CLOSTRIDIUM BROTH
(Reinforced Clostridial Medium)

Dehydrated culture medium

1 - INTENDED USE
For the cultivation and enumeration of clostridia and other anaerobes.

2 - COMPOSITION - TYPICAL FORMULA*
(AFTER RECONSTITUTION WITH 1 L OF WATER)

Yeast extract	3.0 g
Beef extract	10.0 g
Peptone	10.0 g
Glucose	5.0 g
Soluble starch	1.0 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
L-cysteine HCl	0.5 g
Agar	0.5 g

*The formula may be adjusted and/or supplemented to meet the required performances criteria.

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE
Clostridium Broth, formulated by Hirsch and Grinstead in 1954,[1] is also known as Reinforced Clostridial Medium. It is recommended by European Pharmacopoeia[2] for the selective enrichment of clostridia from non-sterile pharmaceutical products and conforms to harmonized USP/EP/JP standards performance specifications.
Clostridium Broth is the basal medium for the preparation of Differential Reinforced Clostridial Medium of Gibbs and Freame,[3] recommended by ISO 6461-1[4] for detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) in water samples.
This medium has been described for the detection of clostridia in food products with the Most Probable Number method.[5]
The medium is very rich and permits the growth of most clostridia, and many other anaerobes and facultative anaerobes. Tryptone and beef extract provide nitrogen, carbon, minerals and amino acids for the microbial growth. Yeast extract is a source of vitamins, particularly of the B-group and glucose is a source of carbon and energy. Sodium chloride maintains the osmotic balance while sodium acetate buffers the medium. L-cysteine, a reducing agent, and agar at low concentration favour the growth of anaerobes. Soluble starch helps to detoxify metabolic by-products. Sodium sulphite and ferric citrate are added to the medium and act as indicators: sulphite reducing clostridia produce sulphide from sulphite, which results in the formation of black medium. According to Hirsch and Grinsted,[1] polymyxin B 0.02 g/L can be added to inhibit Gram-negative bacteria.

4- DIRECTIONS FOR MEDIUM PREPARATION
Suspend 38 g in 1000 ml of cold purified water. Heat to boiling with frequent agitation, distribute into screw-capped bottles, and sterilise by autoclaving at 115°C for 20 minutes.
For the preparation of Differential Clostridium Medium, prepare the Clostridium Broth at double strength reducing the water volume by half and transfer 10 mL and 50 mL aliquots into screw-capped bottles with a capacity slightly more than double. Prepare a 4% solution of sodium sulphite and a 7% solution of ferric citrate; if necessary, heat the ferric citrate solution for 5 minutes to dissolve completely. Sterilise the two solutions by filtration, and store at 2-5 °C in closed bottles; the two solutions are stable for two weeks. The day of analysis, mix equal volumes of the two solutions and, under sterile conditions, add 0.5 mL of reagent to each 25 mL of medium. For the preparation of double strength Clostridium Broth add 0.4 mL of the mixture to each 10 mL and 2 mL to each 50 mL.
If required, add 0.02 g Polymyxin B/litre in form of a filter-sterilized aqueous solution to the pre-cooled medium to 45-50°C.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	pale yellow, fine, homogeneous, free-flowing powder
Solution and prepared tubes appearance	pale yellow, slightly opalescent
Final pH at 20-25 °C	6.8 ± 0.2

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Clostridium Broth	Dehydrated medium	4013042	500 g (13.2 L)

7 - MATERIALS REQUIRED BUT NOT PROVIDED
Autoclave, water-bath, sterile loops and pipettes, incubator and laboratory equipment as required, test tubes, controlled atmosphere generators and jars, Erlenmeyer flasks, sodium sulphite, ferric citrate, ancillary culture media and reagents.

8 - SPECIMENS
Water, food and pharmaceutical samples. When collecting, storing, transporting and preparing samples, follow the rules of good laboratory practice and refer to applicable International Standards and regulations.

9 - TEST PROCEDURE, READING AND INTERPRETATION
For the determination of clostridia in non-sterile pharmaceutical products proceed as follows.[2]
1. Prepare a 1:10 dilution of the sample in Pharmacopoeia Diluent (REF 401395), with a minimum volume of 20 mL, using not less than 2 g or 2 mL of test product.
2. Divide this dilution into 2 aliquots of at least 10 mL each. Heat one aliquot to 80°C for 10 minutes and cool rapidly. Do not heat the second aliquot.

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3. Inoculate 10 mL or the amount corresponding to 1 g or 1 mL of product to be examined from each of the two aliquots into Clostridium Broth.
4. Incubate under anaerobic conditions at 30°C - 35°C for 48 hours.
5. After incubation perform subcultures from each tube/flask on Columbia Agar and incubate under anaerobic conditions at 30°C - 35°C for 48-72 hours.
6. The presence of rods with or without endospores that are negative in the catalase test indicates the presence of clostridia. The culture result must be confirmed by biochemical identification.
7. The test should be considered negative if there are no colonies with the characteristics described above in the sample or if the biochemical identification tests are negative.

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
C. sporogenes ATCC 19404	35-37°C / 48h / AN	growth
C. perfringens ATCC 13124	35-37°C / 48h / AN	growth

AN: anaerobic 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 dehydrated Clostridium Broth, is tested for productivity by comparing the results with a previously approved Reference Batch (RB).
Productivity is tested by dilution to extinction method, by inoculating 1 mL of appropriate decimal dilutions of organisms in test tubes. The strains are incubate (at 35-37°C according to ISO 11133[7] or 30-35° C according to EP) for 48 hours in anaerobic atmosphere and recording the highest dilution showing growth in Reference Batch (Gr_{RB}) and in Test Batch (Gr_{TB}). Productivity is tested with the following strains: *C. perfringens* ATCC 13124, *C. difficile* ATCC 9689, *E. coli* ATCC 25922 according ISO 11133:2014 and *C. sporogenes* ATCC 19404 according to EP. The productivity index Gr_{RB}-Gr_{TB} for each test strain shall be ≤ 1.

12 - LIMITATIONS OF THE METHOD

- Large volume of culture in hermetically sealed glass bottles may explode due to gas production. Refer to ISO 6461-1 or specific texts for precautions to be taken when incubating tubes. [4]
- The medium is not selective: other sporeforming anaerobes as well *C.butyricum*, lactobacilli and streptococci exhibit good growth.[6]
- Clostridium Broth supplemented with sodium sulphite and ferric citrate: other bacteria can produce sulphide; pasteurisation must be done to remove vegetative forms.[6]
- Biochemical, immunological, molecular, or mass spectrometry testing should be performed on isolates, from pure culture, for complete identification.

13 - PRECAUTIONS AND WARNINGS

- This product is for Laboratory use and for professional use only; it is to be used by adequately trained and qualified laboratory personnel, observing approved biohazard precautions and aseptic techniques.
- Dehydrated media must be handled with suitable protection. Before use, consult the Safety Data Sheet.
- This culture medium contains raw materials of animal origin. The *ante* and *post mortem* controls of the animals and those during the production and distribution cycle of the raw materials, cannot completely guarantee that this product doesn't contain any transmissible pathogen. Therefore, it is recommended that the culture medium be treated as potentially infectious, and handled observing the usual specific precautions: do not ingest, inhale, or allow to come into contact with skin, eyes, mucous membranes. Download the TSE Statement from the website www.biolifeitaliana.it, describing the measures implemented by Biolife Italiana for the risk reduction linked to infectious animal diseases.
- Apply Good Manufacturing Practice in the production process of prepared media.
- All laboratory specimens should be considered infectious.
- The laboratory area must be controlled to avoid contaminants such as culture medium or microbial agents.
- 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.
- 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 at +10°C /+30°C away from direct light in a dry place. If properly stored, it may be used up to the expiration date. Do not use beyond this date. Avoid opening the bottle in humid places. After use, the container must be tightly closed. Discard the product if the container and/or the cap are damaged, or if the container is not well closed, or in case of evident deterioration of the powder (colour changes, hardening, large lumps).
The user is responsible for the manufacturing and quality control processes of prepared media and for the validation of the shelf life of the finished products, according to the type (tubes/bottles) and the storage method (temperature and packaging). According to MacFaddin the prepared medium may be stored at +2°C /+8°C for up to 2 weeks.[6]

16 - REFERENCES

1. Hirsch A, Grinsted, E. Methods for the growth and enumeration of anaerobic sporeformers from cheese, with observations on the effect of nisin. J Dairy Res 1954; 21: 101-110.
2. European Pharmacopoeia 11th Edition, 2022, Vol. 1; 2.6.13 Microbiological Examination of non-sterile products: test for specified micro-organisms: 01/2021:20631.
3. Gibbs BM, Freame B. Methods for the recovery of clostridia from foods. J Appl Bacteriol 1965; 28:95-111.
4. ISO 6461-1:1986 Water quality — Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) — Part 1: Method by enrichment in a liquid medium.
5. Mead GC. Principles involved in the detection and enumeration of clostridia in foods. Int J Food Microbiol 1992; 17:135-43.
6. MacFaddin JF. Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria. Baltimore: Williams & Wilkins; 1985.
7. ISO 11133:2014 Microbiology of food, animal feed and water - Preparation, production, storage and performance testing of culture media

TABLE OF APPLICABLE SYMBOLS

REF or REF Catalogue number	LOT Batch code	Manufacturer	Store in a dry place	Use by
Temperature limitation	Contents sufficient for <n> tests	Consult Instructions for Use	Keep away from direct light	

REVISION HISTORY

Version	Description of changes	Date
Revision 1	Updated layout and content	2022/07

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

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