

QUALITY CONTROL PROCEDURES

I INTRODUCTION

Haemophilus Test Medium Agar is for use in the antimicrobial disc diffusion susceptibility procedure for *Haemophilus* species.

II PERFORMANCE TEST PROCEDURE

1. Inoculate representative samples with the cultures listed below.

a. Preparation of inoculum.

Prepare a suspension of the organism in Mueller Hinton Broth or Mueller Hinton II Broth. Adjust the suspension to 0.1 absorbance units at 625 nm. This suspension will contain approximately 1 to 4 x 10⁸ CFU/mL.

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. Using either the 6-place or 8-place **Sensi-Disc** Dispenser for 100 mm style dishes and the 12-place **Sensi-Disc** Designer Dispenser System for 150 mm-style dishes, place the appropriate discs onto the respective cultures. No more than four antimicrobial discs should be placed on a single 100-mm plate and no more than nine antimicrobial discs should be placed on a single 150-mm plate, including not more than six of the following: third generation cephalosporins, aztreonam, imipenem or ciprofloxacin.

f. Within 15 min after the discs are applied, invert the plates and incubate at 35 ± 2°C in an aerobic atmosphere enriched with 5% carbon dioxide.

2. Examine plates after 16 – 18 h and 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; disregard 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 Organisms:

**Haemophilus influenzae* ATCC™ 49247

**Haemophilus influenzae* ATCC 49766

Haemophilus influenzae ATCC 10211

(For verification of growth promotion properties.)

b. Zone sizes should fall within the ranges of acceptable zone diameter quality control limits for *H. influenzae* specified by the Clinical and Laboratory Standards Institute (formerly NCCLS). These limits are published in CLSI Document M100-S17 (M2), which is included with CLSI Document M2-A9, *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed.; Approved Standard.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 box under "RESULTS" in the PRODUCT INFORMATION section for additional information.

c. Growth of *H. influenzae* ATCC 10211 should be moderate to heavy.

*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 at 35 ± 2°C for 72 h and examine for microbial contamination.

PRODUCT INFORMATION

IV INTENDED USE

Haemophilus Test Medium Agar (HTM Agar) is intended for use in the antimicrobial disc diffusion susceptibility procedure for *Haemophilus* spp. as described in the Approved Standard M2-A9, published by the Clinical and Laboratory Standards Institute (CLSI).¹

V SUMMARY AND EXPLANATION

In 1966, Bauer, Kirby and others developed a standardized procedure for the antimicrobial susceptibility testing of common, rapidly growing bacteria in which Mueller Hinton Agar was selected as the test medium.²⁻⁴ This medium is not satisfactory for fastidious organisms such as some streptococci, gonococci and *Haemophilus* species.

Mueller Hinton Agar supplemented with 1% hemoglobin and 1% **IsoVitalex™** Enrichment (Mueller Hinton Chocolate Agar) was the medium previously recommended for *Haemophilus influenzae*.⁵ Extensive studies performed by Jorgensen and colleagues led to the development of Haemophilus Test Medium (HTM).^{6,7} This medium is Mueller Hinton agar or broth supplemented with X factor (hemin or hematin), V factor (nicotinamide adenine dinucleotide, NAD) and yeast extract.

A major advantage of HTM Agar compared with Mueller Hinton Chocolate Agar is optical clarity, permitting zone diameter measurements from the bottom of the dish as is the standard test procedure for nonfastidious organisms on Mueller Hinton Agar. Further, HTM Agar contains low levels of thymidine and is, therefore, suitable for testing trimethoprim/sulfamethoxazole.

Interpretive criteria for the antimicrobial susceptibility testing of *Haemophilus* are provided in the CLSI document M100-S17 (M2),⁸ which is included with CLSI document M2-A9.¹ This document should be consulted for further details.

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. In contrast to earlier methods which used discs of high and low antimicrobial concentrations, and 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).^{1-3,9}

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, resistant or intermediate in its response to the agent is made by comparing zone diameters obtained to those in CLSI document M100-S17 (M2).⁸

Various factors have been identified as influencing disc diffusion susceptibility tests. These include the medium, agar depth, disc potency, inoculum concentration, pH and β -lactamase production by test organisms.^{1,5,9,10}

VII REAGENTS

Haemophilus Test Medium Agar

Approximate Formula* Per Liter Purified Water

Beef Extract	2.0 g	Yeast Extract.....	5.0 g
Acid Hydrolysate of Casein	17.5 g	Hematin	15.0 mg
Starch	1.5 g	Nicotinamide Adenine Dinucleotide	15.0 mg
Agar	17.0 g		

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

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

If excessive moisture is observed, invert 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 time. 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 or other signs of deterioration.

VIII SPECIMEN COLLECTION AND HANDLING

This medium is not intended for direct use with clinical specimens or mixed cultures. The disc diffusion susceptibility test is designed for use with pure cultures. A Gram stain and a presumptive identification of *Haemophilus* are required.

Refer to appropriate texts for details of specimen collection and handling procedures.^{11,12}

IX PROCEDURE

Material Provided: Haemophilus Test Medium Agar

Materials Required But Not Provided:

1. Inoculum broth tubed in 5 mL amounts, such as Mueller Hinton Broth, Mueller Hinton II Broth, or 0.9% saline for preparation of standard inoculum.
2. A 0.5 McFarland barium sulfate standard for adjustment of inoculum (prepared by adding 0.5 mL of 0.048 M BaCl₂ [1.175% w/v BaCl₂ • 2 H₂O] to 99.5 mL of 0.18 M [0.36 N] H₂SO₄ [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.^{13,14}
5. Control cultures - *Haemophilus influenzae* ATCC 49247, ATCC 49766 and ATCC 10211.
6. Paper discs impregnated with specified amounts of antimicrobial agents, such as BBL Sensi-Disc susceptibility test discs.
7. Disc dispensing device, such as the Sensi-Disc Self-Tamping 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 Alternatively, a Sensi-Disc Zone Interpretation Set (Cat. No. 260639), consisting of pattern templates and zone measurement labels, is available for this purpose.
9. A reagent or device for performing a rapid β -lactamase test, such as BBL Cefinase™ Discs.
10. An incubator that produces an atmosphere containing 5% CO₂, or another device that produces a CO₂-enriched aerobic atmosphere.
11. Ancillary culture media, reagents, and laboratory equipment as required.

Test Procedure:

1. Prepare a Gram stain before starting susceptibility testing to confirm culture purity and to confirm tentative identification of *Haemophilus*.
2. Use several well-isolated colonies taken directly from an overnight (preferably 20 – 24 h) Chocolate Agar plate as the source of the inoculum.
3. A rapid β -lactamase test should be utilized for rapid detection of strains that are resistant to penicillin, ampicillin or amoxicillin.
4. Prepare a suspension of the test organism in Mueller Hinton Broth, Mueller Hinton II Broth or 0.9% saline. This suspension should be adjusted to the turbidity of the 0.5 McFarland standard using a photometric device. This suspension will contain approximately 1 to 4 x 10⁸ CFU/mL. Care must be exercised in preparing this suspension because higher inoculum concentrations may lead to false-resistant results with some β -lactam antibiotics, particularly when β -lactamase producing

strains of *H. influenzae* are tested.¹ Periodically perform dilutions and plate counts of inoculum suspensions to confirm that the adjustment method employed produces an inoculum containing approximately 1 to 4 x 10⁸ CFU/mL.

- 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.¹³ This system has also been found to be satisfactory for testing *H. influenzae*.¹⁴
- 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.
- 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.
- The lid may be left ajar for 3 – 5 min and the plate held at room temperature, but no longer than 15 min, to allow any surface moisture to be absorbed before applying the drug-impregnated discs.
- Apply the discs by means of an antimicrobial disc dispenser, using aseptic precautions. Most antimicrobial agents produce larger zones of inhibition when tested against *Haemophilus* compared with other organisms. **Therefore, no more than four antimicrobial discs should be placed on a single 100-mm plate and no more than nine antimicrobial discs should be placed on a single 150-mm plate, including not more than six of the following discs: third generation cephalosporins (e.g., cefotaxime, ceftazidime, ceftriaxone, ceftizoxime), aztreonam, imipenem, or ciprofloxacin.** 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 Self Tamping 12-Place Dispenser (tamper will not descend from holes lacking cartridges).
- Within 15 min after the discs are applied invert the plates and incubate for 16 – 18 h at 35°C in an aerobic atmosphere enriched with 5% carbon dioxide.
- Streak a plate of HTM Agar with *H. influenzae* ATCC 10211 and incubate along with the susceptibility test plates to determine whether the medium supports adequate growth.

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-S17 (M2).⁸

X RESULTS

- Examine the plates after 16 – 18 h of incubation. 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 calipers, a ruler, or a template prepared for this purpose. The measuring device is held on the back of the plate, which is held 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. 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.
- Refer to CLSI document M100-S17 (M2) for interpretation of results obtained with clinical isolates of *Haemophilus*.⁸ Results may be reported as resistant, intermediate or susceptible depending on the zone diameters obtained.

NOTE: Supplemental tables to CLSI Document M2-A9, containing revised tables of antimicrobial discs and interpretive standards are published periodically. The latest tables should be consulted for current recommendations. For information on current publications, call BD Technical Services at (800) 638-8663. The complete standard and informational supplements can be ordered from the Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898. Telephone: (610) 688-1100.

Organisms testing positive for β-lactamase production should be considered resistant to ampicillin regardless of the zone diameters obtained. It should be noted that ampicillin-resistant strains of *H. influenzae* have been described which lack β-lactamase activity.¹⁵ Therefore, if the zone diameter indicates resistance to ampicillin, the isolate should be reported as resistant to that drug, even if the β-lactamase test is negative.

XI LIMITATIONS OF THE PROCEDURE

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 inaccurate 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.

No more than four antimicrobial discs should be placed on a single 100-mm plate and no more than nine antimicrobial discs should be placed on a single 150-mm plate, including not more than six of the following discs: third generation cephalosporins (e.g., cefotaxime, ceftazidime, ceftriaxone, ceftizoxime), aztreonam, imipenem or ciprofloxacin.

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 texts for guidance in the interpretation of results.^{5,10}

XII PERFORMANCE CHARACTERISTICS

A. Reproducibility Study¹⁶

Three production lots of HTM Agar were evaluated using the antimicrobial disc susceptibility test procedure (M2-A4) recommended by the NCCLS at that time.¹⁷ A total of 12 antimicrobial agents (amoxicillin/clavulanate, ampicillin, ampicillin/sulbactam, cefaclor, cefonicid, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, rifampin, tetracycline, trimethoprim/sulfamethoxazole) and 14 strains of *H. influenzae* were used in the study. Of the 14 strains, seven were American Type Culture Collection (ATCC) strains and the remaining seven were stock cultures, which included six clinical isolates and an industrial quality control culture. The zone diameters obtained with each of the three lots of media were compared with the interpretive criteria (Table 2A) in M2-A4.¹⁷ In only six cases were zones obtained with one lot that would produce a different interpretive category compared with the other two lots. Of the six discrepancies, there were four with tetracycline, one with cefaclor and one with cefuroxime. In the case of tetracycline, the result would have

produced an interpretive category of either susceptible or intermediate (minor error). With cefaclor and cefuroxime, the 1 mm difference that occurred would result in either an intermediate or resistant category, also a minor error.

In this study there were five strains of *H. influenzae* that produced β -lactamase. With each of these strains, each HTM lot produced zone diameters with ampicillin that would be interpreted as being resistant. Two of the five strains also produced chloramphenicol acetyltransferase (CAT). The chloramphenicol zones obtained with these two strains would be consistent with chloramphenicol resistance.^{18,19}

B. Comparison with Mueller Hinton Chocolate Agar¹⁶

The NCCLS disc procedure was performed with 213 clinical isolates of *H. influenzae* on HTM Agar and Mueller Hinton Chocolate Agar.^{17,20} The antimicrobial agents tested were ampicillin, ampicillin-sulbactam, amoxicillin-clavulanate and chloramphenicol. These were the only antimicrobial agents for which zone diameter interpretive criteria had been defined for Mueller Hinton Chocolate Agar.^{21,22} The following results were obtained.

		Haemophilus Test Medium Agar		
		S	I	R
Mueller Hinton	S	808	1	2
Chocolate Agar	I	0	0	0
	R	0	0	41

Agreement: 99.6% S = Susceptible I = Intermediate R = Resistant

There were two major errors and one minor error with ampicillin. In both major errors HTM produced a resistant result, whereas Mueller Hinton Chocolate Agar produced a susceptible result. Both of these isolates were β -lactamase positive. Therefore, the resistant result was correct. The one minor error was with an isolate that produced an intermediate result on HTM Agar and a susceptible result on Mueller Hinton Chocolate Agar.

XIII AVAILABILITY

Cat. No.	Description
221954	BBL™ Haemophilus Test Medium Agar, Pkg. of 8 (150 x 15 mm-style) plates
221992	BBL™ Haemophilus Test Medium Agar, Pkg. of 10 (100 x 15 mm-style) plates

XIV REFERENCES

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