BBL™ Mueller Hinton II Broth (Cation-Adjusted)

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QUALITY CONTROL PROCEDURES

I INTRODUCTION

Mueller Hinton II Broth is cation-adjusted for calcium and magnesium ions and is used for quantitative susceptibility testing of gram-negative and gram-positive aerobic bacteria with a variety of antimicrobial agents.

II PERFORMANCE TEST PROCEDURE

1. Inoculate representative samples with the cultures listed below.
   a. Inoculate the tubes using sterile pipettes with a dilution containing approximately 1000 CFU/0.1 mL. Mix well.
   b. Incubate tubes with loosened caps at 35 ± 2 °C in an aerobic atmosphere. Include an uninoculated tube as a growth control.
2. Examine tubes after 18–24 h for growth.
3. Expected Results

Organisms	ATCC®	Recovery
**Enterococcus faecalis*	29212	Growth
**Escherichia coli*	25922	Growth
**Pseudomonas aeruginosa*	27853	Growth
**Staphylococcus aureus*	29213	Growth

*Recommended organism strain for User Quality Control.

III ADDITIONAL QUALITY CONTROL

1. Examine tubes as described under “Product Deterioration.”
2. Visually examine representative tubes 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. Incubate uninoculated representative tubes at 20–25 °C and 30–35 °C and examine after 7 days for microbial contamination.

PRODUCT INFORMATION

IV INTENDED USE

Mueller Hinton II Broth is intended for use in quantitative procedures for susceptibility testing of rapidly-growing aerobic and facultatively anaerobic bacteria isolated from clinical specimens. It is formulated to have a low thymine and thymidine content and is adjusted to the calcium and magnesium ion concentrations recommended in the CLSI standard M7-A7.[1]

V SUMMARY AND EXPLANATION

The development of laboratory tests to determine the activity of antimicrobial agents has paralleled the development of these agents. In 1929, Fleming used a serial dilution technique to measure the lowest concentration of penicillin that prevented growth of a test organism in broth.[2] Ericsson and Sherris published an excellent review of the various methods for susceptibility testing and the relationship of dilution and diffusion methods.[3]

Rammelkamp and Maxon were among the earliest to use the tube dilution test to determine the *in vitro* antimicrobial susceptibility of bacteria isolated from clinical specimens.[4] The development of this test resulted from the need to know why some patients infected with *Staphylococcus aureus* did not respond to penicillin therapy.

The tube dilution test (broth dilution) involves exposing bacteria to decreasing concentrations of antimicrobial agents in liquid media, usually by serial 2-fold dilution.

The mixture, consisting of microorganisms, nutrient medium and antimicrobial agent, is incubated at 35 °C for 16–20 h. The lowest concentration of antimicrobial agent at which no visible growth occurs is defined as the minimal inhibitory concentration (MIC).

Mueller Hinton Broth is the medium usually used for dilution antimicrobial susceptibility tests. This medium is supplemented with calcium and magnesium salts to produce correct MICs with aminoglycosides and *Pseudomonas aeruginosa*.[1]

The term “microdilution” appeared in the literature in 1970 to describe the minimal inhibitory concentration tests performed with volumes of 0.1 mL or less of antimicrobial solution.[5] Correlations between MIC values using microdilution and tube dilution methodologies have been reported to be between 85 and 96%.[6,7]

The qualitative disc diffusion antimicrobial susceptibility procedure has been standardized since 1966.[8] The rationale for an MIC susceptibility test rather than the disc diffusion test is that it gives quantitative information. It provides a relationship between the amount of antimicrobial agent required to inhibit the growth of an organism *in vitro* and the achievable concentrations in the blood, urine, cerebrospinal fluid or bile, under various dosage conditions. It has been suggested that in the treatment of systemic infections, the drug dosage should yield a peak concentration at the site of infection that is two to four times greater than the MIC value, while for urinary tract infections, a peak urine concentration of 10 to 20 times the MIC value should be achieved.[9] However, effective antimicrobial therapy also depends on many other factors.[10]

VI PRINCIPLES OF THE PROCEDURE

The acid hydrolysate of casein and beef extract provide nutrients for growth of test organisms. These ingredients are selected for low thymine and thymidine content as determined by MIC values with *Enterococcus faecalis* and sulfamethoxazole-trimethoprim (SXT). Calcium and magnesium ion concentrations are adjusted to provide the amounts recommended by CLSI[1] to give the correct MIC values with aminoglycosides and *Pseudomonas aeruginosa*. The pH has been adjusted to the specification in M7-A7.

Antimicrobial agents are prepared in serial 2-fold dilutions in Mueller Hinton II Broth and are inoculated with the test culture to give a final concentration of 5 x 10^5 CFU/mL. Following incubation at 35 °C, the presence of turbidity indicates growth of the organism. The lowest concentration of antimicrobial agent showing no growth is the MIC of that organism for that agent.

VII REAGENTS

Mueller Hinton II Broth (Cation-Adjusted)

Approximate Formula* Per Liter Purified Water

Beef Extract	3.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g

*Adjusted and/or supplemented as required with appropriate salts toprovide 20–25 mg/L of calcium and 10–12.5 mg/L of magnesium andas additionally required to meet performance criteria.

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

Tubes with tight caps should be opened carefully to avoid injury due to breakage of glass.

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

Storage Instructions: On receipt, store tubes in the dark at 2–25 °C. Avoid freezing and overheating. Do not open until ready to use. Tubed media stored as labeled until just prior to use may be inoculated up to the expiration date and incubated for the recommended incubation times. Minimize exposure to light.

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

VIII SPECIMEN COLLECTION AND HANDLING

Specimens suitable for culture may be handled using various techniques. For detailed information, consult appropriate texts.[11,12] Specimens should be obtained before antimicrobial agents have been administered. Provision must be made for prompt delivery to the laboratory.

IX PROCEDURE

Material Provided: Mueller Hinton II Broth (Cation-Adjusted)

Materials Required But Not Provided: Ancillary culture media, reagents, quality control organisms and laboratory equipment as required.

Test Procedure: Observe aseptic techniques.

Mueller Hinton II Broth Cation-Adjusted may be used for inoculum preparation for MIC tests and for preparation of antimicrobial dilutions for the microdilution or macrodilution procedure. Details for the preparation of antimicrobial agents are provided in reference 1.

1. Inoculum Standardization
   a. Using aseptic technique, pick three to five isolated colonies of the same organism from an 18- to 24-h **Trypticase™** Soy Agar with 5% Sheep Blood (TSA II) plate and inoculate into 5 mL of Mueller Hinton II Broth.
   b. Incubate 2–6 h at 35 °C. Periodically check turbidity against the McFarland turbidity 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 [0.36N] H_2SO_4 [1% v/v]).
      1) If comparable, go to 3, Inoculation of Antimicrobial Dilutions.
      2) If too turbid, dilute aseptically with additional Mueller Hinton II Broth and repeat turbidity check. If turbidity is comparable to the standard, go to 3, Inoculation of Antimicrobial Dilutions.
      3) If not turbid enough, continue incubation. When turbidity is comparable to the standard, go to 3, Inoculation of Antimicrobial Dilutions.
      4) Suspensions of test organisms must be used within 15 min of standardization.
2. Alternative Direct Inoculum Standardization

   A stationary phase culture may also be used. In this method, skip step number 1b and simply suspend enough colonies in the broth to equal the turbidity of the 0.5 McFarland standard.
3. Inoculation of Antimicrobial Dilutions
   a. The amount of inoculum depends on the procedure used.[1] The standardized inoculum prepared above will contain approximately 1–2 x 10^8 CFU/mL. The final concentration in a well (or tube) should be 5 x 10^5 CFU/mL (*not* CFU/tube or well).
   b. Macrodilution (tube) method

      If the volume of antimicrobial solution in the tube is 1 mL, dilute the standardized inoculum 1:100 in Mueller Hinton II Broth (0.1 mL to a 10-mL tube of broth). Add 1.0 mL of the adjusted inoculum to each tube containing an antimicrobial agent and 2.0 mL to a sterile empty tube for a growth control.
   c. Microdilution method

      In this method, the antimicrobial dilutions are made in sterile plastic trays with round or conical-shaped wells. The volume is either 0.05 or 0.1 mL in each well. If the volume in the well is 0.1 mL, dilute the inoculum 1:10 and add 0.005 mL of the inoculum per well, using a replicator. One well in each tray should contain 0.1 mL of broth without any antimicrobial agent (growth control well).

      If a dropper (0.05 mL) is used for the inoculum and the volume of antimicrobial solution is 0.05 mL, this results in a 1:2 dilution. Therefore, dilute the inoculum 1:100 and add 0.05 mL to each well to obtain the final concentration of 5 x 10^5 CFU/mL (5 x 10^4 CFU/well). Add 0.05 mL of inoculum to a well containing 0.05 mL of broth without any antimicrobial agent (growth control well). After the trays are inoculated, cover with tape or a tight-fitting lid to prevent evaporation.
4. Incubation

   Incubate the tubes or trays (stacked no more than four high) at 35 °C for 16–20 h (do not use a CO_2 incubator).

   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 quality control MIC ranges will be found in M100-S16 (M7), which is included with CLSI Document M7-A7.[1]

   User Quality Control: See “Quality Control Procedures.”

   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 guidance and CLIA regulations for appropriate Quality Control practices.

X RESULTS

The minimal inhibitory concentration (MIC) of an antimicrobial agent for a specific organism is the lowest concentration which will inhibit the growth of the organism. Growth is indicated by turbidity or sediment. Some microorganisms when tested against trimethoprim/sulfamethoxazole or sulfonamides alone do not always give clear-cut endpoints. In the case of doubling dilutions of trimethoprim/sulfamethoxazole, there may be a "trailing" of growth. Such a pattern typically shows an obvious reduction in the amount of growth and, then, either small pellets (usually less than 1 mm in diameter) in the rest of the wells, or an obvious reduction in the amount of growth and then a slight but detectable graduation in the size of the pellets. In these cases, the MIC endpoint should be identified as the lowest concentration of antimicrobial agent beyond which there is no further reduction in the size of the pellet or amount of turbidity.

An organism may be susceptible, intermediate or resistant for a given antimicrobial agent depending on the MIC value. Interpretive standards for MIC values with various drugs may be found in CLSI document M100-S16 (M7)[1] or may be obtained from the drug manufacturer.

NOTE: Supplemental tables to CLSI Document M7-A7, containing revised tables of antimicrobial agents 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 supplements 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

For identification, organisms must be in pure culture. Morphological, biochemical, and/or serological tests should be performed for final identification. Consult appropriate texts for detailed information and recommended procedures.[11-13]

The efficacy of this medium has not been established for all microorganisms that might be isolated from clinical specimens. If growth is inadequate, i.e., turbidity that cannot be seen by the naked eye, the MIC values may *not* be valid. Always include a growth control tube or well that contains the inoculated medium but no antimicrobial agent. If no growth is seen, repeat testing or use an alternative procedure.

Microorganisms that require thymine or thymidine may be encountered in clinical specimens.[14] These organisms may not grow in Mueller Hinton II Broth which is formulated with low levels of thymine and thymidine. Fastidious organisms such as *Haemophilus*, *Neisseria* and certain streptococci also will not grow or will grow poorly in this medium.

Incubation temperatures above the recommended temperature of 35 °C may result in false susceptibility of methicillin-resistant staphylococci (MRSA).[15] These organisms should be tested with oxacillin in broth containing 2% NaCl, using the direct inoculum standardization method and incubated a full 24 h.[16]

The use of an incorrect concentration of bacterial suspension for inoculation of the antimicrobial dilutions may result in incorrect MIC values.

Strains of *S. aureus* and coagulase-negative staphylococci resistant to methicillin should be reported as resistant to cephems and other beta-lactams, regardless of the *in vitro* test result.

Strains of staphylococci and enterococci that produce beta-lactamase may give false penicillin or ampicillin MIC values and should be tested for presence of beta-lactamase.[17] A recommended procedure is the use of **BBL Cefinase™** discs.

In vitro susceptibility of an organism to a specific antimicrobial agent does not mean that it will be effective as a therapeutic agent *in vivo*. Consult appropriate references for details on interpretation of results.[9,10,17-22]

Accurate detection of vancomycin-resistant enterococci requires incubation for a full 24 h; examine tubes or wells carefully for evidence of faint growth.[1]

High-level resistance to aminoglycosides is an indication that an enterococcal isolate will not be affected synergistically by a combination of a penicillin or glycopeptide plus an aminoglycoside. High-concentration gentamicin (500 µg/mL) and streptomycin (1000 µg/mL) tests can be used to screen for this type of resistance. Other aminoglycosides need not be tested because their activities against enterococci are not superior to gentamicin or streptomycin.[1]

For a discussion on the detection of extended-spectrum, ß-lactamase-producing, gram-negative bacilli, refer to CLSI document M7-A7.[1]

Mueller Hinton II Broth described above for the rapidly growing aerobic pathogens is not adequate for susceptibility testing of fastidious organisms. If MIC tests are to be done with fastidious organisms, the medium, quality control procedures and interpretive criteria must be modified to fit each organism; e.g., *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Streptococcus pneumoniae*.[1]

Skipped-Dilution Phenomenon:

In broth dilution susceptibility tests, a skipped well or dilution occasionally occurs. Skipped wells or dilutions result in an interruption in the growth-no growth pattern in a row of wells or series of tubes. As a result, there are multiple endpoints in a dilution series of a specific antimicrobial agent. Skipping may be caused by any of the following: bacterial genetic variability, contamination, deterioration or absence of the antimicrobial agent, or improper technique in inoculation of the wells.

It is recommended that MIC values not be reported for an antimicrobial agent-organism combination that exhibits skips. Consult the physician to determine if a repeat test is needed.

XII PERFORMANCE CHARACTERISTICS

Prior to release, all lots of Mueller Hinton II Broth (Cation-Adjusted) are tested for performance characteristics. Representative samples of the lot are tested with *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212) by inoculating tubes with approximately 1000 CFU/0.1 mL. After 18–24 h incubation at 35 ± 2 °C, all organisms exhibit growth.

Additionally, representative samples of the lot are tested for calcium and magnesium content by atomic absorption assay or ion chromatography.

XIII AVAILABILITY

Cat. No.	Description
297701	**BD BBL™** Mueller Hinton II Broth (Cation-Adjusted), 5 mL, Pkg. of 10 size K tubes
298268	**BD BBL™** Mueller Hinton II Broth (Cation-Adjusted), 5 mL, Ctn. of 100 size K tubes
297310	**BD BBL™** Mueller Hinton II Broth (Cation-Adjusted), 250 mL Bottle
297963	**BD BBL™** Mueller Hinton II Broth (Cation-Adjusted), 400 mL Bottle

XIV REFERENCES

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