

**INSTRUCTIONS FOR USE****LEGIONELLA SELECTIVE AGAR (GVPC)****Ready-to-use plates**

Legionella pneumophila
on Legionella Selective Agar (GVPC)

1 - INTENDED USE

In vitro diagnostic device. Selective medium for the isolation and enumeration of *Legionella* spp. from clinical specimens and 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 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}

For optimal isolation performance of *Legionella* spp. from clinical samples it is advisable to use different types of culture media: 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 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 Edelstein⁷ by introducing α -ketoglutarate. Wadowsky and Yee⁸ 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 Gram-negative bacteria inhibitors, 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 \pm 0.2

5 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Legionella Selective Agar (GVPC)	Ready-to-use plates	549995	2 x 10 plates \varnothing 90 mm primary packaging: 2 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

Legionella Selective Agar (GVPC) is intended for the bacteriological processing of several human clinical specimens including those from the lower respiratory tract, such as sputum, pleural fluid, bronchial aspirates, and bronchial alveolar lavage (BAL) fluid; lung tissue and biopsy specimens are also appropriate for attempting culture.^{1,10} Collect specimens before antimicrobial therapy where possible. Transfer the sample as soon as possible to the laboratory; use a transport medium if the sample cannot be processed immediately.





Non-clinical specimens include all kinds of water samples such as potable, industrial, waste, natural waters and water related samples (e.g. biofilms, sediments, etc.).⁴ Consult the ISO Standard for sampling methods and for sample treatment procedures.⁴ Good laboratory practices for collection, transport and storage of the specimens should be applied.

8 - TEST PROCEDURE

Allow plates to come to room temperature and to dry the surface of the medium.

Clinical specimens^{1,10}

Optimal yield of *Legionella* spp. from clinical specimens usually requires¹:

- That specimen is diluted 1:10 in Tryptic Soy Broth or distilled water to reduce inhibition by tissue and serum factors, as well as antibiotics. If the sputum is very dense, it must be re-suspended with 0.2-1 mL of dithiothreitol-based fluidifying.
- That the specimen is pre-treated to reduce contaminating flora. This is done by diluting 1:10 the specimen with a low pH KCl-HCl buffer (pH 2.2) and incubating at room temperature for 4 minutes. An alternative to specimen acidification is heating at 50°C for 30 min.
- That a variety of media are used: one plate with non selective medium (BCYE) and two with selective media.

Inoculate approximately 0,1 mL on the first quadrant of each plate and streak with a loop over the other quadrants of the plate to obtain well isolated colonies.

Incubate at 35-37°C in humidified air for 14 days. A small amount of CO₂ (2.5%) supplementation may enhance the growth of some of the more fastidious *Legionella* spp. such as *L. saintelensis* and *L. oakridgensis*. This low level of CO₂ supplementation will not harm the growth of *L. pneumophila*, but CO₂ levels higher than 2.5% may inhibit growth.

Environmental samples²

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 Standard⁴ 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 criterion to differentiate *Legionella* 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

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 and then 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 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 specially in the first 2 days of incubation, the edge shows a pink or blue-green iridescence while the centre is opalescent gray with an ground glass like appearance. Observed under UV lamp (366 nm), some species (*L.anisa*, *L.bozemanii*, *L.cherrii*, *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
<i>L. pneumophila</i> ATCC 33152	35-37°C / 44-48 h / A	good growth
<i>L. anisa</i> ATCC 35292	35-37°C / 3-5 days / A	good growth
<i>E. faecalis</i> ATCC 19433	35-37°C / 3 days / A	inhibited
<i>E. coli</i> 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 $Pr \geq 0,5$ 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 reported¹⁴ that BMPA and MWY media yielded significantly higher isolation rates than GVPC and non-selective BCYE media in samples that harboured low *Legionella* 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. If relevant, perform antimicrobial susceptibility testing.
- In clinical microbiology, the diagnosis of legionellosis must be based on an interdisciplinary approach that includes radiological results, cultural results, determination of urinary antigen. Legionella Selective Agar (GVPC) is intended as an aid to the diagnosis of the infection: the interpretation of the results must be made considering the patient's clinical history, the origin of the sample and the results of the microscopic and/or other diagnostic tests.

13 - 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.
- 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.
- 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.

14 - STORAGE CONDITIONS AND SHELF LIFE

Upon receipt, store plates in their original pack at +2°C / +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

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. Pasculle AW, Feeley JC, Gibson RJ et al. Pittsburgh Pneumonia Agent: Direct Isolation from Human Lung Tissue. J Infect Dis 1980; 141:727.
7. Edelstein P.H., Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J Clin Microbiol 1981; 14:298-303
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 P.J.L, 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. Public Health England. UK Standards for Microbiology Investigations. Identification of Legionella species. ID18, Issue no: 3, Issue date: 14.04.15
11. Legionella and the prevention of legionellosis- Edited by: Bartram J, Chartier Y, Lee JV, Pond K, Surman-Lee S. World Health Organization 2007.
12. 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.
13. 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.
14. Descours G, Cassier P, Forey F, Ginevra C, Etienne J, Lina G, Jarraud S. Evaluation of BMPA, MWY, GVPC and BCYE media for the isolation of Legionella species from respiratory samples. J Microbiol Methods 2014; 98:119-21.
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	IVD <i>In vitro</i> 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 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	2024/04

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

