Antimicrobial Susceptibility Testing (AST)

By Disk Diffusion Method (Kirby-Bauer)

According to CLSI (2026)

What is an Antibiogram and why Disk Diffusion?

Definition

An antibiogram is a report of bacterial susceptibility or resistance to various antibiotics that helps clinicians select appropriate therapy.

Disk Diffusion Method

The standardised Kirby-Bauer method: paper disks containing a fixed amount of antibiotic are placed on agar seeded with the test bacterium; the zone of inhibition is measured in millimetres.

CLSI Standards

CLSI M02 defines the test procedure; CLSI M100-Ed36 (January 2026) provides the interpretive breakpoints and quality-control tables.

Required Materials and Equipment



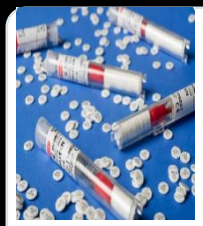
Mueller-Hinton Agar

Depth 4.0 ± 0.5 mm
PH : 7.2-7.4
For non-fastidious bacteria



0.5 McFarland Standard

Approx. 1–2 × 10^8 CFU/mL
For inoculum standardisation



Antibiotic Disks

CLSI-specified content
Store at correct temperature



Caliper or Ruler

Measure zone diameter
to the nearest millimetre



Incubator

35 ± 2 °C
Ambient air or CO_2



QC Reference Strains

E. coli ATCC 25922
and other reference strains

Turbidity Standard

- 0.5 McFarland standard (0.5 ml of 0.048 mol/ml BaCl2 added to 99.5 ml of 0.18 mol/L H2SO4(1% v/v) , density in 625 nm = 0.08 - 0.13
- Keep in 4-6 ml aliquots in screw cap tubes in dark, room temperature
- Verify density monthly



Step-by-Step Procedure

- **Prepare Inoculum** (Pure colonies + 0.5 McFarland standard)
- **Inoculate Plate** (Swab agar surface in three 60° directions)
- **Apply Disks** (Minimum 24 mm centre-to-centre spacing)
- **Incubate** (16–18 h at 35 ± 2 °C)
- **Measure Zones** (Full diameter of inhibition zone in mm)
- **Interpret Results** (Compare with CLSI M100-2026 tables)

Inoculum Preparation and Standardisation

1. **Select Colonies**
   Pick 3–5 similar, pure colonies from an 18–24 h non-selective culture.

2. **Prepare Suspension**
   Suspend the colonies in sterile saline or appropriate broth.

3. **Adjust Turbidity**
   Match the suspension to a 0.5 McFarland standard (acceptable range 0.4–0.6).

4. **Time Limit**
   Use the inoculum within 15 minutes of preparation.

Plate Inoculation and Disk Application

Inoculating the Agar Surface

- Dip a sterile swab into the suspension and remove excess fluid.
- Streak the entire agar surface in three directions, rotating 60° each time.
- Also swab the rim of the plate.
- Allow 3–5 minutes for the surface to dry.
- Apply disks within 15 minutes of inoculation.

Applying the Disks

- Maximum 6 disks on a 100 mm plate
- Maximum 12 disks on a 150 mm plate
- Minimum 24 mm center-to-center spacing
- Gently press each disk to ensure full contact
- Do not move a disk once it has touched the agar

CLSI M02 & M100-Ed36 | Disk Diffusion Antibiogram | 5 / 12

Incubation of Plates

The 15-15-15 minute rule

….. Follow these instructions for disk diffusion:➢

- ❑ Use the inoculum suspension optimally whithin 15 minutes of preparation
- ❑ Apply disks within 15 minutes of inoculation
- ❑Incubate plates whithin 15 minutes of disk application

Incubation Conditions According to CLSI

Non-fastidious bacteria (Enterobacterales, Staphylococcus, Pseudomonas)	**35 ± 2 °C** **Ambient air** **16–18 hours**
Staphylococci (vancomycin and selected agents)	**35 ± 2 °C** **Ambient air** **24 hours**
Haemophilus and certain fastidious organisms	**35 ± 2 °C** **5 % CO_2** **16–18 hours**
Neisseria gonorrhoeae	**36 ± 1 °C** **5 % CO_2** **20–24 hours**

Culture Medium for Antimicrobial Susceptibility Testing (AST)

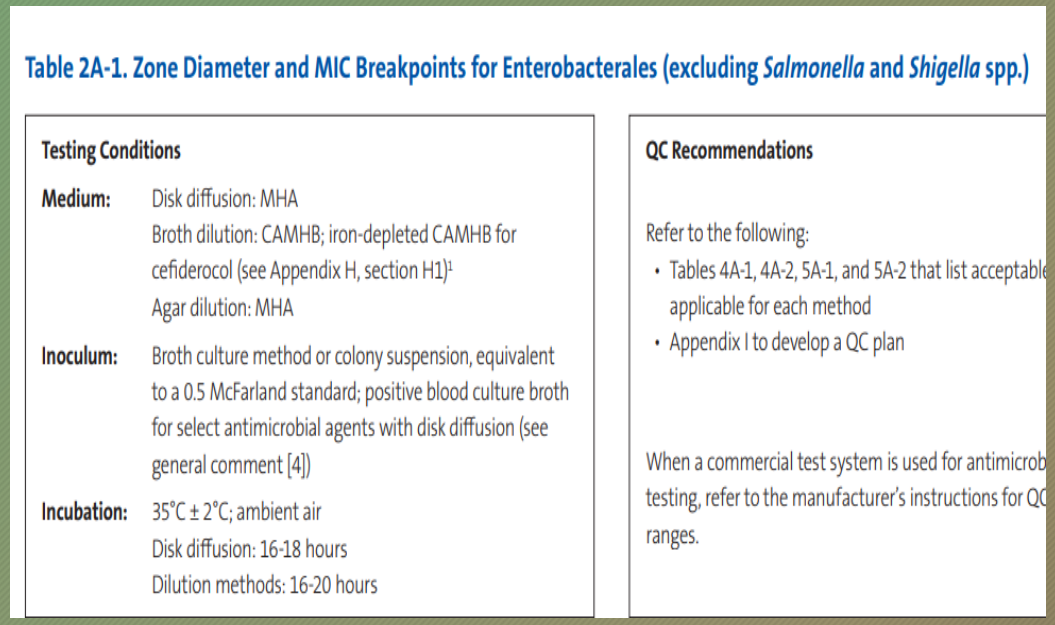


Table 2A-1. Zone Diameter and MIC Breakpoints for Enterobacterales (excluding *Salmonella* and *Shigella* spp.)

Testing Conditions	
Medium:	Disk diffusion: MHA Broth dilution: CAMHB; iron-depleted CAMHB for cefiderocol (see Appendix H, section H1)[1] Agar dilution: MHA
Inoculum:	Broth culture method or colony suspension, equivalent to a 0.5 McFarland standard; positive blood culture broth for select antimicrobial agents with disk diffusion (see general comment [4])
Incubation:	35°C ± 2°C; ambient air Disk diffusion: 16-18 hours Dilution methods: 16-20 hours

QC Recommendations

Refer to the following:

- Tables 4A-1, 4A-2, 5A-1, and 5A-2 that list acceptable applicable for each method
- Appendix I to develop a QC plan

When a commercial test system is used for antimicrob testing, refer to the manufacturer's instructions for QC ranges.

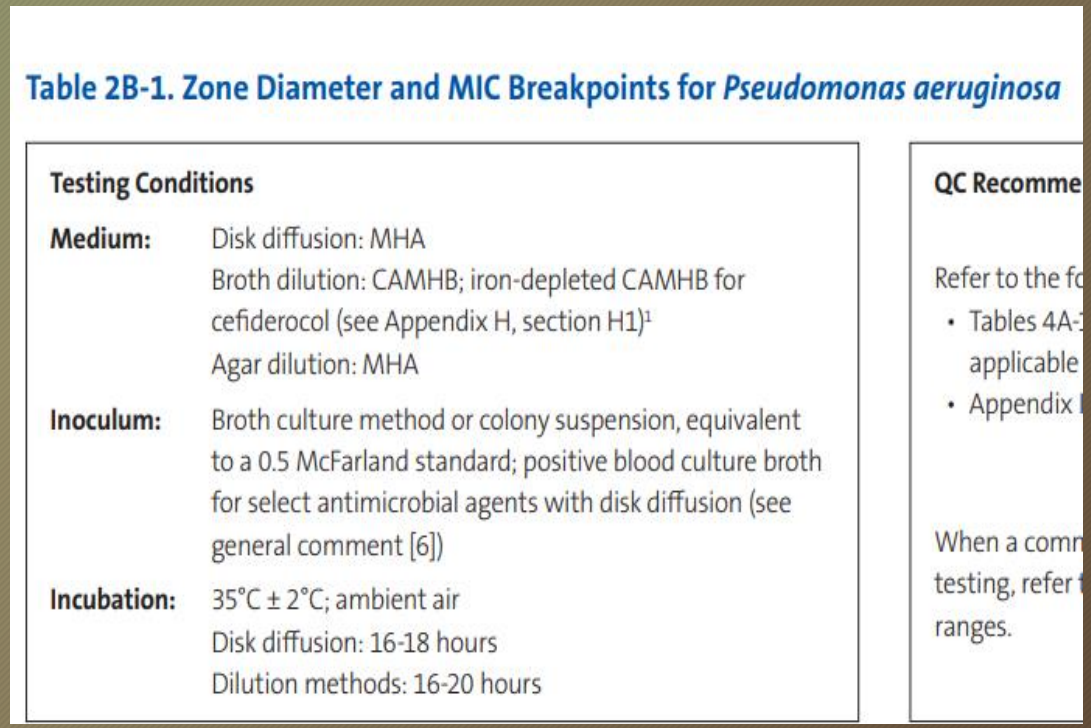


Table 2B-1. Zone Diameter and MIC Breakpoints for *Pseudomonas aeruginosa*

Testing Conditions	
Medium:	Disk diffusion: MHA Broth dilution: CAMHB; iron-depleted CAMHB for cefiderocol (see Appendix H, section H1)[1] Agar dilution: MHA
Inoculum:	Broth culture method or colony suspension, equivalent to a 0.5 McFarland standard; positive blood culture broth for select antimicrobial agents with disk diffusion (see general comment [6])
Incubation:	35°C ± 2°C; ambient air Disk diffusion: 16-18 hours Dilution methods: 16-20 hours

QC Recomme

Refer to the fo

- Tables 4A-
  applicable
- Appendix

When a comr
testing, refer
ranges.

Culture Medium for Antimicrobial Susceptibility Testing (AST)

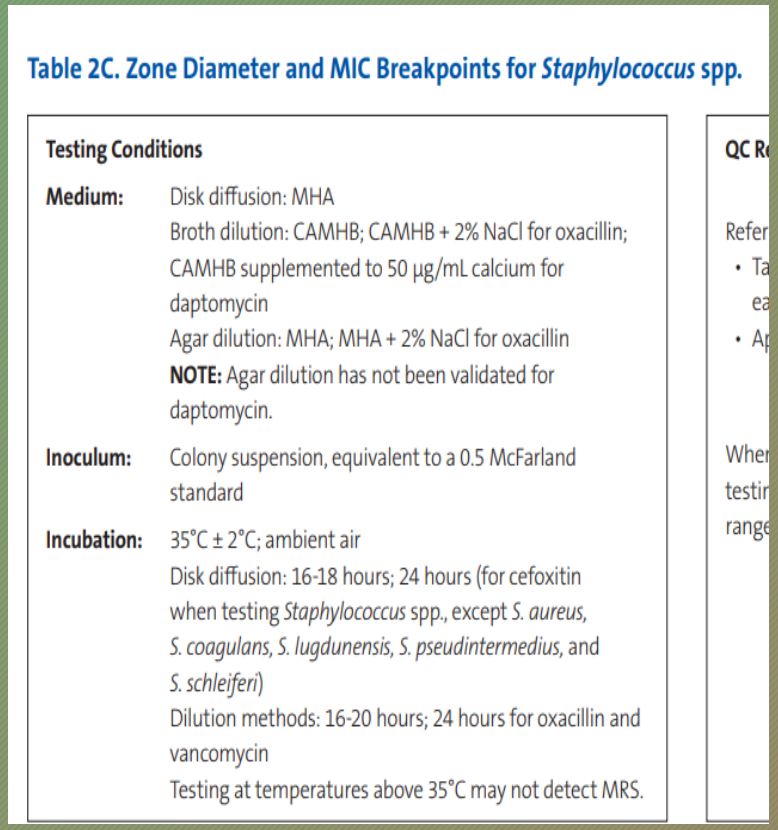


Table 2C. Zone Diameter and MIC Breakpoints for *Staphylococcus* spp.

Testing Conditions	
Medium:	Disk diffusion: MHA Broth dilution: CAMHB; CAMHB + 2% NaCl for oxacillin; CAMHB supplemented to 50 µg/mL calcium for daptomycin Agar dilution: MHA; MHA + 2% NaCl for oxacillin **NOTE:** Agar dilution has not been validated for daptomycin.
Inoculum:	Colony suspension, equivalent to a 0.5 McFarland standard
Incubation:	35°C ± 2°C; ambient air Disk diffusion: 16-18 hours; 24 hours (for cefoxitin when testing *Staphylococcus* spp., except *S. aureus, S. coagulans, S. lugdunensis, S. pseudintermedius,* and *S. schleiferi*) Dilution methods: 16-20 hours; 24 hours for oxacillin and vancomycin Testing at temperatures above 35°C may not detect MRS.

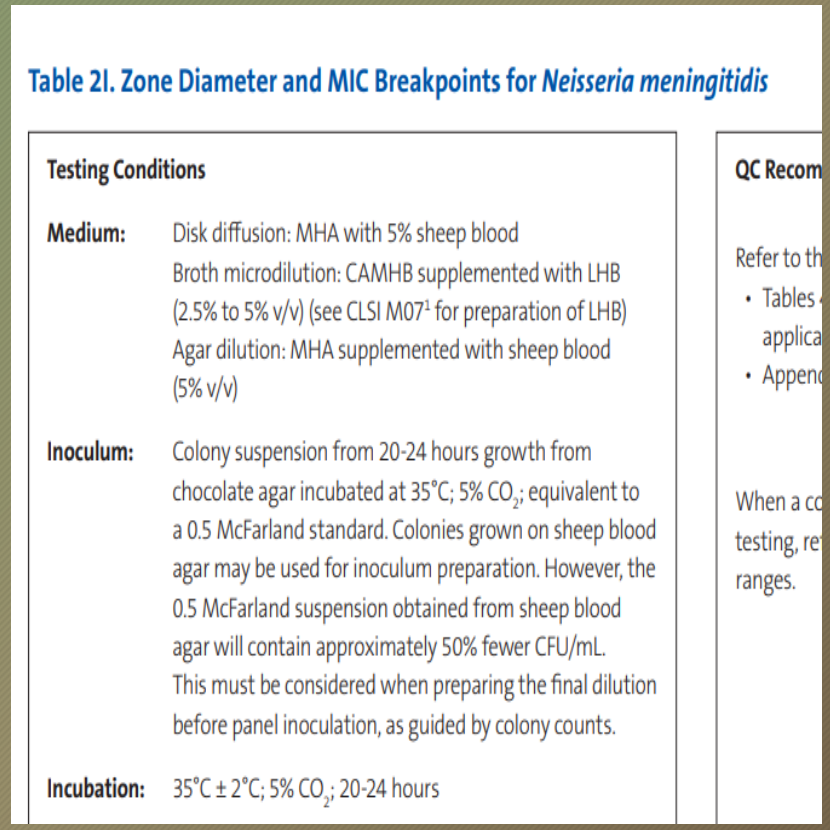


Table 2I. Zone Diameter and MIC Breakpoints for *Neisseria meningitidis*

Testing Conditions	
Medium:	Disk diffusion: MHA with 5% sheep blood Broth microdilution: CAMHB supplemented with LHB (2.5% to 5% v/v) (see CLSI M07[1] for preparation of LHB) Agar dilution: MHA supplemented with sheep blood (5% v/v)
Inoculum:	Colony suspension from 20-24 hours growth from chocolate agar incubated at 35°C; 5% CO_2; equivalent to a 0.5 McFarland standard. Colonies grown on sheep blood agar may be used for inoculum preparation. However, the 0.5 McFarland suspension obtained from sheep blood agar will contain approximately 50% fewer CFU/mL. This must be considered when preparing the final dilution before panel inoculation, as guided by colony counts.
Incubation:	35°C ± 2°C; 5% CO_2; 20-24 hours

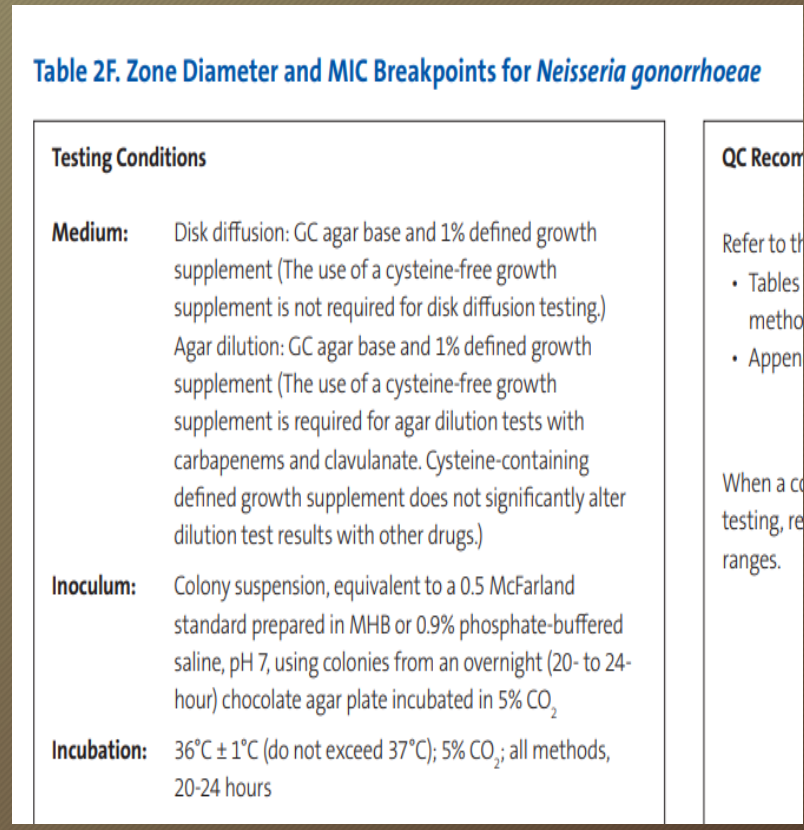


Table 2F. Zone Diameter and MIC Breakpoints for *Neisseria gonorrhoeae*

Testing Conditions	
Medium:	Disk diffusion: GC agar base and 1% defined growth supplement (The use of a cysteine-free growth supplement is not required for disk diffusion testing.) Agar dilution: GC agar base and 1% defined growth supplement (The use of a cysteine-free growth supplement is required for agar dilution tests with carbapenems and clavulanate. Cysteine-containing defined growth supplement does not significantly alter dilution test results with other drugs.)
Inoculum:	Colony suspension, equivalent to a 0.5 McFarland standard prepared in MHB or 0.9% phosphate-buffered saline, pH 7, using colonies from an overnight (20- to 24-hour) chocolate agar plate incubated in 5% CO_2
Incubation:	36°C ± 1°C (do not exceed 37°C); 5% CO_2; all methods, 20-24 hours

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





Swarming…

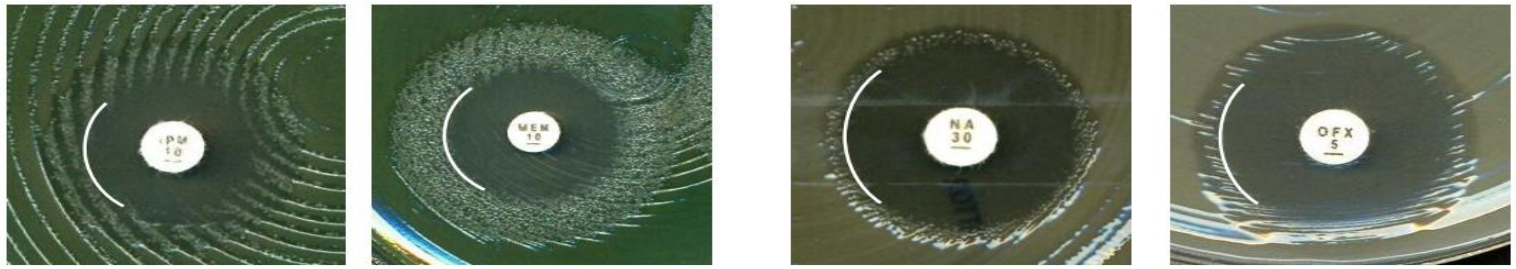
- ✓For *Proteus spp.*,ignore swarming and read inhibition of growth



Double Zones

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.

Growth or Haemolysis

Haemolysis

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.

Zone Measurement and Result Interpretation

Susceptible (S)	Intermediate (I)	Resistant (R)
The organism is inhibited by the antibiotic. Standard dosing is expected to be effective.	Intermediate susceptibility. May respond to higher doses or at certain body sites.	The organism is resistant. The antibiotic should not be used for treatment.

Common errors and how to avoid them

✗ Inoculum too heavy or too light

✓ Always adjust to 0.5 McFarland

✗ Incorrect agar depth

✓ Maintain depth of 4.0 ± 0.5 mm

✗ Disks placed too close together

✓ Minimum 24 mm centre-to-centre

✗ Incorrect incubation time

✓ Follow CLSI table for each organism

✗ Reading zones with poor lighting

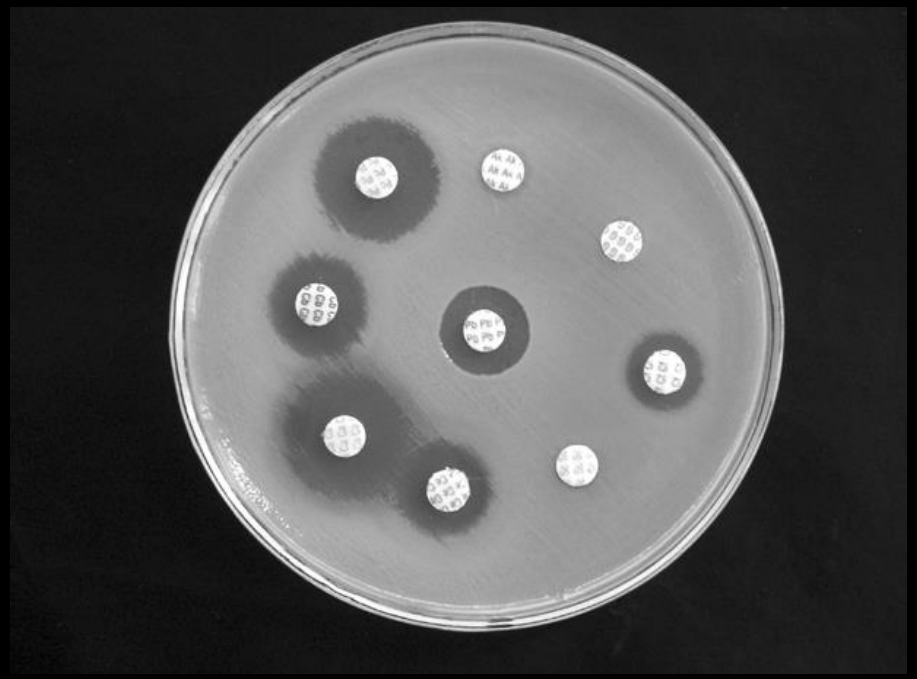
✓ Read from the back against a dark background

✗ Using outdated breakpoints

✓ Always keep M100-Ed36 (2026) current

Quality Control of Antimicrobial Disks

Why is Quality Control of Antibiotic Disks Essential?



Actual disk diffusion plate

Validity of Clinical Results

Ensure inhibition zones are due to true antibiotic activity

Detect System Problems

Identify disk failure, media issues, incubator or operator error

Accreditation Requirements

Compliance with CLSI, EUCAST and ISO 15189

Prevent Diagnostic Errors

Avoid incorrect susceptible/resistant reporting that affects therapy

Standard Quality Control Strains (ATCC)



Commercial QC strain vials

E. coli ATCC 25922

Enterobacterales & Gram-negatives

S. aureus ATCC 25923

Gram-positives (disk diffusion)

P. aeruginosa ATCC 27853

Pseudomonas & non-fermenters

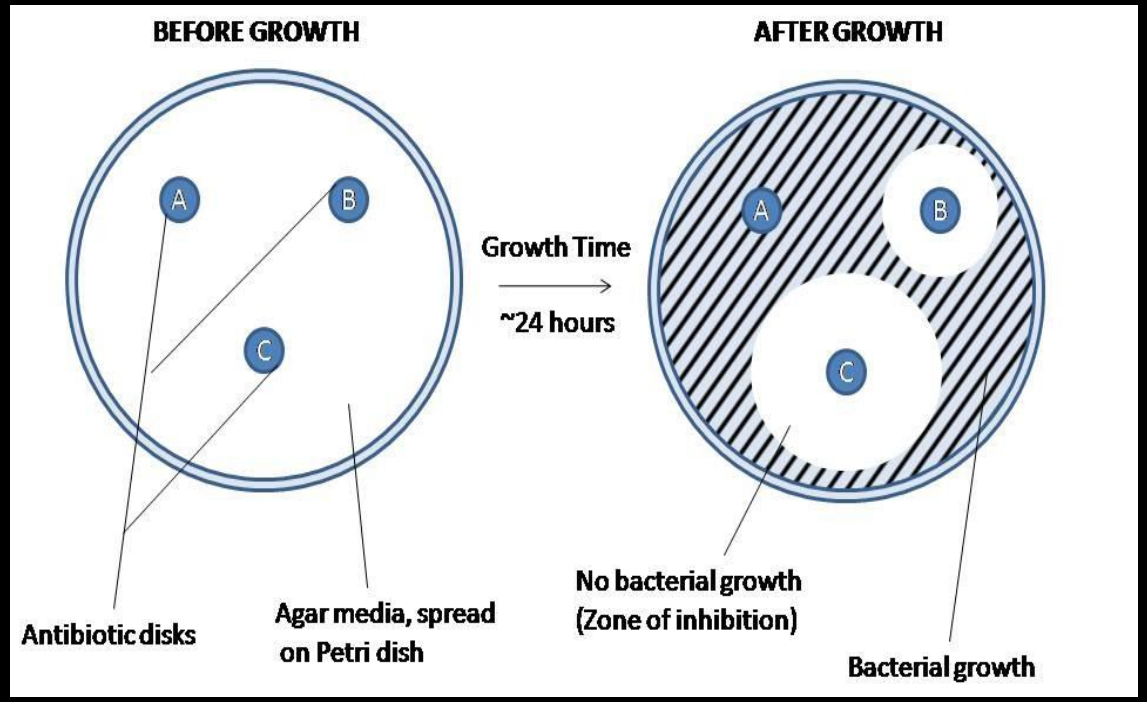
E. faecalis ATCC 29212

Quality Control Strain Maintenance Workflow for Subculturing and Using Reference Strains

Step	Action	Comments
1.	Subculture frozen or freeze-dried stock culture to appropriate agar plate (eg, tryptic soy or blood agar for nonfastidious QC strains or blood agar or enriched chocolate for fastidious QC strains) and incubate under the appropriate conditions for the organism.	This subculture is designated the F1 subculture. ("F" indicates the "frozen" or "freeze-dried" state of the stock culture, while "1" indicates the first passage and "2" the second passage from the stock culture). Store F1 subculture at 2 to 8°C or as appropriate for the organism type for up to 4 weeks.
2.	Subculture from F1 onto an agar slant or agar plate to prepare F2 subculture.	
3.	Incubate the F2 subculture under the appropriate conditions for the organism.	Store the F2 subculture at 2 to 8° C or as appropriate for the organism type for up to one week.
4.	Subsequently, subculture from F2 onto an agar plate (not to broth or agar slant) to prepare F3 subcultures.	The number of F3 subcultures needed depends on the laboratory's QC testing schedule.
5.	Select isolated colonies from fresh F2 or F3 agar plate (eg, overnight incubation) to prepare the QC test inoculum suspension.	
6.	Prepare a new F2 subculture (agar slant or agar plate) each week for weeks 2, 3, and 4.	
7.	After four weeks, prepare a new F1 subculture from frozen or freeze-dried stock culture.	

Abbreviation: QC, quality control.

Practical QC Procedure (Part 1)



Schematic of disk diffusion

1. **Prepare QC strain**

   Fresh ATCC culture (≤5 passages)

2. **0.5 McFarland suspension**

   Acceptable range 0.4–0.6

3. **Inoculate Mueller-Hinton**

   Agar depth 4±0.5 mm, pH 7.2–7.4

Practical QC Procedure (Part 2)



Actual plate with disks

4 **Apply disks**

Max 6 disks on 90 mm or 12 on 150 mm plate

5 **Incubation**

35±2°C for 16–18 h (inverted)

6 **Measure zones**

With ruler or automated system

7 **Compare to CLSI**

Tables 4A and 4B of M100

Quality Control Testing Frequency
Daily Quality Control Testing

- The 15-replicate (3- × 5-day) or 20 days plan
- Three replicates of each applicable QC strain are tested using individual inoculum preparations for five consecutive test days and the results are documented
- Following successful completion of the 15-replicate (3- × 5-day) plan, it is acceptable to go to weekly QC testing.
- If completion of the 15-replicate (3- × 5- day) plan is unsuccessful, corrective action should be taken

Performance Criteria for Reducing Quality Control Frequency to Weekly

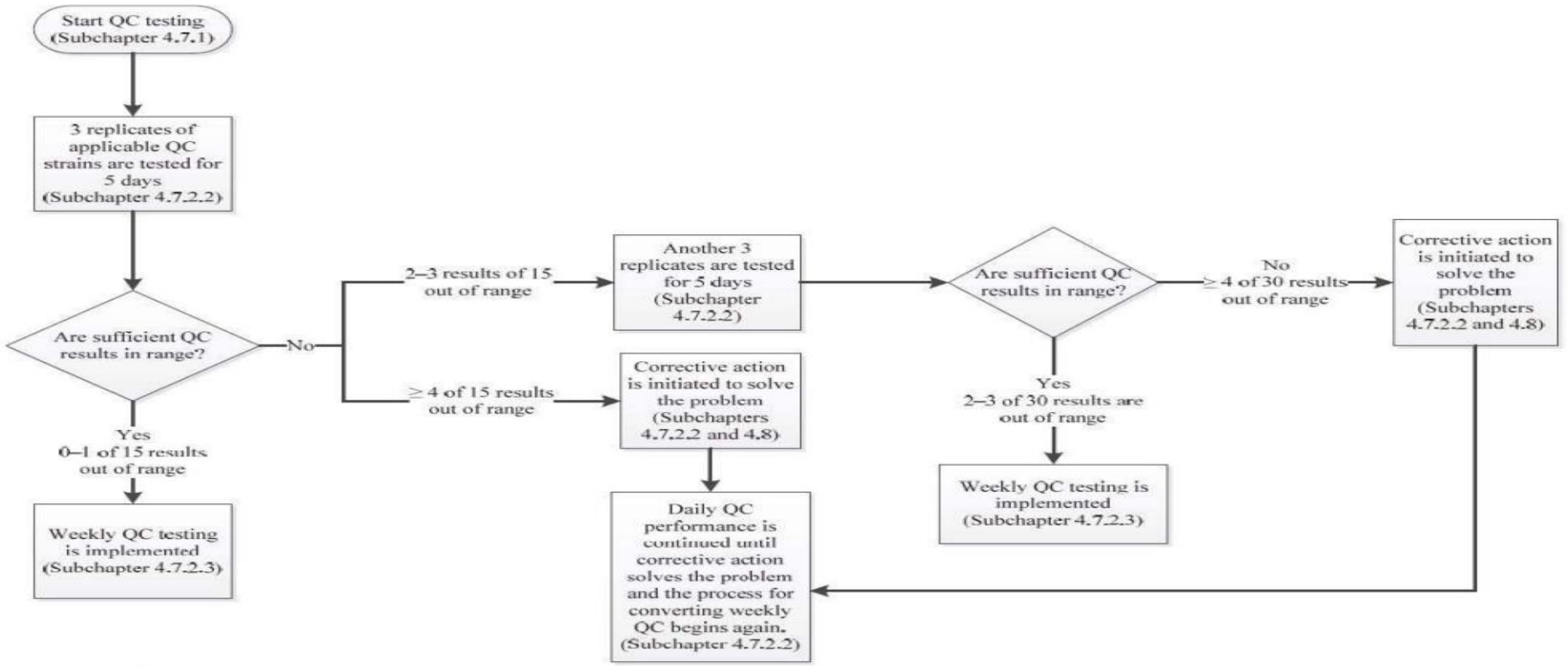
Table 6. 15-Replicate (3-×5-Day) Plan: Acceptance Criteria and Recommended Action*

Number Out of Range With Initial Testing (Based on 15 Replicates)	Conclusion From Initial Testing (Based on 15 Replicates)	Number Out of Range After Repeat Testing (Based on All 30 Replicates)	Conclusion After Repeat Testing
0–1	Plan is successful. Convert to weekly QC testing.	N/A	N/A
2–3	Test another 3 replicates for 5 days.	2–3	Plan is successful. Convert to weekly QC testing.
≥4	Plan fails. Investigate and take corrective action as appropriate. Continue QC each test day.	≥4	Plan fails. Investigate and take corrective action as appropriate. Continue QC each test day.

* Each QC strain and antimicrobial agent combination is assessed separately.

Abbreviations: N/A, not applicable; QC, quality control.

D2 Quality Control Protocol: Conversion From Daily to Weekly Testing (15-Replicate [3- × 5-Day] Plan)



Disk Diffusion QC Ranges for Nonfastidious Organisms

Table 4A-1. Disk Diffusion QC Ranges for Nonfastidious Organisms and Antimicrobial Agents Excluding β-Lactam Combination Agents[a]

Antimicrobial Agent	Disk Content	Disk Diffusion QC Ranges, mm		
		Escherichia coli ATCC®[b] 25922	*Pseudomonas aeruginosa* ATCC® 27853	*Staphylococcus aureus* ATCC® 25923
Amikacin	30 µg	19-26	20-26	20-26
Ampicillin	10 µg	15-22	–	27-35
Azithromycin	15 µg	–	–	21-26
Azlocillin	75 µg	–	24-30	–
Aztreonam	30 µg	28-36	23-29	–
Carbenicillin	100 µg	23-29	18-24	–
Cefaclor	30 µg	23-27	–	27-31
Cefamandole	30 µg	26-32	–	26-34
Cefazolin	30 µg	21-27	–	29-35
Cefdinir	5 µg	24-28	–	25-32
Cefditoren	5 µg	22-28	–	20-28
Cefepime	30 µg	31-37	25-31	23-29
Cefetamet	10 µg	24-29	–	–
Cefiderocol	30 µg	25-31	22-31	–
Cefixime	5 µg	20-26	–	–
Cefmetazole	30 µg	26-32	–	25-34
Cefonicid	30 µg	25-29	–	22-28
Cefoperazone	75 µg	28-34	23-29	24-33
Cefotaxime	30 µg	29-35	18-22	25-31
Cefotetan	30 µg	28-34	–	17-23
Cefoxitin[c]	30 µg	23-29	–	23-29
Cefpodoxime	10 µg	23-28	–	19-25
Cefprozil	30 µg	21-27	–	27-33
Ceftaroline	30 µg	26-34	–	26-35
Ceftazidime	30 µg	25-32	22-29	16-20

Table 4A-1. (Continued)

Antimicrobial Agent	Disk Content	Disk Diffusion QC Ranges, mm		
		Escherichia coli ATCC®[b] 25922	*Pseudomonas aeruginosa* ATCC® 27853	*Staphylococcus aureus* ATCC® 25923
Gatifloxacin	5 µg	30-37	20-28	27-33
Gemifloxacin	5 µg	29-36	19-25	27-33
Gentamicin[g]	10 µg	19-26	17-23	19-27
Gepotidacin	10 µg	18-26	–	23-29
Grepafloxacin	5 µg	28-36	20-27	26-31
Iclaprim	5 µg	14-22	–	25-33
Imipenem[h]	10 µg	26-32	20-28	–
Kanamycin	30 µg	17-25	–	19-26
Lefamulin	20 µg	–	–	26-32
Levofloxacin	5 µg	29-37	19-26	25-30
Levonadifloxacin	10 µg	27-33[e]	17-23[e]	32-39[e]
Linezolid	30 µg	–	–	24-30
Lomefloxacin	10 µg	27-33	22-28	23-29
Loracarbef	30 µg	23-29	–	23-31
Mecillinam	10 µg	24-30	–	–
Meropenem	10 µg	28-35	27-33	29-37
Minocycline	30 µg	20-26	–	25-30
Moxalactam	30 µg	28-35	17-25	18-24
Moxifloxacin	5 µg	28-35	17-25	28-35
Nafcillin	1 µg	–	–	16-22
Nafithromycin	15 µg	–	–	25-31[e]
Nalidixic acid	30 µg	22-28	–	–
Netilmicin	30 µg	22-30	17-23	22-31
Nitrofurantoin	300 µg	20-25	–	18-22
Norfloxacin	10 µg	28-35	22-29	17-28
Ofloxacin	5 µg	29-33	17-21	24-28
Omadacycline	30 µg	22-28	–	22-30

QC recommendation for ESBLs (Extended-Spectrum B lactamase)

Test	Criteria for Performance of ESBL Test				ESBL Test	
Test method	Disk diffusion		Broth microdilution		Disk diffusion	Broth microdilution
QC recommendations	When testing antimicrobial agents used for ESBL detection, *K. pneumoniae* ATCC®[b] 700603 is provided as a supplemental QC strain (eg, for training, competence assessment, or test evaluation). Either strain, *K. pneumoniae* ATCC® 700603 or *E. coli* ATCC® 25922, may then be used for routine QC (eg, daily or per IQCP).		When testing antimicrobial agents used for ESBL detection, *K. pneumoniae* ATCC® 700603 is provided as a supplemental QC strain (eg, for training, competence assessment, or test evaluation). Either strain, *K. pneumoniae* ATCC® 700603 or *E. coli* ATCC® 25922, may then be used for routine QC (eg, daily or per IQCP).		When performing the ESBL test, *K. pneumoniae* ATCC® 700603 and *E. coli* ATCC® 25922 should be used for routine QC (eg, daily or per IQCP).	When performing the ESBL test, *K. pneumoniae* ATCC® 700603 and *E. coli* ATCC® 25922 should be tested routinely (eg, daily or per IQCP).
	E. coli ATCC® 25922 (see acceptable QC ranges in Table 4A-1)		*E. coli* ATCC® 25922 = no growth (see acceptable QC ranges listed in Table 5A-1)		**Acceptable QC:** *E. coli* ATCC® 25922: ≤ 2-mm increase in zone diameter for antimicrobial agent tested in combination with clavulanate vs the zone diameter when tested alone.	**Acceptable QC:** *E. coli* ATCC® 25922: < 3 2-fold concentration decrease in MIC for antimicrobial agent tested in combination with clavulanate vs the MIC of the agent when tested alone.
	K. pneumoniae ATCC® 700603:		*K. pneumoniae* ATCC® 700603 = Growth:		*K. pneumoniae* ATCC® 700603: ≥ 5-mm increase in zone diameter of ceftazidime-clavulanate vs ceftazidime alone; ≥ 3-mm increase in zone diameter of cefotaxime-clavulanate vs cefotaxime alone.	*K. pneumoniae* ATCC® 700603: ≥ 3 2-fold concentration decrease in MIC for an antimicrobial agent tested in combination with clavulanate vs the MIC of the agent when tested alone.
	Cefpodoxime zone	9-16 mm	Cefpodoxime	MIC ≥ 8 µg/mL		
	Ceftazidime zone	10-18 mm	Ceftazidime	MIC ≥ 2 µg/mL		
	Aztreonam zone	10-16 mm	Aztreonam	MIC ≥ 2 µg/mL		
	Cefotaxime zone	17-25 mm	Cefotaxime	MIC ≥ 2 µg/mL		
	Ceftriaxone zone	16-24 mm	Ceftriaxone	MIC ≥ 2 µg/mL		

Abbreviations: ATCC®, American Type Culture Collection; CAMHB, cation-adjusted Mueller-Hinton broth; ESBL, extended-spectrum β-lactamase; FDA, US Food and Drug Administration; h, hour(s); IQCP, individualized quality control plan; MHA, Mueller-Hinton agar; MIC, minimal inhibitory concentration; PK/PD, pharmacokinetic/pharmacodynamic; QC, quality control.

Out-of-Range Results & Corrective Actions

If two consecutive results are out of range or multiple disks fail on one day → do not report patient results until resolved.

Immediate Check

- Re-measure zones
- Check expiry & lot of disks
- Agar depth & pH
- 0.5 McFarland accuracy

System Check

- Incubator calibration
- Media quality
- Inoculation technique
- Disk storage conditions

Further Actions

- Replace disk/media lot
- Repeat with fresh strain
- Full documentation
- Return to daily QC if needed

Common Errors and Effect on Zone Size

Inoculum denser than 0.5 McFarland	Reduced zones (false resistant)
Inoculum lighter than 0.5 McFarland	Increased zones (false susceptible)
Delay placing disks after inoculation	Reduced zones
Delay incubation after placing disks	Increased zones (pre-diffusion)
Loss of disk potency (poor storage)	Reduced zones
Agar depth too high or too low	Zones smaller or larger than true
Incorrect medium pH	Variable effect by antibiotic
Incubation temperature out of range	Altered growth & diffusion rates


Thank You
Thank you for your kindness, as calming as a forest breeze.