INSTRUCTIONS FOR USE

.

MUELLER HINTON BROTH

(CATION-ADJUSTED)

Dehydrated culture medium

1 - INTENDED USE

In vitro diagnostic. Cation adjusted liquid medium for broth dilution antimicrobial susceptibility tests (AST).

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

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

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE

At the end of the twenties of the last century, Alexander Fleming contributed to the development of a broth dilution technique using broth turbidity as an end-point determination.[1] By the end of the 1950s it was apparent that there was a need to standardize AST and several organizations and investigators began addressing this critical issue. The World Health Organization (WHO) published a report on standardization of AST methodology.[3] This has been described as a forerunner of contemporary minimum inhibitory concentration (MIC) methodology. Ericsson and Sherris first used the term 'breakpoint' and published a review of several techniques for susceptibility testing and the relationship between dilution and diffusion methods.[3] Later, in the 1980s, the Clinical and Laboratory Standards Institute consolidated methods and standards for MIC determination and clinical use. The protocols using the Mueller Hinton Broth and microdilution technique and the parameters for defining susceptibility and resistance breakpoints established by CLSI[4] and EUCAST[5-7] are now considered the gold standard in the US and Europe.

With few exceptions, broth microdilution with un-supplemented cation-adjusted Mueller Hinton Broth is the reference method for antimicrobial susceptibility testing of rapidly growing aerobic bacteria.[7] For fastidious organisms, EUCAST recommends testing according to ISO 20776-1,[8] but with the use of Mueller Hinton Broth supplemented with 5% lysed horse blood and 20 mg/L β-NAD (MH-F). The lowest concentration, expressed in mg/L or µg/mL, of antimicrobial agent that inhibits visible growth of a microorganism is defined as the Minimum Inhibitory Concentration (MIC).

In Mueller Hinton Broth, acid digest of casein and beef extract provide nitrogen, carbon, and minerals for bacterial growth. These raw materials are selected with low content of thymine and thymidine as determined by MIC values with *Enterococcus faecalis* and sulfamethoxazole-trimethoprim (SXT). In Mueller Hinton Broth, calcium, magnesium and zinc ions concentrations are adjusted to provide the amounts recommended by ISO Standards[8,9]: Ca^{++}: 20-25 mg/L, Mg^{++}:10-12.5 mg/L, Zn^{++}:< 3 mg/L. Susceptibility testing of *Pseudomonas aeruginosa* with the aminoglycosides and other antibiotics is influenced by the presence of calcium and magnesium ions[10] while zinc ion has an inhibitory activity on carbapenems susceptibility of *P. aeruginosa*[11].

4- DIRECTIONS FOR MEDIUM PREPARATION

Suspend 21 g in 1000 mL of cold purified water. Heat to completely dissolve the powder, distribute and sterilize by autoclaving at 121°C for 10 minutes. Do not overheat.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	yellow, fine, homogeneous, free-flowing powder
Solution and prepared tubes appearance	light straw coloured and clear with no visible precipitate
Final pH at 20-25 °C	7.3 ± 0.1

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Mueller Hinton Broth	Dehydrated medium	4017412	500 g (23.8 L)

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave, water-bath, sterile loops and swabs, incubator and laboratory equipment as required, micro-dilution trays, tubes, Erlenmeyer flasks, ancillary culture media and reagents for the identification of the colonies, antibiotics.

8 - SPECIMENS

Broth dilution antimicrobial susceptibility test is performed with pure culture of strains isolated from clinical specimens. Mueller Hinton Broth 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.

9- TEST PROCEDURE

Mueller Hinton Broth may be used for inoculum preparation for MIC tests and for preparation of antimicrobial dilutions for the micro-dilution or macro-dilution procedures. The micro-dilution procedure here described is a summary of ISO 20776-1 protocol.[8]

Preparation of working solutions and micro-dilution trays

The range of antibiotics concentrations selected for testing depends on the microorganisms and antimicrobial agent. The chosen range shall allow full endpoint MIC determination for appropriate reference strains. A two-fold dilution series based on 1 mg/mL stock solution is prepared in Mueller Hinton Broth. Dilutions should be prepared according to the procedure outlined in Annex C of ISO 20776-1.[8] Working solutions shall be used the same day unless information is available on stability of the solutions under specified storage conditions.

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Working solutions are dispensed into micro-dilution trays at 50 µl per well with double the desired final concentrations of antimicrobial agent, or at 100 µl per well in the desired final concentrations.
At least one well, containing 50 µl or 100 µl of antimicrobial agent-free medium, should be included as a growth control for each strain tested. Likewise, a well containing 100 µl of antimicrobial agent-free medium should be included as an un-inoculated negative control well for each microorganism type tested.

Preparation of inoculum

The inoculum may be prepared by diluting an overnight broth culture or by suspending several morphologically similar colonies cultivated on non-selective agar medium in Mueller Hinton Broth.
In both cases the bacterial suspension is adjusted with saline or broth to give a turbidity equivalent to the 0.5 McFarland standard that contains approximately $1x10^8$ to $2x10^8$ CFU/mL.
The adjusted inoculum prepared as above is diluted in Mueller Hinton Broth to give a final cell number of 5×10^5 CFU/ mL

Inoculation of micro-dilution trays

The trays shall be inoculated within 30 min of standardizing the inoculum suspension
To each well containing 50 µl of diluted antimicrobial agent in broth, a volume of 50 µl of bacterial suspension is added. For tray wells that contain 100 µl of diluted antimicrobial agent in broth, up to 10 µl of diluted inoculum suspension should be added.
Viable counts shall be performed on the test suspension to ensure that test wells contain 5×10^5 CFU/ ml. This shall be done by removing 10 µl from the growth control well immediately after inoculation and diluting it in 10 ml of broth or saline. 100 µl of this dilution is spread over the surface of a suitable agar plate (e.g. Tryptic Soy Agar), which is then incubated overnight (12 h to18 h). Twenty to eighty colonies would be expected from an acceptable test suspension. If this is not achieved corrective action should be taken to ensure proper inoculum preparation.

Macro-dilution (tube) method

If the volume of antimicrobial solution in the tube is 1 mL, dilute the standardized inoculum 1:100 in Mueller Hinton 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.

Incubation

Incubate at 35 ± 1°C in ambient air for 18 ± 2 h for most antimicrobial agent-bacteria combinations.

10 - READING AND INTERPRETATION

Results shall only be read when there is sufficient growth of the test organism (i.e., obvious button or definite turbidity in the positive growth control), when there is no growth in the un-inoculated or negative growth control and when purity and the appropriate cell number concentration of the inoculum has been established.
The amount of growth in each well is compared with that in the positive growth control, and the MIC recorded is the lowest concentration of the agent that completely inhibits visible growth. There are exceptions to this (e.g., trailing endpoints for linezolid, partial inhibition by sulphonamides, incomplete inhibition with some bacteriostatic agents) that will require special attention by the user.

11 - USER QUALITY CONTROL

All manufactured lots of Mueller Hinton Broth are released for sale after the Quality Control has been performed to check the compliance with the specifications. 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. It is recommended that the user refers to pertinent ISO[9] and/or CLSI[4] guidance for appropriate Quality Control practices.

12- PERFORMANCES CHARACTERISTICS

Prior to release for sale a representative sample of all lots of dehydrated Mueller Hinton Broth is tested for Antimicrobial Sensitivity Testing, for productivity properties and for Ca^{++}, Mg^{++}, Zn^{++} detection, by comparing the results with a previously approved Reference Batch.
Productivity is tested by dilution to extinction method, by inoculating 1 mL of appropriate decimal dilutions of organisms in test tubes and incubating at 35 ± 1°C in ambient air for 18 ± 2 h and recording the highest dilution showing growth in Reference Batch (Gr_{RB}) and in Test Batch (Gr_{TB}). Productivity is tested with the following strains: *E.faecalis* ATCC 29212, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853, *S.aureus* ATCC 29213. The productivity index Gr_{RB}-Gr_{TB} for each test strain shall be ≤ 1.
AST is performed with the micro-dilution technique with the following strains: *E.faecalis* ATCC 29212, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853, *S.aureus* ATCC 29213. Antibiotics are chosen batch by batch to ensure rotation.
Concentration of Ca^{++}, Mg^{++} and Zn^{++} is measured for each production batch of dehydrated Mueller Hinton Broth, to assure batch-to-batch reproducibility and the compliance with specifications.

12 - LIMITATIONS OF THE METHOD

- Incorrect inoculum concentration, improper storage of trays, pH out of specifications, improper measurement of endpoints, may produce incorrect results.[12]
- Certain resistance mechanisms may not always be expressed using the standard reference dilution method, e.g., the expression of some β-lactamases, efflux pumps or drug target site modifications. In those cases, the MIC should be interpreted with caution, or other information used instead, to guide clinical therapy.[8]
- *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.[13,14]
- Bacteria requiring thymine or thymidine may not grow satisfactorily on Mueller Hinton Broth because of low levels of thymine or thymidine.[15]
- Un-supplemented Mueller Hinton Broth is not appropriate for slow growing organisms, fastidious organisms such as *Haemophilus*, *Neisseria* and certain streptococci. For these microorganisms, refer to the methods proposed by EUCAST and CLSI.
- Tigecycline shall be tested within 12 h of preparation of the Mueller Hinton Broth.[16]
- For testing dalbavancin, televancin and oritivancin, polysorbate-80 (volume fraction 0.002%) should be added to Mueller Hinton Broth.[17]
- For testing cefiderocol, Mueller Hinton Broth is first depleted of iron with an iron chelating compound. The medium is then supplemented back with standard concentrations of calcium, magnesium and zinc.[18]
- Broth micro-dilution may not reliably detect resistance conferred by the mecA or mecC gene.[8]
- Incorporation of NaCl at a final concentration of 20 g/L in the broth is required for the detection of methicillin resistance in *Staphylococcus* spp. when testing with oxacillin.

- For testing daptomycin Mueller Hinton Broth medium shall be supplemented to a final concentration of 50 mg/L Ca^{++}.[19]
- When testing glycopeptides, the MIC should be read after 24 h incubation to give more consistent and reliable results; examine tubes or wells carefully for evidence of faint growth.[4,8]
- Studies have shown that testing of mecillinam by Mueller Hinton Broth micro-dilution, results in severe trailing endpoints and is therefore not recommended. Agar dilution or disc diffusion provide stable reproducible results.[20]
- Broth micro-dilution may not give reliable results with fosfomycin. Agar dilution should be used as the reference method.[8]
- Since colistin has affinity to plastic, the results obtained by micro-dilution trays may be prone to reproducibility issues or inaccuracy.[21]
- A distinct phenomenon commonly referred as “skipped wells”, characterized by lack of growth in wells with intermediate colistin concentrations followed by growth in wells with higher concentration, has been observed and reported by some studies.[22]
- 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.
- Apply Good Manufacturing Practice in the production process of prepared media
- 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 tubes 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.
- 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 Certificates of Analysis and the Safety Data Sheet of the product 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.

15 - STORAGE CONDITIONS AND SHELF LIFE

Upon receipt, store at +10°C /+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 are damaged, or if the container is not well closed, or in case of evident deterioration of the powder (colour changes, hardening, large lumps). The user is responsible for the manufacturing and quality control processes of prepared media and for the validation of the shelf life of the finished products, according to the type (tubes/bottles/trays) and the storage method applied (temperature and packaging).

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	2022/04
Revision 2	Removal of obsolete classification	2023/04

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

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