



EUCAST

EUROPEAN COMMITTEE
ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

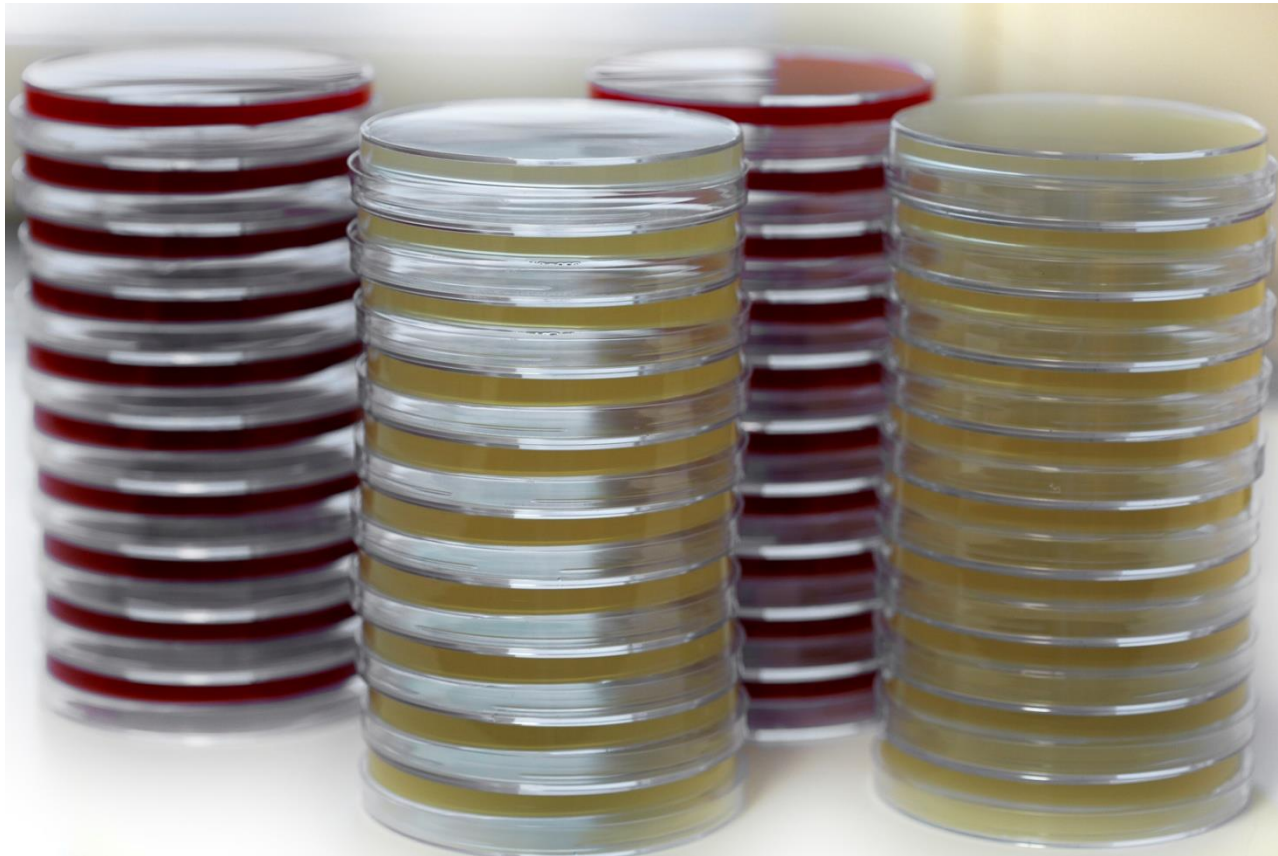
EUCAST disk diffusion method for antimicrobial susceptibility testing

Version 11.0
January 2023

Changes from previous version (v 10.0)

Slide	Change
39	Subculturing scheme for QC strains added.

Susceptibility testing media



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 $\geq 98\%$.

Media for non-fastidious organisms

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

Media for fastidious organisms

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

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.

Quality control of Mueller-Hinton agar

Test each new batch of MH agar to ensure that all zones are within EUCAST QC ranges.

Particular problems:

- High or low concentrations of divalent cations (Ca^{2+} , Mg^{2+}) may be indicated by inhibition zones for aminoglycosides with *P. aeruginosa* ATCC 27853 below/above quality control limits, respectively.
- Excess thymine and thymidine may be indicated by inhibition zones for trimethoprim-sulfamethoxazole and *E. faecalis* ATCC 29212 below quality control limits.

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.

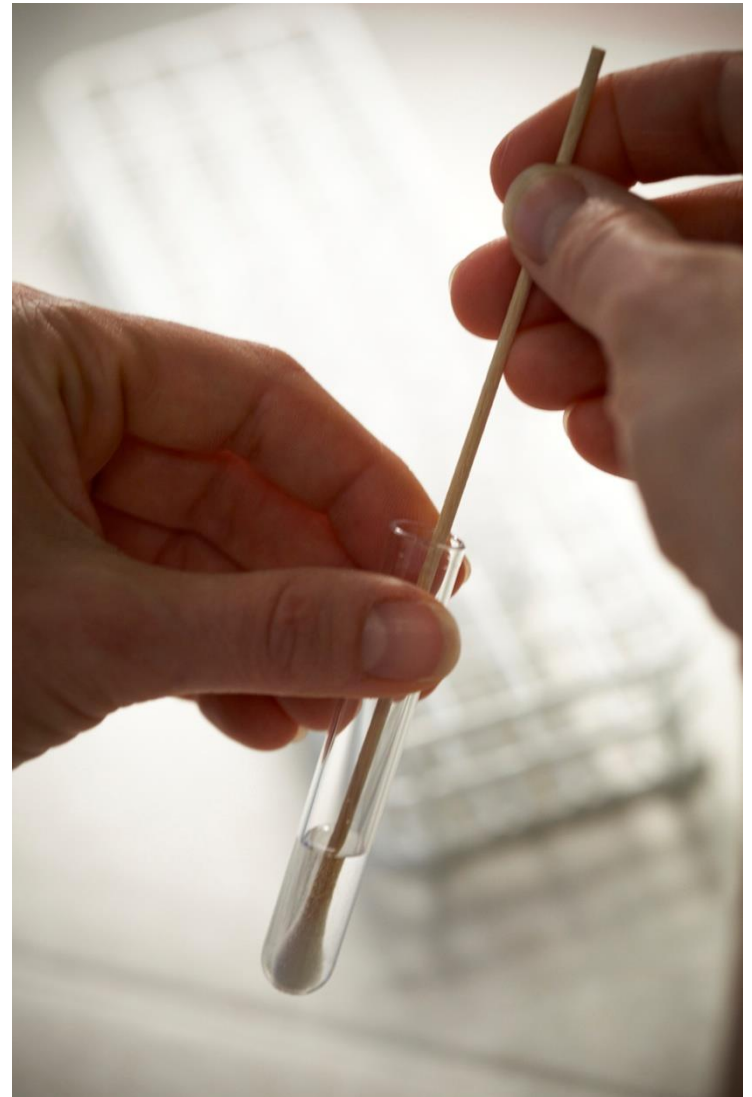
Drying and storage of agar plates

- 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.

Inoculum

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

* Approximately corresponding to $1-2 \times 10^8$ CFU/mL for *E. coli*.



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



Inoculum preparation

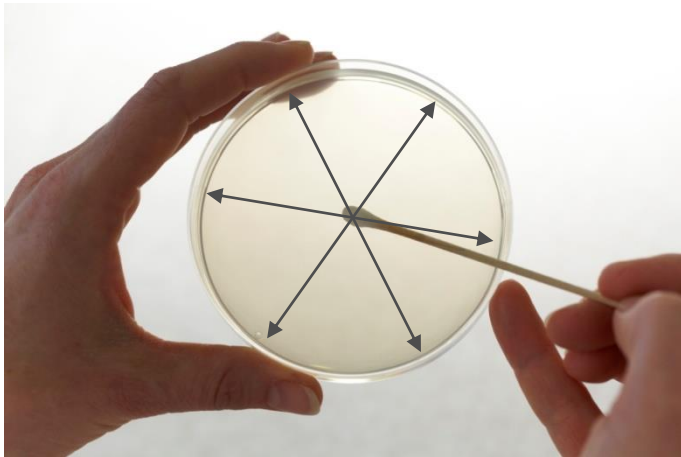
- Use a sterile loop or cotton swab to pick colonies from an overnight culture on non-selective media. If possible, use several morphologically similar colonies to avoid selecting an atypical variant.
- Suspend in saline and mix to an even turbidity.
- Adjust the density of the suspension to 0.5 McFarland 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.

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 $35\pm 1^{\circ}\text{C}$ in air.
- Incubate MH-F plates at $35\pm 1^{\circ}\text{C}$ in air with 4-6% CO_2 (except for *Campylobacter*).

Incubation of plates

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

Incubation of plates

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

The 15-15-15 minute rule

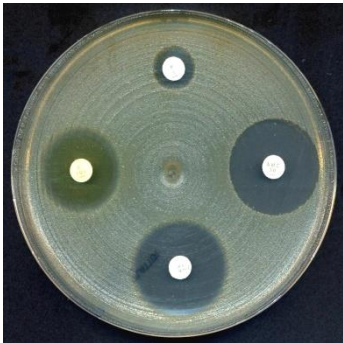
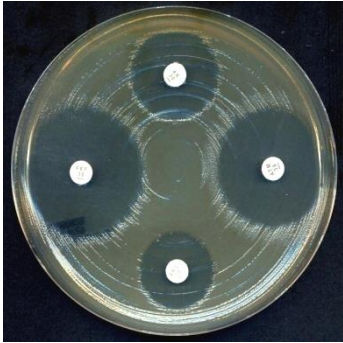
Follow these instructions for disk diffusion:

- 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!

Reading zones

- 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.

Examples:



E. coli
Ciprofloxacin



S. aureus
Erythromycin



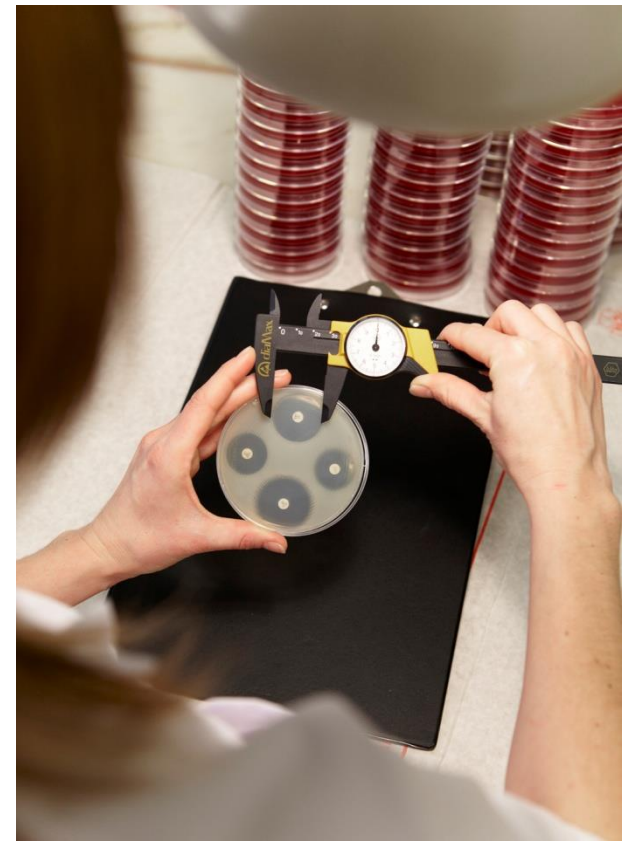
CoNS
Trimethoprim



S. pneumoniae
Rifampicin

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.



Reading zones

- Do not use transmitted light (plate held up to light) or a magnifying glass, unless otherwise stated.
- 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.
- In case of double zones, or 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 zones – exceptions (1)

Organism	Antimicrobial agent	Reading inhibition zones
<i>Enterobacterales</i>	Ampicillin Ampicillin-sulbactam Amoxicillin-clavulanic acid	Ignore fine growth that may appear as an inner zone on some batches of MH agar.
<i>Enterobacterales</i>	Temocillin	Ignore isolated colonies within the inhibition zone.
<i>Enterobacterales</i>	Mecillinam	Ignore isolated colonies within the inhibition zone.
<i>E. coli</i>	Fosfomycin	Ignore isolated colonies within the inhibition zone and read the outer zone edge.
<i>Proteus</i> spp.	Any	Ignore swarming.
<i>S. maltophilia</i> , <i>A. xylosoxidans</i> and <i>B. pseudomallei</i>	Trimethoprim-sulfamethoxazole	Ignore growth within the zone if any zone edge can be seen, even when growth within the zone is substantial.
<i>S. aureus</i>	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.
<i>Enterococcus</i> spp.	Vancomycin	Examine zone edge from the front of the plate with transmitted light (plate held up to light).
<i>Streptococcus</i> spp.	Any	Read inhibition of growth and not the inhibition of haemolysis.
<i>H. influenzae</i>	Beta-lactam agents	Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk.
<i>Aeromonas</i> 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.

Interpreting zones

- Check that zone diameters for quality control strains are within acceptable ranges before interpreting tests.
- Interpret zone diameters into susceptibility categories (S, I and R) according to the current EUCAST Breakpoint Tables (www.eucast.org). Alternatively, a template with EUCAST breakpoints may be used.

Control of susceptibility testing

- Use the recommended routine quality control strains to monitor test performance (see [EUCAST QC Tables](#)).
- For β -lactam-inhibitor combination disks, specific β -lactamase-producing strains are recommended to control the inhibitor component. This should be part of the routine QC. The active component is checked with a susceptible QC strains.
- Quality control strains with defined resistance mechanisms may be used to confirm the ability to detect resistance (Extended QC, see [EUCAST QC Tables](#)).
- Quality control strains may be purchased from culture collections or from commercial sources.

EUCAST routine quality control strains

Organism	Culture collection numbers	Characteristics
<i>E. coli</i>	ATCC 25922; NCTC 12241; CIP 76.24 DSM 1103; CCUG 17620; CECT 434	Susceptible, wild-type
<i>E. coli</i>	ATCC 35218; NCTC 11954; CIP 102181 DSM 5923; CCUG 30600; CECT 943	TEM-1 β -lactamase producer
<i>K. pneumoniae</i>	ATCC 700603; NCTC 13368 CCUG 45421; CECT 7787	ESBL producer (SHV-18)
<i>K. pneumoniae</i>	ATCC BAA-2814	KPC-3, SHV-11 and TEM-1
<i>P. aeruginosa</i>	ATCC 27853; NCTC 12903; CIP 76.110 DSM 1117; CCUG 17619; CECT 108	Susceptible, wild-type
<i>S. aureus</i>	ATCC 29213; NCTC 12973; CIP 103429 DSM 2569; CCUG 15915; CECT 794	Weak β -lactamase producer
<i>E. faecalis</i>	ATCC 29212; NCTC 12697; CIP 103214 DSM 2570; CCUG 9997; CECT 795	Susceptible, wild-type

EUCAST routine quality control strains

Organism	Culture collection numbers	Characteristics
<i>S. pneumoniae</i>	ATCC 49619; NCTC 12977 CIP 104340; DSM 11967 CCUG 33638	Reduced susceptibility to benzylpenicillin
<i>H. influenzae</i>	ATCC 49766; NCTC 12975 CIP 103570; DSM 11970 CCUG 29539	Susceptible, wild-type
<i>Campylobacter jejuni</i>	ATCC 33560; NCTC 11351 CIP 70.2T; DSM 4688 CCUG 11284	Susceptible, wild-type

EUCAST strains for detection of specific resistance mechanisms (extended QC)

Organism	Culture collection numbers	Characteristics
<i>K. pneumoniae</i>	ATCC 700603; NCTC 13368 CCUG 45421; CECT 7787	ESBL producer (SHV-18)
<i>S. aureus</i>	NCTC 12493; CCUG 67181	<i>mecA</i> positive, methicillin resistant (MRSA)
<i>E. faecalis</i>	ATCC 51299; NCTC 13379 CIP 104676; DSM 12956 CCUG 34289	High-level aminoglycoside resistant (HLAR) and vancomycin resistant (<i>vanB</i> positive)
<i>H. influenzae</i>	ATCC 49247; NCTC 12699 CIP 104604; DSM 9999 CCUG 26214	Reduced susceptibility to β -lactam agents due to PBP mutations

Culture Collections

ATCC American Type Culture Collection, USA

<http://www.atcc.org>

NCTC National Collection of Type Cultures, Public Health England, UK

<https://www.phe-culturecollections.org.uk/collections/nctc>

CIP Collection de l'Institut Pasteur, France

<https://www.pasteur.fr/en/public-health/biobanks-and-collections/collection-institut-pasteur-cip>

DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)

<https://www.dsmz.de>

CCUG Culture Collection University of Gothenburg, Sweden

<http://www.ccug.se>

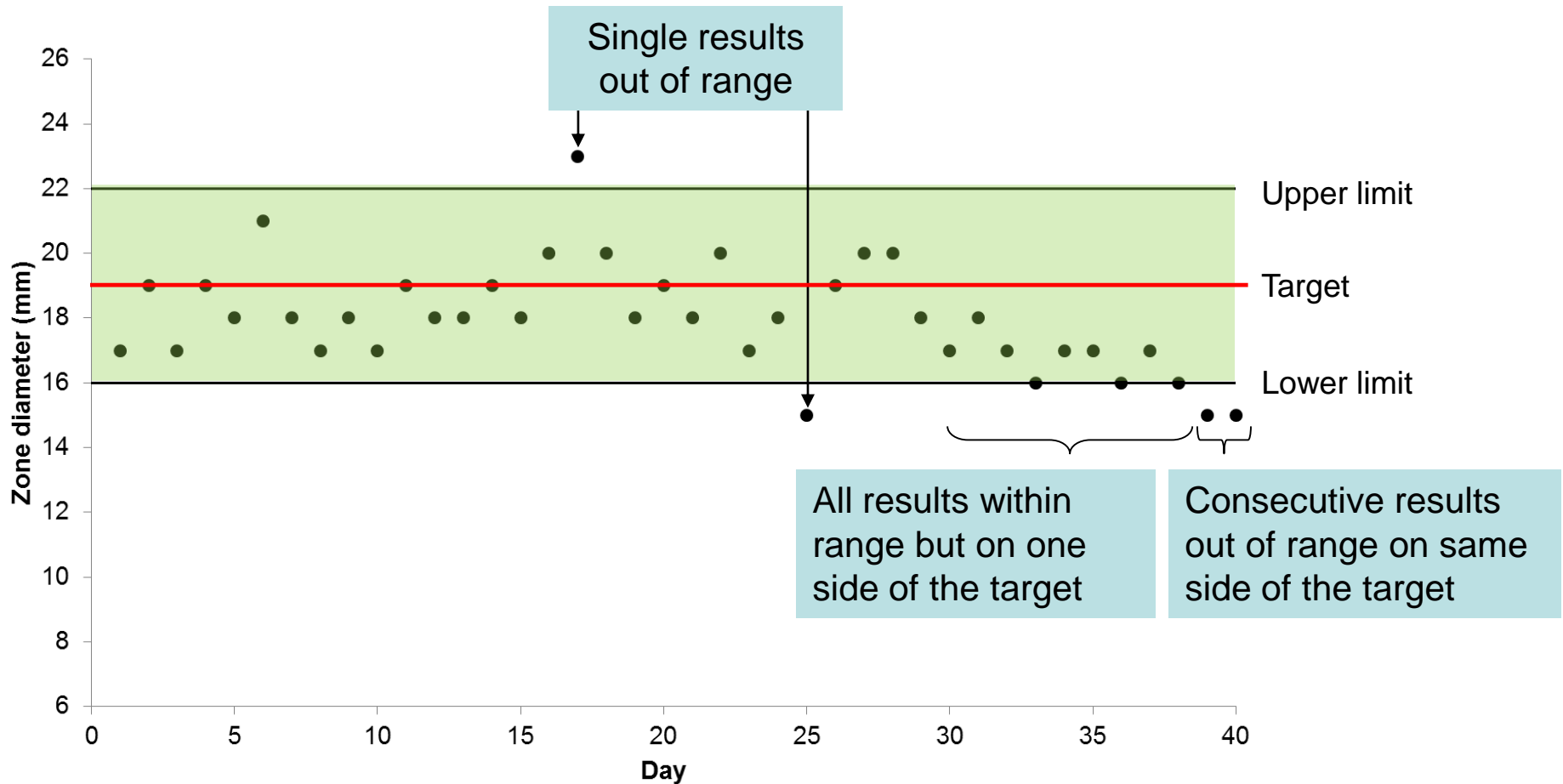
CECT Colección Española de Cultivos Tipo, Spain

<http://www.cect.org>

Use routine quality control strains to assess general performance

- Control tests should be set up and checked daily, or at least four times per week, for antibiotics which are part of routine panels.
- Control tests should always be read and evaluated before reporting results for clinical isolates.
- Each day that tests are set up, examine the results of the last 20 consecutive tests.
- Examine results for trends and for zones falling consistently above or below the target.
- If two or more of 20 tests are out of range investigation is required.

Monitoring test performance



Response to QC results out of range

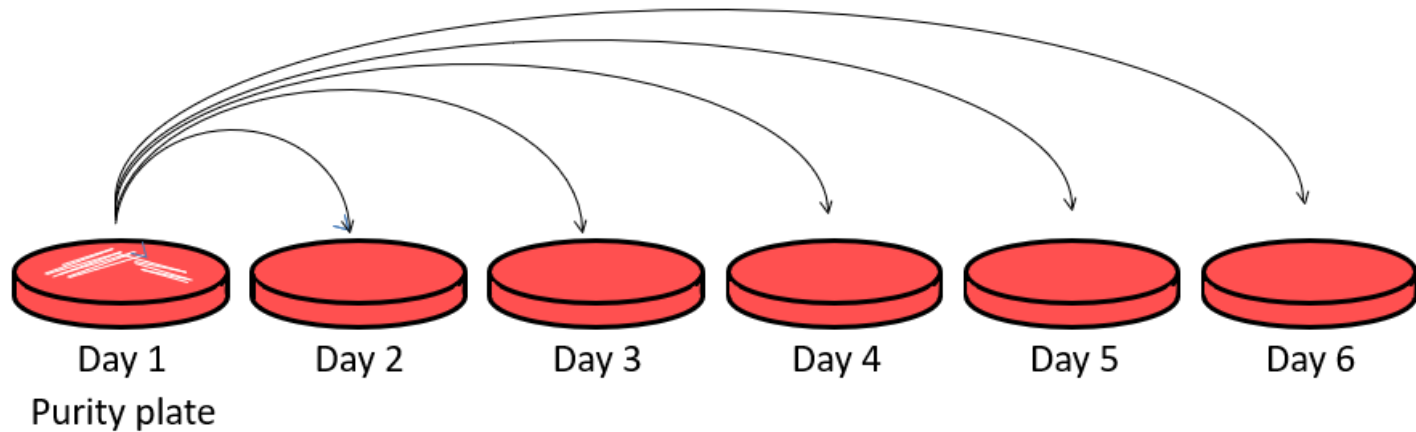
- If two non-consecutive control zone diameters of 20 tests are outside the acceptable range – then report susceptibility test results and investigate.
- If two consecutive control zone diameters of 20 tests are outside the acceptable range – then investigate before reporting susceptibility test results. The tests may have to be repeated.
- If multiple disks (>2) are out of range on one day – then investigate before reporting susceptibility test results. The tests may have to be repeated.
- If resistance in a resistant control strain is not recognised – then suppress susceptibility test results, investigate and retest.

Storage and subculture of control strains

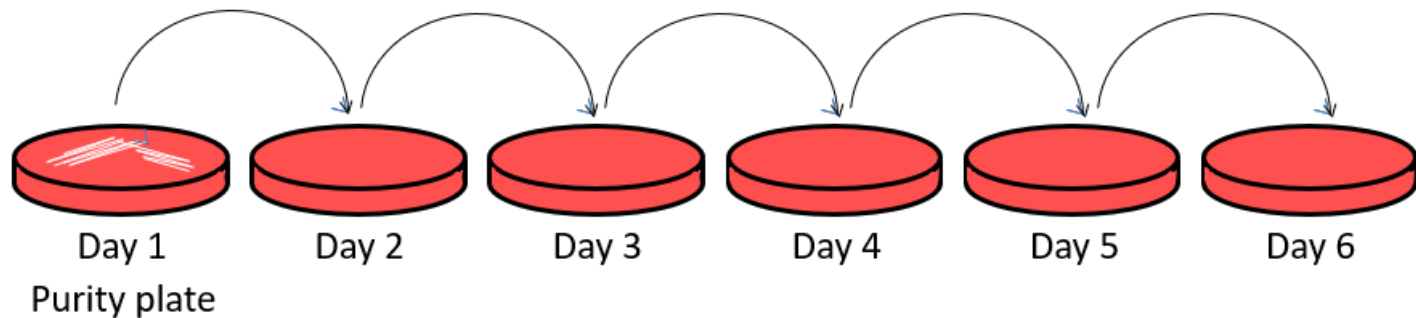
- Store control strains on beads at -70°C in glycerol broth (or commercial equivalent). Store two vials of each strain, one for in-use and one as an archive.
- Each week, subculture from the in-use vial onto appropriate non-selective media and check for purity.
- Each day of the week, prepare a subculture from the purity plate. Use several colonies to avoid selecting a mutant. Fastidious organisms only may be subcultured serially from day to day.
- QC strains may be subcultured for a maximum of 6 days. Then, discard plates and prepare a new purity plate from the frozen in-use vial.
- When the in-use vial is depleted, subculture from the archive vial and prepare another in-use vial from the subculture.

Subculturing of QC strains

Non-fastidious QC strains



Fastidious QC strains



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 minute”-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

EUCAST website

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

www.eucast.org

- Please send any comments and suggestions to erika.matuschek@escmid.org or to the EUCAST secretariat (see website).



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