LEGIONELLA SELECTIVE AGAR (GVPC)

Ready-to-use 55 mm plates

1 - INTENDED USE

Selective medium for the enumeration of *Legionella* spp. in water samples.

2 - COMPOSITION -TYPICAL FORMULA *

Activated charcoal	2.0 g
Yeast extract	10.0 g
Agar	13.0 g
Potassium hydroxide /ACES Buffer	12.8 g
Ferric pyrophosphate	250.0 mg
L-cysteine HCl	400.0 mg
α –ketoglutarate	1.0 g
Glycine	3.0 g
Vancomycin HCl	1.0 mg
Polymyxin B	80,000 IU
Cycloheximide	80.0 mg
Distilled 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, obligate 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 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 detection limit required; 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 Pasculle *et al.*[6] by the addition of ACES buffer and by Edelstein[7] by introducing α-ketoglutarate. Wadowsky and Yee[8] proposed a selective version of BCYE, by introducing in the formulation glycine, vancomycin and polymyxin, 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.

Legionella Selective Agar (GVPC) is prepared according to the formulation recommended by ISO 11731.[4]

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 amino acid and an important energy source for *Legionella* spp. Glycine and polymyxin B are inhibitors of Gram-negative bacteria, vancomycin suppresses the growth of Gram-positive bacteria while cycloheximide is included as an antifungal agent.

4 - PHYSICAL CHARACTERISTICS

Medium appearance	black, homogeneously opaque
Final pH at 20-25°C	6.8 ± 0.2

5 - MATERIALS PROVIDED – PACKAGING

Product	Type	REF	Pack
Legionella Selective Agar (GVPC)	Ready-to-use plates	499995	6 x 5 plates ø 55 mm primary packaging: 6 cellophane sachets secondary packaging: cardboard box

6 - MATERIALS REQUIRED BUT NOT PROVIDED

Sterile loops and swabs, incubator and laboratory equipment as required, reagents for the sample treatment, 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.

8 - TEST PROCEDURE

Keep the plates to room temperature and allow the surface of the medium to dry.

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 involving BCYE-GVPC medium are summarized below.

1. 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***).

2. 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***).
3. 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.

READY-TO-USE PLATES: ^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 criteria 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).
Make sure not to remove the medium along with the colony and inoculate the cysteine-free medium first and then the cysteine medium. Incubate at 36 ± 2°C for 2 to 5 days.[4]

9 - READING AND INTERPRETATION

Examination of the plates
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 14 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 14.[1]
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 helps 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 specially in the first 2 days of incubation, the edge shows a pink or blue-green iridescence while the centre is opalescent gray with a ground glass like appearance. 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* and common Legionellae, normally do not show auto-fluorescence. 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 Standard.[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, the end user can perform his own Quality Control 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.

CONTROL STRAINS	INCUBATION T°/ T / ATM	EXPECTED RESULTS
L. pneumophila ATCC 33152	35-37°C / 44-48 h / A	good growth
L. anisa ATCC 35292	35-37°C / 3-5 days / A	good growth
E. faecalis ATCC 19433	35-37°C / 3 days / A	inhibited
E. coli ATCC 25922	35-37°C / 3 days / A	inhibited

A: aerobic incubation; ATCC is a trademark of American Type Culture Collection

11 - PERFORMANCES CHARACTERISTICS

Prior to release for sale, a representative sample of all lots of ready-to-use plates of Legionella Selective Agar (GVPC) and of the raw material used for the production of prepared plates (dehydrated Legionella Agar Base REF 401582 supplemented with BCYE α-Growth Supplement and Legionella GVPC Selective Supplement) are tested for productivity and selectivity.
Productivity of Legionella Selective Agar (GVPC) (Test Batch-TB) is tested by a quantitative method, comparing the results with a previously approved non selective BCYE Agar batch (Reference Batch-RB), with the following strains: *L. pneumophila* ATCC 33152, *L. pneumophila* clinical isolate and *L. anisa* ATCC 35292. Test batch and Reference batch are inoculated with decimal dilutions in saline of the colonies' suspensions and incubated at 35-37°C for 44-48 hours (*L. pneumophila)* and 3-5 days *(L. anisa)*. The colonies are enumerated on both batches and the productivity ratio (Pr= CFU_{TB}/CFU_{RB}) is calculated. If Pr is ≥ 0,5 and if the colonies' morphology is typical, the results are considered acceptable and conform to the specifications.
Selectivity is evaluated with modified Miles-Misra surface drop method by inoculating the plates with suitable decimal dilutions in saline of a 0.5 McFarland suspension of the following non-target strains: *S. aureus* ATCC 25923, *E. faecalis* ATCC 19433, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 18804. After incubation at 35-37°C for 72 hours the growth of non-target strain is observed and recorded: *S. aureus*, *E. faecalis* and *E. coli* are totally inhibited, while *P. aeruginosa* and *C. albicans* 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.[11]

- Colonies of *Legionella* grown on white membrane filters may have a different appearance to those that develop against a black or dark background filter.
- Feeley *et al.*[5] recommend medium not be incubated with CO2 concentrations higher than 2.5% due to the possibility that *L. pneumophila* growth may be inhibited.
- The glycine-containing medium (GVPC) may inhibit some of the non-*pneumophila* strains.[12]
- Selective BCYE media that contain vancomycin may not support the growth of all *Legionella* spp.[13]
- Culture media performance is a critical factor in the isolation of Legionellae from respiratory samples. It has been reported[14] that BMPA and MWY yielded significantly higher isolation rates than GVPC and BCYE media in samples that harboured low *Legionell*a inocula and high contamination levels.
- Not all the *Legionella* positive samples may be identified by a single culture method. A combination of non-selective and selective media is strongly recommended.[1,10,15]
- The plates with characteristic growth and with colonies presumptively identified as *Legionella*, must undergo confirmation tests with biochemical, immunological, molecular or mass spectrometry techniques.

13 - Precautions and warnings

- This product is for microbiological control 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.
- All laboratory specimens should be considered infectious.
- The laboratory area must be controlled to avoid contaminants such as culture medium or microbial agents.
- When handling *Legionella* spp., it is important to avoid aerosol formation. Thoroughly clean and disinfect all work areas
- 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 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 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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15. 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	Use by	Manufacturer	
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 6	Updated layout and content in compliance with IVDR 2017/746	2020/10
Revision 7	Removal of obsolete classification	2023/04
Revision 8	Update of medium pH range	2024/04

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