BD BBL™ Mueller Hinton Agar with 5% Sheep Blood

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QUALITY CONTROL PROCEDURES

I. INTRODUCTION

BBL™ Mueller Hinton Agar with 5% Sheep Blood is recommended for disc diffusion susceptibility testing of *Streptococcus pneumoniae* with selected agents; i.e., chloramphenicol, erythromycin, ofloxacin, tetracycline and vancomycin, in addition to oxacillin screening for susceptibility to penicillin, as standardized by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS).

II. PERFORMANCE TEST PROCEDURE

1. Inoculate representative samples of the cultures listed below.
   a. Preparation of inoculum.
      The *S. pneumoniae* culture for Mueller Hinton Agar with 5% Sheep Blood is prepared by suspending sufficient growth from an 18- to 20-h culture on **Trypticase™** Soy Agar with 5% Sheep Blood (TSA II) into Mueller Hinton II Broth to achieve the desired turbidity (comparable to a 0.5 McFarland turbidity standard).
   b. Within 15 min after adjusting the turbidity of the inoculum, dip a sterile swab into the broth suspension. Rotate the swab several times on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.
   c. Inoculate the surface of the plate by streaking the swab over the surface of the plate. Repeat this procedure two more times, rotating the plate 60 degrees each time.
   d. Replace the lid of the plate and allow inoculum to be absorbed for at least 3 min, but no longer than 15 min, before applying the **Sensi-Disc™** antimicrobial susceptibility test discs.
   e. Place the appropriate discs onto the respective cultures.
   f. Incubate plates at 35 ± 2°C in 5-7% CO_2 within 15 min after the discs are applied.
2. Examine Mueller Hinton Agar with 5% Sheep Blood plates after 20-24 h. Measure the zone diameters of the complete zones of inhibition to the nearest mm. The endpoint should be taken as the area showing no obvious visible growth, excluding faint growth of tiny colonies which can be detected with difficulty at the edge of the zone of inhibition.
3. Expected Results
   a. Test Organism
      * *Streptococcus pneumoniae* . . . . . ATCC™ 49619
   b. Zone sizes should fall within the ranges of acceptable zone diameter quality control limits specified by the Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS. These limits are published in CLSI Document M100-S23 (M2)[1] Supplemental tables containing revised tables of antimicrobial discs and interpretive standards are published periodically. The latest tables should be consulted for current recommendations. See the "NOTE" under "RESULTS" in the PRODUCT INFORMATION section for additional information.

*Recommended organism strain for User Quality Control.

III. ADDITIONAL QUALITY CONTROL

1. Examine plates as described under "Product Deterioration."
2. Visually examine representative plates to assure that any existing physical defects will not interfere with use.
3. Determine the pH potentiometrically at room temperature for adherence to the specification of 7.3 ± 0.2 for the blood-containing medium.
4. Note the firmness of plates during the inoculation procedure.
5. Incubate uninoculated representative plates at 30 ± 1°C in an aerobic atmosphere for 84h and examine for microbial contamination.

PRODUCT INFORMATION

IV. INTENDED USE

BBL Mueller Hinton Agar with 5% Sheep Blood is recommended for antimicrobial disc diffusion susceptibility testing of *S. pneumoniae* with selected agents; i.e., chloramphenicol, erythromycin, ofloxacin, tetracycline, and vancomycin, in addition to oxacillin screening for susceptibility to penicillin, as standardized by the Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS.[1]

V. SUMMARY AND EXPLANATION

Because clinical microbiology laboratories in the early 1960s were using a wide variety of procedures for determining the susceptibility of bacteria to antibiotics and chemotherapeutic agents, Bauer, Kirby and others developed a standardized procedure in which Mueller Hinton agar was selected as the test medium.[2,3] A subsequent international collaborative study confirmed the value of Mueller Hinton agar for this purpose because of the relatively good reproducibility of the medium, the simplicity of its formula, and the wealth of experimental data that had been accumulated using this medium.[4]

Unsupplemented Mueller Hinton agar, although adequate for susceptibility testing of rapidly growing aerobic pathogens, was not adequate for more fastidious organisms such as *S. pneumoniae*. The CLSI Document M2-A11 recommends Mueller Hinton agar supplemented with 5% defibrinated sheep blood.[1] Details of quality control procedures and interpretive criteria for use with *S. pneumoniae* and other *Streptococcus* spp. Are contained in supplemental tables.[5]

VI. PRINCIPLES OF THE PROCEDURE

The Bauer-Kirby procedure is based on the diffusion through an agar gel of antimicrobial substances which are impregnated on paper discs.[6] In the test procedure, a standardized suspension of the organism is swabbed over the entire surface of the medium. Paper discs impregnated with specified amounts of antibiotic or other antimicrobial agents are then placed on the surface of the medium, the plate is incubated, and zones of inhibition around each disc are measured. For *S. pneumoniae*, the determination as to whether the organism is susceptible, intermediate or resistant to an agent is made by comparing zone sizes to those in the CLSI Document M100-S23 (M2),[5] which is included with CLSI Document M2-A11.[1]

VII. REAGENTS

Mueller Hinton Agar with 5% Sheep Blood

Approximate Formula* Per Liter Purified Water

Beef Extract	2.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Sheep Blood, defibrinated	5.0 %

*Adjusted and/or supplemented as required to meet performance criteria.

Mueller Hinton Agar with 5% Sheep Blood Prepared Plates are medium to dark red in appearance.

Warnings and Precautions: For *in vitro* Diagnostic Use in Taiwan.

If excessive moisture is observed, invert the bottom over an off-set lid and allow to air dry in order to prevent formation of a seal between the top and bottom of the plate during incubation.

Observe aseptic techniques and established precautions against microbiological hazards throughout all procedures. After use, prepared plates, specimen containers and other contaminated materials must be sterilized by autoclaving before discarding.

Storage Instructions: On receipt, store plates in the dark at 2-8°C. Avoid freezing and overheating. Do not open until ready to use. Minimize exposure to light. Prepared plates stored in their original sleeve wrapping at 2-8°C until just prior to use may be inoculated up to the expiration date and incubated for recommended incubation times. Allow the medium to warm to room temperature before inoculation.

Product Deterioration: Do not use plates if they show evidence of microbial contamination, discoloration, drying, cracking or other signs of deterioration.

VIII. SPECIMEN COLLECTION AND HANDLING

This product is not intended for use directly with specimens or mixed cultures. The organism to be tested

must first be in pure culture.

A Gram stain and a presumptive identification of *S. pneumoniae* are recommended.[1]

IX. PROCEDURE

Material Provided: Mueller Hinton Agar with 5% Sheep Blood

Materials Required But Not Provided:

1. Inoculum broth in 5 mL amounts, such as Mueller Hinton II Broth for preparation of standard inoculum.
2. A 0.5 McFarland barium sulfate standard for adjustment of inoculum, or prepared by adding 0.5 mL of 0.048M $BaCl_2$ [1.175% w/v $BaCl_2 \cdot 2H_2O$] to 99.5 mL of 0.18M [0.36N] H_2SO_4 [1% v/v]).
3. A photometric device for adjusting the turbidity of the inoculum suspension to be equivalent to the 0.5 McFarland standard.
4. As an alternative to the above materials (1-3), the **BBL™ Prompt™** Inoculation System (volumetric inoculum preparation device) can be used.[7]
5. Control culture – *S. pneumoniae* ATCC 49619.
6. Paper discs impregnated with specified amounts of antimicrobial agents, such as **BBL Sensi-Disc** susceptibility test discs.
7. Dispensing device, such as the **Sensi-Disc** 6 – or 12 - Place Dispenser.
8. Device for measuring or interpreting zone diameters to the nearest whole millimeter, such as a sliding caliper or a ruler.[1]
9. An incubator that produces an atmosphere containing 5-7% CO_2, or another device that produces a similar CO_2-enriched atmosphere.
10. Ancillary culture media, reagents and laboratory equipment as required.

Test Procedure:

The direct colony suspension method should be used when testing *S. pneumoniae*.[1] Observe aseptic techniques.

1. Suspend growth from an overnight (16-18 h) sheep blood agar plate in saline or broth, such as Mueller Hinton II Broth. Adjust the turbidity to be equivalent to the 0.5 McFarland barium sulfate standard. For the diluent, use sterile broth or sterile saline. The turbidity of the standard and the test inoculum should be compared by holding both tubes in front of a white background with finely drawn black lines or a photometric device can be used.

   Alternative methods of inoculum preparation involving devices that permit direct standardization of inocula without adjustment of turbidity, such as the **BBL Prompt** Inoculation System, have been found to be acceptable for routine testing purposes.[7]
2. Within 15 min of adjusting the turbidity of the inoculum, dip a sterile cotton swab into the properly diluted inoculum and rotate it firmly several times against the upper inside wall of the tube to express excess fluid.
3. Inoculate onto Mueller Hinton Agar with 5% Sheep Blood by streaking the entire agar surface of the plate three times, rotating the plate 60° between streakings to obtain even inoculation. As a final step, swab the rim of the agar bed.
4. Replace the lid of the plate and hold the plate at room temperature for at least 3 min, but no longer than 15 min, to allow surface moisture to be absorbed before applying the drug-impregnated discs. Use no more than nine discs per 150 mm plate, or four discs per 100 mm plate.
5. Incubate for 20-24 h at 35°C in an atmosphere of 5-7% CO_2.

User Quality Control:

The control culture, *S. pneumoniae* ATCC 49619, should be included each time a susceptibility test is performed or weekly if satisfactory performance can be documented according to the CLSI standard.[1] The correct zone diameters will be found in CLSI Document M100-S23 (M2),[5] which is included with CLSI Document M2-A11.[1]

Quality Control requirements must be performed in accordance with applicable local, state and/or federal regulations or accreditation requirements and your laboratory's standard Quality Control procedures. It is recommended that the user refer to pertinent CLSI (formerly NCCLS) guidance and CLIA regulations for appropriate Quality Control practices.

X. RESULTS

With Mueller Hinton Agar with 5% Sheep Blood, the zone of growth inhibition should be measured, not the

zone of inhibition of hemolysis. The zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed. Zone diameters should be compared with those in CLSI Document M100-S23 (M2), which provides interpretive criteria.[5] Results obtained may then be reported as resistant, intermediate or susceptible.

Isolates of *S. pneumoniae* with oxacillin zone diameters of ≥ 20 mm are susceptible (MIC ≤ 0.06 µg/mL) to penicillin. CLSI Document M2-A11 should be consulted for other antimicrobial agents to which penicillin-susceptible isolates may also be considered susceptible.[1] See "Limitations of the Procedure"

NOTE: Supplemental tables to CLSI Document M2-A11, containing revised tables of antimicrobial discs and interpretive standards are published periodically. The latest tables should be consulted for current recommendations. The complete standard and supplemental tables can be ordered from the Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898. Telephone: (610) 688-0100.

XI. LIMITATIONS OF THE PROCEDURE

Isolates of *S. pneumoniae* with oxacillin zone sizes of ≥ 20 mm are susceptible to penicillin (MIC ≤ 0.06 µg/mL), as well as to other β-lactam agents. The disc test does not, however, distinguish penicillin intermediate strains (i.e., MICs = 0.12 to 1.0 µg/mL) from strains that are penicillin resistant (i.e., MICs ≥ 2.0 µg/mL). Furthermore, a small percentage of susceptible strains will also have zone sizes of ≤ 19 mm in the oxacillin screening test; i.e., they will be falsely categorized as resistant or intermediate.[8,9] For these reasons, a penicillin MIC should be determined on all isolates of *S. pneumoniae* with oxacillin zones of ≤ 19 mm.[1]

With some organism-antimicrobial agent combinations, the inhibition zone may not have a sharply demarcated edge, which could lead to incorrect interpretation.

Various factors have been identified as influencing disc diffusion susceptibility tests. These include the medium, agar depth, disc potency, inoculum concentration, age of inoculum, and pH.[4,6]

Incorrect inoculum concentration may produce incorrect results. Zones of inhibition may be too small if the inoculum is too heavy and they may be too large and difficult to measure if the inoculum is too light.

Improper storage of antimicrobial discs may cause a loss of potency and a falsely resistant result.

In vitro susceptibility of an organism to a specific antimicrobial agent does not necessarily mean that the agent will be effective *in vivo*. Consult appropriate references for guidance in the interpretation of results.[6,10]

As indicated in "PERFORMANCE CHARACTERISTICS," the use of cefaclor, cefprozil and trimethoprim/sulfamethoxazole on Mueller Hinton Agar with 5% Sheep Blood when testing *S. pneumoniae* is not recommended.

Alternatively, susceptibility of *S. pneumoniae* to trimethoprim/ sulfamethoxazole can be determined by the broth dilution (MIC) method using Mueller Hinton II Broth (Cation-Adjusted) with Lysed Horse Blood.[11]

XII. PERFORMANCE CHARACTERISTICS

Antimicrobial disc diffusion susceptibility testing using the quality control strain recommended by NCCLS Document M2-A5,[12] *S. pneumoniae* ATCC 49619, was performed in-house with cefaclor, cefprozil, chloramphenicol, erythromycin, ofloxacin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin. Following the test procedures described in M2-A5, twenty tests with the quality control strain and eight antimicrobic discs were performed over a period of 10 test days. For all eight antimicrobics, 100% (160/160) of the zone sizes fell within the expected zone size ranges published in Table 3C of NCCLS Document M100-S6.[13]

The standard deviation for tetracycline was less than 1 mm, and for all other antimicrobics, less than 2 mm.[14]

Reproducibility studies (3x/day for 3 days) were done at two field sites with the antimicrobics listed above against *S. pneumoniae* ATCC 49619 and nine additional well-characterized *S. pneumoniae* strains. Zone diameter interpretive standards from Table 2C of NCCLS Document M2-A5 and Supplement M100-S6 were followed for each antimicrobic. Testing with chloramphenicol, erythromycin, ofloxacin, tetracycline, and vancomycin resulted in over 95% Category Agreement with the NCCLS reference method. Testing with trimethoprim/sulfamethoxazole resulted in 90% Category Agreement with the NCCLS reference method.[14]

Reproducibility could not be determined for cefaclor and cefprozil due to the absence of interpretive standards for these two antimicrobics in Table 2C of NCCLS Document M100-S6.[13]

Based on the studies outlined above, the use of cefaclor, ceprozil or trimethoprim/sulfamethoxazole is not recommended on this medium when testing *S. pneumoniae*. Additionally, references in the literature[15]

report excessive interpretive errors in disc diffusion testing with trimethoprim/sulfamethoxazole on Mueller Hinton sheep blood agar plates.

XIII. AVAILABILITY

Cat. No.	Description
251801	**BD BBL™** Mueller Hinton Agar with 5% Sheep Blood, Ctn. of 24 plates

XIV. REFERENCES

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