INSTRUCTIONS FOR USE

LEGIONELLA AGAR (BCYE)

Ready-to-use plates

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

1 - INTENDED USE

In vitro diagnostic device. Non-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 mg
L-cysteine HCl	400 mg
α–ketoglutarate	1.0 g
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]

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 Pasculle *et al.*[6] by the addition of ACES buffer and by Edelstein[7] by introducing α-ketoglutarate.

Legionella Agar (BCYE) 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.

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 Agar (BCYE)	Ready-to-use plates	549945	2 x 10 plates ø 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 Agar (BCYE) 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, bronchial alveolar lavage (BAL) fluid, lung tissue and biopsy specimens.[1,8] 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[1,8]

Optimal yield of *Legionella* spp. from clinical specimens usually requires[1]:

- That specimen be 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 be 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 be used: one plate with non-selective medium (BCYE) and two with selective media.

Inoculate approximately 0,1 mL onto each plate, with the bulk of inoculums applied to the first quadrant 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 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.

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 involving BCYE with L-cysteine medium 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 Legionella Agar (BCYE) plate 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 Legionella Agar (BCYE) 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 Legionella Agar (BCYE) plate and on plates of one or more selective and highly selective media (BCYE-AB* or BCYE-GVPC** or BCYE-MWY***).

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: *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 Legionella Agar (BCYE) and a plate of Legionella Agar 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 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 3 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 1 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 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* 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 with cysteine but fail to grow on the plate of BCYE without 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 its 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

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 Agar (BCYE) is tested for productivity 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 ≥ 0.7 the results are considered acceptable and conform to the specifications.

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.[9]
- 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 be incubate plates in CO_2 higher than 2.5% due to the possibility that *L.pneumophila* growth may be inhibited.[5]
- 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.[1,8,10]
- 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 2-8°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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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 6	Updated layout and content in compliance with IVDR 2017/746	2020/10
Revision 7	Removal of obsolete classification	2023/03
Revision 8	Update of pH of medium, minor changes in water testing procedure and performances characteristics	2024/04

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

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