

Procedure

Organisms to be tested must first be isolated in pure culture on an appropriate solid medium. Inoculate mucate media with a 3 mm loopful of a broth culture incubated overnight. Incubate mucate media at 35°C for up to 48 hours.

Expected Results

After sufficient incubation, examine tubes for a yellow (acid) reaction indicating utilization of the mucate. The medium remains blue-green if the mucate is not utilized. Consult appropriate texts for information needed to interpret the results.³⁻⁶

References

1. Kauffman and Petersen. 1956. *Acta Pathol. Microbiol. Scand.* 38:481.
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4. Krieg and Holt (ed.). 1984. *Bergey's manual of systematic bacteriology*, vol. 1. Williams & Wilkins, Baltimore, Md.
5. Farmer. 1999. *In Murray, Baron, Pfaller, Tenover and Tenover (ed.)*, *Manual of clinical microbiology*, 7th ed. American Society for Microbiology, Washington, D.C.
6. Flowers, Andrews, Donnelly and Koenig. 1993. *In Marshall (ed.)*, *Standard methods for the examination of dairy products*, 16th ed. American Public Health Association, Washington, D.C.

Availability

BBL™ Mucate Agar

Cat. No. 297709 Prepared Slants (K Tubes) – Pkg. of 10*

BBL™ Mucate Broth

SMD

Cat. No. 296135 Prepared Tubes (K Tubes) – Pkg. of 10*

*Store at 2-8°C.

Mueller Hinton Agars

Mueller Hinton Agar • Mueller Hinton II Agar

Mueller Hinton Agar with 5% Sheep Blood

Intended Use

Each lot of Mueller Hinton Agar and Mueller Hinton II Agar has been tested according to, and meets the acceptance limits of, the current M6 protocol published by the NCCLS.

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 National Committee for Clinical Laboratory Standards (NCCLS).⁴

Mueller Hinton Agar with 5% Sheep Blood is recommended for antimicrobial 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 National Committee for Clinical Laboratory Standards (NCCLS).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 (GC II Agar with BBL™ IsoVitalEX™ Enrichment or equivalent). Interpretive criteria are provided in the NCCLS Document M100-S12 (M2),⁵ which is included with NCCLS 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 NCCLS 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 and Mueller Hinton II Agar are 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

User Quality Control

Identity Specifications

Difco™ Mueller Hinton Agar

Dehydrated Appearance:	Beige, free-flowing, homogeneous.
Solution:	3.8% solution, soluble in purified water upon boiling. Solution is light to medium amber, slightly opalescent, may have a slight precipitate.
Prepared Appearance:	Light to medium amber, slightly opalescent.

Reaction of 3.8% Solution at 25°C:	pH 7.3 ± 0.1
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BBL™ Mueller Hinton II Agar

Dehydrated Appearance:	Fine, dry, homogeneous, free of extraneous material.
Solution:	3.8% solution, soluble in purified water upon boiling. Solution is light to medium, yellow to tan, trace hazy to slightly hazy.
Prepared Appearance:	Light to medium, yellow to tan, trace hazy to slightly hazy.

Reaction of 3.8% Solution at 25°C:	pH 7.3 ± 0.1
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Cultural Response

Difco™ Mueller Hinton Agar or BBL™ Mueller Hinton II Agar

Prepare the medium per label directions. Using the organisms listed below, inoculate plates, add antibiotic disks and incubate as recommended by NCCLS.⁴ Measure zone diameters and compare to the NCCLS recommended zone ranges.⁴

ORGANISM	ATCC™
<i>Enterococcus faecalis</i>	33186
<i>Escherichia coli</i>	25922
<i>Escherichia coli</i>	35218
<i>Pseudomonas aeruginosa</i>	27853
<i>Staphylococcus aureus</i>	25923
<i>Staphylococcus aureus</i>	43300

Mueller Hinton agar complies with requirements of the World Health Organization¹⁴ 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 NCCLS Document M2, *Performance Standards for Antimicrobial Disk Susceptibility Tests*, recommends Mueller Hinton agar supplemented with 5% defibrinated sheep blood. Details of quality control procedures and interpretive criteria for use with *S. pneumoniae* and other *Streptococcus* spp. are contained in supplemental tables.⁵ These documents should be consulted for additional details.^{4,5}

Principles of the Procedure

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 NCCLS 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}

Formulae

Difco™ Mueller Hinton Agar

Approximate Formula* Per Liter	
Beef Extract Powder	2.0 g
Acid Digest of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g

BBL™ Mueller Hinton II Agar

Approximate Formula* Per Liter	
Beef Extract	2.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g

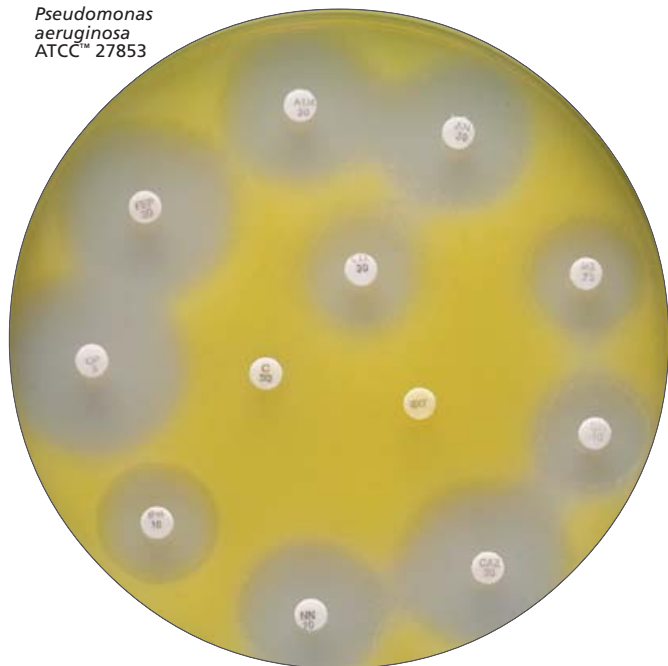
*Adjusted and/or supplemented as required to meet performance criteria.

Directions for Preparation from Dehydrated Product

1. Suspend 38 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes. DO NOT OVERHEAT. OPTIONAL: Cool medium to 45-50°C and aseptically add 5% sterile defibrinated sheep blood.
4. Pour cooled Mueller Hinton agar into sterile Petri dishes on a level, horizontal surface to give a uniform depth of about 4 mm (60-70 mL of medium for 150 mm plates and 25-30 mL for 100 mm plates) and cool to room temperature.⁴
5. Check prepared medium to ensure the final pH is 7.3 ± 0.1 at 25°C.
6. Test samples of the finished product for performance using stable, typical control cultures.

Mueller Hinton II Agar

Pseudomonas aeruginosa
ATCC™ 27853



Mueller Hinton Agar with 5% Sheep Blood

Streptococcus pneumoniae
ATCC™ 49619



Procedure

A. Standard Method⁴

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 2×10^8 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.
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 strains¹⁷ 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 Method⁴

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.

NOTE: 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.¹⁸

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% CO₂.

Expected Results

Zone diameters measured around discs should be compared with those in the NCCLS 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. Zone diameters for the agents specified under “Intended Use” should be compared with those in the NCCLS Document M100 (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 mg/mL) to penicillin. NCCLS Document M100 (M2) should be consulted for other antimicrobial agents to which penicillin-susceptible isolates may also be considered susceptible.⁴

NOTE: Informational supplements to NCCLS Document M2, 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 informational supplements can be ordered from the National Committee for Clinical Laboratory Standards, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898. Telephone: (610) 688-1100.

Refer to other texts for additional information on antimicrobial susceptibility testing.^{19,20} Protocols developed by the NCCLS and used by manufacturers to evaluate the performance of Mueller Hinton Agar in comparison to a reference medium are published in NCCLS document M6-A.²¹

Limitations of the Procedure

1. Numerous factors can affect results: inoculum size; rate of growth; medium formulation and pH, length of incubation and incubation environment; disc content and drug diffusion rate; and measurement of endpoints. Therefore, strict adherence to protocol is required to ensure reliable results.²²
2. When Mueller Hinton agar is supplemented with blood, the zone of inhibition for oxacillin and methicillin may be 2-3 mm smaller than those obtained with unsupplemented agar.²³ Conversely, sheep blood may markedly increase the zone diameters of some cephalosporins when they are tested against enterococci.²⁴ Sheep blood may cause indistinct zones or a film of growth within the zones of inhibition around sulfonamide and trimethoprim discs.²³
3. Mueller Hinton agar deeper than 4 mm may cause false-resistant results, and agar less than 4 mm deep may be associated with a false-susceptibility report.²³
4. A pH outside the range of 7.3 ± 0.1 may adversely affect susceptibility test results. If the pH is too low, aminoglycosides and macrolides will appear to lose potency; others may appear to have excessive activity.²³ The opposite effects are possible if the pH is too high.²³

References

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23. Wood and Washington. 1995. In Murray, Baron, Pfaller, Tenover and Tenover (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
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Availability

Difco™ Mueller Hinton Agar

BAM	BS10	CMPH	ISO	MCM7	NCCLS
Cat. No.	225250	Dehydrated – 500 g			
	225220	Dehydrated – 2 kg			
	225230	Dehydrated – 10 kg			

BBL™ Mueller Hinton II Agar

BAM	BS10	CMPH	ISO	MCM7	NCCLS
Cat. No.	211438	Dehydrated – 500 g			
	211441	Dehydrated – 5 lb (2.3 kg)			
	212257	Dehydrated – 25 lb (11.3 kg)			
	298034	Dehydrated (LitrePak™) – 20 × 1.0 L			

United States and Canada

Cat. No.	221177	Prepared Plates – Pkg. of 20*
	221275	Prepared Plates – Ctn. of 100*
	221994	Prepared Plates (150 × 15 mm) – Pkg. of 8*
	221800	Prepared Plates (150 × 15 mm) – Box of 24*

Europe

Cat. No.	254032	Prepared Plates – Pkg. of 20*
	254081	Prepared Plates – Ctn. of 120*
	255062	Prepared Plates (150 × 15 mm-style) – Pkg. of 20*
	254518	Prepared Plates (square 120 × 120 mm-style) – Pkg. of 20*

Japan

Cat. No.	251177	Prepared Plates – Pkg. of 20*
	251275	Prepared Plates – Ctn. of 100*
	251579	Prepared Plates – Ctn. of 200*
	251800	Prepared Plates (150 × 15 mm-style) – Pkg. of 24*

BBL™ Mueller Hinton Agar with 5% Sheep Blood

BS10 CMPH MCM7 NCCLS

United States and Canada

Cat. No.	221176	Prepared Plates – Pkg. of 20*
	221993	Prepared Plates (150 × 15 mm) – Pkg. of 8*
	221801	Prepared Plates (150 × 15 mm) – Box of 24*

Europe

Cat. No.	254030	Prepared Plates – Pkg. of 20*
	254080	Prepared Plates – Ctn. of 120*
	255080	Prepared Plates (150 × 15 mm-style) – Pkg. of 20*
	254517	Prepared Plates (square 120 × 120 mm-style) – Pkg. of 20*

Japan

Cat. No.	251176	Prepared Plates – Pkg. of 20*
	251262	Prepared Plates – Ctn. of 100*
	251801	Prepared Plates (150 × 15 mm-style) – Pkg. of 24*

*Store at 2-8°C.

Mueller Hinton Chocolate Agar

Intended Use

Mueller Hinton Chocolate Agar is for use in qualitative procedures for the isolation and cultivation of fastidious organisms, particularly *Haemophilus* species. It was formerly recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for antimicrobial susceptibility testing of *H. influenzae*. However, it has been replaced in this procedure by Haemophilus Test Medium (HTM) Agar.

Summary and Explanation

Mueller Hinton Agar was originally developed for the cultivation of pathogenic *Neisseria*.¹ In the 1960s, Bauer, Kirby and others developed a standardized disc diffusion procedure for determining the susceptibility of bacteria to antibiotic and chemotherapeutic agents in which Mueller Hinton Agar was selected as the test medium.²

Because growth of fastidious organisms was poor, the use of Mueller Hinton Agar supplemented with 1% hemoglobin and a defined supplement was adopted for testing of *H. influenzae*.³

Known as Mueller Hinton Chocolate Agar, this formulation was replaced for this purpose by HTM Agar.⁴

The medium is now recommended for routine cultivation of fastidious organisms.

Principles of the Procedure

The primary nutrients in Mueller Hinton Chocolate Agar are beef extract, which provides nitrogenous nutrients, vitamins and minerals required for microbial growth, and acid hydrolysate of casein, which provides amino acids with the exception of cystine (because casein contains little cystine) and tryptophan (which is destroyed by the acid treatment, as are vitamins). The starch neutralizes toxic fatty acids that may be present in the agar. Hemoglobin provides X factor (hemin) for *Haemophilus* species. BBL™ IsoVitalEx™ Enrichment is a defined supplement that provides V factor (nicotinamide adenine dinucleotide, NAD) for *Haemophilus* species and vitamins, amino acids, co-enzymes, dextrose, ferric ion and other factors for improved growth of fastidious organisms; i.e., pathogenic *Neisseria*.