BD™ Mueller Hinton II Agar
BD Mueller Hinton II Agar 150 mm
BD Mueller Hinton II Agar, Square

INTENDED USE

BD Mueller Hinton II Agar, available in several plate formats, is used in the standardized disc diffusion procedure for determining the susceptibility of clinical isolates of rapidly-growing aerobic organisms to antimicrobial agents as standardized by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[1,2]

PRINCIPLES AND EXPLANATION OF THE PROCEDURE

Microbiological method.

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, a medium originally devised for the isolation of gonococci, was selected as the test medium.[1-5] 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.[6]

CLSI and EUCAST have issued performance standards for the Bauer-Kirby procedure and these documents should be consulted for details.[7,8] Other national standards have been developed for antimicrobial susceptibility testing according to the Bauer-Kirby procedure. In those standards, the inoculum densities, the method of inoculation, the resulting zone sizes and the mode of interpretation may vary from the CLSI and EUCAST recommendations.

The Bauer-Kirby procedure is based on the diffusion through an agar gel of antimicrobial substances which are impregnated on paper discs.[9] 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).[1-3,7-9]

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 (S), intermediate (I) or resistant (R) to an agent is made by comparing zone sizes obtained to those in Tables 2A to 2D in the CLSI Document M100 (M2).[10] The determination as to whether the organism is susceptible (S) or resistant (R) to an agent is made by comparing zone sizes obtained to those listed in the EUCAST Breakpoint tables.[11]

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 ß-lactamase production by test organisms.[6-9,12]

BD Mueller Hinton II Agar is manufactured to contain low levels of thymine and thymidine, and controlled levels of calcium and magnesium.[15-17] Thymine and thymidine levels of raw materials are determined using the disc diffusion procedure with trimethoprim-sulfamethoxazole (SXT) discs and *Enterococcus faecalis* 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.
The agar depth of each **BD Mueller Hinton II Agar** plate format was adjusted to meet both CLSI and EUCAST recommendations.[7,8]

REAGENTS

BD Mueller Hinton II Agar

Formula* Per Liter Purified Water

Beef Extract	2.0 g
Acid Hydrolysate of Casein	17.5
Starch	1.5
Agar	17.0

pH 7.3 +/- 0.2
*Adjusted and/or supplemented as required to meet performance criteria.

PRECAUTIONS

IVD . For professional use only.
Do not use plates if they show evidence of microbial contamination, discoloration, drying, cracking or other signs of deterioration. Excessive shrinkage of this medium due to desiccation may lead to false susceptibility results.
Consult **GENERAL INSTRUCTIONS FOR USE** document for aseptic handling procedures, biohazards, and disposal of used product.

STORAGE AND SHELF LIFE

On receipt, store plates in the dark at 2 to 8 °C, in their original sleeve wrapping until just prior to use. Avoid freezing and overheating. The plates may be inoculated up to the expiration date (see package label) and incubated for the recommended incubation times.
Plates from opened stacks of 10 plates can be used for one week when stored in a clean area at 2 to 8 °C.

USER QUALITY CONTROL

For user quality control, the appropriate CLSI[10] and EUCAST[18] or, if applicable, national standards should be consulted. Inoculate representative samples with the following strains on each medium (for details, see **Specimen Types** and **Test Procedure**). Incubate the plates, preferably in an inverted position, according to the below indicated temperature, time and atmospheric condition. **BD Mueller Hinton II Agar** plates and the antimicrobial discs used should be tested at least twice weekly for proper performance.

Table 1: Expected results for inhibition zone diameter ranges of quality control strains according to CLSI[19] and EUCAST[20]

Strain	Antimicrobial agent	Range (mm)	Incubation
Escherichia coli ATCC 25922[1]	Ampicillin (10 µg)	15-22	CLSI: 16-18 h, 35±2 °C EUCAST: 16-20 h, 35±1 °C
	Imipenem (10 µg)	26-32	
	Gentamicin (10 µg)	19-26	
	Amikacin (30 µg)	19-26	
	Ciprofloxacin (5 µg)	30-40	
	Trimethoprim-Sulfamethoxazole SXT (1.25 µg-23.75 µg)	23-29	
Escherichia coli ATCC 35218[1]	Amoxycillin/Clavulanic Acid (30 µg)	17-22	CLSI: 16-18 h, 35±2 °C EUCAST: 16-20 h, 35±1 °C
	Ampicillin/Sulbactam (30 µg)	13-19	

Enterococcus faecalis ATCC 29212[2]	Trimethoprim-Sulfamethoxazole (1.25 µg-23.75 µg)	26-34	CLSI: 16-18 h, 35±2 °C EUCAST: 16-20 h, 35±1 °C
	Ciprofloxacin (5 µg)	19-25	
Staphylococcus aureus ATCC 25923[3]	Tetracycline (30 µg)	24-30	CLSI: 16-18 h, 35±2 °C EUCAST: 16-20 h, 35±1 °C
	Gentamicin (10 µg)	19-27	
	Erythromycin (15 µg)	22-30	
	Clindamycin (2 µg)	24-30	
Staphylococcus aureus ATCC 29213[2]	Tetracycline (30 µg)	23-31	CLSI: 16-18 h, 35±2 °C EUCAST: 16-20 h, 35±1 °C
	Gentamicin (10 µg)	19-25	
	Erythromycin (15 µg)	23-29	
	Clindamycin (2 µg)	23-29	
Pseudomonas aeruginosa ATCC 27853[1]	Aztreonam (30 µg)	23-29	CLSI: 16-18 h, 35±2 °C EUCAST: 16-20 h, 35±1 °C
	Amikacin (30 µg)	18-26	
	Gentamicin (10 µg)	17-23	
	Imipenem (10 µg)	20-28	
Uninoculated	Colorless to light amber		

[1]: Results for quality control strains according to CLSI and EUCAST
[2]: Results for quality control strains according to EUCAST
[3]: Results for quality control strains according to CLSI

PROCEDURE

Materials Provided

BD Mueller Hinton II Agar (provided in several different plate formats; see **Packaging/ Availability**). Microbiologically controlled.

Materials Not Provided

1. According to CLSI: Tubed inoculum broth, such as **BD Trypticase™ Soy Broth** (Soybean-Casein Digest Broth) or Mueller Hinton II Broth (cation-adjusted), for preparation of a standard inoculum, and sterile broth or saline for dilution of inoculum.[7]
   According to EUCAST: 0.9 % saline (5 ml amounts) for preparation of standard inoculum.[8]
2. Barium sulfate comparison standard (0.5 ml of 0.048 M $BaCl_2$ [1.175% w/v $BaCl_2 . 2H_2O$] to 99.5 ml of 0.18 M [0.36 N] H_2SO_4 [1% v/v]) or
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 **BD Prompt™ Inoculation System** (volumetric inoculum preparation device) can be used.[7,8,25]
5. Control cultures according to CLSI: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Klebsiella pneumoniae* ATCC 700603.[10]
   Control cultures according to EUCAST: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, and *Staphylococcus aureus* NCTC 12493.[18]
6. Paper discs impregnated with specified amounts of antimicrobial agents,[7,8] such as **BD Sensi-Disc™** susceptibility test discs.
7. Dispensing device, such as the **BD Sensi-Disc** 6-, 8- or 12-place dispenser. A suitable dispenser is also available for Mueller Hinton II Agar Square plates.
8. Ruler or another device for measuring zone size in millimeters.
9. Ancillary culture media, reagents and laboratory equipment as required.

Specimen Types
This product is used for susceptibility testing of pure cultures that have been isolated from clinical specimens (see also **PERFORMANCE CHARACTERISTICS AND LIMITATIONS OF THE PROCEDURE**). It has been proposed that direct antimicrobial susceptibility testing may be performed on blood cultures and urine cultures; however, tests should be repeated and confirmed with pure cultures.[21-24]

Test Procedure
This methodology describes the direct colony suspension method as recommended by CLSI[7] and EUCAST[8]:

1. Assure that a pure, fresh (=overnight) culture from a non-selective medium such as blood agar is available.
   According to CLSI: For routine susceptibility tests, the inoculum may be prepared by making a direct saline or broth suspension of colonies.
   According to EUCAST: For routine susceptibility tests, the inoculum may be prepared by making a direct saline of several morphologically similar colonies.
2. Immediately adjust this suspension to the turbidity of the barium sulfate standard 0.5 McFarland standard without incubation. 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.
3. Alternative methods of inoculum preparation involving devices that permit direct standardization of inocula without adjustment of turbidity, such as the **BD Prompt Inoculation System**, have been found to be acceptable for routine testing purposes.[25]
4. Use the inoculum optimally within 15 min. The suspension must always be used within 60 min of preparation. Immerse 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 and avoid over-inoculation.
5. Inoculate the entire agar surface of the plate three times, rotating the plate 60° between streakings to obtain even inoculation.
6. According to CLSI: The lid may be left ajar for 3 to 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. Apply the dics by means of an antimicrobial disc dispenser, using aseptic precautions. Deposit discs so that the centers are at least 24 mm apart.
   According to EUCAST: Apply the discs of the dried plate within 15 min of inoculation using aseptic precaution. Desposit a maximum of six discs on the plate.
7. 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 **BD Sensi-Disc** self-tamping dispensers.
8. Within 15 minutes after the discs are applied, invert the plates and place them in the incubator. Plates must not be incubated under an increased concentration of carbon dioxide. For recommended incubation conditions refer to CLSI and EUCAST.[7,8,10,11]

Reading of Results

1. After incubation, confluent "lawn" of growth should be visible. If only isolated colonies grow, the inoculum was too light and the test should be repeated.
2. According to CLSI: 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 callipers, a ruler, or a template prepared for this purpose. The measuring device is held on the bottom of the inverted plate over a black, non-reflecting background, and illuminated from above.[7]
   According to EUCAST: 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 millimetre, using sliding callipers, or a ruler. The measuring device is held on the bottom of the inverted plate over a black background illuminated with reflected light. The plate is held about 30 cm from the eye.[8]

3. 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.
4. According to CLSI: *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 or vancomycin-resistant enterococci.[7,26] 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.
   According to EUCAST: In the case of double zones, the inner zone should be measured unless otherwise specifically stated.[8]

Calculation and Interpretation of Results

According to CLSI:

Zone diameters measured around discs should be compared with those in Tables 2A to 2D in the CLSI Document M100 (M2).[10] Results obtained with specific organisms may then be reported as resistant, intermediate or susceptible.

According to EUCAST**:**

Interpret zone diameters by reference to breakpoint tables.[11] Results obtaines with specific organisms may then be reported as resistant or susceptible. For additional information about specific growth characteristics, interpretation and other guidance documents refer to EUCAST.

PERFORMANCE CHARACTERISTICS AND LIMITATIONS OF THE PROCEDURE

Mueller Hinton Agar is the standard medium used for susceptibility testing of rapidly growing aerobic or facultative anaerobic bacterial bacteria, such as staphylococci, enterococci, members of the *Enterobacteriaceae*, and aerobic gram-negative rods (e.g. *Pseudomonas* spp). Guidance documents for the interpretation of sensitivity are updated annually and the most recent version should be consulted for proper interpretation of the obtained results.[10,11] Different procedures, other media and conditions have been developed for testing fastidious species, i.e., *Haemophilus* spp*., Neisseria* spp. and *Streptococcus pneumoniae* and streptococci.

The CLSI and EUCAST standardized procedure is not applied 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.[7,8] Fastidious organisms (e.g. *Haemophilus influenzae*) should be tested as recommended by CLSI and EUCAST.[7,8]

Internal Performance Evaluation

Performance of **BD Mueller Hinton II Agar** (all plate formats) was internally validated using the recommended quality control (QC) strains including those with characterized resistance mechanisms (*Escherichia coli* ATCC 35218, *Klebsiella pneumonia*e ATCC 700603, *Enterococcus faecalis* ATCC 51299, *Staphylococcus aureus* NCTC 12493, *Staphylococcus aureus* ATCC 29213 with Amoxicillin-Clavulanic acid AMC-3).[19,20,34]
Table 2 summarizes the validated antimicrobial agents for the QC strains and strains with characterized resistance mechanisms. Unless stated otherwise, the determined inhibition zone sizes for the validated antimicrobial agents were within the specified CLSI and EUCAST inhibition zone diameter ranges.[19,20]
Additional performance studies using clinical MRSA isolates harbouring the *mecC*-resistance type indicated that **BD Mueller Hinton II Agar** accurately detects *mecC*-positive MRSA strains.[34,35]

Table 2: Overview of validated antimicrobial agent and quality control strains. Unless stated otherwise, the inhibition zone diameters were within the respective CLSI and/or EUCAST ranges.[19,20]

Antimicrobial agent	Disk content (µg)	*E. coli* ATCC 25922[10,18]	*P. aeruginosa* ATCC 27853[10,18]	*E. faecalis* ATCC 29212[10,18]	*S. aureus* ATCC 29213[18]	*E. coli* ATCC 35218[10,18]	*S. aureus* NCTC 12493[18]	*K. pneumoniae* ATCC 700603[10,18]	*E. faecalis* ATCC 51299[18]
Amikacin	AN-30	✓	✓		✓				
Amoxicillin-clavulanic acid	AMC-3				✓				
	AMC-30	✓				[5]			
Ampicillin	AM-2			[3]	✓				
	AM-10	✓							
Ampicillin-sulbactam	SAM-20	✓				✓			
Aztreonam	ATM-30	✓	✓					✓	
Benzylpenicillin	P-1				✓				
Cefadroxil	CFR-30	✓[1]							
Cefalexin	CN-30	✓[1]							
Cefepime	FEP-30	✓	✓						
Cefexime	CFM-5	✓							
Cefotaxime	CTX-5	✓[2]						✓[2]	
Cefoxitin	FOX-30	✓			✓		✓		
Cefpodoxime	CPD-10	✓						✓	
Ceftaroline	CPT-5	✓[2]			✓				
Ceftazidime	CAZ-10	✓[2]	✓[2]					✓[2]	
Ceftibuten	CFT-30	✓							
Ceftriaxone	CRO-30	✓						✓	
Cefuroxime	CXM-30	✓							
Chloramphenicol	C-30	✓			✓				
Ciprofloxacin	CIP-5	✓	✓	✓	✓				
Clindamycin	CC-2				✓				
Doripenem	DOR-10	✓	✓						
Ertapenem	ETP-10	✓							
Erythromycin	E-15				✓				
Fusidic acid	FA-10				✓				
Gentamicin	GM-10	✓	✓		✓				
	GM-30			✓					✓
Imipenem	IPM-10	✓	✓	✓					
Levofloxacin	LVX-5	✓	✓	✓	✓				
Linezolid	LZD-10			✓	✓				
Mecillinam	MEC-10	✓							
Meropenem	MEM-10	✓	✓						
Minocycline	MI-30				✓				
Moxifloxacin	MXF-5	✓			✓				
Mupirocin	MUP-200				✓				
Nalidixic acid	NA-30	✓							

Table 2: Continued.

Antimicrobial agent	Disk content (µg)	*E. coli* ATCC 25922[10,18]	*P. aeruginosa* ATCC 27853[10,18]	*E. faecalis* ATCC 29212[10,18]	*S. aureus* ATCC 29213[18]	*E. coli* ATCC 35218[10,18]	*S. aureus* NCTC 12493[18]	*K. pneumoniae* ATCC 700603[10,18]	*E. faecalis* ATCC 51299[18]
Netilmicin	NET-10	✓[2]	✓[2]		✓				
Nitrofurantoin	FM-100	✓[2]		✓	✓				
Norfloxacin	NOR-10	✓		✓	✓				
Ofloxacin	OFX-5	✓			✓				
Pefloxacin	PEF-5	✓							
Piperacillin	PIP-30	✓[2]							
Piperacillin-tazobactam	PIP-30/TAZ-6	✓[2]	✓[2]			✓[2]			
Quinupristin-dalfopristin	SYN-15			✓	✓				
Rifampicin	RA-5				✓				
Streptomycin	S-300			[6]					
Teicoplanin	TEC-30			✓					✓
Tetracycline	TE-30				✓				
Ticarcillin	TIC-75	✓							
Ticarcillin-clavulanic acid	TIM-85	✓	✓			✓			
Tigecycline	TGC-15	✓		✓	✓				
Tobramycin	NN-10	✓	✓		✓				
Trimethoprim	TMP-5	✓		✓	[4]				
Trimethoprim-sulfamethoxazole	SXT1.25-23.75	✓		✓	✓				
Vancomycin	VA-5			✓					✓

✓ Indicated zones of inhibition are within the EUCAST and CLSI range.
[1] CLSI does not recommend testing of the antimicrobial agent.
[2] CLSI and EUCAST recommend different concentrations of the antimicrobial agent. EUCAST recommendations were followed.
[3] The inhibition zone of the antimicrobial agent is not within recommended EUCAST range (Mueller Hinton II Agar, 150 mm and square).
[4] The inhibition zone of the antimicrobial agent is not within recommended EUCAST range (Mueller Hinton II Agar, 90 mm).
[5] The inhibition zone of the antimicrobial agent is not within recommended EUCAST and CLSI range (Mueller Hinton II Agar, 90mm).
[6] The inhibition zone of the antimicrobial agent is not within recommended CLSI range (Mueller Hinton II Agar, 90 and 150 mm) but within recommended EUCAST range.

Limitations of the procedure

The disc diffusion susceptibility test is designed for use with pure cultures only. A Gram strain and a presumptive identification of the isolate are recommended before the susceptibility test is prepared.

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

Various factors have been identified as influencing disc diffusion susceptibility test. These includes the medium, agar depth, disc potency, inoculum concentration, age of inoculum, and pH.[31]

Inappropriate 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. Therefore, it is strongly recommended to follow the CLSI and EUCAST recommendations on handling of the inoculum and the inoculated plates to minimize the potential risk of incorrect results due to improper handling. Improper storage of antimicrobial discs may cause a loss of potency and a falsely resistant result. Excessive shrinkage of the medium due to improper storage may lead to falsely sensitive results.

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.[10,11,32,33]

Bacteria requiring thymine or thymidine may be encountered.[27,28] 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 mg) and streptomycin (300 mg) 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.[7,28,29]

For complete discussions on the detection of MRSA, resistant enterococci, extended-spectrumß-lactamase-producing gram-negative bacilli and other test limitations refer to CLSI documents M2 and M7 and EUCAST.[7,8,10,11,30]

Mueller Hinton II Agar was shown to be reliable in detecting MRSA which produce a hazy zone of inhibition around oxacillin discs.[26] When in doubt, an additional method, such as the **BD Oxacillin Screen Agar** should be used.

The method of inoculation, interpretation, recommendations and the limits of zone sizes given in the present document and recommended by CLSI and EUCAST standard may differ from national standards.[7,8,31]

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FURTHER INFORMATION

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