INSTRUCTIONS FOR USE

LEGIONELLA BCYE AGAR BASE

Ready-to-use flasks

Legionella Agar (BCYE): colonies of *L. pneumophila* and of contaminating flora

1 - INTENDED USE

In vitro diagnostic. medium base to be used with growth and selective supplements 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
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]

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 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 *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.

The culture media and supplements described here comply with the requirements of ISO Standard 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, cefazolin is active against Gram-positive bacteria and some Gram-negative bacteria, vancomycin suppress the growth of Gram-positive bacteria while cycloheximide, natamycin and anisomycin are used as antifungal agents.

4- DIRECTIONS FOR MEDIA PREPARATION

Liquefy the contents of the flask in an autoclave set at 100 ± 2°C or in a temperature-controlled water bath (100°C). Alternatively, the bottle may be placed into a jar containing water, which is placed on a hot plate and brought to boiling. Slightly loosen the cap before heating to allow pressure exchange. Cool to 47-50°C and pour the medium into sterile Petri dishes, under aseptic conditions. Cool to 47-50°C and add the suitable growth and selective supplements. After supplements addition, keeping the medium under stirring, distribute into sterile Petri dishes (90 mm or 55 mm).

HIGHLY SELECTIVE MEDIUM BCYE-GVPC

To 180 mL of medium base cooled to 47-50°C, add 20 mL of Legionella BCYE α-Growth Supplement (REF 423210) reconstituted with 50 mL of sterile purified water and 4 mL of Legionella GVPC Selective Supplement (REF 423215) reconstituted with 10 mL of sterile purified water.

SELECTIVE MEDIUM BCYE-AB

To 180 mL of medium base cooled to 47-50°C, add 20 mL of Legionella BCYE α-Growth Supplement (REF 423210) reconstituted with 50 mL of sterile purified water and 2 mL of Legionella AB Selective Supplement (REF 423225), reconstituted with 5 mL of sterile purified water.

HIGHLY SELECTIVE MEDIUM BCYE-MWY (WITH ANISOMYCIN)
To 180 mL of medium base cooled to 47-50°C, add 20 mL of Legionella BCYE α-Growth Supplement (REF 423210) reconstituted with 50 mL of sterile purified water and 4 mL of Legionella MWY Selective Supplement (ISO) (REF 423220), reconstituted with 10 mL of sterile purified water.

NON-SELECTIVE MEDIUM WITH CYSTEINE: BCYE W/ L-CYSTEINE
To 180 mL of medium base cooled to 47-50°C add 20 mL of Legionella BCYE α-Growth Supplement (REF 423210), reconstituted with 50 mL of sterile purified water.

NON-SELECTIVE MEDIUM WITHOUT CYSTEINE: BCYE W/O L-CYSTEINE
To 180 mL of medium base cooled to 47-50°C add 20 mL of Legionella BCYE α-Growth Supplement w/o Cysteine (REF 423212), reconstituted with 50 mL of sterile purified water.

5 - PHYSICAL CHARACTERISTICS

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

6 - MATERIALS PROVIDED – PACKAGING

Product	Type	REF	Pack
Legionella BCYE Agar Base	Ready to use flasks	5115824	6 x 180 mL; 6 glass bottles with flat bottom and aluminium screw-cap; packaging: cardboard box.

7 - MATERIALS REQUIRED BUT NOT PROVIDED
Autoclave, water bath or hot plate, incubator and laboratory equipment as required, selective and enrichment supplements, sterile plastic Petri dishes, sterile loops, needles and swabs, ancillary culture media and reagents for the identification of the colonies.

8 - SPECIMENS
Legionella BCYE Agar Base supplemented with Legionella BCYE α-Growth Supplement and the selective supplement 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.[1,12] 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.
Legionella BCYE Agar Base supplemented with Legionella BCYE α-Growth Supplement and one of the selective supplements GVPC, MWY-ISO and AB is intended for the bacteriological processing of non-clinical specimens: 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 11731 for sampling methods and for sample treatment procedures.[4] Good laboratory practices for collection, transport and storage of the specimens should be applied.
Legionella BCYE Agar Base supplemented with Legionella BCYE α-Growth Supplement w/o Cysteine, must be inoculated with colonies cultivated on selective or non-selective isolation media for the presumptive confirmation of *Legionella* colonies.

9 - TEST PROCEDURE
Keep the plates to room temperature and allow the surface of the medium to dry.
Isolation from clinical specimens[1,12]
Optimal yield of *Legionella* spp. from clinical specimens usually requires[1]:
- 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 different 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. A small amount of CO_2 (2.5%) supplementation may enhance the growth of some of the more fastidious *Legionella* spp. such as *L.sainthelensi* and *L.oakridgensis*. This low level of CO_2 supplementation will not harm the growth of *L.pneumophila*, but CO_2 levels higher than 2.5% may inhibit growth.
Colonies are normally microscopically visible after 2 days and, macroscopically, after 3-5 days.
Enumeration in environmental samples[4]
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.

PLATES PREPARED AS DESCRIBED ABOVE OR BIOLIFE 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 *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]

10 - 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 help in the *Legionella* and contaminants colonies recognition.
Legionella colonies, in principle, appear white-grey, 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 a 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]

11 - 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. The choice of *Legionella* strains and non-target microorganisms must be made depending on of the prepared, selective or non-selective, media and the field of application (clinical or water analysis). Consult the quoted literature for the details of the quality control procedures.[13,14,15]

12 - PERFORMANCES CHARACTERISTICS

Prior to release for sale, a representative sample of all lots ready-to-use flasks and of the raw material for the production (dehydrated Legionella Agar Base REF 401582 (Test Batch-TB) supplemented with BCYE α-Growth Supplement and Legionella GVPC Selective Supplement is tested for productivity and selectivity, comparing the results with a previously approved batch (Reference Batch-RB)
Productivity is tested by a quantitative method, 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 water 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 ≥ 0.7 and if the colonies morphology is typical, the results are considered acceptable and conform to the specifications.
The productivity of Legionella BCYE Agar Base REF 401582 is also evaluated with the addition of only BCYE α-Growth Supplement with the target strain *L. pneumophila* ATCC 33152 with the same acceptance criteria described above for the GVPC medium.
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.

13 - 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.
- Feeley *et al.*[5] recommend not to incubate the medium with CO_2 concentrations 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 and cefamandole may not support the growth of all *Legionella* spp.[18]
- Culture media performance is a critical factor in the isolation of legionellae from respiratory samples. It has been reported[3] that MWY medium yielded significantly higher isolation rates than GVPC and non-selective BCYE media, with samples containing small numbers of *Legionella* and high levels of contaminants.

- 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.
- 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 BCYE Agar Base and supplements are 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.

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.
- The medium base and the supplements shall be used in association, according to the described directions.
- This product is not classified as dangerous according to current European legislation.
- All laboratory specimens should be considered infectious.
- When handling *Legionella* spp., it is important to avoid aerosol formation. Thoroughly clean and disinfect all working areas.
- The laboratory area must be controlled to avoid contaminants such as culture medium or microbial agents.
- Be careful when opening screw cap flasks to prevent injury due to breakage of glass.
- When using a hot plate and/or a water bath, boil sufficiently long to dissolve the whole medium.
- Wear heat-protective gloves during medium liquefaction. Do not place the hot flasks into an ice bath or in cold water to accelerate cooling as this might cause cracks in the glass.
- The time required for complete liquefaction of the medium may vary considerably and depends on the actual temperature of the heating device, its wattage, the size and volume of the bottle.
- Once the bottled medium is liquefied, it cannot be solidified and dissolved a second time.
- Ready-to-use flasks of Legionella BCYE Agar Base are subject to terminal sterilisation in a steam autoclave.
- 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.
- Do not use the culture medium as active ingredient for pharmaceutical preparations or as production material intended for human and animal consumption.
- The Certificates of Analysis and the Safety Data Sheet of the product are available on the website www.biolifeitaliana.it.
- Notify Biolife Italiana S.r.l (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.

15 - STORAGE CONDITIONS AND SHELF LIFE

Upon receipt, store flasks in their original pack at +2/+8°C away from direct light. If properly stored, the flasks may be used up to the expiration date. Do not use the flasks beyond this date. Flasks from opened secondary packages can be used up to the expiration date. Opened flasks must be used immediately. Before use, check the closing and the integrity of the screw cap. Do not use flasks with signs of deterioration (e.g. microbial contamination, abnormal turbidity, precipitate, atypical colour).

The user is responsible for the correct preparation of the plates and validation of their shelf-life, depending on the storage method applied (temperature and packaging) and the supplements used.
According ISO 11731[4] the prepared plates may be stored at 5 ± 3 °C in airtight containers in the dark for:
BCYE-with cysteine: up to 3 months
BCYE-without cysteine: up to 3 months
BCYE-GVPC: up to 4 weeks
BCYE-MWY: up to 4 weeks
BCYE-AB: up to 3 months

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	For single use only	Fragile, handle with care

REVISION HISTORY

Version	Description of changes	Date
Instructions for Use (IFU) - Revision 0	First edition in compliance with IVDR 2017/746	2021/09
Revision 1	Removal of obsolete classification	2023/04
Revision 2	Update of pH of complete media, minor changes in the chapter on precautions and warnings, inclusion of storage conditions for plates prepared by users.	2024/04

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

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