LEGIONELLA SELECTIVE AGAR AB

Ready to use plates

1 - INTENDED USE

For the enumeration of *Legionella* 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
Cefazolin	9.0 mg
Pimaricin (natamycin)	70.0 mg
Polymyxin B	80,000 UI
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* species share growth dependence for L-cysteine and growth enhancement by iron. 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 desired lower limit of detection level; a decision matrix for choosing an appropriate method is described in ISO 11731.[4]

Buffered Charcoal Yeast Extract (BCYE) agar was developed by Feeley *et al.*[5] and then further modified by Pasculle *et al.*[6] with the addition of ACES buffer and by Edelstein[7] with the introduction of α-ketoglutarate. Wadowsky and Yee[8] devised a selective version of BCYE by including glycine, vancomycin and polymyxin (GVP medium) in the formulation. Finally, in 1984 Dennis *et al.*[9] proposed the introduction of cycloheximide making the medium even more selective for Legionella, resulting in the GVPC medium.

Legionella Selective Agar AB is one of the culture media recommended by ISO 11773 for the enumeration of *Legionella* from water samples and consists of BCYE medium supplemented with a mixture of antimicrobials.

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. Polymyxin B is inhibitor of Gram-negative bacteria, cefazolin is active against Gram-positive and some Gram-negative bacteria and pimaricin (natamycin) is used as antifungal agent

4 - PHYSICAL CHARACTERISTICS

Prepared plates appearance	black, opaque
Final pH at 20-25 °C	6.8 ± 0.2

5 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Legionella Selective Agar AB	Ready-to-use plates	549947	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, 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.).[5] Consult the Standard ISO 11731 for sampling methods and sample handling procedures.[5] Apply good laboratory practices for specimen collection, transport and storage.

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[5] 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-AB 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 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***).

Allow the inoculum to absorb well then incubate the inverted plates in a humid atmosphere at 36 ± 2°C for 7-10 days
The procedural elements reported above are entirely schematic. For details of *Legionella* counting techniques in water, refer to the ISO 11731 Standard[5] 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 *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 and a plate of BCYE w/o cysteine.
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.
Inspect the plates for the first time either on day 2, 3, 4 or 5 followed by a final inspection at the end of the incubation period.
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 grey with an appearance similar to ground glass. 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 colonies appear green, opaque, often tinged with yellow. The colour 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
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.

CONTROL STRAINS		INCUBATION T°/ T / ATM	EXPECTED RESULTS
L. pneumphila	ATCC 33152	35-37 °C / 44-48 H / A	growth, grey/bluish-white colonies
L. anisa	ATCC 35292	35-37 °C / 3-5 days / A	growth, grey/bluish-white colonies
E. coli	ATCC 25922	35-37 °C / 3 days / A	partially inhibited
E. faecalis	ATCC 319433	35-37 °C / 3 days / A	totally 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 Legionella Selective Agar AB is tested for productivity and selectivity by comparing the results with the non-selective BCYE Agar (Reference Batch-RB).
Productivity is tested by a quantitative method, with the following strains: *L. pneumophila* ATCC 33152 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 ≥ 0.5 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: *E. faecalis* ATCC 19433, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853. After incubation at 35-37°C for 3 days the growth of non-target strain is observed and recorded: *P. aeruginosa* and *E. coli* are partially inhibited, while *E. faecalis* is totally inhibited.

12 - LIMITATIONS OF THE METHOD

- Some Legionellae cannot be grown on normal culture media and have been defined as Legionella-like amoebal pathogens (LLAPs), because they grow in some 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.[8]
- Not all *Legionella*-positive samples may be identified by a single culture method. A combination of non-selective and selective media is strongly recommended.[9]
- 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.
- 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 culture medium 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

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TABLE OF APPLICABLE SYMBOLS

REF or REF Catalogue number	LOT Batch code	Manufacturer	Fragile, handle with care	Use by
Temperature limitation	Contents sufficient for <n> tests	Consult Instructions for Use	For single use only	

REVISION HISTORY

Version	Description of changes	Date
Revision 0	First issue	2024/04

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