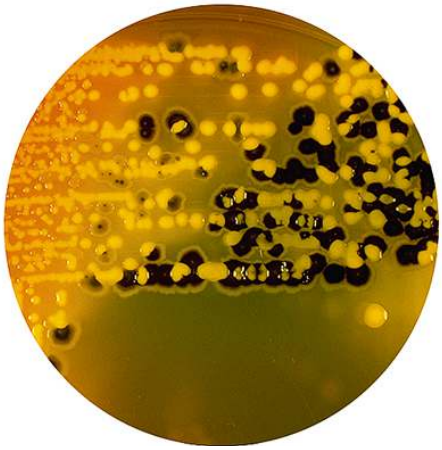
INSTRUCTIONS FOR USE

HEKTOEN ENTERIC AGAR

Ready-to-use flasks



HEA: *Salmonella* colonies with large black centre and yellow-orange *K.pneumoniae* colonies.

1-INTENDED USE

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

2- COMPOSITION - TYPICAL FORMULA *

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.

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE

In the first half of the twentieth century, several culture media were developed and proposed for the isolation of enteric pathogens from faeces and other materials. Some of them were moderately selective and allowed the growth of faecal contaminants, others showed excessive toxicity for the growth of pathogens, especially of *Shigella*.[1]

Sylvia King and William I. Metzger, working at the Hektoen Institute in Chicago, formulated HE agar in 1968[2] with the goal to increase the recovery of *Shigella* species from mixed cultures. They enriched SS Agar formulation, evaluated in 1941 by Catherine Mayfield and Maud Gober[3], with extra amounts of carbohydrates and peptones to offset the inhibitory effects of the bile salts. The two dyes added to the medium, bromothymol blue and acid fuchsin, have lower toxicity than other dyes, thus pathogens recovery was improved.[4]

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 non-clinical specimens.[5] Hektoen Enteric Agar is recommended by ISO 21567[6] as plating medium for the detection of *Shigella* and by FDA-BAM[7] 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 inhibit 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.[1]

Lactose, sucrose and salicin are fermented by coliforms, that are able to grow in the presence of 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 causes the release of a sulphide molecule from the sodium thiosulfate present in the medium. This sulphide molecule couples with a hydrogen ion to form H_2S gas that reacts with the ferric ammonium citrate, forming a precipitate, resulting in colonies that are black or have a black centre.

4- METHOD OF PREPARATION

Liquefy the contents of the flask in an autoclave set at 100 ± 2°C or in a temperature-controlled water bath (100°C). Alternatively, the bottle may be placed into a jar containing water, which is placed on a hot plate and brought to boiling. Slightly loosen the cap before heating to allow pressure exchange. Cool to 47-50°C and pour the medium into sterile Petri dishes, under aseptic conditions.

5 - PHYSICAL CHARACTERISTICS

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

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Hektoen Enteric Agar	Ready to use flasks	5115412	6 x 100 mL; 6 glass bottles with flat bottom and aluminium screw-cap; packaging: cardboard box.
		5115413	6 x 200 mL; 6 glass bottles with flat bottom and aluminium screw-cap; packaging: cardboard box.

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave, water bath or hot plate, incubator and laboratory equipment as required, sterile plastic Petri dishes, sterile loops, needles and swabs, ancillary culture media and reagents for the identification of the colonies.

8 - SPECIMENS

Hektoen Enteric Agar is intended for the bacteriological processing of clinical specimens such as faeces and rectal swab[8,9] 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] Collect specimens before antimicrobial therapy where possible. Consult appropriate standard methods for details on food sample collection and preparation.[6,7]

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.
Maximal recovery of *Salmonella* from faecal specimens is obtained by using an enrichment step in Selenite Broth followed by subculture on Hektoen Enteric Agar and on a second plating medium.[9]
For *Shigella* isolation from faecal specimens, the enrichment in GN Broth is advised, followed by subculture in two different selective media: Hektoen Enteric Agar and a second less selective medium (Mac Conkey Agar).[9]
Incubate inoculated Hektoen Enteric Agar 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.[6,7]

10 - READING AND INTERPRETATION

After incubation, observe the bacterial growth and record the specific morphological and chromatic characteristics of isolated colonies. Do not examine areas of confluent growth as false negative fermentation reactions may occur.
The different colour characteristics of isolates may be interpreted as follows:[1]
Greenish-blue, light green, or transparent colonies with black centres: no fermentation present, H_2S production present: suspect *Salmonella*.
Greenish-blue, light green, or transparent colonies: no fermentation present, H_2S production absent: suspect *Shigella* or H_2S 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, H_2S production absent: not likely to be *Salmonella* or *Shigella*.
Yellow, salmon to orange colonies with black centre: fermentation of lactose or sucrose, or salicin, H_2S 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 H_2S positive *Salmonella* colonies, it could be difficult to choose the colonies for further biochemical and serological identification.
It is advised to screen the colonies by flooding the plate with one drop of MUCAP reagent (REF 191500) and observing after 3 to 5 min for the development of fluorescence under Wood's lamp, produced in the presence of the C_8 esterase enzyme, typical of *Salmonella* spp.[10]

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.[11]

CONTROL STRAINS			INCUBATION T°/ T / ATM	EXPECTED RESULTS
S.Typhimurium	ATCC	14028	35-37°C / 18-24h / A	growth, light green colonies with black centre
S.flexneri	ATCC	12022	35-37°C / 18-24h / A	growth, light green colonies
E.faecalis	ATCC	29212	35-37°C / 18-24h / A	inhibited
E.coli	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

12 - PERFORMANCES CHARACTERISTICS

Prior to release for sale, a representative sample of all lots of ready-to-use flasks and the raw material for the production (dehydrated Hektoen Enteric Agar REF 401541) 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 35-37°C for 18-24 hours, with 6 target strains: S.Enteritidis NCTC 5188, S.Typhimurum ATCC 14028, S.Gallinarum clinical isolate, *S.arizonae* clinical isolate, *S.flexneri* ATCC 12022 and *S.sonnei* ATCC 9290. *Salmonella* colonies are light 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 from 10^{-1} to 10^{-4} of a 0.5 McFarland suspension of the non-target Gram-positive strain *E.faecalis* ATCC 29212 and with decimal dilutions in saline from 10^{-1} to 10^{-6} of 6 non-target Gram negative strains: *P.mirabilis* ATCC 10005, *P.vulgaris* ATCC 9484, *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 inhibited at the dilution 10^{-1}, the growth of Gram-negative non-target strains are partially inhibited and the colonies show typical chromatic characteristics, according to the specifications.
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]

13 - LIMITATIONS OF THE METHOD

- Be aware that carbohydrates non-fermenting strains of *Proteus* spp. may or may not be inhibited and colonies may resemble *Salmonella.*[5] Rapid differentiation between very similar colonies may be performed with MUCAP test.[10]
- Some lactose-fermenting *Shigella* and *Salmonella* strains may resemble coliforms and are not recognized on Hektoen Enteric Agar.
- Do not incubate longer than 24 hours since a loss of yellow/salmon colour may occur due to the utilisation of peptones for growth with the productions of alkaline end-products.[1]
- A single medium is only rarely useful to recover all pathogens contained in a specimen. Therefore, additional media for the isolation of *Salmonella* and/or *Shigella*, with lower selectivity such as Mac Conkey Agar and with higher selectivity such as SS Agar, should be used; other media for the isolation of other enteric pathogens must be inoculated with the specimen.[8,9]
- Over time and during the shelf-life, bile salts in Hektoen Enteric Agar plates may crystallize and form a precipitate in the medium. This does not affect the performance of the medium.

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- 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.
- This product is not classified as dangerous according to current European legislation.
- 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 product 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.
- Be careful when opening screw cap flasks to prevent injury due to breakage of glass.
- When using a hot plate and/or a water bath, boil sufficiently long to dissolve the whole medium.
- Wear heat-protective gloves during medium liquefaction. Do not place the hot flasks into an ice bath or in cold water to accelerate cooling as this might cause cracks in the glass.
- The time required for complete liquefaction of the medium may vary considerably and depends on the actual temperature of the heating device, its wattage, the size and volume of the bottle.
- Once the bottled medium is liquefied, it cannot be solidified and dissolved a second time.
- Ready-to-use flasks of Hektoen Enteric Agar are subject to terminal heating to 100°C.
- 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 flasks in their original pack at 2-8°C away from direct light. If properly stored, the flasks may be used up to the expiration date. Do not use the flasks beyond this date. Flasks from opened secondary packages can be used up to the expiration date. Opened flasks must be used immediately. Before use, check the closing and the integrity of the screw cap. Do not use flasks with signs of deterioration (e.g. microbial contamination, abnormal turbidity, precipitate, atypical colour).
The user is responsible of the correctness of plates preparation. The user is responsible of the validation of plates shelf-life, according to the method of storage (temperature and packaging) .

15 - References

1. Jan Hudzicki. Hektoen Enteric Agar Protocol. American Society for Microbiology. 11 November 2010.
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3. Mayfield CR, M Gober M. Comparative efficiency of plating media for the isolation of Shigella dysenteriae. Am J Public Health 1941; 31:363–368.
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5. MacFaddin JF. Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria. Baltimore: Williams & Wilkins; 1985.
6. ISO 21567 :2005. Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Shigella spp.
7. U.S. Food and Drug Administration. Bacteriological Analytical Manual (BAM) Chapter 5: Salmonella. Rev 12/2019.
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9. Strockbine NA, Bopp CA, Fields PI, Kaper JB, Nataro JP. Escherichia, Shigella and Salmonella. In Jorgensen JH, Carrol KC, Funke G et al. editors. Manual of clinical microbiology,11th ed. Washington,DC: American Society for Microbiology; 2015. p.685.
10. Ruiz J, Sempere MA, Varela C, Gomez J. Modification of the methodology of stool culture for Salmonella detection, J Clin Microbiol 1992; 30:525-526.
11. CLSI (formerly NCCLS) Quality Control of Commercially Prepared Culture Media. Approved Standard, 3rd edition. M22 A3 vol. 24 n° 19, 2004.
12. King S. Department of Microbiology, Cook County Hospital, Chicago. Personal communication.1968.

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	For single use only	Fragile, handle with care

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REVISION HISTORY

Version	Description of changes	Date
Revision 3	Method of preparation, precautions and warnings, storage conditions and shelf life	2021/09
Revision 4	Removal of obsolete classification	2023/04

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

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