CLOSTRIDIUM PERFRINGENS AGAR BASE
KANAMYCIN POLYMYXIN B ANTIMICROBIC SUPPLEMENT
D-CYCLOSERINE ANTIMICROBIC SUPPLEMENT

Dehydrated culture medium and selective supplements

1 - INTENDED USE
For the isolation and enumeration of *Clostridium perfringens* from foods, waters and other materials.

2 – COMPOSITION*
CLOSTRIDIUM PERFRINGENS AGAR BASE
TYPICAL FORMULA (AFTER RECONSTITUTION WITH 1 L OF WATER)

Tryptose	15 g
Beef extract	5 g
Soy peptone	5 g
Yeast extract	5 g
Sodium metabisulphite	1 g
Ferric (III) ammonium citrate	1 g
Agar	13 g

KANAMYCIN POLYMYXIN B ANTIMICROBIC SUPPLEMENT
(VIAL CONTENT FOR 500 ML OF MEDIUM)

Kanamycin sulphate	6 mg
Polymyxin B	15,000 U.I.

D-CYCLOSERINE ANTIMICROBIC SUPPLEMENT
(VIAL CONTENT FOR 500 ML OF MEDIUM)

D-cycloserine	200 mg

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

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE
Food poisoning caused by *Clostridium perfringens* may occur when foods such as raw meats, poultry, dehydrated soups and sauces, raw vegetables, and spices are cooked and held without maintaining adequate heating or refrigeration before serving.[1] The enumeration of *C.perfringens* in food samples plays a key role in the epidemiological investigation of food-borne disease outbreaks and for this purpose various culture media have been proposed since the 1950s.
Clostridium Perfringens Agar Base is prepared according to a modification of Shahidi-Ferguson-perfringens (SFP) agar[2] by the addition of 5 g/L of beef extract to the original formulation and corresponds to the formulation described by USDA-FSIS.[4]
For the isolation and enumeration of *C. perfringens* in food, the most common methods in continental Europe and North America are those using the medium of Harmon et al. (Tryptose Sulfite Cycloserine - TSC - Agar)[5] or Shahidi-Ferguson's medium[2] with kanamycin and polymyxin B (SFP Agar), which have replaced Angelotti's (Sulfite Polymyxin Sulphadiazine Agar) and Marshall's (Tryptone Sulfite Neomycin Agar) media, which were found to be inhibitory for some strains of *C. perfringens*.[2]
TSC Egg Yolk Agar is prepared by adding to Clostridium Perfringens Agar Base, 400 mg/L of D-Cycloserine and Egg Yolk Emulsion enrichment. The Shahidi and Ferguson Perfringens (SFP) medium is prepared by adding kanamycin and polymyxin B and Egg Yolk Emulsion enrichment to the basal medium.
Tryptose, beef extract and soy peptone provide nitrogen, carbon, minerals and amino acids for the microbial growth. The yeast extract is a source of vitamins particularly of the B-group. Ferric ammonium citrate and sodium metabisulfite are indicators of sulphite reduction by *C. perfringens* which produces black colonies. Egg yolk is the substrate to detect lecithinase activity. D-cycloserine or the mixture kanamycin and polymyxin help in the selective isolation of *C.perfringens* by inhibiting accompanying flora.

4- DIRECTIONS FOR DEHYDRATED MEDIUM PREPARATION
SFP Agar[2]: suspend 22.5 g in 450 mL of cold purified water, heat to boiling with frequent agitation and sterilize by autoclaving at 121°C for 15 minutes. Cool to 47-50°C and add 50 mL of Egg Yolk Emulsion (REF 42111601) and the contents of one vial of Kanamycin Polymyxin B Antimicrobic Supplement (REF 4240005), reconstituted with 5 mL of sterile distilled water. Mix well and pour into sterile Petri dishes.
TSC Egg Yolk Agar[4]: suspend 22.5 g in 475 mL of cold purified water, heat to boiling with frequent agitation and sterilize by autoclaving at 121°C for 15 minutes. Cool to 47-50°C and add 25 mL of Egg Yolk Emulsion (REF 42111601) and the contents of one vial of D-Cycloserine Antimicrobic Supplement (REF 4240002), reconstituted with 5 mL of sterile purified water. Mix well and pour into sterile Petri dishes.
TSC Agar and SFP Agar w/o Egg Yolk: prepare the media as described above by omitting the addition of Egg Yolk Emulsion and weighting 22.5 g in 500 mL of water.

5 - PHYSICAL CHARACTERISTICS

Clostridium Perfringens Agar Base	
Dehydrated medium appearance	beige, fine, homogeneous, free-flowing powder
Solution appearance	yellow, limpid
Final pH at 20-25 °C	7.6 ± 0.2
D-Cycloserine Antimicrobic Supplement	
Freeze-dried supplement appearance	short, dense, white pellet
Reconstituted supplement appearance	colourless limpid
Kanamycin Polymyxin B Antimicrobic Supplement	
Freeze-dried supplement appearance	short, dense, white pellet
Reconstituted supplement appearance	colourless limpid

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6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Clostridium Perfringens Agar Base	Dehydrated medium	4013072	500 g (11.1 L)
Kanamycin Polymyxin B Antimicrobic Supplement	Freeze-dried supplement	4240005	10 vials, each for 500 mL of medium
D-Cycloserine Antimicrobic Supplement	Freeze-dried supplement	4240002	10 vials, each for 500 mL of medium

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave, water-bath, sterile loops, swabs and pipettes, incubator and laboratory equipment as required, Erlenmeyer flasks, sterile Petri dishes, controlled atmosphere generators and jars, ancillary culture media and reagents.

8 - SPECIMENS

Foods and animal feeding stuffs. For sample collection, storage, transport and preparation, follow good laboratory practice and refer to applicable International Standards and regulations.

9 - TEST PROCEDURE

The presumptive enumeration of *C. perfringens* is performed by spreading the sample on TSC Agar or on SFP Agar with egg yolk and adding a second layer of medium without egg yolk on top of the inoculated layer.

- Inoculate the plates with 0.1 mL of the initial suspension and decimal dilutions of the prepared sample, distributing on the surface with a sterile glass rod spreader. Allow to dry 5-10 minutes.
- Cover the inoculated layer with 10 mL of medium prepared without egg yolk. Allow to solidify.
- Incubate the plates for 20-24 hours at 37°C in an anaerobic atmosphere (5% CO_2, 10% H_2, 85% N_2).

10 - READING AND INTERPRETATION

After incubation, observe the bacterial growth and record the specific morphological and chromatic characteristics of the colonies.
For enumeration of *C. perfringens* choose plates containing 20 to 200 typical colonies.
C. perfringens usually produce black or grey to yellow brown colonies as a result of the reduction of sulphite to sulphide which reacts with a ferric salt in the medium, surrounded by an opaque halo of egg yolk precipitation.

11 - USER QUALITY CONTROL

All manufactured lots of the products 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. perfringens ATCC 13124	37°C/ 18-24 H / AN	growth, black colonies surrounded by an opaque halo
E.coli ATCC 25922	37°C/ 18-24 H / AN	totally inhibited

AN: anaerobic incubation; ATCC is a trademark of American Type Culture Collection

12 - PERFORMANCES CHARACTERISTICS

Prior to release for sale, a representative sample of all lots of dehydrated Clostridium Perfringens Agar Base supplemented with Egg Yolk 50% Emulsion, with and without D-Cycloserine Antimicrobic Supplement (Test Batch:TB), is tested for productivity and selectivity by comparing the results with a previously approved Reference Batch (RB).
Productivity is tested by a quantitative technique, with the target strains *C. perfringens* ATCC 13124 and ATCC 12916, incubating at 37°C for 20 hours in anaerobic atmosphere. The colonies are enumerated on both batches and the productivity ratio (Pr: UFC_{TB}/UFC_{RB}) is calculated. If Pr is ≥ 0.7 and if the colonies morphology and colour are typical (black colonies with opaque halo) the results are considered acceptable and conform to the specifications.
Selectivity is tested with modified Miles-Misra method with the following non-target strains: *E. coli* ATCC 25922, *P. mirabilis* ATCC 10005 and *P. aeruginosa* ATCC 27853. After incubation *E. coli* is totally inhibited while *P. mirabilis* and *P. aeruginosa* are partially inhibited.
Clostridium Perfringens Agar Base without D-cycloserine is tested by ecometry with *E. coli* ATCC 25922 which exhibits a good growth.

13 – LIMITATIONS OF THE METHOD

- *C. perfringens* colonies may produce an opaque zone in the surrounding medium due to lecithinase activity, but this phenomenon is not a distinguishing feature of all *C.perfringens* strains after overnight incubation. Both lecithinase-positive and lecithinase-negative black colonies must be tested for confirmation.[6]
- Lecithinase-positive facultative anaerobes can grow on SFP Agar to produce completely opaque plates that mask the egg yolk reaction of *C. perfringens*.
- Black colonies must be confirmed as *C. perfringens* by appropriate tests: motility (-), nitrate reduction (+), acid and gas from lactose (+), gelatin liquefaction (+).

14 - PRECAUTIONS AND WARNINGS

- The medium base and the supplements are for microbiological control and for professional use only; they are 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. Apply Good Manufacturing Practice in the production process of prepared media.
- Dehydrated media and antibiotics containing supplements must be handled with suitable protection. Kanamycin Polymyxin B Antimicrobic Supplement is classified as dangerous. Before use, consult the Material Safety Data Sheets.
- 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 the 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.

- Be careful when opening the metal ring of the supplement vial to avoid injury.
- The supplements are sterilized by membrane filtration.
- All laboratory specimens should be considered infectious.
- The laboratory area must be controlled to avoid contaminants such as medium powder and supplement or microbial agents.
- Sterilize all biohazard waste before disposal. Dispose the unused medium and supplement and the inoculated plates with samples or microbial strains in accordance with current local legislation.
- Do not use the culture medium and the supplements as active ingredients for pharmaceutical preparations or as production materials intended for human and animal consumption.
- The Certificates of Analysis and the Safety Data Sheets 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

Dehydrated medium

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).

Freeze-dried supplements

Upon receipt, store the product in the original package at 2-8°C away from direct light. If properly stored, the product may be used up to the expiry date printed on the label; do not use beyond this date. Once the vial has been opened and the lyophilised product has been reconstituted, the resulting solution should be used immediately. Before use, examine the lyophilized and reconstituted product and discard if there are obvious signs of deterioration (e.g., contamination, atypical colour or other abnormal characteristics).

The user is responsible for the manufacturing and quality control processes of prepared media and the validation of their shelf life, according to the type (plates/bottles) and the applied storage conditions (temperature and packaging). According to ISO 14189[7] the plated medium with D-cycloserine may be stored at 2-8°C for up to 7 days; the basal medium (without D-cycloserine) may be stored at 2-8°C and used within 4 weeks of preparation.

16 - REFERENCES

1. U.S. Food and Drug Administration. Bacteriological Analytical Manual (BAM). Chapter 16: Clostridium perfringens.
2. Shahidi SA, Ferguson AR. New quantitative, qualitative, and confirmatory media for rapid analysis of food for Clostridium perfringens. Appl. Microbiol. 1971; 21:500-506.
3. Atlas R. Parks LC. Handbook of Microbiological Media. 2nd edition. CRC Press,1997
4. United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science. Laboratory Guidebook Notice of Change. MLG Appendix 1.10. Effective Date: 03/07/22.
5. Harmon SM, Kautter DA, Peeler JT. Improved medium for enumeration of Clostridium perfringens. Appl Microbiol 1971; 22:688-92.
6. Hauschild AH, Hilsheimer R. Evaluation and modifications of media for enumeration of Clostridium perfringens. Appl Microbiol 1974; 27:78-82.
7. ISO 14189:2013 Water quality — Enumeration of Clostridium perfringens — Method using membrane filtration

TABLE OF APPLICABLE SYMBOLS

REF or REF Catalogue number	LOT Batch code	Manufacturer	This side up	Store in a dry place	Fragile
Temperature limitation	Content sufficient for <n> tests	Consult Instructions for Use	Use by	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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