INSTRUCTIONS FOR USE

.

MUELLER HINTON AGAR II

Dehydrated culture medium

Mueller Hinton Agar II*: P.aeruginosa* ATCC 27853

1 - INTENDED USE

In vitro diagnostic. Culture medium for Antimicrobial Susceptibility Testing (AST) by disk diffusion method of common, aerobic, rapidly growing bacteria.

2- COMPOSITION - TYPICAL FORMULA * (AFTER RECONSTITUTION WITH 1 L OF WATER)

Beef extract	2.0 g
Acid digest of casein	17.5 g
Starch	1.5 g
Agar	17.0 g

*The formula may be adjusted and/or supplemented to meet the required performances criteria.

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE

The development of bacterial resistance to antimicrobials in the first half of the twentieth century, resulted in the need for physicians to request the microbiology lab to test a patient's pathogen against various concentrations of a given antimicrobial to determine susceptibility or resistance to that drug.[1] William M.M. Kirby and his colleagues proposed a single disk method for antimicrobial susceptibility testing, and thereafter Kirby and Bauer, extensively reviewed the susceptibility testing literature, consolidated and updated all the previous descriptions of the disk diffusion method and published their findings.[2]

This publication led the World Health Organization to form a committee in 1961 to lay the groundwork for the development of a defined procedure for single antimicrobial disk susceptibility testing. The result was a standardized procedure for the disk diffusion susceptibility test, henceforth called at first the Anderson and later the Kirby-Bauer disk diffusion test.[3]

The culture medium proposed for Kirby-Bauer method was Mueller Hinton Agar, originally developed by Howard Mueller and Jane Hilton in 1941 for the isolation of gonococcus and meningoccus.[4]

Currently, the Clinical Laboratory Standards Institute (CLSI) for USA and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) for Europe are responsible for updating and modifying the original procedure through a global consensus process.[5,6] Interpretative guidelines for inhibition zone sizes are included in their publications.[5,7]

Mueller Hinton agar is considered the best medium to use for Antimicrobial Susceptibility Testing and is recommended by both CLSI[5] and EUCAST[6]. It is suitable and standardized by EUCAST for testing the more common rapidly growing bacteria: *Enterobacteriaceae*, *Pseudomonas* spp., *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Staphylococcus* spp., *Enterococcus* spp., *Aeromonas*, *Burkolderia pseudomallei*.[6]

Variations in performances of Mueller-Hinton Agar between and with manufacturers' batches/lots, involving different causes, have been observed.[8,9] Concentration of divalent cations Mg^{++} and Ca^{++} influences susceptibility of *Pseudomonas* spp. to tetracycline, gentamicin, polymyxin B, and carbenicillin[10]; calcium concentration modifies daptomycin inhibition zones of Gram-positive bacteria.[11] Variation in thymine and thymidine content, affects sulphonamide and trimethoprim values.[12,13]. The concentration of $zinc_{?}$ influences resistance interpretations with carbapenems against *P.aeruginosa*,[14] and manganese levels affect resistance interpretations with tigecycline against *Enterobacteriaceae* and *A.baumannii*.[15]

Biolife Mueller Hinton Agar II shows good batch-to-batch reproducibility for susceptibility testing, is low in sulphonamide and trimethoprim inhibitors (thymine and thymidine), supports satisfactory growth of Gram-positive and Gram-negative non-fastidious pathogens and contains controlled and adjusted levels of calcium and magnesium, low level of zinc and very low level of Mn, to guarantee optimal inhibition zones, within the quality control ranges.

In the EUCAST evaluation[9] of 21 brands of Mueller–Hinton media, Biolife Mueller Hinton Agar II and five other brands demonstrated excellent performance, with ≥99% of zone diameter readings within QC ranges and ≥70% on target ±1 mm.

4- DIRECTIONS FOR MEDIUM PREPARATION

Suspend 38 g in 1000 mL of cold purified water. Heat to boiling stirring constantly and sterilize by autoclaving at 121°C for 15 minutes. Cool to 47-50°C and pour into sterile Petri dishes.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	yellow, fine, homogeneous, free-flowing powder
Solution and prepared plates appearance	pale yellow, limpid or slightly opalescent
Final pH at 20-25 °C	7.3 ± 0,1

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6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Mueller Hinton Agar II	Dehydrated medium	4017402 4017404	500 g (13.1 L) 5 kg (131 L)

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave, water-bath, sterile loops and swabs, incubator and laboratory equipment as required, Petri dishes, Erlenmeyer flasks, ancillary culture media and reagents for the identification of the colonies, antimicrobial susceptibility paper disks.

8 - SPECIMENS

AST by disk diffusion method is designed to use with pure culture of strains isolated from clinical specimens.
Mueller Hinton Agar II is not intended for microbial isolation directly from clinical specimens.
A Gram stain and a preliminary bacterial identification are required for choosing the appropriate antimicrobial agents to be tested.
EUCAST has published a method for rapid AST (reading at 4, 6 or 8h incubation) directly from positive blood culture bottles, validated for selected organisms; consult the EUCAST document for the test procedure, reading and interpretation of inhibition zones.[16]

9- TEST PROCEDURE

The test procedure and the reading and interpretation of inhibition zones here described are a summary of EUCAST documents.[6,7,17]

- The surface of the agar should be dry before use. No drops of water should be visible on the surface of the agar or inside the lid. If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min. Do not over-dry plates.
- Use a sterile loop or a cotton swab to pick colonies from an overnight culture on non-selective media. Use several morphologically similar colonies (when possible) to avoid selecting an atypical variant. Suspend the colonies in saline and mix to an even turbidity. Adjust the density of the organism suspension to 0.5 McFarland by adding saline or more bacteria. The suspension must always be used within 60 min of preparation.
- Dip a sterile cotton swab into the suspension. To avoid over-inoculation of Gram-negative bacteria, remove excess fluid by pressing and turning the swab against the inside of the tube. For Gram-positive bacteria, do not press or turn the swab against the inside of the tube.
- Plates can be inoculated either by swabbing in three directions or by using an automatic plate rotator. Spread the inoculum evenly over the entire agar surface ensuring that there are no gaps between streaks.
- Allow disks to reach room temperature before opening cartridges or containers used for disk storage.
- Apply disks firmly to the surface of the inoculated agar plate within 15 minutes of inoculation. Disks must be in close and even contact with the agar surface and must not be moved once they have been applied as the initial diffusion of antimicrobial agents from disks is very rapid.
- The number of disks on a plate should be limited to avoid overlapping of zones and interference between agents. It is important that zone diameters can be reliably measured. The maximum number of disks depends on the organism and the selection of disks. Normally 6 and 12 disks are the maximum possible number on a 90 and 150 mm circular plate, respectively.
- To be able to detect inducible clindamycin resistance in staphylococci and streptococci, the erythromycin and clindamycin disks must be placed at a distance of 12-20 mm from edge to edge for staphylococci and 12-16 mm from edge to edge for streptococci.
- Invert agar plates and make sure disks do not fall off the agar surface. Incubate plates within 15 min of disk application. If the plates are left at room temperature after disks have been applied, pre-diffusion may result in erroneously large zones of inhibition.
- Incubate at 35 ± 1°C in air for 18 ± 2 h (24 h for glycopeptides and *Enterococcus*).

10 - READING AND INTERPRETATION

After incubation, read plates from the back with reflected light and the plate held above a dark background.
A correct inoculum and satisfactorily streaked plates should result in a confluent lawn of growth. If individual colonies can be seen, the inoculum is too light and the test must be repeated.
The growth should be evenly distributed over the agar surface to achieve uniformly circular (non-jagged) inhibition zones.
Check that inhibition zones for quality control strains are within acceptable ranges.
For all agents, the zone edge should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye. Holding the plate at a 45-degree angle to the work bench may facilitate reading when zone edges are difficult to define.
Measure the inhibition zone diameters to the nearest millimetre with a ruler or a calliper.
For specific reading instructions consult the EUCAST document.[6]
Interpret zone diameters into susceptibility categories according to the current breakpoint tables.[7]

11 - USER QUALITY CONTROL

All manufactured lots of Mueller Hinton Agar II plates are released for sale after the Quality Control has been performed to check the compliance with the specifications, according to EUCAST rules[6,17]. However, the end user can perform its own Quality Control in accordance with the local applicable regulations, in compliance with accreditation requirements and the experience of the Laboratory. Use the quality control strains specified by EUCAST, summarized here below, to monitor the performance of the test. The principal recommended control strains are typical susceptible strains, but resistant strains can also be used to confirm that the method will detect resistance mediated by known resistance mechanisms. Check that results for control strains are within acceptable ranges in EUCAST QC tables.[17]

Escherichia coli ATCC 25922 - susceptible, wild-type
Escherichia coli ATCC 35218 TEM-1 ß-lactamase, ampicillin resistant (for the control of the inhibitor component of β-lactam-inhibitor combination disks)
Klebsella pneumoniae ATCC 700603 ESBL-producing strain (SHV-18) (for the control of the inhibitor component of β-lactam-inhibitor combination disks)
Pseudomonas aeruginosa ATCC 27853 - susceptible, wild-type
Klebsiella pneumoniae ATCC BAA-2814 - KPC-3, SHV-11 and TEM-1
Staphylococcus aureus ATCC 29213 - weak β-lactamase producer
Enterococcus faecalis ATCC 29212 - susceptible, wild-type.
Staphylococcus aureus NCTC 12493 - mecA+, methicillin resistant (MRSA)

Enterococcus faecalis ATCC 51299 - HLAR, vanB+ High level aminoglycoside resistant (HLAR) and vancomycin resistant (vanB positive)

For details about the choice of antibiotics, the control strains, the frequency of the controls and the tables of the acceptability ranges, consult the EUCAST documents.[6,17]

12- PERFORMANCES CHARACTERISTICS

Prior to release for sale a representative sample of all lots of dehydrated Mueller Hinton Agar II is tested by Antimicrobial Sensitivity Testing, for productivity properties and by Ca++ ad Mg++ detection, by comparing the results with a previously approved Reference Batch.

Productivity is tested by semi-quantitative ecometric technique with the following target strains: *E.coli* ATCC 25922, *S.aureus* ATCC 25923 and *P.aeruginosa* ATCC 27853. After aerobic incubation at 35-37°C for 18-24 hours, the amount of growth is evaluated and recorded. All strains must show a good growth. AST is performed according to EUCAST procedure[6] with the following strains and antimicrobial disks: *E.faecalis* ATCC 29212: TRS, CIP, DAP (MIC strips); *E.coli* ATCC 35218: AMS, PIT; *E.coli* ATCC 25922: AMI, AMC, AMP, CTZ, CXI, CTA, CIP, CHL, GEN, IMI, TRS; *S.aureus* ATCC 25923: APM, CHL, CIP, CLI, ERY, GEN, LIN, BEN, QUD, TET; *P.aeruginosa* ATCC 27853: AMI, AZT, CEP, CTZ, CIP, GEN, IMI, PIT, TOB. After incubation the inhibition zones are measured, recorded and evaluated to be within the quality control ranges reported by EUCAST and/or CLSI.[5,17] Concentration of Ca^{++} and Mg^{++} are measured for all production batches of dehydrated Mueller Hinton Agar II, to assure batch-to-batch reproducibility.

During 2018-2019 EUCAST evaluated the performance of 21 internationally available brands of dehydrated Mueller-Hinton agar from 17 manufacturers.[9] Testing included 4 test strains (*E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853, *S.aureus* ATCC 29213, *E.faecalis* ATCC 29212) and 18 antimicrobial disks, chosen to represent different agent classes and to include agents that could reveal effects of varying pH and contents of cations and thymidine. All brands were tested blindly and in parallel. The agar depth, pH and concentration of five cations (Mg, Ca, Zn, Mn, Fe) were measured for all brands. Each brand was given a total rating based on how mean values (30 per agar) from triplicate tests related to the respective QC criteria in the EUCAST QC Tables. Biolife Mueller Hinton Agar II demonstrated excellent performance, with 99% of zone diameter readings within QC ranges and 81% on target ±1 mm.

12 - LIMITATIONS OF THE METHOD

- EUCAST has evaluated the disk potency of 16 strategically important antibiotic disks from nine manufacturers of disks for antimicrobial susceptibility testing. The study disclosed some good and some poor quality among disks and manufacturers. It is the responsibility of laboratories to perform quality control to guarantee that the material used performs to the standards of the laboratory and the health care system.[18]
- Incorrect inoculum concentration, improper storage of antimicrobial disks, improper storage of the plates resulting in an agar depth and pH out of specifications, excessive moisture, improper measurement of endpoints, may produce incorrect results.[19,20] Therefore, strict adherence to protocol is required to ensure reliable results.
- Antimicrobial susceptibility testing of colistin has been fraught with difficulties. A joint EUCAST and CLSI subcommittee issued recommendations confirming that broth microdilution is so far the only valid method and that disk diffusion does not work because of the poor diffusion of the large colistin molecule.[21]
- Bacteria requiring thymine or thymidine may not grow satisfactorily on Mueller Hinton Agar II because of low levels of thymine or thymidine.[22]
- Mueller Hinton Agar II is not appropriate for assay by disk-diffusion method with slow growing organisms, anaerobes and capnophiles.[20]
- Consult the EUCAST and/or CLSI papers for the details of disk diffusion methodology, reading and interpretations of inhibition zones, warnings, guidance documents in susceptibility testing, guidelines for detection of resistance mechanisms, clinical breakpoints.
- Mueller Hinton Agar II can be used for the determination of Minimum Inhibiting Concentrations (MICs) with strips containing antimicrobial gradients. To perform this method, it is required to follow the instructions for use of the supplier of strips and to validate the work procedure in the laboratory.
- This culture medium is intended as an aid in the treatment of infectious diseases; the interpretation of the results must be made considering the patient's clinical history, the origin of the sample and the results of other diagnostic tests.

14 - PRECAUTIONS AND WARNINGS

- This product is a qualitative *in vitro* diagnostic, for professional use only; it is to be used by adequately trained and qualified laboratory personnel, observing approved biohazard precautions and aseptic techniques.
- Dehydrated media must be handled with suitable protection. Before use, consult the Safety Data Sheet.
- This culture medium contains raw materials of animal origin. The *ante* and *post mortem* controls of the animals and those during the production and distribution cycle of the raw materials, cannot completely guarantee that this product doesn't contain any transmissible pathogen. Therefore, it is recommended that the culture medium be treated as potentially infectious, and handled observing the usual specific precautions: do not ingest, inhale, or allow to come into contact with skin, eyes, mucous membranes. Download the TSE Statement from the website www.biolifeitaliana.it, describing the measures implemented by Biolife Italiana for the risk reduction linked to infectious animal diseases.
- All laboratory specimens should be considered infectious.
- The laboratory area must be controlled to avoid contaminants such as culture medium or microbial agents.
- Sterilize all biohazard waste before disposal. Dispose the unused medium and the sterilized plates inoculated with samples or microbial strains in accordance with current local legislation.
- Do not use the culture medium as active ingredient for pharmaceutical preparations or as production material intended for human and animal consumption.
- The Certificates of Analysis and the Safety Data Sheet of the product are available on the website www.biolifeitaliana.it.
- Notify Biolife Italiana Srl (complaint@biolifeitaliana.it) and the relevant Authorities of any serious incident occurring in connection with the use of the *in vitro* diagnostic.
- The information provided in this document has been defined to the best of our knowledge and ability and represents a guideline for the proper use of the product but without obligation or liability. In all cases existing local laws, regulations and standard procedures must be observed for the examination of samples collected from human and animal organic districts, for environmental samples and for products intended for human or animal consumption. Our information does not relieve our customers from their responsibility for checking the suitability of our product for the intended purpose.

15 - STORAGE CONDITIONS AND SHELF LIFE

Upon receipt, store at 10-30°C away from direct light in a dry place. If properly stored, it may be used up to the expiration date. Do not use beyond this date. Avoid opening the bottle in humid places. After use, the container must be tightly closed. Discard the product if the container and/or the cap were damaged or in case of evident deterioration of the powder (colour changes, hardening, large lumps).

16 - REFERENCES

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TABLE OF APPLICABLE SYMBOLS

REF or REF Catalogue number	LOT Batch code	IVD *In vitro* Diagnostic Medical Device	Manufacturer	Use by
Temperature limitation	Contents sufficient for <n> tests	Consult Instructions for Use	Keep away from direct light	Store in a dry place

REVISION HISTORY

Version	Description of changes	Date
Revision 1	Updated layout and content	2020/05
Revision 2	Modification of "precautions and warnings", "storage conditions and shelf life".	2022/01
Revision 3	Removal of obsolete classification	2023/04

Note: minor typographical, grammatical, and formatting changes are not included in the revision history.

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