BD BBL™ Mueller Hinton II Agar

111-251177-N-02, October 2020

QUALITY CONTROL PROCEDURES

I. INTRODUCTION

Mueller Hinton II Agar is used in the standardized disc diffusion procedure for determining the susceptibility of rapidly-growing aerobic organisms to antimicrobial agents.

II. PERFORMANCE TEST PROCEDURE

1. Inoculate representative samples with the cultures listed below.
   a. Preparation of inoculum.

   Growth Method

   1) Use fresh **Trypticase™** Soy Broth cultures prepared by picking three to five colonies from **Trypticase** Soy Agar with 5% Sheep Blood (TSA II) plates and suspending them in the broth. Incubate for 2-6 h, or until the desired turbidity is achieved.
   2) Adjust the turbidity of all broth cultures to a 0.5 McFarland standard.

   Direct Colony Suspension Method

   1) Select three to five colonies from fresh TSA II plates and suspend in **Trypticase** Soy Broth.
   2) Adjust the turbidity of all broth cultures to a 0.5 McFarland standard.

   b. Within 15 min after adjusting the turbidity of the inoculum, dip a sterile cotton 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. 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.
   f. Incubate plates aerobically at 35 ± 2°C within 15 min after the discs are applied.
2. Examine Mueller Hinton II Agar plates after 16-18 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. With trimethoprim and the sulfonamides, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter.
3. Expected Results
   a. Test Organism
      * *Staphylococcus aureus* . . . . . ATCC™ 25923
      * *Escherichia coli* . . . . . ATCC 25922
      * *Pseudomonas aeruginosa* . . . . . ATCC 27853
      * *Enterococcus faecalis* . . . . . ATCC 29212
   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 Table 4 of CLSI Document M100-S30 (M2)[5] Supplemental tables containing revised tables of antimicrobial discs and interpretive standards are published periodically. The latest tables should be consulted for current recommendations. See "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 ± 0.1.
4. Note the firmness of plates during the inoculation procedure.
5. Incubate uninoculated representative plates aerobically at 30 ± 1°C for 60 h and examine for microbial contamination.

PRODUCT INFORMATION

IV. INTENDED USE

Mueller Hinton Agar is recommended for antimicrobial disc diffusion susceptibility testing of common, rapidly growing bacteria by the Bauer-Kirby method,[2-4] as standardized by the Clinical and Laboratory Standards Institute (CLSI).[1]

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 II Agar with **IsoVitaleX™** Enrichment. Interpretive criteria are provided in the CLSI Document M100-S30,[5] which is included with CLSI Document M2-A13[1]

V. 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.[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.[7]

The CLSI has written a performance standard for the Bauer-Kirby procedure and this document should be consulted for additional details.[1] 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 II 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 ATCC 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]

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.[14] 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 minimum inhibitory concentrations (MIC).[1-3,7]

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 Table 4 in the CLSI Document M100-S30.[5]

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.[7,13,14]

VII. REAGENTS

Mueller Hinton II Agar

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

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

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

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

The disc diffusion susceptibility test is designed for use with pure cultures. A Gram stain and tentative identification are required in order to determine the appropriate antimicrobial agents against which the isolate is to be tested.[1]

It has been suggested that direct antimicrobial susceptibility testing may be performed on blood cultures and urine cultures;[15-18] however, tests should be repeated and confirmed with a pure culture.

IX. PROCEDURE

Material Provided: Mueller Hinton II Agar

Materials Required But Not Provided:

1. Inoculum broth, such as **Trypticase** Soy Broth (Soybean-Casein Digest Broth) or Mueller Hinton II Broth (cation-adjusted), tubed in 5 mL volumes for preparation of a standard inoculum, and sterile broth or saline for dilution of inoculum.
2. Barium sulfate comparison standard (0.5 mL of 0.048 M $BaCl_2$ [1.175% w/v $BaCl_2 \cdot 2H_2O$] to 99.5 mL of 0.18 M [0.36 N] 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.[19]
5. Control cultures - *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *E. faecalis* ATCC 33186 or ATCC 29212.
6. Paper discs impregnated with specified amounts of antimicrobial agents,[1] such as **Sensi-Disc** susceptibility test discs.
7. Dispensing device, such as the **Sensi-Disc** 6 –or 12-place dispenser.
8. Device for measuring zone size to the nearest whole millimeter, such as a sliding caliper or a ruler.[1]
9. Ancillary culture media, reagents and laboratory equipment as required.

Test Procedure: Observe aseptic techniques.

A. Standard Method[1]

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 h). This results in a suspension containing approximately 1 to 2 x 10^8

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.

   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.[19]
5. Within 15 min 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 min and the plate held at room temperature for no longer than 15 min 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.
9. Within 15 min 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 CO_2.
10. Examine plates after 16-18 h incubation. A full 24 h incubation is recommended for *S. 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. *S. 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[20] or vancomycin-resistant enterococci.[1] 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. Alternative Inoculum Standardization Procedures

1. Direct Method

   For routine susceptibility tests, the inoculum may be prepared by making a direct saline or broth suspension of colonies selected from an 18- to 24-h nonselective nutrient agar plate; e.g., blood agar. Immediately adjust this suspension to the turbidity of the 0.5 McFarland barium sulfate standard without incubation. This alternative procedure is the method of choice for testing staphylococci for potential methicillin or oxacillin resistance and for species that grow poorly in broth media and is a convenient alternative to the growth method.[1]
2. **Prompt** Inoculation System
   a. Remove the required number of **Prompt** inoculation tubes from the box and place in a test tube rack.
   b. Remove an inoculation wand from the box.
   c. Holding the wand tip perpendicular to the agar surface, touch five isolated colonies greater than

1 mm in diameter. (As a reference, the tip of the wand is 2 mm in diameter.) Do not penetrate the agar. Do not scrape or drag the tip across the colonies.

NOTE: If the colonies are small (0.5-1 mm in diameter), touch ten instead of five. For very small, pinpoint colonies, continue incubation of the primary plate until they reach a diameter of approximately 0.5-1 mm. If the colony diameter is not likely to reach 0.5 mm (e.g., some streptococci), the direct method for inoculum preparation should be used.

d. While holding the inoculation wand with one hand, remove a **Prompt** inoculation tube from the rack.
e. Bend the cap of the tube sideways until it snaps off.
f. Place the inoculation wand into the tube and press down with a twisting motion to assure a tight seal.
g. Vortex the tube vigorously for 10 s to release the bacteria from the wand tip. If the organisms are not released from the wand, let the solution sit for 5 min and vortex again.
h. The bacterial suspension should be used within 6 h of preparation. If not used immediately after preparation, shake vigorously to resuspend the bacteria just prior to use.
i. Remove the inoculation wand from the tube just prior to inoculating the agar plate.

User Quality Control: See "Quality Control Procedures."

Control cultures 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 M100-S23 (M2).

X. RESULTS

Zone diameters measured around discs should be compared with those in Table 4 in the CLSI Document M100-S30. Results obtained with specific organisms may then be reported as resistant, intermediate or susceptible.

NOTE: Supplemental tables to CLSI Document M2-A13, 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

The CLSI standardized procedure is not recommended for testing obligately anaerobic organisms, organisms that demonstrate a poor or slow growth rate on Mueller Hinton Agar, or organisms that show marked strain-to-strain variation with regard to growth rate.[1] Fastidious organisms should be tested as directed in section 11 of CLSI Document M7-A11,[21] or section 11 of M2-A13.[1]

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

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.[13,14]

Bacteria requiring thymine or thymidine may be encountered.[22,23] These organisms may not grow satisfactorily on Mueller Hinton Agar that contains low levels of thymine or thymidine.

New procedures have been developed utilizing high-content gentamicin (120 µg) and streptomycin (300 µg) discs to screen for high-level resistance to aminoglycosides as an indication that an enterococcal isolate will not be affected synergistically by a combination of a penicillin or glycopeptide plus an aminoglycoside.[1,21,24]

For complete discussions on the detection of MRSA, resistant enterococci, extended-spectrum β -lactamase-producing gram negative bacilli and other test limitations, refer to CLSI documents M2-A13[1] and M7-A11.[21]

BBL Mueller Hinton II Agar was shown to be reliable in detecting MRSA which produce a hazy zone of inhibition around oxacillin discs.[20] When in doubt, an additional method, such as the **BBL** MRSA Screen Agar test should be used.

XII. AVAILABILITY

Cat. No.	Description
251177	**BD BBL™** Mueller Hinton II Agar, Pkg. of 20 plates
251275	**BD BBL™** Mueller Hinton II Agar, Ctn. of 100 plates

XIII.REFERENCES

1. Clinical and Laboratory Standards Institute. 2018. Approved standard: M2-A13. Performance standards for antimicrobial disk susceptibility tests, 13th ed. Clinical and Laboratory Standards Institute, Wayne, Pa.
2. Bauer, A.W., W.M.M. Kirby, J.C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493-496.
3. Ryan, K.J., F.D. Schoenknecht, and W.M.M. Kirby. 1970. Disc sensitivity testing. Hospital Practice 5:91-100.
4. Barry, A.L., F. Garcia, and L.D. Thrupp. 1970. An improved single-disk method for testing the antibiotic susceptibility of rapidly-growing pathogens. Am. J. Clin. Pathol. 53:149-158.
5. Clinical and Laboratory Standards Institute. 2020. CLSI disk diffusion supplemental tables, M100-Ed30. Clinical and Laboratory Standards Institute, Wayne, Pa.
6. Mueller, J.H., and J. Hinton. 1941. A protein-free medium for primary isolation of the gonococcus and meningococcus. Proc. Soc. Exp. Biol. Med. 48:330-333.
7. Ericsson, H.M., and J.C. Sherris. 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sec. B, Suppl. 217.
8. Koch, A.E., and J.J. Burchall. 1971. Reversal of the antimicrobial activity of trimethoprim by thymidine in commercially prepared media. Appl. Microbiol. 22:812-817.
9. Ferone, R., S.R.M. Bushby, J.J. Burchall, W.D. Moore, and D. Smith. 1975. Identification of Harper-Cawston factor as thymidine phosphorylase and removal from media of substances interfering with susceptibility testing to sulfonamides and diaminopyrimidines. Antimicrob. Agents Chemother. 7:91-98.
10. Reller, L.G., F.D. Schoenknecht, M.A. Kenny, and J.C. Sherris. 1974. Antibiotic susceptibility testing of Pseudomonas aeruginosa: selection of a control strain and criteria for magnesium and calcium content in media. J. Infect. Dis. 130:454-463.
11. Pollock, H.M., B.H. Minshew, M.A. Kenny, and F.D. Schoenknecht. 1978. Effect of different lots of Mueller-Hinton agar on the interpretation of the gentamicin susceptibility of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 14:360-367.
12. D.Amato, R.F., and C. Thornsberry. 1979. Calcium and magnesium in Mueller-Hinton agar and their influence on disk diffusion susceptibility results. Current Microbiol. 2:135-138.
13. Thornsberry, C., T.L. Gavan, and E.H. Gerlach. 1977. Cumitech 6, New developments in antimicrobial agent susceptibility testing. Coordinating ed., J.C. Sherris. American Society of Microbiology, Washington, DC.
14. Jorgensen, J.H., J.D. Turnidge, and J.A. Washington. 1999. Antibacterial susceptibility tests: dilution and disk diffusion methods, p. 1526-1543. In P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, and R.C. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, DC.
15. Wegner, D.L., C.R. Mathis, and T.R. Neblett. 1976. Direct method to determine the antibiotic susceptibility of rapidly growing blood pathogens. Antimicrob. Agents Chemother. 9:861-862.
16. Johnson, J.E., and J.A. Washington II. 1976. Comparison of direct and standardized antimicrobial susceptibility testing of positive blood cultures. Antimicrob. Agents Chemother. 10:211-214.
17. Waterworth, P.M., and M. Del Piano. 1976. Dependability of sensitivity tests in primary culture. J. Clin. Pathol. 29:179-184.
18. Hollick, G.E., and J.A. Washington II. 1976. Comparison of direct and standardized disk diffusion susceptibility testing of urine cultures. Antimicrob. Agents Chemother. 9:804-809.
19. Baker, C.N., C. Thornsberry, and R.W. Hawkinson. 1983. Inoculum standardization in antimicrobial susceptibility testing: evaluation of overnight agar cultures and the rapid inoculum standardization system. J. Clin. Microbiol. 17:450-457.
20. Hindler, J.A., and C.B. Anderbied. 1985. Effect of the source of Mueller-Hinton agar and resistance frequency on the detection of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 21:205-210.
21. Clinical and Laboratory Standards Institute. 2018. Approved standard: M7-A11. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 11th ed. Clinical and Laboratory Standards Institute, Wayne, Pa.
22. Maskell, R., O.A. Okubadejo, R.H. Payne, and L. Pead. 1977. Human infections with thymine-requiring bacteria. J. Med. Microbiol, 11:33-45.
23. Haltiner, R.C., P.C. Migneault, and R.G. Robertson. 1980. Incidence of thymidine-dependent enterococci detected on Mueller-Hinton agar with low thymidine content. Antimicrob. Agents Chemother. 18:365-368.
24. Murray, B.E. 1990. The life and times of the *Enterococcus*. Clin. Microbiol. Rev. 3:46-65.

For further information please contact your local BD representative.

Nippon Becton Dickinson Company, Ltd.
1 Aza Gotanda, Tsuchifune, Fukushima City, Fukushima, Japan
e-mail : BD-eDial@bd.com
WEB : www.bd.com/jp/