INSTRUCTIONS FOR USE

MUELLER HINTON AGAR II

Ready-to-use plates

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

1 - INTENDED USE

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

2 - COMPOSITION - TYPICAL FORMULA *

Beef Extract	2.0 g
Acid Digest of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Purified water	1000 mL

*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 of 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 both by 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] Variation 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 tygecycline against *Enterobacteriaceae* and *A. baumannii*.[15] Biolife Mueller Hinton Agar II shows good batch-to-batch reproducibility for susceptibility testing, it is low in sulphonamide and trimethoprim, inhibitors (thymine and thymidine), it supports satisfactory growth of Gram positive and Gram negative non-fastidious pathogens, it contains controlled and adjusted levels of calcium, magnesium and zinc, 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 - PHYSICAL CHARACTERISTICS

Medium appearance	pale yellow, limpid
Final pH at 20-25 °C	7.3 ± 0,1
Agar depth	4.0 ± 0,5 mm

5 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Mueller Hinton Agar II CND: W0104010403 EDMA: 14.01.04.03– RDM: 1444954/R	Ready-to-use plates	541740	2 x 10 plates ø 90 mm primary packaging: 2 cellophane sachets secondary packaging: cardboard box
Mueller Hinton Agar II – 150 mm CND: W0104010403 EDMA: 14.01.04.03– RDM: 1458976/R	Ready-to-use plates	501740P	5 plates ø 150 mm primary packaging: 1 cellophane sachets secondary packaging: cardboard box

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6 - MATERIALS REQUIRED BUT NOT PROVIDED

Sterile loops and swabs, incubator and laboratory equipment as required, antimicrobial susceptibility paper discs.

7 - 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 8 hours 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]

8- 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*).

9 - 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]

10 - 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 it is responsibility of the end-user to perform Quality Control testing 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 and summarized here below, to monitor the performance of the test. 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)

ATCC is a trademark of American Type Culture Collection; NCTC is a trademark of National Collection of Type Culture

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]

1- PERFORMANCES CHARACTERISTICS

Prior to release for sale a representative sample of all lots of ready-to-use plates of Mueller Hinton Agar II 90 mm and 150 mm and of the raw material used for the production of prepared plates (dehydrated Mueller Hinton Agar II REF 401740) are 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 discs: *E.faecalis* ATCC 29212: TRS, DAP (Etest), CIP; *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 raw material 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 discs, improper storage of the plates resulting in an agar depth and pH out of the 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 disc 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 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.

13 - PRECAUTIONS AND WARNINGS

- Mueller Hinton Agar II 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.
- Mueller Hinton Agar II is not classified as dangerous according to current European legislation.
- 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 these products do not contain any transmissible pathogen. Therefore, it is recommended that the ready-to use plates 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 S.r.l. for the risk reduction linked to infectious animal diseases.
- All laboratory specimens should be considered infectious.
- Each plate of this culture medium is for single use only.
- Ready-to-use plates are not to be considered a "sterile product" as they are not subject to terminal sterilization but a product with controlled bio contamination, within the limits of defined specifications reported on the Quality Control Certificate.
- Sterilize all biohazard waste before disposal. Dispose the unused medium and the plates inoculated with samples or microbial strains in accordance with current local legislation.
- The laboratory area must be controlled to avoid contaminants such as culture medium or microbial agents.
- The Certificates of Analysis and the Safety Data Sheet are available on the website www.biolifeitaliana.it.
- 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.

14 - STORAGE CONDITIONS AND SHELF LIFE

Upon receipt, store plates in their original pack at 2-8°C away from direct light. If properly stored, the plates may be used up to the expiration date. Do not use the plates beyond this date. Plates from opened plastic sachet can be used for 7 days when stored in a clean area at 2-8°C. Do not use the plates if the plastic sachet is damaged or if the dish is broken. Do not use the plates with signs of deterioration (e.g. microbial contamination, dehydration, shrinking or cracking of the medium, atypical colour, excess of moisture).

15 - 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	For single use only	Fragile, handle with care

REVISION HISTORY

Version	Description of changes	Date
Instructions for Use (IFU) - Revision 1	Updated layout and content in compliance with IVDR 2017/746	2020/05

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

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