Kirby-Bauer Method for Susceptibility Testing - including the following organisms:

Mucoid *Pseudomonas*; Small Colony Variant *Staphylococcus aureus*; *N. meningitidis.*

***Haemophilus* species susceptibility testing is currently not available by Kirby Bauer method and should be sent out if required.**

Purpose	This procedure provides instruction for the performance of Kirby Bauer Disk Diffusion Susceptibility Testing.
Principal	A standardized inoculum of bacteria is swabbed onto the surface of a Mueller-Hinton agar plate. Filter paper disks impregnated with antimicrobial agents are placed on the surface of the agar. After overnight incubation, the diameter of the zone of inhibition is measured around each disk. Using the tables in the CLSI disk diffusion standard, a qualitative report of susceptible, intermediate and resistant is obtained.
Policy Statements	This procedure applies to Microbiologists who perform antimicrobial susceptibility testing
Work-up Code	KBS

	Supplies	**Equipment**	**Media**
Materials	--Sterile cotton tip swabs --12 x 75 polystyrene tubes	Disk Dispenser (s) --Gram positive --EBAC, HAE, NMEN --PSAR NF Vitek Densichek	Agar plates: store at 2-8ºC. --Mueller-Hinton agar (MH) --MH with 5% sheep blood (MHSB) --Saline-0.45-0.9% --Trypticase Soy Broth (TSB)

Specimen	Prepare inoculum from 4 or 5 isolated colonies of similar colony morphology. 1. For direct colony inoculum (stationary-phase): use colonies grown overnight on nonselective medium (e.g. SB or CHOC). Always use direct colony inoculum for staphylococci. 2. For log-phase-growth inoculum: use colonies grown for 1 or 2 days on non-selective or selective (e.g. MAC, CNA) medium. 3. Subculture QC stock, frozen, or lyophilized isolates 2 times prior to testing.
Special Safety Precautions	Microbiologists are subject to occupational risks associated with specimen handling. Refer to the safety policies: • Biohazard Containment • Safety in the Microbiology Laboratory • Biohazardous Spills
Quality Control/ QC Strains	**Gram-negative disks—EBAC HAE NMEN and PSAR NF dispensers: Mueller Hinton agar** --*Escherichia coli* ATCC 25922 --*Pseudomonas aeruginosa* ATCC 27853

	Gram-positive disks: Mueller Hinton Agar --*Staphylococcus aureus* ATCC 25923 --*Enterococcus faecalis* ATCC 29212 (only use when troubleshooting new lots of MH for unacceptable levels of thymidine when trimeth/sulfa is tested) **Gram positive disks: Mueller Hinton Agar with 5% SB** --*Streptococcus pneumoniae* ATCC 49619
Monthly QC Testing	1. Perform QC monthly on the first Thursday of each month. 2. Document QC set up on Kirby-Bauer QC Review Log. 3. Perform QC with each new lot or shipment of MH or MHSB plates and antimicrobial disks before put into service. Document QC set-up on Kirby Bauer QC Review Log. 4. Record results in QC manual. Record tech, date, "Pass/Fail" on Kirby Bauer QC Review Log. 5. If there is a QC failure, document observation, notify Technical Specialist and proceed with corrective action. Do not report patient results until the problem is resolved. Record on Kirby Bauer QC Review Log.
Monthly QC Review	1. Each month QC data will be reviewed and assessed by the Micro Technical Specialist or designee. 2. Person assessing will initial the log for monthly review and notify Micro Technical Specialist of any ongoing or critical issues.
Out of Control Results due to Obvious Error	**Out- of -control results due to obvious error**. Possible errors include: • Use of wrong disk • Use of wrong control strain • Contamination • Wrong incubation temperature or conditions 1. Document the reason and retest the strain on the day 2. If the repeated result is within range, no further corrective action is necessary
Out of Control Results Not due Obvious Error	**Out-of-control results not due to an obvious reason**. Investigate possible procedural problems: • Correct zone measurements • Standardization of the inoculum • Storage and expiration dates of the disks • Incubation conditions • Control strain was not contaminated • Control organism was more than 24 h old 1. Perform alternate test method until the problem is resolved. 2. Suppress the results for the individual antimicrobial agent. 3. Investigate potentially affected patient results performed since the last successful QC event. 4. Retest the strain on the same day. 5. If the repeated result is within range, no further corrective action is necessary. 6. If the repeated results is not within range, test the antimicrobial agent for 5 consecutive days. Record all results. 7. If all 5 zone diameters are within range, no additional corrective action is necessary. 8. If the problem is not resolved (1 or more diameters out of range), daily QC testing must be done until the problem is resolved. 9. In order to return to monthly testing, satisfactory performance must be demonstrated by testing for 30 days QC plan or 3 X 5 QC plan.

10. It may be necessary to obtain a new QC organism either from the frozen stock or from BD.
11. Call BD technical service at 1-800-638-8663 if it may be a manufacturer problem.

Reporting patient results

1. Perform alternate test method until the problem is resolved.
2. Suppress the results for the individual antimicrobial agent.

KBS Procedure

1. **Bring plates and dispensers to RT before use.** It is essential for the dispensers to be at room temperature to prevent moisture condensation, and loss of antibiotic potency.
    a. Dispensers need at least 30 minutes to warm up.
    b. Invert plates to equilibrate so that the condensation does not fall onto the agar.
    c. Agar plates can be put in the 35ºC ambient air incubator to warm (no longer than 30 minutes, though, to prevent agar dehydration).
2. **Label plate** with patient's accession number and date of set up.

Inoculum preparation:

3. **Stationary Phase (direct colony)** – Preferred Method
    a. Pick isolated colonies from 18-24 h growth on non-selective media (SB or CHOC)
    b. Emulsify in 1.8 ml of 0.45% sterile saline.
    c. Using the Vitek Densichek, obtain a reading of 0.5 - 0.55, (**not** up to 0.62 as for Vitek methods).
    d. Use the adjusted inoculum suspension to inoculate AST test plate within 15 minutes.
4. **Log Phase (growth method) –**This method can be used alternatively and is preferable when colony growth is difficult to suspend directly and a smooth suspension cannot be made or when it grows poorly, e.g., mucoid Pseudomonas. It can also be used for non-fastidious organisms (except staphylococci) when fresh (24-hour) colonies or from non-selective media are not available.
    a. Select 4-5 well isolated colonies with a sterile swab and transfer to 2-5 mL of TSB.
    b. Incubate at 35ºC for 2 – 6 h until growth reaches the turbidity standard or above of a 0.5 McFarland standard.
    c. Using the Vitek Densichek, obtain a reading of 0.5 - 0.55, (**not** up to 0.62 as for Vitek methods).
    d. Avoid extremes in inoculum density. Never use an undiluted overnight broth culture.
    e. Use the adjusted inoculum suspension to inoculate AST test plate within 15 minutes.
5. **Inoculation of Test Plates**
    a. Dip sterile swab into the suspension. Rotate swab against the wall of the tube above the liquid to remove excess inoculum.
    b. Inoculate the dried surface of the MH plate. First streak of swab should go down the middle of the plate. Swab across the entire agar surface at a 90º angle.
    c. Repeat this procedure 3 times, rotating the plate approximately 60º between streaking to ensure even distribution. Avoid hitting the sides of the plate to prevent aerosols.
    d. Run the swab around the rim of the agar to remove excess moisture.
    e. Allow plate to stand 3-5 minutes, (no more than 15) before applying the disks. This allows excess moisture to be absorbed before applying the drug impregnated disks.
6. **Application of Disks to Inoculated Agar Plates**
    a. Apply the disks using the self-tamping dispenser.
    b. Press each disk down to ensure complete contact with the agar surface even though the self-tamping dispenser is used, to prevent disks from falling off when plates are inverted for incubation.
    c. Do not put more than 12 disks on 150 mm plate or more than 6 disks on a 100mm plate.
    d. Because some of the drug diffuses almost instantaneously, do not relocate disks once they have made contact with the plate.
    e. **If performing the D-zone test** for inducible clindamycin resistance, the CC (clinda) and E (erythro) disks must be dispensed by hand, spaced 15-26 mm apart for staphylococcus or 12 mm apart for *S. pneumoniae* and β- hemolytic streptococci
7. **Incubation**

KBS Procedure continued.

a. Invert plates and incubate at 35ºC in an ambient air incubator within 15 minutes after the disks are applied.
b. Incubate *Neisseria meningitidis* (MHSB), small colony variant *Staph aureus* (MHSB), *S. pneumoniae* and β- hemolytic streptococci (MHSB) in CO_2 incubator.
c. Incubate mucoid *P. aeruginosa* for 24 hours.
d. Incubate small colony variant *S. aureus* and other *Staphylococci* for 16-20 hours.
e. Incubate *N. meningitidis*, *S. pneumoniae* and β- hemolytic streptococci for 20-24 h.
f. Incubate Enterobacterales, and *Enterococci for* 16 to 18 h.
g. Incubate Coag neg Staph (cefoxitin) and *Enterococci* (vancomycin) for 24 h.

8. Reading Plates

a. Read the plates after incubation only if the lawn of growth is confluent. If individual colonies are apparent, the inoculum concentration was too light and the test must be repeated.
b. Examine plate for any possible contamination.
c. Measure zones of complete inhibition and record the diameters to the nearest millimeter, including the diameter of the disk,
d. Use sliding calipers or a ruler, which are held on the back of the inverted Petri dish.
e. Use reflected light, and hold the Petri plate a few inches above a black surface.
f. For MHSB, (blood agar base) measure the zones from the upper surface of the agar with reflected light, and with the cover removed.
g. For MHSB, measure the zone of growth inhibition, not the zone of hemolysis.
h. Do not hold plates up to the light to read, using transmitted light.
i. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye.
j. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth.
k. For staphylococci, examine closely for small colonies. Consider any growth resistant.
l. Discrete colonies within the zone may represent a mixed culture or resistant variants. Subculture a single colony from the primary plate and retest. If the discrete colonies are present, measure the colony-free inner zone.
m. Disregard swarming of *Proteus* sp.
n. When measuring zones of trimethoprim/sulfa, disregard light growth (20%), and measure the more obvious margin to determine the zone diameter.

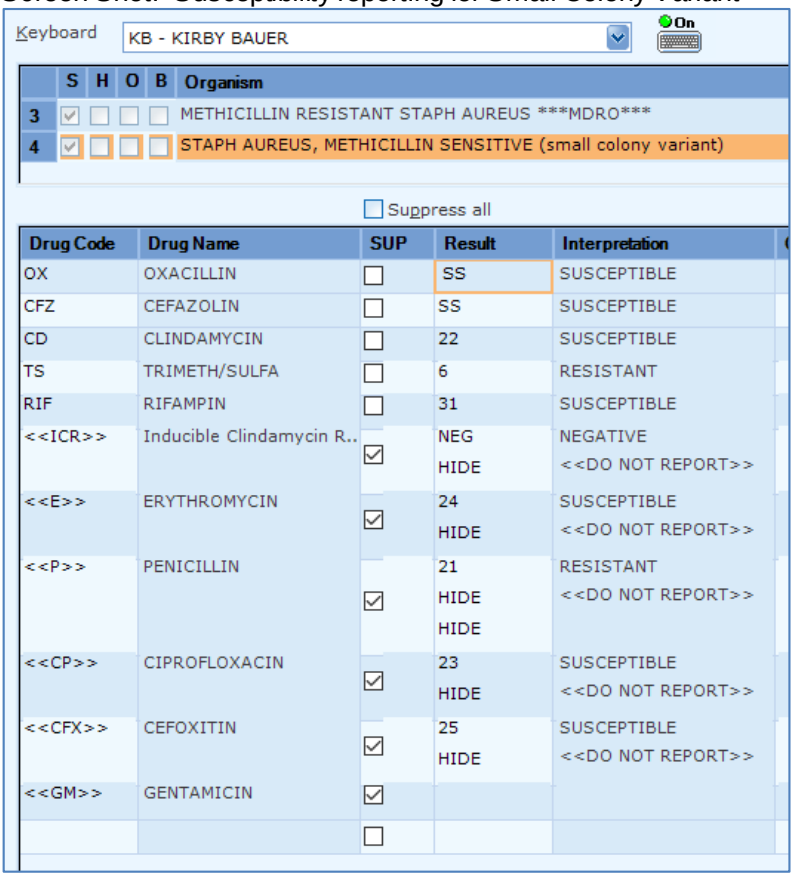
Small Colony Variant Staphylococcus aureus

Medium: Mueller Hinton with SB
Disk Dispenser: Gram Positive

1. Use the Stationary phase- Direct suspension method from isolated colonies grown on SB to make McFarland 0.5 suspension as directed in procedure step 3, above.
2. Inoculate the MHSB plate as directed in procedure steps 5 and 6 above, removing the CC and E disks before stamping.
3. **The D-zone test for inducible Clindamycin resistance must be performed**; The CC (clindamycin) and E (erythromycin) disks must be dispensed by hand, spaced 15-26 mm apart.
4. **Vancomycin testing cannot be performed by Kirby Bauer**. Set up an Etest for Vancomycin. See MC 6.60 Etest procedure for instructions.
5. Incubation: 35ºC, in 5-10% CO_2, 16 –20 hours
6. Read zone sizes as directed in procedure step 8, above.
7. Enter zone sizes into Sunquest in MRE, Susceptibility tab, KB keyboard, in Sunquest.
8. OX and CFZ interpretations are reported based on the FOX interpretation. FOX is a surrogate for Oxacillin to detect *mec-A* gene. Perform PBP2a on isolates susceptible to FOX.
9. If FOX is susceptible, result OX and CFZ as “SS”
10. If FOX is resistant, result OX and CFZ as “R”.
11. Enter the results of the D test as ICR, (inducible clindamycin resistance) -- POS or NEG.

12. Result CD as R when ICR is Positive.

Screen Shot: Susceptibility reporting for Small Colony Variant

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Organism #4   -   STAPH AUREUS, METHICILLIN SENSITIVE (small colony variant)
- KB -
                SS          CD(22),RIF(31),OX(SS),CFZ(SS)
                R           TS(6)
                HIDE        ICR(NEG),E(24-SS),P(21-R),CP(23-SS),CFX(25-SS)
- MMIC -
                HIDE        VA(1-SS)
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Neisseria meningitidis
[Process in BSC]

Medium: Mueller Hinton with SB (MHSB)

Disks

Disks dispenser: EBAC HAE NMEN. Add MEM if needed.
SXT--Trimethoprim sulfamethoxazole (1.25/23.75 mcg)
CRO--Ceftriaxone (30 mcg)
MEM--Meropenem (10 mcg)
CIP --Ciprofloxacin (5 mcg) do not report in CSF

Procedure

1. Send isolates from blood to U of M for Penicillin. Enter MIC results under the **MMIC** keyboard.
2. Use the Stationary phase- Direct suspension method from isolated colonies grown on CHOC **(process in BSC)** to make McFarland 0.5 suspension as directed in procedure step 3.
3. Inoculate the MHSB plate and dispense disks as directed in procedure steps 5 and 6, above.
4. Incubation: 35ºC, 5-10% CO_2, 20-24 hours

Process in BSC!

5. Read zone sizes as directed in procedure step 8, above.

Sunquest Result Reporting

1. Criteria specified by CLSI are used to interpret the zone diameters.
2. Record the zone diameter in Sunquest function MRE by clicking on the susceptibility tab.
3. Use the drop-down arrow to select the **KB** keyboard. Highlight the organism #. Enter the zone diameter at the appropriate drug prompt.
4. If there is no zone, growth up to disk, enter 6 mm, the diameter of the disk.
5. The computer will automatically interpret the results.
6. Display results to make sure they are correct by clicking the summary button.
7. Click on the File button to file results.

Reporting Rules

1. MRSA is resistant to all penicillins, all cephems, carbapenems, beta-lactam and beta-lactam inhibitor combinations such as amoxicillin-clavulanic acid, ampicillin-sulbactam, piperacillin-tazobactam, and ticarcillin-clavulanic acid.
2. Salmonella isolates from extraintestinal sites: Report ampicillin, ciprofloxacin, trimethoprim-sulfa and ceftriaxone results.
3. Perform D Test on staphylococci and beta-hemolytic streptococci that are resistant to erythromycin for inducible clindamycin resistance.
4. Perform D Test on *Strep pneumoniae*.
5. **Do not** report misleading results for the following organisms:
    a. *Salmonella and Shigella*: first and second generation cephalosporins and aminoglycosides
    b. Staph sp.: do not report CF; AM; AMC-- the beta-lactam drugs—(except Penicillin)
    c. ESBL-producing *E. coli, Proteus* sp., and *Klebsiella* sp.: cephalosporins, penicillins, and aztreonam
    d. *Enterococcus* sp.: cephalosporins, trimeth/sulfa, clindamycin, and aminoglycosides
    e. CSF: DO NOT report per CLSI-- Agents administered by oral route only; or 1st -and 2nd-generation cephalosporins (except Cefuroxime parenteral) and cephamycins Clindamycin; Erythromycin; Tetracyclines; Fluoroquinolones (e.g. Ciprofloxacin, and the other “floxacins”).

Procedure Notes

1. **Disks:** Working supplies of disks, properly stored, can be used for at least one month; verify acceptability by monthly QC.
2. **Last Disc**: The last disc in each cartridge is marked with an ‘X’. A green plastic plug has been incorporated in each cartridge after the ‘X’ marked disc. This prevents the dispenser from operating when an empty cartridge is in place. Watch for the appearance of discs marked ‘X’ and insert a full cartridge.
3. **Instructions for use:**
    - Lift dispenser and place over agar plate.
    - Depress knob firmly until sound of the tampers is heard. Partial depression of the knob may cause discs to jam in the dispenser.
    - Allow knob to return to the upper position and lift dispenser from the agar plate.
    - Inspect the agar plate for the ‘X’ on any of the dispensed discs and replace empty cartridges.
4. **Jammed disks:**
    - Slide the black button to Unlock position
    - Remove all the cartridges and reinsert into the dispenser upside down.
    - Place the black button in the Lock position. Carefully inspect the end of each cartridge for deformed discs. Deformed discs must be manually removed and discarded.

- With the cartridges upside down in the dispenser, dry cycle the dispenser several times to assure that it is operating freely.

5. Media:

a. Thymidine can interfere with the performance of sulfonamides and trimeth/sulfa. MHA with excessive amounts of thymidine will yield smaller zones or no zone at all which may result in false resistant reports. If problems with QC of sulfonamides and trimethoprim occur, monitor by testing *E. faecalis* ATCC 29212 and trimeth/sulfa (zone of ≤20 mm is acceptable).

b. An increased cation content (Ca^{2+}, $Mg2^{+}$) in the media results in decreased zone sizes with the aminoglycosides for *P. aeruginosa* and increased zone sizes with tetracycline. Decreased cation content has the opposite effect.

c. Increased zinc ions may cause decreased zones with carbapenems.

Limitations

1. This method applies to rapid growing aerobes.
2. Some bacteria may become resistant during antimicrobial therapy. Repeat testing on subsequent isolates should be performed every 3 days.

References

1. Hindler, Janet and Humphries, Romney, Section editor, Antimicrobial Susceptibility Testing, 5.1, "Disk Diffusion Test" in *Clinical Microbiology Procedures Handbook,* Amy Leber, editor, 2016, ASM Press, Washington, D.C.
2. BD BBL Sensi-Disc Designer Dispenser 260640 2015-03 Becton, Dickinson and Company 7 Loveton Circle Sparks, MD 21152 bbl_sensidisc_designer_dispenser.pdf
3. Clinical and Laboratory Standards Institute (CLSI) M100 Performance Standards for Antimicrobial Susceptibility Testing, 35 edition, 2025.

Training Plan/ Competency Assessment

Training Plan	Initial Competency Assessment
-Employee must read the procedure -Employee will observe trainer performing the procedure. -Employee will demonstrate the ability to perform procedure, record results and document corrective action after instruction by the trainer.	1. Direct observation.

Historical Record

Version	Written/Revised by:	Effective Date:	Summary of Revisions
1	Pat Ackerman	1978	Initial Version
1.1	Pat Ackerman	1/29/1992	Re-format
1.2	Pat Ackerman	8/12/05 PA	Re-format
1.3	Pat Ackerman	11/14/06 PA	Appendix 5. ESBL reporting modified. Added Appendix 4 Dtest for beta-hemolytic streptococci. Added information regarding extraintestinal Salmonella. Clarified when Enterococcus faecalis QC should be performed in Procedure Notes #2.
1.4	Pat Ackerman	3/31/07 PA	Appendix 2. *Neisseria meningitidis* Disk diffusion testing updated with the new 2007 CLSI Standards. MH with 5% SB replaces MH agar. Interpretations added for ceftriaxone, meropenem, trimeth/sulfa and ciprofloxacin. For *Salmonella* isolates, Nalidixic acid used for surveillance of reduced fluoroquinolone susceptibility.
1.5	Becky Carlson	3/15/2009 BJC	Changed CDDT reporting rules to be the same as Vitek AST-67 verbiage –ICR Removal of CHOC MH from materials field. Neisseria gonorrhoeae AST will be referred for AST to MML.
1.6	Becky Carlson	10/23/2012 BJC	MRSA SCV KBS testing instruction added.
2	Becky Carlson	6/6/2015 BJC	Reformatted for CMS load. Re-numbered from MC 1101
3	Becky Carlson	9/24/2015	Added Kirby Bauer QC Review Log for recording performance and review of QC results.

	4	Susan DeMeyere	4/20/2018	Reformatted, Removed NA testing. Update references. Removed requirements for log phase growth with mucoid pseudomonas.
	5	Susan DeMeyere	**8/28/2019**	Added Examine plate for any possible contamination.
	6	Susan DeMeyere	**6/22/2021**	Added instructions for Saur-SCV testing of vancomycin
	7	Susan DeMeyere	**6/2/2025**	Changed to monthly QC, instead of weekly