCAST Disk Diffusion - Reading Guide](#) (9 January, 2017)

For translations to other languages - see → [Translations](#).

For previous versions of documents - see → [Previous versions](#).



Potential sources of error (1)

Medium	Storage of plates
	Not prepared according to instructions
	Batch to batch variation or change of supplier of agar
	Supplements (batch to batch variations, incorrect amount or expired)
	pH
	Agar depth/Agar volume
	Expiry date
Test conditions	“15-15-15”-rule not adhered to (suspension used within 15 min, disks applied within 15 min, incubation within 15 min)
	Incubation (temperature, atmosphere and time)
	Incorrect inoculation (too light, too heavy or uneven)
	Reading conditions (background, light)
	Reading zone edges

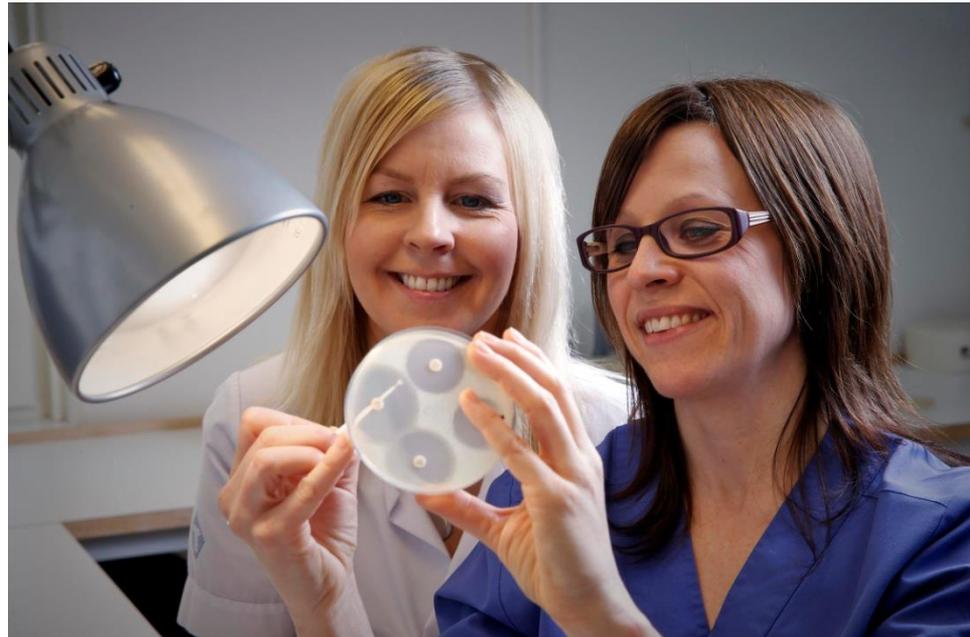
Potential sources of error (2)

Disks	Incorrect disk (wrong agent or wrong disk strength)
	Disk potency (incorrect storage, labile agent, expiry date)
	Disks not at room temperature when containers opened
	Too many disks on plate (interference between agents)
Control organisms	Incorrect QC strain
	Mutation
	Contamination
	Age of culture

Questions and/or problem?

Contact us!

erika.matuschek@escmid.org





EUCAST

EUROPEAN COMMITTEE
ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Frequently Asked Questions (FAQ)

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Frequently Asked Questions (FAQ)

The EUCAST secretariat receives many questions on subjects ranging from how we determine breakpoints, the MIC-distribution website, and the new 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 2016-03-29

Previous version of FAQ:

 [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

Sub-headings in the FAQ document

- EUCAST Disk Diffusion Test
 - Medium
 - Disks
 - Inoculum preparation
 - Reading zones of inhibition
 - General methodology
- Breakpoints –general
- Breakpoints –zone diameter
- Quality Control
- Other questions



Questions? Please contact
erika.matuschek@escmid.org

EUCAST Frequently Asked Questions

Click on question
to read answer



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 \$\beta\$ -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 \pm\$ 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?](#)



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1 Which manufacturer of Mueller-Hinton agar does EUCAST recommend?

EUCAST does not recommend a particular manufacturer of Mueller-Hinton agar. We have tested batches of Mueller-Hinton agar from four manufacturers (BBL, Oxoid, bioMérieux and Bio-Rad) repeatedly and have evaluated other media occasionally. We have also tested batches of pre-poured commercial MH-F (Mueller-Hinton Fastidious organisms; which is Mueller-Hinton agar with 5% mechanically defibrinated horse blood and 20 mg/L β -NAD) from the manufacturers mentioned above. Irrespective of the manufacturer used, each user should ensure that batches of media meet the internal quality control ranges published by EUCAST. These ranges have been checked with media from several manufacturers. For users of pre-poured commercial plates, it should be noted that the plate manufacturer not necessarily is the same as the Mueller-Hinton powder manufacturer.



2 What is the difference between Mueller-Hinton agar and Mueller-Hinton II agar?

The original specification of Mueller-Hinton agar did not define cation content, which is known to affect the activity of several agents, particularly aminoglycosides. Furthermore, the content of thymidine, which affects trimethoprim and trimethoprim-sulfamethoxazole activity, was undefined. Mueller-Hinton II agar is manufactured to contain a low concentration of thymidine and controlled concentrations of calcium and magnesium ions. Today, all Mueller-Hinton agars for susceptibility testing should be produced to meet the current CLSI performance standard (soon to be superseded by an ISO standard). Therefore, all Mueller-Hinton agars that yield inhibition zones within the acceptable ranges for EUCAST internal quality control strains can be used and EUCAST does not distinguish between MH and MH II.

Warnings on www.eucast.org

- When AST products (disks, media, gradient tests etc.) do not perform to the expected standard
 - EDL informs the manufacturer and publish a warning on www.eucast.org
- EDL does not systematically test all products
 - The lack of a warning does not imply that there is no problem with the product in question
- Contact the EDL for advice if you suspect that problems with AST is related to a particular product!

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... Warnings!

www.eucast.org

EUCAST warnings concerning antimicrobial susceptibility testing products or procedures.

The EUCAST disk diffusion development laboratories, a network of laboratories coordinated from the EUCAST development laboratory in Växjö, Sweden, from time to time discover products (disks, media batches, gradient tests or procedures) which are not performing to the expected standard. When this is the case we inform the manufacturer and publish a warning on this page.

We do not systematically test all products so the lack of a warning does not imply that there is no problem with the product in question.

Laboratories which experience problems with a susceptibility test method, and suspect that this may be related to a particular product, may contact EUCAST for advice.

1. **Problems with piperacillin-tazobactam gradient tests from two manufacturers** - the warning issued 2015 will be removed when problem is resolved.
2. **Wide variation in disk quality in 16 disks from nine manufacturers** - the warning issued in 2015 and reiterated 2016 will be removed when issue is resolved.
3. **Problems with colistin gradient tests from both bioMérieux and Liofilchem** - the warning issued July 2016 will be removed when issue is resolved.

1. Problems with piperacillin-tazobactam gradient tests from two manufacturers.

This warning, issued 2015, will be removed when problem is solved.

Following reports of problems related to piperacillin-tazobactam gradient tests, several batches of tests from bioMérieux and Liofilchem (from both single packs and multipacks) were evaluated at the EUCAST Development Laboratory, Växjö, Sweden (January 2016). Enterobacteriaceae and Pseudomonas aeruginosa (type strains and strains with resistance mechanisms) and broth micro dilution were used.

Gradient tests from both manufacturers gave variable and unreliable results. The difference between gradient tests and broth micro dilution was not systematic, some values were two dilutions too high, others too low. There was also considerable variation between batches from the same manufacturer.

We urge manufacturers to seriously consider these problems and users to introduce internal quality control of each procedure. We have detected no reason