Directions for Preparation from Dehydrated Product

MPN Procedure

1. For an inoculum of 1 mL or less, suspend 56.4 g of the powder in 1 L of purified water. Mix thoroughly.
   For an inoculum of 10 mL, suspend 84.6 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. For an inoculum of 1 mL or less, dispense 10 mL amounts into culture tubes.
   For an inoculum of 10 mL, dispense 20 mL amounts into culture tubes.
4. Autoclave at 121°C for 10 minutes.
5. Test samples of the finished product for performance using stable, typical control cultures.

Membrane Filter Procedure

1. Suspend 56.4 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Dispense in 100 mL amounts into flasks and autoclave at 121°C for 10 minutes.
4. Cool to 60°C and add 1 mL TTC Solution 1% per 100 mL of medium.
5. Test samples of the finished product for performance using stable, typical control cultures.

Procedure

MPN Procedure

1. Inoculate tubes of the KF Streptococcus Broth with the appropriate amount of inoculum.
2. Incubate tubes at 35 ± 1°C, with loosened caps, for 46-48 hours.

Membrane Filter Procedure

1. Place a sterile absorbent pad in each sterile Petri dish.
2. Saturate the pads with the sterile medium containing TTC.
3. Place an inoculated membrane filter, inoculated side up, on the saturated pad.
4. Incubate at 35 ± 1°C in an atmosphere saturated with water vapor for 46-48 hours.

Expected Results

MPN Procedure

MPN tubes positive for enterococci are turbid with growth that appears yellow in color and does not produce foaming. When foaming occurs, confirmation for enterococci should be made by Gram staining.

Membrane Filter Procedure

All red or pink colonies visible with 15× magnification are counted as enterococci colonies.

Limitations of the Procedure

1. Many strains of *S. bovis* and *S. equinus* are inhibited by azide.
2. Overheating may lower the pH, resulting in a decrease in productivity of the medium.

References

1. Kenner, Clark and Kabler. 1960. Am. J. Public Health *50*:1553.
2. Kenner, Clark and Kabler. 1961. Appl. Microbiol. 9:15.
3. MacFaddin. 1985. Media for isolation-cultivation-identification-maintenance of medical bacteria, vol. 1. Williams & Wilkins, Baltimore, Md.
4. Facklam and Moody. 1970. Appl. Microbiol. *20*:245.

Availability

Difco™ KF Streptococcus Broth

Cat. No. 212226 Dehydrated – 500 g

Difco™ TTC Solution 1%

Cat. No. 231121 Tube – 30 mL
264310 Bottle – 25 g

KL Virulence Agar
KL Virulence Enrichment • KL Antitoxin Strips

Intended Use

KL (Klebs-Loeffler) Virulence Agar is used with KL Virulence Enrichment, Tellurite Solution 1% and KL Antitoxin Strips in differentiating virulent (toxigenic) from nonvirulent strains of *Corynebacterium diphtheriae*.

Summary and Explanation

Elek[1] was the first to describe the agar plate diffusion technique for demonstrating the *in vitro* toxigenicity (virulence) of *Corynebacterium diphtheriae*. King, Frobisher and Parsons[2] expanded on Elek's technique and, by using a carefully standardized medium, obtained results in agreement with animal inoculation tests. These authors demonstrated that Proteose Peptone possessed properties essential for toxin production. Incorporating Proteose Peptone into the test medium assured consistent results. The authors used rabbit, sheep and horse serum as enrichments, finding human serum to be unsatisfactory. To overcome irregularities encountered in previous formulations, Hermann, Moore and Parsons[3] refined the medium used for the *in vitro* KL Virulence Test, simplifying the basal medium and developing a nonserous enrichment. The medium and enrichment described by these authors have been standardized for use in the KL Virulence Test.

KL Virulence Agar and KL Virulence Enrichment are prepared according to the formulation of Hermann, Moore and Parsons.[3]

Principles of the Procedure

Peptone provides the carbon and nitrogen sources required for good growth of a wide variety of organisms and for toxin production. Sodium chloride maintains the osmotic balance of the medium. Agar is incorporated as the solidifying agent.

KL Virulence Enrichment provides a source of nonserous enrichment. Casamino acids are derived from acid-hydrolyzed casein that has low sodium chloride and iron concentrations. The low iron concentration is beneficial because iron is known to prevent the production of diphtheria toxin when present in more than minute amounts. Glycerol (glycerin) contains no heavy metals and is used by bacteria as a source of carbon. Polysorbate 80 improves growth of certain strains of *Corynebacterium diphtheriae*.

Toxin produced by bacteria and diffused into the medium is detected by precipitation with the antitoxin present on the KL Antitoxin Strip. Tellurite Solution 1% (1% potassium tellurite solution) inhibits gram-negative and most gram-positive bacteria except *Corynebacterium* spp., *Streptococcus mitis*, *S. salivarius*, enterococci and possibly *Staphylococcus epidermidis*. This permits direct testing of mixed primary cultures.

Formulae

Difco™ KL Virulence Agar

Approximate Formula* Per Liter

Proteose Peptone	20.0	g
Sodium Chloride	2.5	g
Agar	15.0	g

Difco™ KL Virulence Enrichment

Approximate Formula* Per 100 mL

Casamino Acids	1.0	g
Glycerol	1.0	mL
Polysorbate 80	1.0	mL

BBL™ Taxo™ KL Antitoxin Strips

KL Antitoxin Strips are 1 × 7 cm filter paper strips containing antitoxin to diphtheria toxin.

**Adjusted and/or supplemented as required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Suspend 37.5 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.
4. Cool in a water bath to 55-60°C.
5. Aseptically dispense 10 mL of KL Virulence Agar into a Petri dish containing 2 mL KL Virulence Enrichment and 0.5 mL Tellurite Solution 1%. Mix thoroughly.

User Quality Control

Identity Specifications

Difco™ KL Virulence Agar

Dehydrated Appearance:	Light beige with some small dark specks, free-flowing, homogeneous.
Solution:	3.75% solution, soluble in purified water upon boiling. Solution is light to medium amber, very slightly to slightly opalescent with precipitate.
Prepared Appearance:	Light medium amber, slightly opalescent, may have a slight precipitate.
Reaction of 3.75% Solution at 25°C:	pH 7.8 ± 0.2

Difco™ KL Virulence Enrichment

Appearance:	Colorless to very light amber, clear liquid.

BBL™ Taxo™ KL Antitoxin Strips

Appearance:	White, filter paper strips, 1 x 7 cm.

Cultural Response

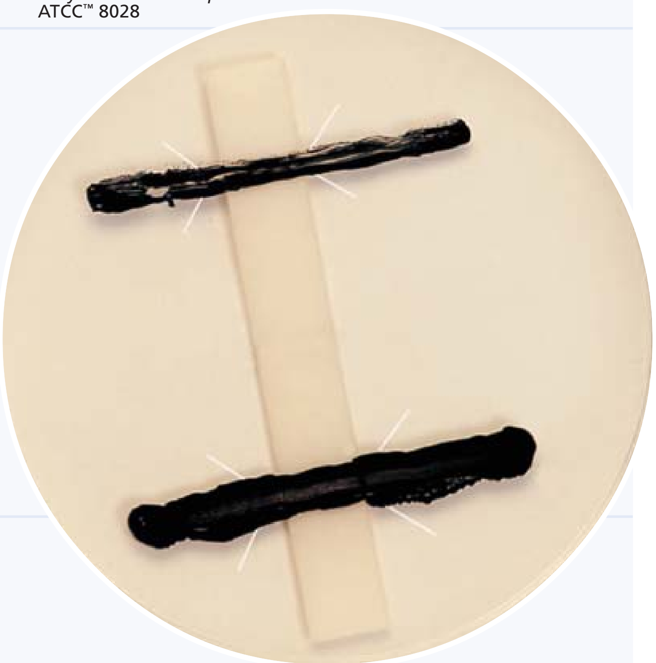
Difco™ KL Virulence Agar

Prepare the medium per label directions, including KL Virulence Enrichment, Tellurite Solution 1% and one KL Antitoxin Strip per plate. Inoculate with a single line streak across the plate perpendicular to the strip and incubate at 35 ± 2°C under CO_2 for up to 72 hours.

ORGANISM	ATCC™	REACTION
Bacillus subtilis	6633	–
Corynebacterium diphtheriae biotype gravis	8028	+
Corynebacterium diphtheriae biotype intermedius	8032	+
Corynebacterium diphtheriae biotype mitis	8024	+
Staphylococcus aureus	25923	–

+ = positive, line of precipitation at 45° angle to the strip
– = negative, no line of precipitation

Corynebacterium diphtheriae
ATCC™ 8028



Precipitate lines are graphically enhanced for demonstration purposes (see Expected Results).

6. Submerge a KL antitoxin strip or equivalent beneath the agar prior to solidification.
7. Test samples of the finished product for performance using stable, typical control cultures.

Procedure

Process the specimen according to accepted practices.[4] Inoculate the complete medium by streaking a loopful of a 24-hour culture in a single line across the plate perpendicular to (right angle to) the antitoxin strip. (Do not touch the actual strip itself.) As many as eight cultures may be tested on a single plate.[5] Place test isolates about 1 cm apart. Also inoculate a toxigenic (positive control) and a nontoxigenic (negative control) *C. diphtheriae* strain approximately 1 cm on either side of the test isolates.[5] Incubate the inverted plates under CO_2 at 37°C for 72 hours. Examine at 24-, 48- and 72-hour intervals.

Expected Results

Toxigenic (virulent) cultures of *C. diphtheriae* will show fine lines of precipitation at approximately 45° angles from the culture streak. This line forms where toxin (from the bacteria) combines with antitoxin from the strip. Primary precipitin lines form an arc of identity with the precipitin line produced by an adjacent positive control strain.[6] Nontoxigenic strains of *C. diphtheriae* will show no lines of precipitation.

Limitations of the Procedure

1. False-positive reactions may be seen after 24 hours as weak bands near the antitoxin strip. These can be recognized when compared with the positive control.[7]
2. *Corynebacterium ulcerans* and *C. pseudotuberculosis* may also produce lines of toxin-antitoxin.[8]

References

1. Elek. 1948. Br. Med. J. *1*:493.
2. King, Frobisher and Parsons. 1949. Am. J. Public Health *39*:1314.
3. Hermann, Moore and Parsons. 1958. Am. J. Clin. Pathol. *29*:181.
4. Funke and Bernard. 1999. *In* Murray, Baron, Pfaller, Tenover and Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.
5. MacFaddin. 1985. Media for isolation-cultivation-identification-maintenance of medical bacteria, vol. 1. Williams & Wilkins, Baltimore, Md.
6. Washington. 1981. Laboratory procedures in clinical microbiology. Springer-Verlag, New York, N.Y.
7. Lennette, Balows, Hausler and Truant (ed.). 1980. Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
8. Branson. 1972. Methods in clinical bacteriology. Charles C. Thomas, Springfield, Ill.

Availability

Difco™ KL Virulence Agar

Cat. No. 212192 Dehydrated – 500 g

Difco™ KL Virulence Enrichment

Cat. No. 298610 Tube – 12 × 20 mL*

BBL™ Taxo™ KL Antitoxin Strips

Cat. No. 231740 Vial – 12 strips*

BBL™ Tellurite Solution 1%

Cat. No. 211917 Tube – 20 mL

**Store at 2-8°C.*

Kligler Iron Agar

Intended Use

Kligler Iron Agar is used for the differentiation of members of the *Enterobacteriaceae* on the basis of their ability to ferment dextrose and lactose and to liberate sulfides.

Summary and Explanation

In 1911, Russell described a new double sugar tube medium for the isolation of typhoid bacilli from urine and feces.[1] Six years later, Kligler developed a simple lead acetate medium for the differentiation of the typhoid-paratyphoid group.[2] Subsequently, Kligler evaluated culture media used in the isolation and differentiation of typhoid, dysentery and allied bacilli and endorsed Russell's medium.[3] Bailey and Lacey substituted phenol red for the Andrade indicator previously used as a pH indicator.[4]

The current formulation of Kligler Iron Agar combines features of Kligler's lead acetate medium with those of Russell's double sugar agar.

Principles of the Procedure

Kligler Iron Agar, in addition to casein and meat peptones, contains lactose and dextrose which enable the differentiation of species of enteric bacilli due to color changes of the phenol red pH indicator in response to the acid produced during the fermentation of these sugars. The dextrose concentration is only 10% of the lactose concentration. The combination of ferric ammonium citrate and sodium thiosulfate enables the detection of hydrogen sulfide production.

Lactose nonfermenters (e.g., *Salmonella* and *Shigella*) initially produce a yellow slant due to acid produced by the fermentation of the small amount of dextrose. When the dextrose supply is exhausted in the aerobic environment of the slant, the reaction reverts to alkaline (red slant) due to oxidation of the acids. The reversion does not occur in the anaerobic environment in the butt, which remains acid (yellow butt). Lactose fermenters produce yellow slants and butts because enough acid is produced in the slant to maintain an acid pH under aerobic conditions. Organisms incapable of fermenting either carbohydrate produce red slants and butts.

Hydrogen sulfide production is evidenced by a black color either throughout the butt, or in a ring formation near the top of the butt. Gas production (aerogenic reaction) is detected as individual bubbles or by splitting or displacement of the agar.