LEGIONELLA SELECTIVE AGAR MWY ISO

Ready to use plates

1 - INTENDED USE

For the isolation and enumeration of *Legionella* spp. in water (ISO 11731).

2 - COMPOSITION - TYPICAL FORMULA *

Activated charcoal	2.0 g
Yeast extract	10.0 g
Agar	13.0 g
ACES Buffer/Potassium hydroxide	12.8 g
α–ketoglutarate	1.0 g
Ferric pyrophosphate	250.0 mg
L-Cysteine HCl	400.0 mg
Glycine	3.0 g
Vancomycin HCl	1.0 mg
Polymyxin B	50,000 UI
Anisomycin	80.0 mg
Bromothymol blue	10.0 mg
Bromocresol purple	10.0 mg
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

Legionellae are mesophilic, motile, a-saccharolytic, obligately aerobic, nutritionally fastidious, Gram-negative, non-spore-forming gammaproteobacteria.[1] *Legionella pneumophila*, the most widely studied species, displays pleomorphism, demonstrating coccoid, bacillary and/or long filamentous forms that are influenced by temperature, available nutrients or metabolites, growth environment and medium type.[2] *Legionella* species share growth dependence for L-cysteine and growth enhancement by iron.[1] Legionellae grow on several types of complex artificial media, however, the most successful medium is buffered charcoal yeast extract (BCYE) agar containing ferric pyrophosphate, α-ketoglutarate and L-cysteine.[2]

The culture is the reference technique for laboratory diagnosis: it has 100% specificity and a variable sensitivity depending on the characteristics of the sample, on the experience and technical proficiency of laboratory personnel, as well as on the delays in respiratory sample processing, the prior use of antimicrobial therapies and culture overgrowth by other oropharyngeal bacteria.[2,3]

Optimal yield of *Legionella* spp. from clinical specimens usually requires that a variety of media be used: one plate with non-selective medium (BCYE) and two with selective media.[1]

The choice of the method used for the enumeration of *Legionella* spp. in water depends on the origin and characteristics of the sample, the reason of sampling or investigation, the expected concentration of interfering microorganisms and the desired lower limit of detection level; a decision matrix for choosing an appropriate method is described in ISO 11731.[4]

Buffered Charcoal Yeast Extract Agar (BCYE) was developed by Feeley et al.[5] and then further modified by Edelstein[6] by introducing α-ketoglutarate, and by Pasculle et al.[7] by the addition of ACES buffer.

Wadowsky and Yee[8] proposed a selective version of BCYE, by introducing in the formulation glycine, vancomycin and polymyxin B, resulting in the formation of GVP medium. Another modification in 1984 by Dennis et al.[9] made the medium even more selective for *Legionella* by the addition of cycloheximide, resulting in GVPC medium.

Vickers et al.[10] introduced 0.001% of bromocresol purple and bromothymol blue into BCYE agar for the differentiation between members of the family *Legionellaceae*. Edelstein in 1982[11] proposed MWY medium as a modification of the GVP medium of Wadowsky and Yee, including bromothymol blue and bromocresol purple and an antifungal agent.

Yeast extract is a source of nitrogen, carbon, and vitamins for microbial growth. Activated charcoal removes hydrogen peroxide and other toxic products. ACES Buffer is used for pH stabilisation, α-ketoglutarate and ferric pyrophosphate stimulate *Legionella* growth. L-cysteine, is an essential aminoacid and an important energy source for *Legionella* spp. Glycine and polymyxin B are inhibitors of Gram-negative bacteria, vancomycin suppress the growth of Gram-positive bacteria while anisomycin is used as antifungal agent. Bromothymol blue and bromcresol purple are included as an aid in the identification of legionellae.

4 - PHYSICAL CHARACTERISTICS

Prepared plates appearance	black, opaque
Final pH at 20-25 °C	6.9 ± 0.1

5 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Legionella Selective Agar MWY ISO	Ready-to-use plates	549948	2 x 10 plates ø 90 mm

6 - MATERIALS REQUIRED BUT NOT PROVIDED

Sterile loops and swabs, incubator and laboratory equipment as required, ancillary culture media and reagents for the identification of the colonies.

7 - SPECIMENS

The medium is intended for the enumeration of *Legionella* in different types of water: drinking, natural, industrial, wastewater and in water-related samples (for example biofilm, sediments, etc.). [4] Consult the Standard ISO 11731 for sampling methods and sample handling procedures. Apply good laboratory practices for specimen collection, transport and storage.

Biolife Italiana S.r.l., Viale Monza 272, 20128 Milan, Italy.
Tel. +39 02 25209.1, Fax +39 02 2576428
E-mail: export@biolifeitaliana.it; web: www.biolifeitaliana.it

8 - TEST PROCEDURE

Allow plates to come to room temperature and to dry the surface of the medium.
The work procedures described in the ISO 11731 Standard differ in relation to the origin of the sample, its characteristics, the purposes of the research and in relation to the expected concentrations of the target microorganism and the contaminating flora.
Schematically, the different possibilities of treatment and inoculation of the samples are summarized below.

1. For samples with a high number of *legionellae* and a low number of contaminants: direct inoculation of the sample on a non-selective BCYE w/L-cysteine^ medium and on a selective BCYE-AB* medium plate.
2. For samples with a low number of legionellae and a low number of contaminants: membrane filtration and positioning of the untreated filter on a BCYE w/L-cysteine^ non-selective medium plate, positioning of the filter(s) treated with acids on one or more selective or highly selective medium plates (BCYE-AB* or BCYE-GVPC** or BCYE-MWY***); wash the untreated and acid or heat treated membrane and inoculate 0.1-0.5 mL on a non-selective medium plate and on plates of one or more selective and highly selective media (BCYE-AB* or BCYE-GVPC** or BCYE-MWY***).
3. For samples with a high number of contaminants: inoculate the non-concentrated, concentrated and diluted 1:10 sample; divide each sub-sample into three aliquots: one untreated, one treated with heat and one treated with acids; inoculate 0.1-0.5 mL of each aliquot on a selective medium plate (BCYE-GVPC** or BCYE-MWY***).
4. For samples with a very high number of contaminants: inoculate the un-concentrated and diluted sample 1:10 and 1: 100 after a pre-treatment with a combination of heat followed by the acid solution. Prepare dilutions with the appropriate diluent after acid treatment. After vortexing, inoculate 0.1-0.5 mL of each aliquot on a selective medium (BCYE-GVPC** or BCYE-MWY***) plate.

Allow the inoculum to absorb well then incubate the inverted plates in a humid atmosphere at 36 ± 2°C for 7-10 days, observing the plates at days 2, 3, 4, 5 and then at the end of the incubation period.
The procedural elements reported above are entirely schematic. For details of *Legionella* counting techniques in water, refer to the ISO 11731 Standard[4] or other applicable guidelines.

^ 549945 LEGIONELLA AGAR (BCYE); *549947 LEGIONELLA AB SELECTIVE AGAR; **549995 or 499995 LEGIONELLA SELECTIVE AGAR-GVPC *** 549948 LEGIONELLA SELECTIVE AGAR MWY-ISO

Confirmation of the colonies
A first criterion to differentiate *Legionell*a colonies is their inability to grow, with rare exceptions (*L.oakridgensis*, *L.jordanis*, and *L.nagasakiensis, L.spiritensis*)[2,4,12], on medium lacking L-cysteine.
When there is only one colony type, pick three presumptive colonies; if more morphological different types of presumptive colonies of *Legionella* are growing on the plate, take at least one colony from each type.[4]
Subculture onto a plate of BCYE w/cysteine (REF 549945) and a plate of BCYE w/o cysteine (REF 549943).
Be careful not to carry over any culture media with the colony and first inoculate a plate of Legionella Agar w/o Cysteine.
Incubate at 36 ± 2°C for 2 to 5 days.[4]

9 - READING AND INTERPRETATION

Isolation and enumeration
After incubation, observe the bacterial growth and record the specific morphological and chromatic characteristic of the colonies.
Legionella spp. colonies begin to appear on culture plates on day 2 of incubation. It is very unusual for the bacterial colonies to appear on plates after 5 days of incubation. Some very rarely isolated *Legionella* spp. may require up to 10 days of incubation before growth appears; this is an extremely rare event. Regardless, it is reasonable to inspect culture plates on days 2 to 5 ad than again at day 10.[4]
In the first 24-36 hours of incubation the observation of the plate under a low power binocular microscope with incident light illuminating the agar surface at an acute angle may help in the *Legionella* and contaminants colonies recognition.
Legionella colonies, in principle, appear white-gray, with entire, shiny edges, rounded with a diameter of 1 to 4 mm. Generally, and especially in the first 2 days of incubation, the edge shows a pink or blue-green iridescence while the centre is opalescent gray with an appearance similar to ground glass. Observed under UV lamp (366 nm), some species (*L.anisa, L.bozemanii, Lcherrii, L.dumoffii, L.gormanii, L.gratiana, L.parisiensis, L.steigerwaltii* and *L.tucsonensis*) show a blue-white auto-fluorescence, others (*L.erythra and L.rubrilucens*) a bright red auto-fluorescence.
L. pneumophila colonies appear green, opaque, often tinged with yellow. The color of the fluorescence can help differentiate colonies in samples containing different Legionella species.
With the prolongation of the incubation time, the colonies become wider, the centre assumes a creamy white colour and lose much of their iridescence. A common feature of *Legionella* colonies is the difficulty in taking them with the loop from the surface of the agar.
For the details of *Legionella* spp. enumeration in water samples consult the ISO 11731.[4]
Confirmation of the colonies
After incubation, observe the bacterial growth on both inoculated plates. Regard as *Legionella* those colonies which grow on the plate of BCYE w/cysteine but fail to grow on the plate of BCYE w/o cysteine.
Presumptive identification should be completed by Gram staining prepared from cysteine containing agar only: *Legionella* cells are Gram-negative poorly/faintly staining thin rods, which may be filamentous in older cultures.[4]

10 - USER QUALITY CONTROL

All manufactured lots of the product are released for sale after the Quality Control has been performed to check the compliance with the specifications. 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. Here below are listed some test strains useful for the quality control. [13,14,15]

11 – PERFORMANCES CHARACTERISTICS

Prior to release for sale, a representative sample of all lots of Legionella Selective Agar MWY ISO ready to use plate medium is tested for productivity and selectivity by comparing the results with the BCYE (Reference Batch-RB) non-selective medium.
The productivity of the Batch-TB Test is assessed with a quantitative method with the following target strains: *L.pneumophila* ATCC 33152, *L.anisa* ATCC 35292. The test lot and the reference lot are inoculated with appropriate decimal dilutions in water solution of the suspensions colonies and incubated at 35-37 ° C for 44-48 hours (*L. pneumophila*) and 3-5 days (*L.anisa*). Colonies are enumerated on both lots and the productivity ratio (Pr = CFUTB / CFURB) is calculated. If Pr is ≥ 0.5 the results are considered acceptable and conform to specifications. To evaluate the selectivity of the medium, appropriate dilutions from an initial suspension ~~of~~ McFarland 0.5 of the following non-target strains are inoculated with the modified Miles-Misra surface drop method: *E.faecalis* ATCC 19433, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853

After incubation at 35-37 ° C for 3 days in an aerobic atmosphere, the growth of *E. coli* is completely inhibited while the other non-target strains are partially inhibited.

12 - LIMITATIONS OF THE METHOD

- Some legionellae cannot be grown on routine Legionella culture media and have been termed Legionella-like amoebal pathogens (LLAPs), because they grow in certain host species of amoeba.[16]
- Colonies of *Legionella* grown on white membrane filters may have a different appearance to those that develop against a black or dark background filter.
- Do not incubate the medium in CO_2 higher than 2.5% due to the possibility that *L.pneumophila* growth may be inhibited.
- The glycine contained in the medium may inhibit some of non-*pneumophila* strains.[17]
- Selective BCYE media that contain vancomycin may not support the growth of all *Legionella* spp.[18]
- Not all *Legionella*-positive samples may be identified by a single culture method. A combination of non selective and selective media is strongly recommended.[1,12,19]
- The plates with characteristic growth and with colonies presumptively identified as *Legionella*, must undergo confirmation tests with biochemical, immunological, molecular or mass spectrometry techniques. If relevant, perform antimicrobial susceptibility testing.

13 - PRECAUTIONS AND WARNINGS

- This product is for microbiological control only and for professional use only; it is to be used by adequately trained and qualified laboratory personnel, observing approved biohazard precautions and aseptic techniques.
- This product is not classified as dangerous according to current European legislation.
- Treat all specimens as potentially infectious.
- When handling Legionella cultures, it is important to avoid aerosol formation. Thoroughly clean and disinfect all work areas.
- The laboratory environment must be controlled to avoid contamination with soil and microbial agents.
- The single plate of the product described here 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 sterilized plates inoculated with samples or microbial strains in accordance with current local legislation.
- 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.

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 after the expiration date. 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

1. Edelstein PH, Luck C. *Legionella*. *In* Jorgensen JH, Carrol KC, Funke G et al. editors. Manual of clinical microbiology,11th ed. Washington, DC: American Society for Microbiology; 2015.
2. Mercante JW, Winchell JM. Current and Emerging Legionella Diagnostics for Laboratory and Outbreak Investigations. Clin Microbiol Rev. 2015; 28:95-147
3. Descours G, Cassier P, Forey F , Ginevra C, Etienne J, G. Jarraud LS. Evaluation of BMPA, MWY, GVPC and BCYE media for the isolation of Legionella species from respiratory samples. J Microbiol Meth 2014; 98:119–121
4. ISO 11731:2017 Water quality — Enumeration of Legionella
5. Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB, Charcoal-yeast extract agar: primary isolation medium for Legionella pneumophila, J Clin Microbiol 1979; 10:437-441.
6. Edelstein P.H., Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J Clin Microbiol 1981; 14:298-303
7. Pasculle AW, Feeley JC, Gibson RJ et al. Pittsburgh Pneumonia Agent: Direct Isolation from Human Lung Tissue. J Infect Dis 1980; 141:727.
8. Wadowsky RM, Yee RB.. Glycine-Containing Selective Medium for Isolation of Legionellaceae from Environmental Specimens. Appl Environ Micro 1981; 42:768-772
9. Dennis PJL, Bartlett CLR, Wright AE. 1984. Comparison of Isolation Methods for Legionella spp. In Thronsbury, C. et al. (ed.) Legionella: Proceedings of the 2nd International Symposium. Washington, D.C. ASM.; 294- 296.
10. Vickers RM, Brown A, Garrity GM. Dye-containing BCYE medium for differentiation of members of the family Legionellaceae. J Clin Micriobiol 1981;13:380.
11. Edelstein PH Comparative Study of Selective Media for Isolation of Legionella pneumophila from Potable Water.J Clin Microbiol 1982; 16:697.
12. Public Health England. UK Standards for Microbiology Investigations. Identification of Legionella species. ID18, Issue no: 3, Issue date: 14.04.15
13. ISO 11133:2014. Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media
14. CLSI (formerly NCCLS) Quality Control of Commercially Prepared Culture Media. Approved Standard, 3rd edition. M22 A3 vol. 24 n° 19, 2004.
15. The Australian Society for Microbiology. Guidelines for Assuring Quality of Medical Mycological Culture Media. 2012
16. Legionella and the prevention of legionellosis- Edited by: Bartram J, Chartier Y, Lee JV, Pond K, Surman-Lee S. World Health Organization 2007.
17. Lück PC, Igel L, Helbig JH, Kuhlisch E, Jatzwauk L. Comparison of commercially available media for the recovery of Legionella species. Int J Hyg Environ Healt 2004; 207(6):589-93.
18. Lee TC, Vickers RM, Yu VL, Wagener MM. Growth of 28 Legionella species on selective culture media: a comparative study. J Clin Microbiol 1993;31(10):2764-8.
19. Kusnetsov JM, Jousimies-Somer HR, Nevalainen AI, Martikainen PJ. Isolation of Legionella from water samples using various culture methods. J Appl Bacteriol. 1994 76(2):155-62.

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
Revision 0	First edition	2022/07

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

Biolife Italiana S.r.l., Viale Monza 272, 20128 Milan, Italy.
Tel. +39 02 25209.1, Fax +39 02 2576428
E-mail: export@biolifeitaliana.it; web: www.biolifeitaliana.it