INSTRUCTIONS FOR USE

TRIPLE SUGAR IRON AGAR

Dehydrated culture medium

Triple Sugar Iron Agar – from left: uninoculated tube, S.Typhimurium, *E.coli.*

1 - INTENDED USE

In vitro diagnostic. For the differentiation of *Enterobacteriaceae*, especially *Salmonella*, based on carbohydrate fermentation and production of hydrogen sulphide.

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

Peptocomplex	20.000 g
Lactose	10.000 g
Sucrose	10.000 g
Glucose	1.000 g
Ferrous Ammonium Sulphate	0.200 g
Sodium Chloride	5.000 g
Sodium Thiosulphate	0.200 g
Agar	14.000 g
Phenol Red	0.025 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

The formulation of Triple Sugar Iron Agar medium is based on several microbiologists' attempts to develop a medium to aid in the identification of intestinal gram-negative bacilli: Russel[1], Kliger,[2] Krunweide and Kohn[3]. In 1940, Sulkin and Willet[4] modified the triple sugar medium of Krunweide and Kohn by the addition of H_2S indicators. The current formulation of triple sugar iron medium is essentially a modification of Haja[5] to Sulkin and Willet triple sugar ferrous sulphate medium.

Triple Sugar Iron (TSI) Agar is intended for the differentiation of *Enterobacteriaceae*, especially *Salmonella* spp., grown on primary isolation media, based on the fermentation of glucose, lactose and sucrose, with production of acids and gas, and the production of hydrogen sulphide.[6] The medium is included in the FDA-BAM[7] procedures for the identification of *Salmonella* from food, together with other biochemical tests. TSI Agar proposed by the ISO Standard 6579 for *Salmonella* identification has a different formulation and corresponds to Biolife medium Triple Sugar Iron Agar ISO Formulation (REF 402141S).

The fermentation of the three carbohydrates can take place both on the surface of the slant and in the butt with or without the presence of gas (CO_2 + H_2) and 3 reaction models can be registered:

1-fermentation of glucose; 2-fermentation of glucose, lactose and/or sucrose; 3-no fermentation.

In the first case, after 18-24 hours of incubation, an alkaline reaction on the slant and an acid reaction in the butt is observed. The complete consumption of glucose, present at a concentration of 0.1%, on the surface, where aerobic conditions exist, after 18-24 hours induces the oxidative degradation of peptones, with production of ammonia, alkalinity and a red colour change of phenol red (reversal of the acid-alkaline reaction). However, in the anaerobic butt the bacteria metabolize the glucose producing ATP and pyruvate, which is converted into stable acid end-products with a colour change of the indicator to yellow (acid pH).

In the second case, the microorganisms ferment glucose and one or both lactose and sucrose: after 18-24 hours of incubation an acid reaction is recorded on the slant and in the butt. This is due to the high concentration of lactose and sucrose: after 18-24 hours their degradation is not exhausted on the surface and therefore there is no utilisation of peptones and therefore no reversal of the reaction.

In the third model an alkaline reaction is recorded both on the slant and in the butt. This behavior is not typical of *Enterobacteriaceae* but of some non-enteric non fermenting Gram-negative bacteria that can utilise the peptones for growing (*Alcaligenes faecalis, Acinetobacter, Pseudomonas*). If the degradation of the peptones is anaerobic the indicator will turn to red (alkaline pH) both on the surface and in the butt, if the degradation is aerobic, there is no colour change of phenol red in the butt.

Ferrous ammonium sulphate is an indicator of the formation of hydrogen sulphide. Thiosulphate reductase producing organisms cause the release of a sulphide molecule from the sodium thiosulfate. The hydrogen sulphide will react with ferric ions in the medium to produce iron sulphide, a black insoluble precipitate.

4- DIRECTIONS FOR MEDIUM PREPARATION

Suspend 60.4 g in 1000 mL of cold purified water. Heat to boiling with frequent agitation to dissolve completely. Distribute into tubes and sterilize by autoclaving at 121°C for 15 minutes. Cool in a slanted position to obtain deep butts and short slopes.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	pink, fine, homogeneous, free-flowing powder
Solution and prepared tubes appearance	red-orange, limpid
Final pH at 20-25 °C	7.3 ± 0.2

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Triple Sugar Iron Agar	Dehydrated culture medium	4021412	500 g (8.2 L)

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7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave and water-bath, sterile needles, screw capped tubes, incubator and laboratory equipment as required, ancillary culture media and reagents for complete identification of the culture.

8 - SPECIMENS

Triple Sugar Iron Agar Medium is not intended for primary isolation from clinical specimens; it is inoculated with pure colonies from a culture on solid media, isolated from clinical specimens or other materials.

9 - TEST PROCEDURE

With an inoculating needle, pick the centre of a single pure colony, inoculate the slant by first stabbing the butt to the bottom; withdraw the needle, and then streak the surface of the slant. Loosen the cap of the tube before incubating.
Incubate aerobically at 35-37°C for 18 to 24 hours.

10 - READING AND INTERPRETATION

Three kinds of data may be obtained from the reactions.[8]

Sugar fermentations

Acid (yellow) butt, alkaline (red) slant: glucose fermented, sucrose or lactose not fermented.
Acid (yellow) butt, acid (yellow) slant: glucose, lactose and/or sucrose fermented.
Alkaline (red) butt, alkaline (red) slant: neither glucose, lactose, nor sucrose fermented.

Gas production

Presence of bubbles in the butt. With large amounts of gas, the agar may be cracked and displaced.

Hydrogen sulphide production

Hydrogen sulphide production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of H_2S with the ferric ions to form black ferrous sulphide. Formation of H_2S requires an acidic environment; sometimes the butt will be entirely black; in such a case, it is assumed that butt portion of the tube is acid (yellow colour is masked by H_2S production).
All combinations of the reactions described above can be observed on Triple Sugar Iron Agar, therefore it is important to record the results of all the reactions (sugar fermentations, gas production, H_2S production). The following table, taken from MacFaddin[9] shows the reaction patterns of some *Enterobacteriaceae*.

Microorganism	Lac	Suc	Glu	Gas	H_2S
Edwarsiella	-	-	A	+	+
Escherichia coli	A [1]	V	A	V^+	-
Shigella	V^- [3]	V^- [1]	A	V^- [2]	-
Klebsiella	A	A	A	+	-
Enterobacter	V	V^+	A	V^- [6]	-
Hafnia	V^-	V^-	A	V^+	-
Serratia	V^-	A	A	V^-	-
Morganella	-	-	A	V^+	-
Proteus mirabilis	-	V^{-1}	A	+	+
Proteus vulgaris	-	A	A	V[7]	+
Salmonella	- [4]	-	A	V^+	+[5]
Salmonella arizonae	V^{+1}	V^-	A	+	+
Citrobacter amalonaticus	V	V^-	A	+	-
Citrobacter diversus	V	V^-	A	+	-
Citrobacter freundii	A[1]	V^-	A	+	+
Yersinia	-	V	A	V	-

Notes
Lac: lactose fermentation; Suc: sucrose fermentation; Glu: glucose fermentation; A: acid reaction; V: variable; V^+: variable, usually positive; V^-: variable, usually negative.
1: the reaction may by delayed; 2: *S.flexneri* ser.6 gas production positive (slight amount); 3: usually negative except *S.sonnei* (acid reaction may be delayed); 4: although rare, lactose positive variants of *S.*Typhi exist; 5: *S.*Typhi may have a ring of H_2S but its presence is not diagnostic. *S.*Paratyphi A if positive may be weak.; 6: *E.agglomerans* gas production variable; 7: if gas produced, a slight amount.

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.

E.coli ATCC 25922: growth, yellow slant, yellow butt, gas + H_2S -
S.flexneri ATCC 12022: growth, red slant, yellow butt, gas - H_2S -
S.Typhimurium ATCC 14028: growth, red slant, yellow butt, gas + H_2S +
Aerobic incubation at 35-37°C for 18-24 h.

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 Triple Sugar iron Agar is tested for performances characteristics comparing the results with a previously approved Reference Batch.
Pure colonies cultivated on Tryptic Soy Agar of 7 *Enterobacteriaceae* strains are inoculated into the tubes: *E.coli* ATCC 25922, *C.freundii* ATCC 8090, *P.rettgeri* ATCC 39944, *S.*Enteritidis ATCC 13076, S.Thyphimurium ATCC 14028, *S.flexneri* ATCC 12022, *S.sonnei* ATCC 9290. After aerobic incubation at 35-37°C for 18-24 hours, the colour changes on the slant and in the butt, the gas and H_2S production are observed and recorded. All strains show reactivity according to the specifications for both batches tested.

13 - LIMITATIONS OF THE METHOD

- It is necessary to inoculate the medium with a microbiological needle without breaking the agar (do not use loops).
- Perform the reading between 18 and 24 hours of incubation; early readings can induce false acidity results of the A/A type or there is not enough time for the sugar fermentation with consequent color change of the indicator; delayed readings can give false K/K results due to the use of peptones and alkaline change of the medium.[9]
- H_2S production can mask the acid reaction in the butt, however the production of H_2S requires acidic conditions therefore the butt must be considered acid when there is blackening.
- Hydrogen sulphide production may be evident on Kligler Iron Agar but negative on Triple Sugar Iron Agar. Studies by Bulmash and Fulton[10] showed that the utilization of sucrose could suppress the enzymatic mechanisms responsible for H_2S production. Padron and Dockstader[11] found that not all H_2S-positive *Salmonella* are positive on TSI.
- An H_2S producing organism may exhibit blackening on SIM medium (positive) but none on TSI medium.[9]
- The medium does not contain inhibitors therefore a large variety of microorganisms can grow on it; for this reason, before inoculation, make sure that the organisms are catalase positive, Gram-negative bacilli.
- The addition of sucrose allows the earlier detection of coliform bacteria that ferment sucrose more rapidly than lactose. Adding sucrose also aids the identification of certain Gram-negative bacteria that could ferment sucrose but not lactose.[8]
- A pure culture is essential when inoculating the medium. If the culture is not pure, irregular results may be obtained.
- Some organisms such as the *Klebsiella-Enterobacter* group produce such an abundance of gas that the medium may be completely displaced by gas resulting in the medium being blown up into the cap. If this occurs, handle the culture with caution when sub-culturing to avoid contaminations.
- Make sure that the caps are loosened during incubation since for a correct medium performance a free exchange of air is necessary. If the caps are too closed, an acid reaction occurs only on the slant even in the presence of glucose fermentation.[9]
- 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.
- 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 preparation process of tubed or bottled 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.
- 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 period of validity of the finished products, according to the type (tubes/bottles) and the storage method applied (temperature and packaging).

16 - REFERENCES

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10. Bulmash JM, Fulton MD. Discrepant tests for hydrogen sulfide. J Bacteriol 1964; 88(2):1813
11. Padron AP, Dockstader WB. Selective medium for hydrogen sulfide production Appl Microbiol 1972; 23:1107

TABLE OF APPLICABLE SYMBOLS

REF Or REF Catalogue number	LOT Batch code	IVD *In vitro* Diagnostic Medical Device	Manufacturer	Use by
Temperature limitation	Σ 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”	2022/01
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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