MUELLER HINTON AGAR

INTENDED USE

Mueller Hinton Agar is recommended for antimicrobial disc diffusion susceptibility testing of common, rapidly growing bacteria by the Bauer-Kirby method,[1-3] as standardized by the Clinical and Laboratory Standards Institute (CLSI).[4]

NOTE: The recommended medium for disc diffusion susceptibility testing of *Streptococcus pneumoniae* is Mueller Hinton agar with 5% sheep blood. The recommended medium for *Haemophilus influenzae* is Haemophilus Test Medium (HTM) Agar. The recommended medium for *Neisseria gonorrhoeae* is GC Agar with 1% defined growth supplement. Interpretive criteria are provided in the CLSI Document M100 (M2),[5] which is included with CLSI Document M2, Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard.[4]

SUMMARY AND EXPLANATION

Mueller Hinton Agar was originally developed for the cultivation of pathogenic *Neisseria*.[6] However, these organisms are now commonly isolated on selective media.

Because clinical microbiology laboratories in the early 1960s were using a wide variety of procedures for determining the susceptibility of bacteria to antibiotic and chemotherapeutic agents, Bauer, Kirby and others developed a standardized procedure in which Mueller Hinton Agar was selected as the test medium.[1,2] 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.[7]

The CLSI has written a performance standard for the Bauer-Kirby procedure and this document should be consulted for additional details.[4] The procedure is recommended for testing rapidly growing aerobic or facultatively anaerobic bacterial pathogens, such as staphylococci, members of the *Enterobacteriaceae*, aerobic gram-negative rods; e.g., *Pseudomonas* spp. and *Acinetobacter* spp., enterococci and *Vibrio cholerae*. The procedure is modified for testing fastidious species; i.e., *H. influenzae*, *N. gonorrhoeae* and *S. pneumoniae* and other streptococci.

Mueller Hinton Agar is manufactured to contain low levels of thymine and thymidine[8,9] and controlled levels of calcium and magnesium.[10-12] Thymine and thymidine levels of raw materials are determined using the disc diffusion procedure with trimethoprim-sulfamethoxazole (SXT) discs and *Enterococcus faecalis* ATCC™ 33186 and/or 29212. Calcium and magnesium levels are controlled by testing raw materials and supplementing with sources of calcium and/or magnesium as required to produce correct zone diameters with aminoglycoside antibiotics and *Pseudomonas aeruginosa* ATCC 27853.[13]

Mueller Hinton agar complies with requirements of the World Health Organization[14] and is specified in the FDA Bacteriological Analytical Manual for food testing.[15]

Unsupplemented Mueller Hinton agar, although adequate for susceptibility testing of rapidly growing aerobic pathogens, is not adequate for more fastidious organisms such as *S. pneumoniae*. The CLSI Document M2, Performance Standards for Antimicrobial Disk Susceptibility Tests, recommends Mueller Hinton agar supplemented with 5% defibrinated sheep blood.

PRINCIPLE

Acid hydrolysate (digest) of casein and beef extract supply amino acids and other nitrogenous substances, minerals, vitamins, carbon and other nutrients to support the growth of microorganisms. Starch acts as a protective colloid against toxic substances that may be present in the medium. Hydrolysis of the starch during autoclaving provides a small amount of dextrose, which is a source of energy. Agar is the solidifying agent.

The Bauer-Kirby procedure is based on the diffusion through an agar gel of antimicrobial substances which are impregnated on paper discs.[16] In contrast to earlier methods which used discs of high and low antimicrobial concentrations and which used the presence or absence of inhibition zones for their interpretation, this method employs discs with a single concentration of antimicrobial agent and zone diameters are correlated with minimal inhibitory concentrations (MIC).[1,2,4,7,16]

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. The determination as to whether the organism is susceptible, intermediate or resistant to an agent is made by comparing zone sizes obtained to those in the CLSI Document M100(M2).[4]

Various factors have been identified as influencing disc diffusion susceptibility tests. These include the medium, excess surface moisture on the medium, agar depth, disc potency, inoculum concentration, pH and β-lactamase production by test organisms. [7,13,16]

REAGENTS (FORMULA)

Beef Extract	2.0	g
Acid Digest of Casein	17.5	g
Starch	1.5	g
Agar	17.0	g
Deionized Water	1000.0	ml

PROCEDURE

A. Standard Method

1. Perform a Gram stain before starting a susceptibility test to confirm culture purity and to determine appropriate test battery.

2. Select at least three to five well-isolated similar colonies and transfer with an inoculation needle or loop into 4-5 mL of suitable broth.

3. Incubate the broth at 35°C until it achieves or just exceeds the turbidity of the 0.5 McFarland barium sulfate standard (usually 2-6 hours). This results in a suspension containing approximately 1 to 2 × 108 CFU/mL (for *E. coli* ATCC 25922).

4. Adjust the turbidity to be equivalent to the 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.

5. Within 15 minutes after adjusting the turbidity of the inoculum, immerse 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.

6. Inoculate 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.

7. The lid may be left ajar for 3-5 minutes and the plate held at room temperature for no longer than 15 minutes to allow any surface moisture to be absorbed before applying the antimicrobial agent-impregnated discs.

8. Apply the discs by means of an antimicrobial disc dispenser, using aseptic precautions. Deposit discs so that the centers are at least 24 mm apart. It is preferable to deposit penicillin and cephalosporin discs so that they are not less than 10 mm from the edge of the Petri dish, and their centers are at least 30 mm apart. Avoid placing such discs adjacent to one another. After discs have been placed on the agar, tamp them with a sterile needle or forceps to make complete contact with the medium surface. This step is not necessary if the discs are deposited using the Sensi-Disc™ 12-place self-tamping dispenser.

9. Within 15 minutes after the discs are applied, invert the plates and place them in a 35°C incubator. With nonfastidious organisms, plates should not be incubated under an increased concentration of carbon dioxide.

10. Examine plates after 16-18 hours incubation. A full 24 hours incubation is recommended for *Staphylococcus aureus* with oxacillin to detect methicillin-resistant *S. aureus* (MRSA) and for *Enterococcus* spp. when tested with vancomycin to detect vancomycin-resistant strains. Growth within the apparent zone of inhibition is indicative of resistance.

A confluent "lawn" of growth should be obtained. If only isolated colonies grow, the inoculum was too light and the test should be repeated. Measure the diameter of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disc, to the nearest whole millimeter, using sliding calipers, a ruler, or a template prepared for this purpose. The measuring device is held on the back of the inverted plate over a black, non-reflecting background, and illuminated from above.

The endpoint should be taken as the area showing no obvious visible growth that can be detected with the unaided eye. Disregard faint growth of tiny colonies which can be detected with difficulty near the edge of the obvious zone of inhibition. *Staphylococcus aureus* when tested with oxacillin discs is an exception, as are enterococci when tested with vancomycin. In these cases, transmitted light should be used to detect a haze of growth around the disc which is shown by "occult resistant" MRSA strains[17] or vancomycin-resistant enterococci.[4] With *Proteus* species, if the zone of inhibition is distinct enough to measure, disregard any swarming inside the zone. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.

B. Direct Method

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

1. Suspend growth from an overnight (16-18 hour) sheep blood agar plate in saline or broth, such as Mueller Hinton 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.

2. Within 15 minutes of adjusting the turbidity of the inoculum, dip a sterile 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 minutes, but no longer than 15 minutes, 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 hours at 35°C in an atmosphere of 5% CO_2.

EXPECTED RESULTS

Zone diameters measured around discs should be compared with those in the CLSI Document M100 (M2). Results obtained with specific organisms may then be reported as resistant, intermediate or susceptible.

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.

QUALITY CONTROL

All lot numbers have been tested and have been found to be acceptable. Customers can test products using the following quality control organisms. Testing of control organisms should be performed in accordance with established laboratory quality control procedures. If aberrant quality control results are noted, sample results should not be reported.

Organisms
Enterococcus faecalis ATCC 33186
Escherichia coli ATCC 25922
Pseudomonas aeruginosa ATCC 27853
Staphylococcus aureus ATCC 25923

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