



INSTRUCTIONS FOR USE

**CHROMOGENIC SALMONELLA AGAR
HEKTOEN ENTERIC AGAR**

Ready-to-use bi-plates

1-INTENDED USE

In vitro diagnostic device. Selective and differential media for the isolation of Gram-negative enteric pathogens, especially *Salmonella* and *Shigella*, from clinical and non clinical specimens.

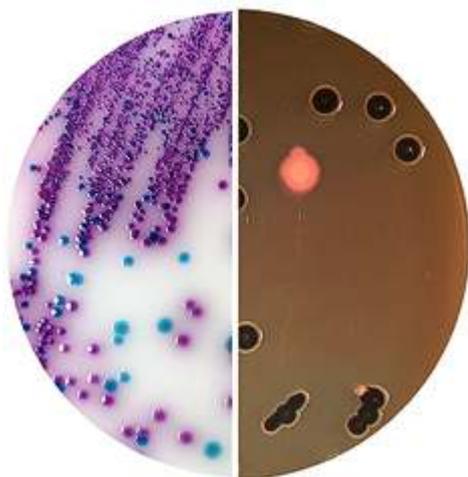
2 - COMPOSITIONS -TYPICAL FORMULAS**CHROMOGENIC SALMONELLA AGAR***

Peptones	10.0 g
Selective compounds	12.0 g
Cefsulodin	5.0 mg
Chromogenic mixture	0.9 g
Emulsifying agents	11.4 g
Opacifier	10.0 g
Agar	15.0 g
Purified water	1000 mL

HEKTOEN ENTERIC AGAR*

Tryptose	12.000 g
Yeast extract	3.000 g
Bile salts n° 3	9.000 g
Lactose	12.000 g
Sucrose	12.000 g
Salicin	2.000 g
Sodium chloride	5.000 g
Sodium thiosulphate	5.000 g
Fe-ammonium citrate	1.500 g
Bromothymol blue	0.065 g
Acid fuchsin	0.100 g
Agar	15.000 g
Purified water	1000 mL

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



Salmonella sp. on Chromogenic Salmonella Agar at left (magenta colonies) and on Hektoen Enteric Agar at right (black colonies)

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE

The bi-plate with Chromogenic Salmonella Agar and Hektoen Enteric Agar culture media is indicated in clinical and industrial microbiology when determination in *Salmonella* spp. (including *S.Typhi* and *Salmonella lac +* strains) and *Shigella* spp is required.

CHROMOGENIC SALMONELLA AGAR

Chromogenic Salmonella Agar is a selective and diagnostic medium useful for the isolation of *Salmonella* spp. from clinical and non clinical specimens and for the presumptive identification of the colonies. Chromogenic Salmonella Agar is included by ISTISAN Report¹ in the plating media range for the detection of *Salmonella* spp. and chromogenic media are included as the second plating medium in ISO Standards for detection of *Salmonella* in food and water.^{2,3}

Peptones provide carbon, nitrogen, vitamins and trace elements for bacterial growth. The selective compounds incorporated in the medium are the following: cefsulodin, a third generation cephalosporin antibiotic that has very specific activity against *P. aeruginosa* and *S.aureus*, sodium desoxycholate that suppresses the growth of Gram-positive and some Gram-negative bacteria and Tergitol 4, active mainly against the growth of *Proteus* spp.

Differentiation of *Salmonella* from the other organisms that grow is achieved by:

- a chromogenic substrate for C₈ esterase enzyme, that is split by *Salmonella* spp. with the release of an insoluble magenta-red chromophore.
- a chromogenic glucopyranoside derivative which is split by β-glucosidase with the release of an insoluble blue-green chromophore.

Some *Enterobacteriaceae*, including *Klebsiella* and *Enterobacter*, but not *Salmonella*, are β-glucosidase positive and if growing will form blue-green or dark blue colonies, even if they are esterase positive, which make them easy to differentiate from magenta-red *Salmonella* colonies. The chromogenic and selective compounds of the medium also allow the detection of the rare lactose positive *Salmonella* strains, missed on traditional media based on lactose fermentation. Chromogenic Salmonella Agar is useful also for the detection of *S.Typhi* and *S.Paratyphi*. The ready-to-use plates include a compound that gives a white opaque background to the medium and enhances the colour of the colonies.

HEKTOEN ENTERIC AGAR

Hektoen Enteric Agar is a selective and differential medium intended for the isolation of Gram-negative enteric pathogens, especially *Salmonella* and *Shigella* from clinical and nonclinical specimens.⁴ Hektoen Enteric Agar is recommended by ISO 21567⁵ as plating medium for the detection of *Shigella* and by FDA-BAM⁶ for detection of *Salmonella*, in food.

Animal peptone and yeast extract provide carbon, nitrogen, vitamins and trace elements for bacterial growth; the high concentration of bile salts n°3 and dyes inhibits Gram-positive organisms and most of the non-pathogenic coliform flora of the intestinal tract. Since the enteric pathogens *Salmonella* and *Shigella* can tolerate these inhibitory substances, they generally grow faster and larger than the coliforms.⁷ Lactose, sucrose and salicin are fermented by coliforms, that are able to grow in the presence of the bile salts, and by some *Proteus* species with production of acids. The acid condition causes the bromothymol blue indicator to change from its neutral green colour to an orange-yellow colour and to bile salts to precipitate appearing as a hazy zone around the colonies. Ferric ammonium citrate is an indicator of the formation of hydrogen sulphide. *Salmonella* spp. produce thiosulphate reductase that cause the release of a sulfide





molecule from the sodium thiosulfate present in the medium. This sulfide molecule couples with a hydrogen ion to form H₂S gas that reacts with the ferric ammonium citrate, forming a precipitate, resulting in colonies that are black or have a black centre.

4 - PHYSICAL CHARACTERISTICS

CHROMOGENIC SALMONELLA AGAR

Medium appearance whitish opaque
Final pH at 20-25 °C 7.2 ± 0.2

HEKTOEN ENTERIC AGAR

Medium appearance dark green, limpid or lightly opalescent
Final pH at 20-25 °C 7.5 ± 0.2

5 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Biosector Chromogenic Salmonella Agar / Hektoen Enteric Agar CND: W0104010407, EDMA: 14.01.04.05; RDM: 1458967/R	Ready-to-use bi-plates	495350	2 x 10 plates ø 90 mm with 2 sectors 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

Bisector Chromogenic Salmonella Agar / Hektoen Enteric Agar is intended for the bacteriological processing of clinical specimens such as faeces and rectal swab⁸ and non clinical specimens such as food and animal feeding stuffs^{6,7}. Good laboratory practices for collection, transport and storage of clinical specimens should be applied.⁸ Consult appropriate standard methods for details on food sample collection and preparation.^{2,3,5}

8 - 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 media surfaces to obtain well isolated colonies. 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.

Maximal recovery of *Salmonella* from faecal specimens is obtained by using an enrichment step in Selenite Broth followed by subculture on Chromogenic Salmonella Agar and Hektoen Enteric Agar and on a second plating medium.⁹

For *Shigella* isolation from faecal specimens, the enrichment in GN Broth is advised.⁹

Incubate inoculated bi-plates with the specimen or with a specimen enriched in liquid medium, in aerobic conditions at 35-37°C for 18-24 hours.

Consult appropriate references for the detection of *Shigella* and *Salmonella* in non clinical specimens.^{2,3,5}

9 - READING AND INTERPRETATION

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

The different microorganisms grow on Chromogenic Salmonella Agar with the following characteristics:

Microorganism	Growth characteristics
<i>Salmonella</i> spp.	good growth, magenta-red colonies
<i>Salmonella</i> spp. lac ⁺	good growth, magenta-red colonies
<i>Salmonella Typhi</i>	good growth, magenta-red colonies
<i>E.coli</i>	poor growth with colourless colonies
<i>Enterobacter</i> spp.	growth with blue-green colonies
<i>Klebsiella</i> spp.	poor growth with blue-green colonies
<i>Pseudomonas</i> spp.	inhibited
<i>Proteus</i> spp.	poor growth with pale brown or green colonies
Gram-positive bacteria	inhibited

The different colour characteristics of isolates on Hektoen Enteric Agar may be interpreted as follows:⁷

Greenish-blue, light green, or transparent colonies with black centres: no fermentation present, H₂S production present: suspect *Salmonella*.

Greenish-blue, light green, or transparent colonies: no fermentation present, H₂S production absent: suspect *Shigella* or H₂S negative *Salmonella*.

Yellow colonies with an orange-yellow precipitate: fermentation of lactose, sucrose, or salicin: not likely to be *Salmonella* or *Shigella*.

Salmon to orange colonies: fermentation of salicin, H₂S production absent: not likely to be *Salmonella* or *Shigella*.

Yellow, salmon to orange colonies with black centre: fermentation of lactose or sucrose, or salicin, H₂S production present: not likely to be *Shigella* or *Salmonella* (other than rare lactose positive *Salmonella*).

Since some *Proteus* spp. may grow with greenish blue colonies with black centre and if *Proteus* colonies are mixed with H₂S positive *Salmonella* colonies, it could be difficult to choose the colonies for further biochemical and serological identification.

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
CHROMOGENIC SALMONELLA AGAR		
S.Typhimurium ATCC 14028	35-37°C / 18-24h / A	growth, magenta-red colonies
S. Enteritidis ATCC 13076	35-37°C / 18-24h / A	growth, magenta-red colonies
<i>E. aerogenes</i> ATCC 13048	35-37°C / 18-24h / A	growth, blue-green colonies
<i>P. aeruginosa</i> ATCC 27853	35-37°C / 18-24h / A	inhibited
HEKTOEN ENTERIC AGAR		
S.Typhimurium ATCC 14028	35-37°C / 18-24h / A	growth, green colonies with black centre
<i>S. flexneri</i> ATCC 12022	35-37°C / 18-24h / A	growth, light green colonies
<i>E. faecalis</i> ATCC 29212	35-37°C / 18-24h / A	inhibited
<i>E. coli</i> ATCC 25922	35-37°C / 18-24h / A	partially inhibited, yellow to salmon colonies

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

11 - PERFORMANCES CHARACTERISTICS

Prior to release for sale, representative samples of all lots of ready to use bi-plates of Chromogenic Salmonella Agar / Hektoen Enteric Agar and of the raw materials used for the production of bi-plates (dehydrated Chromogenic Salmonella Agar REF 405350 supplemented with Salmonella Selective Supplement REF 4240013 and Hektoen Enteric Agar, REF 401541) are tested for productivity and selectivity by comparing the results with a previously approved Reference Batch.

CHROMOGENIC SALMONELLA AGAR

Productivity is tested by a quantitative test with the target strains *S. Enteritidis* ATCC 13076 and *S. Typhimurium* ATCC 14028; Chromogenic Salmonella Agar plates are inoculated with decimal dilutions in saline of a suspension of colonies and incubated at 35-37°C for 18-24 hours. The colonies are enumerated on Test Batch (TB) and Reference Batch (RB) and the productivity ratio ($Pr = \text{UFC}_{\text{TB}} / \text{UFC}_{\text{RB}}$) is calculated. If Pr is $\geq 0,7$ and if the colonies' colour is typical (magenta red colonies) the results are considered acceptable and conform to the specifications.

Specificity is evaluated with by semi-quantitative ecometric technique with the following non-target strains: *S. flexneri* ATCC 12022, *E. aerogenes* ATCC 13048, *E. coli* ATCC 8739; *E. aerogenes* grows with blue-green colonies, the growth of *S. flexneri* is not inhibited and the colonies are colourless, *E. coli* is partially inhibited with colourless colonies. 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 the non-target strains *P. vulgaris* ATCC 13315, *A. calcoaceticus* ATCC 19606, *P. aeruginosa* ATCC 27853, *A. hydrophila* ATCC 7966, *E. faecalis* ATCC 19433, *Mucor* CBM1. *A. hydrophila* is partially inhibited and grows with magenta colonies; the growth of other non-target strains is totally inhibited at the dilution 10^{-1} .

HEKTOEN ENTERIC AGAR

Productivity is tested by semi-quantitative ecometric technique, by incubating at 35-37°C for 18-24 hours, with 6 target strains: *S. Enteritidis* NCTC 5188, *S. Typhimurium* ATCC 14028, *S. Gallinarum* clinical isolate, *S. arizonae* clinical isolate, *S. flexneri* ATCC 12022 and *S. sonnei* ATCC 9290. *Salmonella* colonies are green with black centre, *Shigella* colonies are light green; 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 of a 0.5 McFarland suspension of the non-target Gram positive strain *E. faecalis* ATCC 29212 and of 6 non target Gram negative strains: *P. mirabilis* ATCC 10005, *P. vulgaris* ATCC9484, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 27736, *C. freundii* ATCC 8090 and *E. aerogenes* ATCC 13048. The growth of non-target strain *E. faecalis* is totally inhibited, the growth of Gram negative non target strains are partially inhibited and the colonies show typical chromatic characteristics, according to the specifications.

Chromogenic Salmonella Agar was evaluated by Babic-Ergeg et al.¹⁰ on 3,000 stool specimens, 45 of which positive for *Salmonella*, including SS Agar as the reference medium. The authors reported a sensitivity of 100% and a specificity of 99% in the isolation and preliminary identification of *Salmonella* colonies.

In another independent study¹¹, 50 pure cultures of *Salmonella* of clinical origin, gave all the specific chromatic reactions; among the other 80 strains of Gram negative bacteria tested, not belonging to the *Salmonella* genus, 3 out of 3 strains of *P. aeruginosa* and 1 out of 3 strain of *A. baumannii* provided chromatic results similar to *Salmonella* spp. (red-pink colonies), the remaining 76 strains of *Enterobacteriaceae* gave non-typical chromatic reactions; 20 out of 20 strains of Gram positive bacteria were totally inhibited.

Chromogenic Salmonella Agar performance was evaluated with an in-house study, compared to Hektoen Enteric Agar (HEA). Productivity, selectivity and specificity have been evaluated by semi-quantitative ecometric technique, incubating at 35-37°C for 18-24 hours, using 43 bacterial strains: 8 target strains and 35 non target strains. 8 *Salmonella* strains, including 2 *S. Typhi*, showed a good growth with magenta colonies; 3 *Shigella* strains showed a poorer growth than on HEA with colourless colonies; 22 *Enterobacteriaceae* strains belonging to 9 genera showed a poorer growth than on HEA with colourless or blue-green colonies; 4 *P. aeruginosa* strains were totally inhibited; 2 non fermenters strains were totally inhibited and *A. hydrophila* grew with magenta red colonies; 1 Gram positive strain was totally inhibited and 1 yeast strain was partially inhibited showing colourless colonies.

Dehydrated Hektoen Enteric Agar prepared by Biolife has been tested by Silvia King for the isolation of *Salmonella* and *Shigella* from faecal specimens, with results comparable to the medium prepared in her laboratory.¹²

12 - LIMITATIONS OF THE METHOD

- Some strains of *Pseudomonas*, *Acinetobacter* and *Aeromonas*, resistant to antimicrobial agents of Chromogenic Salmonella Agar, may grow with red-pink colonies, differentiable from *Salmonella* with oxidase test.
- The growth rate on the plates also depends on the nutritional requirements of *Salmonella*. It is possible that some strains with particular metabolic characteristics may not grow on Chromogenic Salmonella Agar or grow colourless (e.g., *Salmonella enterica* serovar Dublin grows with white colonies).
- Do not examine areas of confluent growth on Hektoen Enteric Agar as false negative fermentation reactions may occur.
- Be aware that carbohydrates non fermenting strains of *Proteus* spp. may or may not be inhibited on Hektoen Enteric Agar and colonies may resemble *Salmonella*.⁴ Rapid differentiation between very similar colonies may be performed on Chromogenic Salmonella Agar.
- Some lactose fermenting *Shigella* and *Salmonella* strains may resemble coliforms and not recognized on Hektoen Enteric Agar.
- Do not incubate longer than 24 hours since a loss of yellow/salmon colour may occur on Hektoen Enteric Agar due to the utilisation of peptones for growth with the productions of alkaline end products.⁷





- Over time and during the shelf-life, bile salts in Hektoen Enteric Agar sector may crystallize and form a precipitate in the medium. This does not affect the performance of the medium.
- 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 disease; 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.

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.
- These culture media contain 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 these products do not contain any transmissible pathogen. Therefore, it is recommended that the ready-to use plates 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 S.r.l. 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.
- Each bi-plate is for single use only.
- Ready-to-use bi-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.
- 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 <i>In vitro</i> 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 0	First edition, in compliance with IVDR 2017/746	2021/09

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

