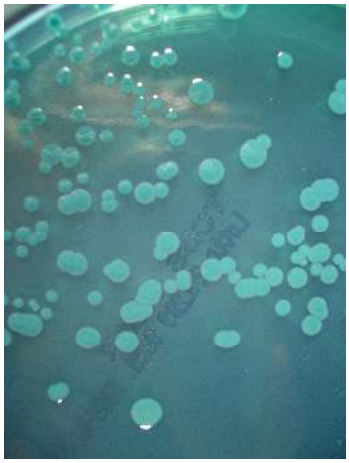
INSTRUCTIONS FOR USE

TCBS KOBAYASHI AGAR

Dehydrated culture medium



V.parahaemolyticus on TCBS Agar

1 - INTENDED USE

In vitro diagnostic. Selective and differential medium for the isolation of *Vibrio* spp. from clinical specimens and other materials.

2- COMPOSITION

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

Peptone	10.00 g
Yeast extract	5.00 g
Sodium thiosulphate	10.00 g
Sodium citrate	10.00 g
Sodium chloride	10.00 g
Oxgall	8.00 g
Sucrose	20.00 g
Ferric citrate	1.00 g
Thymol blue	0.04 g
Bromothymol blue	0.04 g
Agar	16.00 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

Members of the genus *Vibrio* are Gram-negative, asporogenous rods, straight or with a single, rigid curve. They are motile; most have a single polar flagellum, when grown in liquid medium. Most produce oxidase and catalase, and ferment glucose without producing gas. *Vibrio* spp. are natural inhabitants of brackish and salt water. Several species are pathogenic to humans and are usually associated with the ingestion of contaminated water or seafood. The species that most frequently cause diarrhoea are *Vibrio cholerae* (the causative agent of cholera), *Vibrio parahaemolyticus, Vibrio fluvialis* and *Vibrio mimicus*. *Vibrio vulnificus* does not cause diarrhoea, but has been isolated from extra-intestinal sites of septic patients.[1]

TCBS (Thiosulphate-Citrate-Bile-Sucrose) agar was developed by Kobayashi, Enomoto, Sakazaki and Kuwahara in 1963,[2] who modified the selective isolation medium of Nakanishi[2]. TCBS Kobayashi Agar is used for the selective and differential isolation of *Vibrio* spp. from clinical specimens, environmental samples and foodstuffs.[4] TCBS Agar is recommended by ISO 21872[5] and by FDA-BAM[6] for the isolation of *Vibrio* spp. from foodstuffs.

Peptone and yeast extract provide nitrogen, carbon, vitamin B complex and other essential growth nutrients for bacterial growth. Inhibition of Gram-positive bacteria and coliforms is achieved by the strong alkalinity and by the incorporation of bile salts (oxgall), sodium thiosulphate and sodium citrate. Sodium chloride is incorporated to provide optimum growth of halophilic *Vibrio* spp. Sucrose is a fermentable carbohydrate for the metabolism of vibrios: sucrose fermenting bacteria produce acid end-products that makes the pH indicators (bromothymol blue and thymol blue) turn yellow. Inclusion of sucrose allows preliminary differentiation of *Vibrio* spp., with *V.cholerae, V.fluvialis* and *V.alginolyticus* producing yellow colonies, while *V.parahaemolyticus, V.mimicus* and most strains of *V.vulnificus* produce green colonies (sucrose non fermented). Using thiosulfate as a sulphur source, the production of hydrogen sulphide is visualized in the presence of ferric citrate. The alkaline pH of the medium improves the recovery of *V.cholerae*. Enteric bacteria are strongly inhibited on TCBS Agar; the rare colonies of some strains of *Proteus* and enterococci are easily distinguished by their reduced size and absence of colour.

4- DIRECTIONS FOR MEDIUM PREPARATION

Suspend 90 g in 1000 mL of cold purified water. Heat to boiling with frequent agitation to dissolve completely. Cool to 47-50ºC, mix well and pour into sterile Petri dishes. Do not sterilise in the autoclave.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	grey-green, fine, homogeneous, free-flowing powder
Solution and prepared plates appearance	dark green, limpid
Final pH at 20-25 °C	8.6 ± 0.2

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
TCBS Kobayashi Agar	Dehydrated medium	4021062	500 g (5.6L) 5 kg (56 L)

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Water-bath, incubator and laboratory equipment as required, sterile loops and swabs, Petri dishes, Erlenmeyer flasks, ancillary culture media and reagents for the identification of the colonies.

8 - SPECIMENS

TCBS Kobayashi Agar is used for the bacteriological processing of clinical specimens such as faeces, rectal swab, vomitus[1,7] and non-clinical samples such as environmental samples, seafoods, foodstuffs[4,5,6,]. Collect clinical specimens before antimicrobial therapy where possible. Stool specimens for detecting *Vibrio* spp. should be inoculated within 2-4 hours; for more prolonged storage use transport media such as Cary Blair, because *Vibrio* spp. are particularly susceptible to drying.[7] Special methods for the collection and processing of extra-intestinal specimens (blood, wounds etc.) are not required as vibrios, as a rule, are isolated in pure culture from these sites.[7]

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Good laboratory practices for collection, transport and storage of clinical specimens should be applied.[1,7] Consult appropriate standard methods for details of collection and preparation of non-clinical samples.[5,6]

9 - TEST PROCEDURE

Allow plates to come to room temperature and to dry the surface of the medium.
Inoculate and streak the specimen with a loop over the four quadrants of the plate to obtain well isolated colonies, ensuring that sections 1 and 4 do not overlap. Alternatively, if the material is being cultured directly from a swab, roll the swab over a small area of the surface at the edge; then streak from this inoculated area.
Faeces may be diluted 1:4 in appropriate diluent prior to inoculation of culture medium. It has been shown that dilution significantly reduces the amount of competing flora without compromising isolation of low numbers of pathogens.[1]
In acute diarrhoeal disease, stool enrichment is generally not required; however, when enrichment is necessary, Alkaline Peptone Water is the most commonly used enrichment broth for human specimens. It should be incubated at 35-37°C and sub-cultured at 18 hours on TCBS Agar.
Incubate inoculated TCBS Agar plates with the specimen or with a specimen enriched in liquid medium, in aerobic conditions at 35-37°C for 18-24 hours.
According to ISO 21872[5] method, the detection of potentially enteropathogenic *Vibrio* spp. *(V. parahaemolyticus, V. cholerae* and *V. vulnificus)* in products intended for human consumption and the feeding of animals and environmental samples in the area of food production and food handling, requires four successive phases:

1. selective enrichment in Alkaline Peptone Water with incubation at 41.5 °C for 6 h and/or 37°C for 6 h.;
2. secondary enrichment in a selective liquid medium (Alkaline Peptone Water) with incubation at 41.5 °C for 18 h and/or 37°C for 18 h;
3. inoculation of two solid selective media: TCBS Kobayashi Agar incubated at 37°C for 24 hours and another appropriate solid selective medium, left to the choice of the laboratory;
4. Presumptive colonies of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* are sub-cultured and confirmed by means of appropriate biochemical and/or polymerase chain reaction (PCR) tests.

Recovery of certain *Vibrio* spp. from foodstuffs may be improved by the use of different incubation temperatures depending upon the target species or state of the food matrix.[5]
Consult appropriate references for the details of the procedures for the detection of *Vibrio* spp. in food.[5,6]

10 - READING AND INTERPRETATION

After incubation, observe the bacterial growth and record the specific morphological and chromatic characteristics of isolated colonies. Interpretation of colonies' colours:

V.cholerae	Yellowish-brown colonies surrounded by yellow zones in medium
V.alginolyticus	Large yellow colonies
V.fluvialis, *V.furnissii*	Yellow or translucent colonies.
V.metschnikovii	Yellow (reduced growth).
V.parahaemolyticus	Colourless or green colonies with blue-green centre; the medium does not turn or turns slightly blue.
V.mimicus	Green colonies
V.vulnificus	Green (85%) or yellow (15%).
V.hollisae	Green (poor growth).
Proteus /Enterococci	Partial to complete inhibition. If growth, small, yellow to translucent colonies.
Pseudomonas / *P.shigelloides*	Partial to complete inhibition. If growth, blue colonies.
Aeromonas hydrophila	Partial to complete inhibition. If growth some strains produce yellow colonies
Escherichia coli	Partial to complete inhibition. If growth, translucent colonies

After subculture on Nutrient Agar, Tryptic Soy Agar or Blood Agar the suspected colonies are submitted to oxidase test and to the agglutination tests with specific antisera.

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 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.[8]

CONTROL STRAINS			INCUBATION T°/ T / ATM	EXPECTED RESULTS
V. parahaemolyticus	ATCC	17802	36-38°C / 18-24H / A	good growth, green colonies
V.furnissii	NCTC	11218	36-38°C / 18-24H / A	good growth, yellow colonies
P.mirabilis	ATCC	12453	36-38°C / 18-24H / A	partially inhibited, yellow-green colonies with black centre
E.coli	ATCC	25922	36-38°C / 18-24H / A	inhibited

A: aerobic incubation; ATCC is a trademark of American Type Culture Collection; NCTC: National Collection of Type Cultures

12 - PERFORMANCES CHARACTERISTICS

Prior to release for sale a representative sample of all lots of dehydrated TCBS Kobayashi Agar is tested for productivity and selectivity by comparing the results with a previously approved Reference Batch.
Productivity is tested by semi-quantitative ecometric technique, by incubating at 36-38°C for 18-24 hours, with 3 target strains: *V.parahaemolyticus* NCTC 10885, *V.parahaemolyticus* ATCC 17802, *V.furnissii* NCTC 11218. *V.parahaemolyticus* colonies are colourless with blue-green centres, *V.furnissii* colonies are yellow; the amount of growth on the plates is evaluated and shall be comparable in both batches.
Selectivity is evaluated with modified Miles-Misra surface drop method by inoculating the plates with decimal dilutions in saline from 10^{-1} to 10^{-4} of a 0.5 McFarland suspension of non-target strains**:** *P.mirabilis* ATCC 10005, , *E.coli* ATCC 25922*, P.aeruginosa* ATCC 27853*, A.hydrophila* ATCC 7966*, E.faecalis* ATCC 29212. The growth of non-target strains *E.coli, A.hydrophila* and *E.faecalis* is inhibited at the dilution 10^{-1}. The growth of Gram-negative non-target strains *P.mirabilis* and *P.aeruginosa* is partially inhibited and the colonies show typical chromatic characteristics, according to the specifications.

13 - LIMITATIONS OF THE METHOD

- On initial isolation, *V.parahaemolyticus* may be confused with *Aeromonas hydrophila*, *Plesiomonas shigelloides* and *Pseudomonas* species.[4]
- A few strains of *V.cholerae* may appear green or colourless on TCBS due to delayed sucrose fermentation.[4]
- Some strains of *Proteus* and enterococci may exhibit growth and form small colourless colonies; however, these organisms are easily distinguished. Any coliforms that may grow do not metabolize sucrose.[4]
- Oxidase and agglutination tests are unreliable when performed directly on colonies growing on this medium. Growth from a non-sugar containing medium, such as blood agar or nutrient agar should be used for oxidase and agglutination testing.[4,7]
- It should be noted that yellow colonies may convert to green if plates are examined after more than 24 hours or are refrigerated after incubation.[7]
- A single medium is only rarely useful to recover all pathogens contained in a specimen. Therefore, for the isolation of *Vibrio* spp. additional media such us chromogenic media should be used. For enhanced recovery of *V.vulnificus*, a medium containing derivatives of cellobiose-polymyxin B-colistin has been shown to be effective.[5]
- Inoculate extra-intestinal specimens also on non-selective blood agar plates and on other selective plates to identify other pathogens possibly involved in the infection.
- Some strains of *V.vulnificus* produce better recovery at 30°C.
- Even if the microbial colonies on the plates are differentiated on the basis of their morphological and chromatic characteristics, it is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on isolates, from pure culture, for complete identification. If relevant, perform antimicrobial susceptibility testing.
- This culture medium is intended as an aid in the diagnosis of infectious diseases; the interpretation of the results must be made considering the patient's clinical history, the origin of the sample and the results of 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.
- Dehydrated media must be handled with suitable protection. Before use, consult the Safety Data Sheet.
- Apply Good Manufacturing Practice in the production process of prepared media.
- 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.
- 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 media 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 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.

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 (plates/tubes/bottles) and the storage method (temperature and packaging).

16 - REFERENCES

1. Public Health England. Investigations of Faecal Specimens for Enteric Pathogens. UK Standards for Microbiology Investigations. 2014, B 30 Issue 8.1.
2. Kobayashi T, Enomoto S, Sakasaki R, Kuwahara S. A new selective isolation medium for the *Vibrio* group; on a modified Nakanishi's Medium (TCBS Agar Medium). Jap J Bacteriol 1963;18:392-397
3. Nakanishi Y. An isolation agar medium for cholerae and enteropathogenic halophilic vibrios. Modern Media 1963; 9:246.
4. MacFaddin JF. Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria. Baltimore: Williams & Wilkins; 1985.
5. ISO 21872:2017. Microbiology of the food chain -Horizontal method for the determination of Vibrio spp. - Part 1:Detection of potentially enteropathogenic Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus.
6. U.S. Food and Drug Administration. Bacteriological Analytical Manual (BAM) Chapter 9: Vibrio. Content current as of:12/16/2019
7. Tarr CI, Bopp CI, Farmer III JJ. Vibrio and related organisms. In Jorgensen JH, Carrol KC, Funke G et al. editors. Manual of clinical microbiology,11th ed. Washington,DC: American Society for Microbiology; 2015.
8. The Australian Society for Microbiology. Guidelines for Assuring Quality of Medical Microbiological Culture Media. 2nd ed. 2012

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	Keep away from direct light	Store in a dry place

REVISION HISTORY

Version	Description of changes	Date
Revision 1	Updated layout and content	2020/06
Revision 2	Update of “precautions and warnings” and “storage conditions and shelf life”	2022/03
Revision 3	Removal of obsolete classification	2023/04

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

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