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

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

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| **Purpose****Principal** | This procedure provides instruction for the performance of KIRBY – BAUER DISK DIFFUSION SUSCEPTIBILITY TESTING.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 |
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|  | **Supplies** | **Equipment** | **Media** |
| **Materials** | --Sterile cotton tip swabs--12 x 75 polystyrene tubes | Disk Dispenser (s) --Gram positive--Enteric gram negative (Urine; Haemophilus) --Mucoid Pseudo (NLF)DensiCHEK Plus® (Vitek) | Agar plates: store at 2-8ºC.--Mueller-Hinton agar (MH)--MH with 5% sheep blood (MHB)*--Haemophilus* Test Medium (HTM)--Saline-0.45-0.9%--Trypticase Soy Broth (TSB) |
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|  | Prepare inoculum from 4 or 5 isolated colonies of similar colony morphology. |
| Specimen | 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.
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| **Special Safety Precautions** | Microbiologists/virologists are subject to occupational risks associated with specimen handling. Refer to the safety policies:1. [Biohazard Containment](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.1%20Biohazard%20Containment.docx)
2. [Safety in the Microbiology/Virology Laboratory](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.2%20Safety%20in%20the%20Microbiology%20Lab.docx)
* [Biohazardous Spills](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.4%20Biohazardous%20Spills.docx)
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| **Quality Control/****QC Strains****Weekly****QC Testing****Monthly QC Review****Out of Control Results** | **Gram-negative disks—Enteric and Mucoid Pseudo (NLF):** **Mueller Hinton agar***--Escherichia coli* ATCC 25922*--Pseudomonas aeruginosa* ATCC 27853*--Haemophilus influenzae* ATCC 49247**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 496191. Perform QC weekly.
2. Perform QC with each new lot or shipment of MH, MHSB or HTM plates and antimicrobial disks before put into service. Document QC set-up on Kirby Bauer QC Review Log.
3. Record results in QC manual. Record tech, date, “Pass/Fail” on Kirby Bauer QC Review Log.
4. If there is a QC failure, document observation, notify supervisor and proceed with corrective action. Do not report patient results until the problem is resolved. Record on Kirby Bauer QC Review Log.

 1. Each month QC data will be reviewed and assessed by the Micro Supervisor or designee.
2. Person assessing will initial the log for monthly review and notify Lab Director of any ongoing or critical issues.

**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 day2. If the repeated result is within range, no further corrective action is necessary. **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. Retest the strain on the same day.2. If the repeated result is within range, no further corrective action is necessary. 3. Test the antimicrobial agent for 5 consecutive days. Record all results.4. If all 5 zone diameters are within range, no additional corrective action is necessary. 5. If the problem is not resolved (1 or more diameters out of range), daily QC testing  must be done until the problem is resolved.6. It may be necessary to obtain a new QC organism either from the frozen stock or from BD.7. 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. |
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| **KBS Procedure****KBS Procedure continued.****KBS Procedure continued.****MRSA****Small colony Variant**  | 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.
	1. Dispensers need at least 30 minutes to warm up.
	2. ESBL dispenser (stored frozen) should equilibrate to RT for at least an hour.
	3. Invert plates to equilibrate so that the condensation does not fall onto the agar.
	4. Agar plates can be put in the 35ºC ambient air incubator to warm (no longer than 30 minutes, though, to prevent agar dehydration).

**Inoculum preparation:** 1. **Stationary Phase (direct colony)** – Preferred Method
	1. Pick isolated colonies from 18-24 h growth on non-selective media (SB or CHOC)
	2. Make a direct saline suspension.
2. **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 nonfastidious organisms (except staphylococci) when fresh (24-hour) colonies or from non-selective media are not available.
	1. Select 4-5 well isolated colonies with a sterile swab and transfer to 2-5 mL of TSB.
	2. Incubate at 35ºC for 2 – 8 h until growth reaches the turbidity standard or above of a 0.5 McFarland standard.
	3. Using the Vitek DensiCHEK Plus®, obtain a reading of 0.5 - 0.55, (**not** up to 0.62 as for Vitek methods).
	4. Avoid extremes in inoculum density. Never use an undiluted overnight broth culture.
	5. Use the adjusted inoculum suspension to inoculate AST test plate within 15 minutes.
3. **Inoculation of Test Plates**
	1. Dip sterile swab into the suspension. Rotate swab against the wall of the tube above the liquid to remove excess inoculum.
	2. 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.
	3. 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.
	4. Run the swab around the rim of the agar to remove excess moisture.
	5. To ensure purity of the organism, prepare a purity plate by streaking the inoculum to a blood or chocolate agar plate and incubating overnight.
	6. 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.
4. **Application of Disks to Inoculated Agar Plates**
	1. Apply the disks using the self-tamping dispenser.
	2. 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.
	3. Do not put more than 12 disks on 150 mm plate or more than 6 disks on a 100mm plate.
	4. Because some of the drug diffuses almost instantaneously, do not relocate disks once they have made contact with the plate.
	5. **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
5. **Incubation**
	1. Invert plates and incubate at 35ºC in an ambient air incubator within 15 minutes after the disks are applied.
	2. Incubate *Haemophilus* (HTM media), *Neisseria meningitidis* (MHSB)*,* small colony variant *Staph aureus* (MHSB), *S. pneumoniae* and β- hemolytic streptococci (MHSB) in CO2 incubator.
	3. Incubate mucoid *P. aeruginosa* for 24 h
	4. Incubate small colony variant *S. aureus* and other *Staphylococci* for 16-20 hours.
	5. Incubate *N. meningitidis*, *S. pneumoniae* and β- hemolytic streptococci *for* 20-24 h.
	6. Incubate *Haemophilus, Enterobacteriaceae,* and *Enterococci* for 16 to 18 h.
	7. Incubate Coag neg Staph (cefoxitin) and *Enterococci* (vancomycin) for 24 h.
6. **Reading Plates**
	1. 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.
	2. Examine purity plate for any possible contamination.
	3. Measure zones of complete inhibition and record the diameters to the nearest millimeter, including the diameter of the disk,
	4. Use sliding calipers or a ruler, which are held on the back of the inverted Petri dish.
	5. Use reflected light, and hold the Petri plate a few inches above a black surface.
	6. For MHSB, (blood agar base) measure the zones from the upper surface of the agar with reflected light, and with the cover removed.
	7. For MHSB, measure the zone of growth inhibition, not the zone of hemolysis.
	8. Do not hold plates up to the light to read, using transmitted light.
	9. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye.
	10. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth.
	11. For staphylococci, examine closely for small colonies. Consider any growth resistant.
	12. 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.
	13. Disregard swarming of *Proteus* sp.
	14. When measuring zones of trimeth/sulfa, disregard light growth (20%), and measure the more obvious margin to determine the zone diameter.

**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 2, 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. Incubation: 35ºC, in 5-10% CO2, 16 –20 hours
5. Read zone sizes as directed in procedure step 8, above.
6. Enter zone sizes into Sunquest in MRE, Susceptibility tab, KBS keyboard, in Sunquest.
7. Do not report **(do not enter results)** AM; AMC; or CF; (beta-lactam drugs)
8. OX zone size is reported with the FOX zone measurement as a surrogate for Oxacillin to detect *mec-A* gene. Perform PBP2a on isolates susceptible to FOX.
9. If FOX is resistant, result CFZ as “R”, (deduced).
10. Enter the results of the D test as ICR, (inducible clindamycin resistance) -- POS or NEG. CDDT should not be resulted.
11. Unhide the RIF and the TS.
12. The CFZ result will be automatically hidden, because of the OX = R. Unhide with CD as in step 13.
13. If the erythromycin interpretation is I or R, the CD results will be automatically hidden. CAUTION: It cannot be released before doing a file and save. You must re-access the culture, and then un-hide the CD, so that the pop-up box reads, “the suppression has been removed”.

Screen Shot: Susceptibility reporting for Small Colony Variant  |
| ***Haemophilus*** **species****Procedure*****Neisseria meningitidis*****[Process in BSC]****Disks** **Procedure****Process in BSC!** | **Medium: HTM Plate Disk Dispenser: Urine / Enteric GNR / Haemophilus disks****Perform β lactamase testing**1. Use the Stationary phase- Direct suspension method from isolated colonies grown on CHOC to make McFarland 0.5 suspension as directed in procedure step 2, above.
2. Inoculate the HTM plate and dispense disks as directed in procedure steps 5 and 6, above.
3. Incubation: 35ºC, 5-10% CO2, 16 –18 hours
4. Read zone sizes as directed in procedure step 8, above.
5. Beta lactamase positive *Haemophilus* sp. must always be reported resistant to ampicillin regardless of the zone size.
6. Occasional isolates are beta-lactamase negative and ampicillin resistant (BLNAR). These isolates must be reported resistant to ampicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefonicid, cefuroxime, and pipercillin-tazobactam.
7. For isolates of Haemophilus from CSF, only report ampicillin, third generation cephalosporin’s, and meropenem.

**Medium: Mueller Hinton with SB (MHSB) (weekly QC)****Perform β lactamase testing** Disk diffusion tests with penicillin and ampicillin are unreliable**Disks:** Pick from gram pos and urine dispenser 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 RA--Rifampin (5 mcg)1. 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 2.
2. Inoculate the MHSB plate and dispense disks as directed in procedure steps 5 and 6, above.
3. Incubation: 35ºC, 5-10% CO2, 20-24 hours
4. Read zone sizes as directed in procedure step 8, above.

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| **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.
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| **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, pipercillin-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. **Do not** report misleading results for the following organisms:
	1. *Salmonella and Shigella*: first and second generation cephalosporins and aminoglycosides
	2. Staph sp.: do not report CF; AM; AMC-- the beta-lactam drugs—(except Penicillin)
	3. ESBL-producing *E. coli, Proteus* sp., and *Klebsiella* sp.: cephalosporins, penicillins, and aztreonam
	4. *Enterococcus* sp.: cephalosporins, trimeth/sulfa, clindamycin, and aminoglycosides
	5. 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”).
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| **Procedure Notes**  |  1. Disks: Working supplies of disks, properly stored, can be used for at least one week; verify acceptability by weekly QC.
2. Media:
	1. 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).
	2. An increased cation content (Ca2+, Mg2+) in the media results in decreased zone sizes with the aminoglycosides and increased zone sizes with tetracycline for *P. aeruginosa*.
3. Decreased cation content has the opposite effect.
	1. Increased zinc ions may cause decreased zones with carbapenems
	2. Variation in calcium ions affects the results of daptomycin.
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| **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.
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| **References** | 1. Hindler, J.F., Section editor, Antimicrobial Susceptibility Testing, 5.1.6, “Disk Diffusion Test” in *Clinical Microbiology Procedures Handbook,* Lynne Garcia, editor, 2010, ASM Press, Washington, D.C.
2. CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Thirteenth Edition*, CLSI document M02-A13, Wayne, PA: Clinical and Laboratory Standards Institute; 2018,
3. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*, *Twenty-Eight Edition*, CLSI document M100-S28, Wayne PA: Clinical and Laboratory Standards Institute, 2018
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| **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.
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| **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 –ICRRemoval 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 inoculate a purity plate and examine the following day.  |