APPENDIX VOL 1 & 2 (Revision # 1; 1/10/01)

MEDIA AND REAGENTS

B. P. Dey

All media and reagents necessary for each analysis are listed in each chapter. The formulations and procedures for preparing the special media (M) and Reagents (R) used throughout this Guidebook are presented in alphabetical order in this appendix. The very specialized media, used to a limited extent for the genus *Yersinia*, are presented as a separate addendum in Chapter 9. Formulations and preparations for basic media that may be used for general microbiological procedures, which are not listed in this appendix, may be obtained by consulting readily available reference materials such as general microbiology textbooks, commercially available media formulation handbooks, FDA's Bacteriological Analytical Manual, and APHA's Compendium of Methods for the Microbiological Examination of Foods.

The ingredients and the chemicals used for preparing media and reagents may be the product of any manufacturer if comparative tests show satisfactory results. The carbohydrates (sugars) should be chemically pure and suitable for biological use; inorganic chemicals used as reagents should be American Chemical Society (ACS) grade; and dyes must be certified by the "Biological Stain Commission" for use in media.

For convenience, dehydrated media of any brand equivalent to the formulation may be used unless instructions indicate otherwise. However, each lot of medium should be tested for sterility and ability to support growth of suitable organisms (e.g., inoculate media containing lactose with a non-pathogenic coliform and *Staphylococcus* media with a nonpathogenic *Staphylococcus*, etc).

Hydrogen ion concentration (pH) of media should be determined using an electronic pH meter which is standardized against known buffers, prepared according to the Official Methods of Analysis of the Association of Official Analytical Chemists (16th Edition). If necessary the pH of a medium should be adjusted by adding sufficient 1 N sodium hydroxide or 1 N hydrochloric acid.

Unless otherwise indicated, a medium should be sterilized by steam under pressure at 121°C (15 lb.) for 15 minutes.

I. MEDIA

M 1. A-K AGAR #2 (SPORULATING AGAR)

Pancreatic Digest of Gelatin	6.0 g
Pancreatic Digest of Casein	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Manganous Sulfate ($MnSO_4.7H_2O$)	0.3 g
Distilled water	1.0 L

Suspend above ingredients and boil gently for 1 minute to completely dissolve. Dispense and autoclave at 121°C for 15 minutes. Final pH 6.6 ± 0.2 at 25°C.

M 2. ANTIBIOTIC MEDIUM #1

Peptone	6.0 g
Pancreatic Digest of Casein	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	1.0 L

Boil to dissolve. Dispense in 100 ml quantities and autoclave. Final pH 6.5 - 6.6.

M 3. ANTIBIOTIC MEDIUM #2

Peptone	6.0 g
Beef Extract	1.5 g
Yeast Extract	3.0 g
Agar	15.0 g
Distilled water	1.0 L

Boil gently 1-2 minutes to dissolve. Dispense in 100 ml volumes and autoclave. When cooled but still liquid (60-65°C), add sterile dextrose solution to a final concentration of 1 g/L. Final pH 6.6.

M 4. ANTIBIOTIC MEDIUM #5

Peptone	6.0 g
Beef Extract	1.5 g
Yeast Extract	3.0 g
Agar	15.0 g
Distilled water	1.0 L

Boil gently 1-2 minutes to dissolve. Dispense in 100 ml volumes and autoclave. Final pH 7.9 - 8.1

M 5. ANTIBIOTIC MEDIUM #8

Peptone	6.0 g
Beef Extract	1.5 g
Yeast Extract	3.0 g
Agar	15.0 g
Distilled water	1.0 L

Boil gently 1-2 minutes to dissolve. Dispense in 100 ml volumes and autoclave. Final pH 5.7 - 5.9.

M 6. ANTIBIOTIC MEDIUM #11 (NEOMYCIN ASSAY AGAR)

Gelsate Peptone	6.0 g
Trypticase Peptone	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Distilled water	1.0 L

Autoclave 121°C for 15 minutes. Refrigerate. Final pH 7.9 - 8.1.

M 7. APT AGAR

Pancreatic digest of casein	12.5 g
Glucose	10.0 g
Yeast Extract	7.5 g
Sodium Chloride	5.0 g
K_2HPO_4	5.0 g
Sodium Citrate	5.0 g
Na_2CO_3	1.25 g
$MnCl_2.4H_2O$	0.14 g
$MgSO_4.7H_2O$	0.8 g
Polysorbate 80	0.2 g
$FeSO_4.7H_2O$	0.04 g

Thiamine Hydrochloride	1.0	mg
Agar	15.0	g
Distilled water	1.0	L

Add components to distilled water, bring volume to 1.0 L and mix thoroughly. Gently heat and bring to boiling. Distribute into tubes or flasks and sterilize by autoclaving at 118°C - 121°C at 13 psi for 15 minutes. Avoid excessive heating. Pour into sterile Petri dishes or leave in tubes. Final pH 6.7 ± 0.2 at 25°C.

M 8. BAIRD-PARKER MEDIUM

Basal Medium

Tryptone	10.0 g
Beef Extract	5.0 g
Yeast Extract	1.0 g
Sodium Pyruvate	10.0 g
Glycine	12.0 g
Lithium Chloride $6H_20$	5.0 g
Agar	20.0 g
Distilled water	950.0 ml

Suspend ingredients in water. Heat to boiling to dissolve completely. Dispense 95 ml portions in screw-capped bottles. Autoclave at 121°C for 15 minutes. Final pH 6.8 - 7.2.

Complete medium.

a. Add 5 ml prewarmed (45-50°C) Bacto EY tellurite enrichment to 95 ml molten basal medium, which has been adjusted to 45-50°C.
b. Mix well (avoiding bubbles) and pour 15-18 ml into sterile 100 x 15 mm Petri dishes.
c. Plates of complete medium should be stored in refrigerator for no longer than 48 h before use.
d. Plates should be dried before use by any of the following procedures:

    i. In a laminar flow hood with lids removed and agar surface upward;
    ii. In a forced air oven or incubator for 2 h at 50°C, with lids on and the agar surface upward;

iii. In an incubator for 4 h at 35°C, with lids on and agar surface upward;

or

iv. On laboratory bench for 16-18 h at room temperature with lids on and agar surface upward.

M 9. BC (BACILLUS CEREUS) MOTILITY MEDIUM

Trypticase	10.0 g
Yeast Extract	2.5 g
Glucose	5.0 g
Disodium Hydrogen Phosphate	2.5 g
Agar	3.0 g
Distilled water	1.0 L

Dissolve the ingredients in distilled water and heat to boiling to completely dissolve the agar. Mix thoroughly and dispense 2.0 ml into 13 X 100 mm tubes. Autoclave for 15 minutes at 121°C. Final pH should be 7.4 ± 0.2. Allow the medium to solidify and store at room temperature for up to 2 or 3 days for best results.

M 10. BILE ESCULIN AGAR

Beef Extract	3.0 g
Peptone	5.0 g
Esculin	1.0 g
Oxgall	40.0 g
Ferric Citrate	0.5 g
Agar	15.0 g
Distilled water	1.0 L

Heat with agitation to dissolve. Dispense 4 ml volumes into 13 x 100 mm screw-capped tubes, autoclave 15 minutes at 121°C, and slant until solidified. Final pH, 6.6 $\pm$ 0.2.

M 11. BRAIN HEART INFUSION (BHI) AGAR

Calf Brain (infusion from)	200.0 g
Beef Heart (infusion from)	250.0 g
Proteose peptone or gelysate	10.0 g
NaCl	5.0 g
Na_2HP0_4	2.5 g
Dextrose	2.0 g
Agar	15.0 g
Distilled water	1.0 L

Dissolve ingredients in distilled water by heating to boiling. Dispense as desired and autoclave at 121°C for 15 minutes. Final pH 7.4 ± 0.2.

M 12. BRAIN HEART INFUSION (BHI) BROTH

Prepare same as above except omit the 15.0 g agar.

Dispense 5 ml per 13 x 100 mm screw capped tubes and autoclave at 121°C for 15 minutes. Final pH 7.4 ± 0.2.

M 13. BRILLIANT GREEN SULFA AGAR (OSBORN AND STOKES)

Yeast Extract	3.0	g
Polypeptone	10.0	g
Sodium Chloride	5.0	g
Lactose	10.0	g
Sucrose	10.0	g
Phenol Red	0.08	g
Agar	20.0	g
Sulfapyridine	1.0	g
Brilliant Green	0.0125	g
Distilled water	1.0	L

Mix thoroughly, adjust pH to 7.3 with 8 N NaOH, and heat with frequent agitation to dissolve. Dispense in bottles or flasks and autoclave at 121°C for 15 minutes. Cool to 50°C and pour approximately 20 ml into sterile 100 x 15 mm Petri dishes.

M 14. BROMCRESOL PURPLE (BCP) DEXTROSE BROTH

Peptone	10.0	g
Beef Extract (optional)	3.0	g
Sodium Chloride	5.0	g
Bromcresol Purple	0.04	g
Distilled water	1.0	L

Combine the above ingredients with 5 g glucose per liter. (Other carbohydrates such as adonitol, arabinose, mannitol, maltose, sucrose, lactose, sorbitol, cellobiose, salicin or trehalose may also be used individually at a quantity of 5 g per liter to prepare these individual BCP carbohydrate fermentation broths). Adjust to pH 7.0. Dispense 8.0 ml aliquots into 16 x 150 mm tubes containing inverted 12 x 75 mm fermentation tubes. Autoclave for 10 minutes at 121°C. Final pH should be 6.8 - 7.0.

NOTE: Dehydrated prepared medium not available commercially.

M 15. BRUCELLA-FBP (BFBP) AGAR

Bacto Peptamin	20.0 g
Bacto Dextrose	1.0 g
Bacto Yeast Extract	2.0 g
Sodium Chloride	5.0 g
Sodium Bisulfite	0.1 g
Bacto Agar	15.0 g
Ferrous Sulfate	0.25 g
Sodium Metabisulfite	0.25 g
Sodium Pyruvate	0.25 g
Distilled water	1.0 L

Brucella agar (dehydrated; Difco), 43.0 g, may be substituted for the first six ingredients above. Suspend the dehydrated ingredients in distilled water, heat with frequent agitation, and boil for 1 minute to dissolve completely. Autoclave at 121°C for 15 minutes. Cool to 50°C and add 4 ml filter-sterilized ferrous sulfate-sodium metabisulfite-sodium pyruvate (FBP) solution or 2 vials of Oxoid FBP supplement (See M 30 for FBP supplement preparation). Mix thoroughly and pour into sterile petri dishes (approximately 20 ml/100 x 15 mm plate). Dry the agar surfaces prior to inoculating by placing the plates on a bench top (protected from light) overnight.

M 16. BRUCELLA-FBP (BFBP) BROTH

Bacto Tryptone	10.0 g
Bacto Peptamin	10.0 g
Bacto Dextrose	1.0 g
Bacto Yeast Extract	2.0 g
Sodium Chloride	5.0 g
Sodium Bisulfite	0.10 g
Ferrous Sulfate	0.25 g
Sodium Metabisulfite	0.25 g
Sodium Pyruvate	0.25 g
Distilled water	1.0 L

Brucella broth (dehydrated; Difco), 28.0 g, may be substituted for the first six ingredients above. Dissolve the dehydrated ingredients in distilled water and

autoclave at 121°C for 15 minutes. Cool the medium to room temperature and add filter-sterilized FBP solution. (Use Oxoid FBP supplement SR84 or the FBP solution, prepared as described under M 30). Aseptically dispense 10 ml aliquots of broth into sterile 16 x 150 mm screw-capped tubes.

M 17. BUFFERED PEPTONE WATER

Peptone	10.0 g
Sodium Chloride	5.0 g
Sodium Phosphate, dibasic	3.5 g
Potassium Phosphate, monobasic	1.5 g
Distilled water	1.0 L

Dissolve dry ingredients in distilled water, dispense into appropriate containers, and sterilize in the autoclave at 121°C for 15 minutes. Final pH 7.2 ± 0.2.

M 18. CARBOHYDRATE FERMENTATION BROTH (EWING)

Fermentation Broth Base

Peptone	10.0 g
Meat Extract	3.0 g
Sodium Chloride	5.0 g
Andrade's indicator	10.0 ml
Distilled water	1.0 L

Adjust reaction to pH 7.1 - 7.2. Dispense in tubes with inverted insert tubes and sterilize at 121°C for 15 minutes.

(See exceptions)

Glucose, lactose, sucrose, and mannitol are employed in a final concentration of 1%. Other carbohydrates such as galactitol, salicin, etc., may be used in a final concentration of 0.5%. Glucose, mannitol, galactitol, salicin, adonitol, and inositol may be added to the basal medium prior to sterilization. Medium containing neutral glycerol should be sterilized at 121°C for 10 minutes. Disaccharides such as lactose, sucrose, and cellobiose (10% solution in distilled water, neutral pH) should be sterilized by filtration or at 121°C for 10 minutes and added to previously sterilized basal medium. Arabinose, xylose, and rhamnose also should be sterilized

separately. If basal medium is tubed in 3.0-ml amounts, add 0.3 ml of sterile aqueous carbohydrate solution, i.e., one-tenth the volume. The natural occurring forms of the carbohydrates are used.

M 19. COLUMBIA AGAR (COLUMBIA BLOOD AGAR BASE)

Pantone	10.0 g
Bitone	10.0 g
Tryptic Digest of Beef Heart	3.0 g
Corn Starch	1.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Autoclave 121°C for 15 minutes. If desired, cool to 50°C and add 5% sterile defibrinated sheep blood and swirl. Avoid bubble formation. Pour 15 to 20 ml into sterile 100 x 15 mm Petri dishes.

M 20. DECARBOXYLASE MEDIUM (MOELLER; Ewing)

Peptone	5.0 g
Meat Extract	5.0 g
Bromcresol Purple (1.6%)	0.625 ml
Cresol Red (0.2%)	2.5 ml
Glucose	0.5 g
Pyridoxal	5.0 g
Distilled water	1.0 L

Adjust pH to 6 or 6.5. Divide into four portions. Tube one portion without addition of amino acid, for control purposes. To one of the remaining portions, add l% L-lysine dihydrochloride, to another add l% L-arginine hydrochloride and to the third portion, add l% L-ornithine dihydrochloride. Readjust the pH, if necessary. Tube (3-4 ml per 13 x 100 mm screw-capped tube). Sterilize at 121°C for 10 minutes. (If D, L-amino acids are used, use 2% concentration).

After inoculation, layer with sterile mineral oil. Examine daily for four days.

M 21. DOUBLE MODIFIED LYSINE IRON AGAR (DMLIA)

Lysine Iron Agar	34.0 g
Bile Salts No. 3	1.5 g
Lactose	10.0 g

Sucrose	10.0	g
Sodium Thiosulfate	6.76	g
Ferric Ammonium Citrate	0.3	g
Distilled water	1.0	L
Sodium Novobiocin	0.015	g

Suspend all ingredients except Sodium Novobiocin in 1.0 L distilled water and heat to boiling. DO NOT AUTOCLAVE. Cool to 50°C and add Sodium Novobiocin from a filter-sterilized stock solution. Pour plates. DMLIA plates may be stored in a refrigerator for up to 3 weeks. This medium is also commercially available as a dehydrated powder with a separate novobiocin supplement.

M 22. EC BROTH

Tryptose or Trypticase	20.0	g
Bacto Bile Salt #3 or Bile salts mixture	1.5	g
Lactose	5.0	g
K_2HPO_4	4.0	g
KH_2PO_4	1.5	g
NaCl	5.0	g
Distilled water	1.0	L

Dispense 10 ml/tube into 16 x 150 mm straight tubes with inverted 10 x 75 mm fermentation tubes. Autoclave at 121°C for 15 minutes. Final pH 6.9 ± 0.1.

M 23. ENRICHED SEMISOLID BRUCELLA MEDIUM

Bacto Tryptone	10.0	g
Bacto Peptamin	10.0	g
Bacto Dextrose	1.0	g
Bacto Yeast Extract	2.0	g
Sodium Chloride	5.0	g
Sodium Bisulfite	0.10	g
Agar	5.0	g
Distilled water	1.0	L
Sterile defibrinated sheep blood	100.0	ml

Brucella broth (dehydrated; Difco), 28.0 g, may be substituted for the first six ingredients above. Dissolve by boiling dehydrated ingredients in distilled water and autoclave at 121°C for 15 minutes. Cool to 50°C and add the blood. Dispense 4-ml amounts into sterile 13 x 100 mm screw-capped tubes.

M 24. EOSIN METHYLENE BLUE (EMB) AGAR

Levine formula of EMB agar prepared according to manufacturer's instructions. Add enough additional agar to bring the final concentration to 3%. This will prevent Proteus swarming.

M 25. EY-FREE TRYPTOSE SULFITE CYCLOSERINE (TSC) AGAR

The above medium is made exactly as that shown for M 76 (Tryptose Sulfite Cycloserine (TSC) Agar) except, omit the 50 ml addition of sterile egg yolk emulsion. Add 50 ml distilled water instead of the egg yolk emulsion.

M 26. FLUID THIOGLYCOLLATE MEDIUM

Pancreatic Digest of Casein (Trypticase)	15.0	g
l-Cystine	0.5	g
Dextrose	5.0	g
Yeast Extract	5.0	g
Sodium Chloride	2.5	g
Sodium Thioglycollate	0.5	g
Resazurin	0.001	g
Agar	0.75	g
Distilled water	1.0	L

Dissolve by boiling and distribute 15 ml per 20 x 150 mm screw-capped tube. Autoclave at 121°C for 15 minutes. Tighten caps. Store in dark cool place. (Do not refrigerate).

M 27. FRASER BROTH

Proteose Peptone	5.0	g
Tryptone	5.0	g
Lab Lemco Powder (Oxoid)	5.0	g
Yeast Extract	5.0	g
NaCl	20.0	g
KH_2PO_4	1.35	g
Na_2HPO_4	12.0	g
Esculin	1.0	g
Naladixic Acid (2% in 0.1 M NaOH)	1.0	ml
Lithium Chloride	3.0	g
Distilled water	1.0	L

Mix well to resuspend the media and dispense 10 ml into 20 X 150 mm test tubes. Sterilize at 121°C for 15

minutes. DO NOT OVERHEAT; COOL AT ONCE AFTER REMOVAL FROM THE STERILIZER. Store in the refrigerator. Just before use, add 0.1 ml of 2.5 mg/ml of filter sterilized acriflavin (Sigma) and 0.1 ml filter sterilized 5% stock solution of ferric ammonium citrate (Sigma) in distilled water to each 10 ml tube.

M 28. GN BROTH (HAJNA)

Pancreatic digest of casein	10.0 g
Peptic digest of animal tissue	10.0 g
Sodium Chloride	5.0 g
Sodium Citrate	5.0 g
K_2HPO_4	4.0 g
D-Mannitol	2.0 g
KH_2PO_4	1.5 g
Dextrose	1.0 g
Sodium Deoxycholate	0.5 g
Distilled water	1.0 L

Add components to distilled water and bring volume to 1.0 L. Mix thoroughly. Gently heat and bring to boiling. Dispense 12 ml per 16 x 150 mm straight tubes. Autoclave at 118°C at 13 psi for 15 minutes or steam for 30 minutes at 100°C. Final pH 7.0 ± 0.2 at 25°C.

M 29. HORSE BLOOD OVERLAY MEDIUM (HL)

Base Layer:

Columbia Blood Agar Base	1.0 L

Prepare according to manufacturer's specifications and sterilize at 121°C for 15 minutes. Pour 10 ml per 100 mm diameter Petri dish. Allow to solidify and while still warm, overlay with blood agar as described below.

Top Layer:

Add 4% sterile horse blood to a portion of melted Columbia Blood Agar Base which has been cooled to 46°C. Stir or swirl to mix evenly. Quickly place 5 or 6 ml on top of the base layer and tilt the plates to spread top layer evenly. Store plates in the refrigerator. Discard any plates which become discolored.

M 30. HUNT ENRICHMENT BROTH (Hunt, 1992)

a. Basal Broth

Nutrient broth #2 (Oxoid CM 67)	25.0 g
Yeast Extract (Oxoid L 21)	6.0 g
Distilled water	950.0 ml

Dissolve the nutrient broth #2 and yeast extract in distilled water. Autoclave at 121°C for 15 minutes.

Cool media and add supplements (FBP, filter-sterilized antibiotics, and horse blood) just before use and mix thoroughly.

b. FBP Supplement

Ferrous Sulfate	0.25 g
Sodium Metabisulfite	0.25 g
Sodium Pyruvate	0.25 g

FBP Stock Solution

Ferrous Sulfate	6.25 g
Sodium Metabisulfite	6.25 g
Sodium Pyruvate	6.25 g

Dissolve ingredients in distilled water in a 100 ml volumetric flask, bring to volume and filter sterilize. Dispense in 4 ml aliquots and store at -20°C. Use 4 ml for each liter of enrichment broth. Discard frozen FBP stock solution after 2 months.

Alternatively, use Oxoid FBP (Campylobacter Growth Supplement; SR84). Rehydrate the supplement with 2 ml sterile distilled water and add to the cooled medium. Add 2 vials for each liter of broth.

c. Antibiotics

Vancomycin-Hydrochloride (Sigma)	10.0 mg

Vancomycin Stock Solution

In a 100 ml volumetric flask, dissolve 0.25 g vancomycin in distilled water, bring to volume, mix well, and filter sterilize. Store at 4°C. Use 4 ml for each liter of

enrichment broth. Discard the vancomycin solution after 2 months.

Trimethoprim Lactate (Sigma) 12.5 mg

Trimethoprim Lactate Stock Solution

In a 100 ml volumetric flask, dissolve 0.3125 g trimethoprim lactate in distilled water, bring to volume, mix well, and filter sterilize. Store at 4°C. Use 4 ml for each liter of enrichment broth. Discard the trimethoprim lactate solution after 12 months.

Cefoperazone-Sodium (Sigma) 15.0 mg

Cefoperazone Stock Solution

In a 100 ml volumetric flask, dissolve 0.375 g cefoperazone in distilled water, bring to volume, mix well, and filter sterilize. Store at -70°C in 4 ml aliquots. Initially, use 4 ml for each liter of enrichment broth (for the first four hours, incubation is at 37°C). After four hours, add an additional 4 ml/liter, to bring the final concentration to 30 mg/liter, and increase the incubation temperature to 42°C. Discard the frozen cefoperazone solution after 5 months.

Cycloheximide (Sigma) 100.0 mg

Cycloheximide Stock Solution

Prepare as a 10% solution in 50% ethanol. In a 50 ml volumetric flask, dissolve 5 g cycloheximide in 50 ml 50% ethanol, mix, and bring to volume. Filter sterilize and store at 4°C indefinitely. Use 1 ml for each L of broth.

d. Sterile, lysed horse blood 50.0 ml

Lyse horse blood by subjecting it to two freeze/thaw cycles. Store frozen and discard blood after 12 months.

M 31. KCN BROTH (MOELLER)

Base

Proteose Peptone #3
or
Orthana Special Peptone 3.0 g

Disodium Phosphate	5.64 g
Monopotassium Phosphate	0.225 g
Sodium Chloride	5.0 g
Distilled water	1.0 L

Sterilize 100 ml amounts in the autoclave at 121°C for 15 minutes. Cool to below 20°C. Prepare 0.5% KCN with cold sterile distilled water. Using a sterile syringe or bulb pipet, add 1.5 ml KCN† solution to each 100 ml of base. Distribute 1 to 2 ml to small sterile 13 x 100 mm straight tubes and stopper quickly with corks sterilized by heating in paraffin. Store the finished medium in the refrigerator not more than two weeks.
(CAUTION: KCN IS A DEADLY POISON).

M 32. KF BROTH

Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g
Yeast Extract	10.0 g
Sodium Chloride	5.0 g
Sodium Glycerophosphate	10.0 g
Maltose	20.0 g
Lactose	1.0 g
Na_2CO_3	0.636 g
Sodium Azide†	0.4 g
Phenol Red	0.018 g
Distilled water	990.0 ml

Stock 2,3,5-triphenyltetrazolium chloride solution:

Place 0.1 g 2,3,5 triphenyltetrazolinum chloride in distilled water to make a total volume of 10 ml. Filter sterilize through a 0.2 µm filter.

Place the above components, except for the 2,3,5 triphenyltetrazolium chloride solution, in distilled water, bring volume to 990.0 ml and mix thoroughly. Gently heat and bring to boiling. Autoclave for 15 minutes at 121°C. Cool to 45 - 50°C and aseptically add the 10 ml sterile, stock 2,3,5 triphenyltetrazolium chloride solution to the base medium. Mix thoroughly. Aseptically distribute in 5 - 8 ml volumes in sterile tubes. Final pH 7.2 ± 0.2 at 25°C.

M 33. LACTOSE GELATIN MEDIUM

Tryptose	15.0	g
Yeast Extract	10.0	g
Lactose	10.0	g
Gelatin	120.0	g
Phenol red (as solution)	0.05	g
Distilled water	1.0	L

Suspend the ingredients except the gelatin and phenol red in 400 ml of distilled water, and dissolve by heating gently while stirring. Suspend the gelatin in 600 ml of cold distilled water, and dissolve by heating in a waterbath at 50 to 60°C with frequent stirring. When the gelatin is dissolved, combine with the other dissolved ingredients and adjust the pH to 7.5 with l N sodium hydroxide. Add the phenol red, mix well, and dispense 10 ml portions into 16 x 125 mm screw-capped tubes. Sterilize by autoclaving for 10 minutes at 121°C. If the medium is not used within 8 h, deaerate by holding in a waterbath at 50 to 70°C for 2 to 3 h before use.

M 34. LAURYL SULFATE TRYPTOSE (LST) BROTH

Trypticase or tryptose	20.0	g
NaCl	5.0	g
Lactose	5.0	g
K_2HPO_4	2.75	g
KH_2PO_4	2.75	g
Sodium Lauryl Sulfate	0.1	g
Distilled water	1.0	L

Dispense 10 ml/tube into 20 x 150 mm straight test tubes containing inverted fermentation tubes (10 x 75 mm). Autoclave at 121°C for 15 minutes. Final pH 6.8 $\pm$ 0.1.

M 35. LYSINE IRON AGAR (EDWARDS AND FIFE)

Peptone	5.0	g
Yeast Extract	3.0	g
Glucose	1.0	g
L-lysine	10.0	g
Ferric Ammonium Citrate	0.5	g
Sodium Thiosulfate	0.04	g
Bromcresol Purple	0.02	g
Agar	15.0	g
Distilled water	1.0	L

Dispense 4 ml/tube in 13 x 100 mm tubes and autoclave for 12 minutes at 121°C. Slant with deep butt and short slant.

M 36. MACCONKEY SORBITOL AGAR WITH BCIG (MSA-BCIG)

MacConkey sorbitol agar (MSA; DIFCO Cat. # 0079-17-7)	50.0 g
BCIG (5-bromo-4-chloro-3-indolyl-β-D-glucuronide, sodium salt; Biosynth International, Skokie, Ill.)	0.1 g
Distilled water	1.0 L

Autoclave at 121°C for 15 minutes. Temper and pour into 100 x 15 mm petri dishes (20 ml/plate) and/or into 150 x 15 mm petri dishes (60-80 ml/plate). Leave at room temperature overnight to dry.

NOTE: BCIG is a new compound and any long term toxic reactions are unknown. Please take precautions when handling it. Read and follow the MSDS (Material Safety Data Sheet) for the compound.

M 37. MALONATE BROTH (LEIFSON, MODIFIED)

Yeast Extract	1.0 g
Ammonium Sulfate	2.0 g
Dipotassium Phosphate	0.6 g
Monopotassium Phosphate	0.4 g
Sodium Chloride	2.0 g
Sodium Malonate	3.0 g
Glucose	0.25 g
Bromthymol Blue	0.025 g
Distilled water	1.0 L

Dispense 4 ml/tube into 13 x 100 mm screw-capped test tubes and sterilize at 121°C for 15 minutes.

NOTE: The commercially prepared media may require addition of yeast extract and glucose to meet the above formula.

M 38. MANNITOL YOLK POLYMYXIN (MYP) AGAR

Preparation A:

Beef Extract	1.0	g
Peptone	10.0	g
D-Mannitol	10.0	g
NaCl	10.0	g
Phenol Red	0.025	g
Agar	15.0	g
Distilled water	900.0	ml

Preparation B:

Colbeck's Egg Yolk Broth or Concentrated Egg Yolk Emulsion. This is available from Difco Laboratories or Oxoid.

Preparation C:

Polymyxin B Sulfate - Dissolve 500,000 units of sterile polymyxin B sulfate (Burroughs Welcome Co., Research Triangle Park, NC) in 50.0 ml of sterile distilled water.

Mix the ingredients (Preparation A) in distilled water, adjust pH to 7.2 ± 0.1, heat to boiling to dissolve completely and dispense 225.0 ml portions into 500 ml flasks. Autoclave at 121°C for 20 minutes, cool to 50°C in a waterbath, and add 12.5 ml of Preparation B and 2.5 ml of Preparation C to each flask containing 225.0 ml of medium. Mix well, pour into Petri dishes, allow to solidify, and dry for 24 h at room temperature. Plates may be stored at 4°C for 7 days.

M 39. MODIFIED CAMPYLOBACTER CHARCOAL DIFFERENTIAL AGAR (MCCDA), (Hutchinson and Bolton, 1984)

Nutrient broth No. 2 (Oxoid)	25.0	g
Bacteriological charcoal	4.0	g
Casein Hydrolysate	3.0	g
Sodium Deoxycholate	1.0	g
Ferrous Sulfate	0.25	g
Sodium Pyruvate	0.25	g
Agar	12.0	g
Sodium Cefoperazone	0.032	g
Distilled water	1.0	L

MCCDA is available commercially (in two parts) from Oxoid and is prepared as follows:

Suspend 22.75 g of Campylobacter Blood-Free Selective Agar Base (Modified CCDA-Preston; Oxoid CM739) in 500 ml of distilled water and bring to a boil to dissolve. Sterilize by autoclaving at 121°C for l5 minutes. Cool to 50°C. Aseptically add one vial of Cefoperazone Selective Supplement (Oxoid SR125) reconstituted with 2 ml of sterile distilled water. Mix well and pour approximately 20 ml/plate into sterile 100 x 15 mm petri dishes. Dry the agar surfaces prior to streaking by placing the plates on a bench top (protected from light) overnight.

M 40. MODIFIED COOKED MEAT MEDIUM

a. Cooked Meat Medium (dehydrated prepared medium available commercially)

Beef Heart	454.0 g
Proteose Peptone	20.0 g
Dextrose	2.0 g
Sodium Chloride	5.0 g

b. Diluent (not available commercially)

Trypticase or Tryptone	10.0 g
Sodium Thioglycollate	1.0 g
Soluble Starch	1.0 g
Dextrose	2.0 g
Neutral Red (1% aqueous)	5.0 ml
Distilled water	1.0 L

Adjust to pH 6.8. Add about 1 gram of (a) and 16 ml of (b) to screw-capped tubes no smaller than 20 x 150 mm. Tighten caps, vortex tubes to disperse meat, loosen caps, and autoclave at 121°C for 15 minutes. Wait about 10 minutes after completion of the autoclave cycle before opening the door in order to prevent loss of liquid from the tubes.

M 41. MODIFIED EC BROTH WITH NOVOBIOCIN (mEC+n)

Tryptone (Difco 0123-01-2)	20.0 g
Bile Salts #3 (Difco 0130-01-2)	1.12 g
Lactose	5.0 g
K_2HPO_4	4.0 g

KH_2PO_4	1.5 g
NaCl	5.0 g
Distilled water	1.0 L

If necessary, adjust pH to 6.9 ± 0.1 with 1 N HCl before autoclaving. Autoclave at 121°C for 15 minutes and cool. Add 5 ml of a filter sterilized, aqueous solution of 4mg/ml sodium novobiocin (adjusted for potency; Sigma N1628) for each liter of medium (20 mg/L).

M 42. MODIFIED OXFORD MEDIUM (MOX)

MOX Agar Base:

Columbia Blood Agar Base (depending on brand)	39-44.0 g
Agar	2.0 g
Esculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Lithium Chloride (Sigma L0505)	15.0 g
1% Colistin Solution	1.0 ml
Distilled water	1.0 L

Rehydrate commercial Columbia Blood Agar Base with constant stirring using a magnetic mixer and adjust pH to 7.2, if necessary. Autoclave this base at 121°C for 10 minutes, mix again, and cool rapidly to 46°C in a water bath. Add 2 ml of 1% filter sterilized Moxalactam Solution to make the complete MOX medium, mix well, and pour 12 ml per plate.

CAUTION: DO NOT use the Oxford Supplement or any other supplement with this formula.

1% Colistin Solution:

Colistin, Methane Sulfonate (Sigma C1511)	1.0 g
0.1 M Potassium Phosphate Buffer, pH 6.0	100.0 ml

Colistin solution is not sterile; store frozen in small aliquots (3-5 ml) at -20°C or below.

1% Moxalactam Solution:

Sodium (or Ammonium) Moxalactam (Sigma M1900)	1.0 g
0.1 M Potassium Phosphate Buffer, pH 6.0	100.0 ml

Dissolve, sterilize by filtration, dispense in 2 ml

quantities and store in freezer at -20°C or below.

M 43. MODIFIED UVM BROTH

Proteose Peptone	5.0 g
Tryptone	5.0 g
Lab Lemco Powder (Oxoid)	5.0 g
Yeast Extract	5.0 g
NaCl	20.0 g
KH_2PO_4	1.35 g
Na_2HPO_4	12.0 g
Esculin	1.0 g
Naladixic Acid (2% in 0.1 M NaOH)	1.0 ml
Acriflavin	12.0 mg
Distilled water	1.0 L

Sterilize at 121°C for 15 minutes. DO NOT OVERHEAT; COOL AT ONCE AFTER REMOVAL FROM THE STERILIZER. IF THE MEDIUM BLACKENS OR DARKENS, IT HAS BEEN OVERHEATED AND MUST BE DISCARDED. Store in the refrigerator.

M 44. MOTILITY-NITRATE MEDIUM (BUFFERED)

Beef Extract	3.0 g
Peptone	5.0 g
Potassium Nitrate	1.0 g
Disodium Phosphate	2.5 g
Agar	3.0 g
Galactose	5.0 g
Glycerol	5.0 g
Distilled water	1.0 L

Dissolve the ingredients, except agar, in distilled water, and adjust the pH to 7.4. Add the agar, and heat to boiling with stirring to dissolve completely. Dispense 11 ml portions into 16 x 125 mm tubes. Sterilize the dispensed medium by autoclaving for 15 minutes at 121°C, and cool quickly in cold water. If the medium is not used within 4 h after preparation, heat for 10 minutes in boiling water or flowing steam and chill in cold water before use.

M 45. MOTILITY TEST MEDIUM (EWING)

Meat Extract	3.0 g
Peptone	10.0 g
Sodium Chloride	5.0 g
Agar	4.0 g

Distilled water	1.0 L

Adjust to pH 7.4. Add agar. Heat to dissolve. Dispense 5 ml/tube into 13 x 100 mm screw-capped tubes and sterilize at 121°C for 15 minutes.

M 46. MR-VP MEDIUM (EWING)

Buffered Peptone	7.0 g
Dextrose	5.0 g
K_2HPO_4	5.0 g
Distilled water	1.0 L

Dispense 5 ml/tube into 13 x 100 mm screw-capped tubes and sterilize at 121°C for 15 minutes. Final pH 6.9.

M 47. MUELLER HINTON AGAR

Beef Extract	2.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1.0 L

Suspend ingredients and heat to a boil for 1 minute to completely dissolve. Dispense and autoclave at 121°C for 15 minutes. Final pH 7.3 ± 0.1.

M 48. NITRATE BROTH

Beef Extract	3.0 g
Peptone	5.0 g
Potassium Nitrate	1.0 g
Distilled water	1.0 L

Suspend above ingredients in distilled water and heat to boiling to dissolve completely. Dispense 5 - 7 ml volumes into tubes and autoclave for 15 minutes at 121°C. Final pH 7.0 ± 0.2 at 25°C.

M 49. NUTRIENT AGAR

Beef Extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Heat to boiling to dissolve ingredients. Dispense into tubes or flasks. Autoclave 15 minutes at 121°C. Final pH, 6.8 ± 0.2.

M 50. NUTRIENT GELATIN

Beef Extract	3.0 g
Peptone	5.0 g
Gelatin	120.0 g
Distilled water	1.0 L

Warm to 50°C to dissolve completely. Dispense into tubes and autoclave at 121°C for 15 minutes. Cool promptly in cold running water.

M 51. O/F (OXIDATIVE/FERMENTATIVE) MEDIUM, WITH 1% GLUCOSE

Tryptone	2.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	0.3 g
Brom Thymol Blue	0.08 g
Agar	2.0 g
Distilled water	1.0 L

Suspend above ingredients in distilled water and heat to boiling to dissolve completely. Distribute in 100 ml amounts and autoclave for 15 minutes at 121°C. To 100 ml sterile medium, aseptically add 10 ml of a sterile 10% glucose solution and mix thoroughly. Aseptically dispense in 5 ml amounts into sterile 13 x 100 mm culture tubes. Final pH 6.8 ± 0.2 at 25°C.

M 52. PHENOL RED CARBOHYDRATE FERMENTATION BROTHS

Proteose Peptone No. 3	10.0 g
Beef Extract	1.0 g
Sodium Chloride	5.0 g
Phenol Red	0.018 g
Distilled water	1.0 L

Suspend the above ingredients in distilled water, add 5 to 10 g (0.5 - 1.0%) of the individually desired carbohydrate, such as rhamnose, xylose, mannitol or others, per liter of base medium and mix to dissolve completely. Dispense into tubes containing inverted fermentation vials and autoclave at 121°C for 15 minutes. If preferred, the base may be prepared and autoclaved

without added carbohydrates. Filter sterilized carbohydrate solutions may then be added aseptically after the medium has cooled. The addition of some carbohydrates may result in an acid reaction. In this case use sterile 0.1 N NaOH added dropwise to restore the original color, taking care not to obtain a too deep red or cerise color. Final pH 7.4 ± 0.2 at 25°C.

M 53. PHENOL RED SORBITOL AGAR WITH MUG (PRS-MUG)

Phenol Red broth base (DIFCO Cat. # 0092-02-7)	16.0 g
D-Sorbitol	5.0 g
MUG (methyl umbelliferyl β-D-glucuronide; Biosynth International, Skokie, Ill.)	0.05 g
Agar	20.0 g
Distilled water	1.0 L

Dissolve the first three ingredients in distilled water. Adjust pH to 7.4 (final pH of 6.8 to 6.9 after autoclaving). Add agar. Autoclave at 121°C for 15 minutes. Temper and pour into 100 x 15 mm petri dishes (40 ml/plate to make deep dishes). Leave at room temperature overnight to dry.

M 54. PHENOL RED TARTRATE AGAR (JORDAN AND HARMON)

Peptone	10.0 g
Sodium Potassium Tartrate	10.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Phenol Red	0.024 g
Distilled water	1.0 L

Dissolve with gentle heat. Dispense 4.5 ml in 13 x 100 mm tubes. Autoclave for 15 minutes at 121°C. Cool tubes promptly in upright position.

M 55. PHENYLALANINE AGAR (EWING)

Yeast Extract	3.0 g
Dipotassium Phosphate	1.0 g
Sodium Chloride	5.0 g
l-phenylalanine	1.0 g
or	
d, l-phenylalanine	2.0 g

Agar	12.0 g
Distilled water	1.0 L

Boil to dissolve, dispense 4 ml/tube in 13 x 100 mm screw-capped tubes, and sterilize at 121°C for 15 minutes. Allow to solidify as a slant.

M 56. PLATE COUNT AGAR (STANDARD METHODS AGAR)

Pancreatic digest of casein USP	5.0 g
Yeast Extract	2.5 g
Dextrose	1.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend ingredients in distilled water. Heat to boiling until all ingredients are dissolved. Sterilize at 121°C for 15 minutes. Final pH 7.0 ± 0.1.

M 57. PURPLE BROTH WITH CARBOHYDRATES

Proteose Peptone No. 3	10.0 g
Beef Extract	1.0 g
Sodium Chloride	5.0 g
Brom Cresol Purple	0.015 g
Distilled water	1.0 L

Suspend the above ingredients in distilled water, add 5 to 10 g (0.5 - 1.0%) of the individually desired carbohydrate, such as salicin, xylose, sucrose, trehalose, rhamnose or others, per liter of base medium and mix to dissolve completely. Dispense into tubes as desired and autoclave at 121°C for 15 minutes. Alternatively, the base may be prepared using 900 ml distilled water and autoclaved without added carbohydrates. A 100 ml aliquot of a filter sterilized 5 - 10% carbohydrate solution may then be added aseptically after the base medium has been cooled to 45 - 50°C. The addition of some carbohydrates may result in an acid reaction. In this case use sterile 0.1 N NaOH added dropwise to restore the proper pH. Final pH 6.8 ± 0.2 at 25°C.

M 58. RAPPAPORT VASSILIADIS (RV) BROTH

Peptone from casein	4.0 g
Peptone from soymeal	1.0 g
Magnesium Chloride Hexahydrate	29.0 g

Sodium Chloride	8.0	g
Dipotassium Phosphate	0.4	g
Monopotassium Phosphate	0.6	g
Malachite Green	0.036	g
Distilled water	1.0	L

Dissolve ingredients in distilled water. Heat gently if necessary. Autoclave for 15 minutes at 115°C (12 lbs.). DO NOT OVERHEAT. NOTE: Use dehydrated prepared medium available commercially as Merck cat. # 7700 (distributed by GENE-TRAK Systems Corp., Framingham, MA) or equivalent.

M 59. SELENITE BRILLIANT GREEN SULFA (SBGS) BROTH

Yeast Extract	5.0	g
Peptone	5.0	g
D-mannitol	5.0	g
Sodium Taurocholate	1.0	g
Sodium Sulfapyridine	0.5	g
Sodium Selenite	4.0	g
Dipotassium Phosphate	2.65	g
Monopotassium Phosphate	1.02	g
Brilliant Green	0.005	g
Distilled water	1.0	L

Suspend dry ingredients in water and heat to boiling to dissolve completely. Sterilize by boiling for 5-10 minutes. DO NOT AUTOCLAVE. Dispense into sterile tubes and allow to cool. Final pH 7.2 ± 0.2.

M 60. SEMISOLID BRUCELLA GLUCOSE MEDIUM (Holdeman et al., 1977)

Pancreatic digest of casein	15.0	g
Peptic digest of animal tissue	5.0	g
Dextrose	1.0	g
Yeast Extract	2.0	g
Sodium Chloride	5.0	g
Sodium Bisulfite	0.1	g
Agar	1.6	g
Glucose	10.0	g
Phenol Red	0.02	g
Distilled water	1.0	L

Brucella broth (Albimi; dehydrated; BBL), 28.0 g, may be substituted for the first six ingredients above. Suspend all ingredients except phenol red and agar in distilled water, and adjust pH to 7.4 with 8 N NaOH. Add the agar, heat with frequent agitation, and boil for 1 minute to dissolve completely. Cool to 55°C and add 2.5 ml of phenol red stock solution (0.08 g/10 ml of 0.1 N NaOH). Readjust pH to 7.4 if necessary, dispense 10-ml aliquots into 16 x 125 mm screw-capped tubes, and autoclave at 121°C for 10 minutes. Final pH 7.0 ± 0.2.

M 61. SIMMONS CITRATE AGAR

Magnesium Sulfate	0.2 g
Monoammonium Phosphate	1.0 g
Dipotassium Phosphate	1.0 g
Sodium Citrate	2.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Bromthymol Blue	0.08 g
Distilled water	1.0 L

Heat to dissolve. Dispense 4 ml/tube into 13 x 100 mm screw-capped test tubes. Autoclave at 121°C for 15 minutes. Slant. Streak slants from a colony or culture without introducing a carbon source with the inoculum.

M 62. SOB + A MEDIUM

Bacto-tryptone	20.0 g
Bacto-yeast extract	5.0 g
NaCl	0.5 g
Bacto-agar (For SOB agar only)	15.0 g
Deionized water	950.0 mL

Shake and mix until all solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of deionized water.) Adjust the pH to 7.0 with 5 N NaOH (about 0.2 ml). Adjust the volume of the solution to 1 liter with deionized water. Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle.

To the autoclaved and tempered medium, add 5 ml of a sterile solution of 2 M $MgCl_2$, 10 ml of a sterile solution of 2M $MgSO_4$, and a filter sterilized solution of ampicillin (sodium salt) to give a final concentration of 100 µg/ml.

2 M $MgCl_2$: Dissolve 19 g of $MgCl_2$ in 90 ml deionized water. Adjust the volume of the solution to 100 ml with deionized water. Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle.

2 M $MgSO_4$: Dissolve 24.1 g $MgSO_4$ in 90 ml deionized water. Adjust the volume of the solution to 100 ml with deionized water. Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle.

M 63. SPRAY'S FERMENTATION MEDIUM (for *C. perfringens)*

Trypticase	10.0 g
Neopeptone	10.0 g
Agar	2.0 g
Sodium Thioglycollate	0.25 g
Distilled water	1.0 L

Dissolve all ingredients except the agar in distilled water and adjust the pH to 7.4. Add the agar and heat while stirring until the agar is dissolved. Mix well and dispense 9.0 ml portions into 16 x 125 mm tubes. Autoclave for 15 minutes at 121°C. Before use, heat the tubed medium in boiling water or flowing steam for 10 minutes, and add 1.0 ml of 10% sterile carbohydrate solution (salicin or raffinose) to each tube.

M 64. STARCH-AMPICILLIN (SA) AGAR (PALUMBO et al, 1985)

Phenol Red agar base (Difco 0098-01-2)	31.0 g
Soluble Starch (Difco 0178-15-9)	10.0 g
Distilled water	1.0 L
Ampicillin	10.0 mg

Heat all ingredients except ampicillin to boiling to dissolve completely. Sterilize at 121°C for 15 minutes. Temper the sterile medium to 50°C and add 1 ml of sterile sodium ampicillin stock solution per liter of medium (prepare stock solution as described below for TSBA, M 72). Pour into sterile 15 x 100 mm petri dishes and allow to harden.

M 65. STRONG'S SPORULATING MEDIUM (MODIFIED)

Proteose Peptone	15.0 g
$Na_2HPO_4.7H_2O$	10.0 g
Raffinose	4.0 g
Yeast Extract	4.0 g
Sodium Thioglycollate	1.0 g

Distilled water	1.0 L

Dissolve ingredients in distilled water and bring volume to 1.0 L. Mix thoroughly , gently heat and bring to a boil. Dispense in 17 ml amounts in 20 x 150 mm screw-capped tubes or 4 ml amounts in 13 x 100 mm screw-capped tubes. Sterilize at 121°C for 15 minutes. Adjust pH to 7.8 with filter sterilized 0.66 M Na_2CO_3. Pore into sterile Petri dishes or leave in tubes. Final pH 7.8 ± 0.2 at 25°C. Store at room temperature.

M 66. TOLUIDINE BLUE DNA AGAR

Agar	10.0	g
Sodium Chloride	10.0	g
Tris(hydroxymethyl)aminomethane buffer	6.1	g
Deoxyribonucleic acid	0.3	g
Toluidine Blue O	0.083	g
$CaCl_2$, anhydrous	1.1	mg
Distilled water	1.0	L

Add tris(hydroxymethyl)aminomethane buffer to distilled water and bring volume to 1.0 L. Mix thoroughly and adjust pH to 9.0. Add the remaining components, except for the Toluidine Blue O, and mix thoroughly. Gently heat and bring to a boil. Add the Toluidine Blue O and mix thoroughly. If used the same day, sterilization is not necessary. Cool to 50°C and pour into sterile Petri dishes or distribute into tubes. Final pH 9.0 ± 0.2 at 25°C.

M 67. TRIPLE SUGAR IRON (TSI) AGAR

Beef Extract	3.0	g
Yeast Extract	3.0	g
Peptone	15.0	g
Proteose Peptone	5.0	g
Lactose	10.0	g
Sucrose	10.0	g
Dextrose	1.0	g
Ferrous Sulfate	0.2	g
Sodium Chloride	5.0	g
Sodium Thiosulfate	0.3	g
Agar	12.0	g
Phenol Red	0.024	g
Distilled water	1.0	L

Heat to dissolve. Dispense 4 ml/tube into 13 x 100 mm tubes. Sterilize at 121°C for 15 minutes. Slant tubes for generous butt.

M 68. TRYPTICASE PEPTONE GLUCOSE YEAST EXTRACT BROTH (BUFFERED)

Pancreatic digest of casein	50.0 g
Peptone	5.0 g
Yeast Extract	20.0 g
Glucose	4.0 g
Disodium Phosphate	5.0 g
Sodium Thioglycollate	1.0 g
Distilled water	1.0 L

Dissolve the ingredients in distilled water, adjust the pH to 7.3, and dispense 15 ml into 20 x 150 mm culture tubes. Sterilize the dispensed medium by autoclaving at 121°C for 8 minutes (15 minutes for larger volumes), and refrigerate until used.

M 69. TRYPTICASE SOY AGAR (TS BLOOD AGAR)

Trypticase	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend ingredients in water and heat to boiling with stirring. Sterilize at 121°C for 15 minutes. If desired, cool to 50°C, add 5% sterile, defibrinated, sheep blood and swirl. Avoid bubble formation. Pour 15 ml quantities into sterile 100 x 15 mm Petri dishes and allow to harden.

M 70. TRYPTICASE SOY AGAR-YEAST EXTRACT (TSA-YE)

Trypticase	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Yeast Extract	6.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend the above ingredients in distilled water and dissolve completely by heating to boiling while stirring.

Autoclave for 15 minutes at 121°C. Temper the medium to 45 - 50°C and pour into sterile Petri dishes.

M 71. TRYPTICASE SOY BROTH

Trypticase	17.0 g
Phytone	3.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1.0 L

Dispense into tubes and sterilize at 121°C for 15 minutes.

M 72. TRYPTIC SOY BROTH WITH AMPICILLIN (TSBA)

Prepare Tryptic soy broth (Difco 0370-01-1) according to the manufacturer's instructions. Cool the sterilized broth to at least 50°C and add 1 ml of sterile sodium ampicillin stock solution per liter of medium. The final concentration of ampicillin is 10 μg/ml.

NOTE: Prepare stock solution by dissolving 1.06 g sodium ampicillin (Sigma; A-9518) in 100 ml distilled water. Sterilize by filtration through a 0.2 μm filter.

M 73. TRYPTICASE SOY BROTH (TSB) WITH 10% SODIUM CHLORIDE AND 1% SODIUM PYRUVATE (PTSBS)

Sodium Chloride	100.0 g
Trypticase (Pancreatic Digest of Casein)	17.0 g
Phytone (Papaic Digest of Soya Meal)	3.0 g
K_2HPO_4	2.5 g
Dextrose	2.5 g
Sodium Pyruvate	10.0 g
Distilled water	1.0 L

To make from commercial TSB, add 95 g of NaCl to 30 g of dry ingredients, and dissolve in 1.0 L distilled water. Dispense into tubes. Sterilize at 121°C for 15 minutes. Final pH 7.3 $\pm$ 0.1.

NOTE: Dehydrated complete medium not available commercially.

M 74. TRYPTONE BROTH

Tryptone or Trypticase	10.0 g
Distilled water	1.0 L

Dispense into tubes and sterilize at 121°C for 15 minutes.

M 75. TRYPTOSE BROTH

Tryptose	20.0 g
Sodium Chloride	5.0 g
Dextrose	1.0 g
Thiamine Hydrochloride	0.005 g
Distilled water	1.0 L

Suspend ingredients in water and heat to boiling with stirring. Sterilize at 121°C for 15 minutes.

M 76. TRYPTOSE SULFITE CYCLOSERINE (TSC) AGAR

Tryptose	15.0 g
Agar	14.0 g
Beef Extract	5.0 g
Pancreatic digest of soybean meal	5.0 g
Yeast Extract	5.0 g
Ferric Ammonium Citrate	1.0 g
$Na_2S_2O_5$	1.0 g
Egg Yolk Emulsion	50.0 ml
Cycloserine Solution	10.0 ml
Distilled water	940.0 ml

Egg Yolk Emulsion:

Chicken egg yolks	11
Whole chicken egg	1

Soak whole eggs with 1:100 dilution of saturated mercuric chloride solution for 1 minute. Crack eggs and separate yolks from whites. Mix egg yolks with one chicken egg.

Cycloserine Solution:

D-Cycloserine	0.4 g
Distilled water	10.0 ml

Add cycloserine to distilled water, bring volume up to 10.0 ml, mix thoroughly and filter sterilize through a 0.2 µm filter.

To prepare this medium, add the above components, except for the egg yolk emulsion and the cycloserine solution, to distilled water and bring volume up to 940.0 ml. Mix thoroughly. Gently heat and bring to boiling. Autoclave for 15 minutes at 121°C. Cool to 45 - 50°C and aseptically add 50 ml of the prepared egg yolk emulsion and the sterile 10 ml cycloserine solution. Mix thoroughly and pour into sterile Petri dishes. Final pH 7.6 ± 0.2 at 25°C.

See M 25 for preparation of Egg Yolk Free Tryptose Sulfite Cycloserine Agar (EY-free TSC).

M 77. TT BROTH (HAJNA AND DAMON, 1956)

Yeast Extract	2.0 g
Tryptose	18.0 g
Glucose	0.5 g
d-Mannitol	2.5 g
Sodium Desoxycholate	0.5 g
Sodium Chloride	5.0 g
Sodium Thiosulfate	38.0 g
Calcium Carbonate	25.0 g
Brilliant Green	0.01 g
Distilled water	1.0 L

Dissolve and heat to boiling. DO NOT AUTOCLAVE. Cool below 50°C. Add 40 ml iodine solution. (Dissolve 8 g potassium iodide in 20 ml distilled water. Add 5 g iodine crystals and shake to dissolve. Add distilled water to volume of 40 ml). Shake to mix. Do not heat after the addition of iodine. Dispense into sterile containers and use the day it is prepared. The basal medium without the iodine may be stored indefinitely.

M 78. UREA AGAR (CHRISTENSEN)

Agar	15.0 g
Distilled water	900.0 ml

Autoclave and cool to 50°C. Add filter-sterilized urea base. Mix and distribute into sterile tubes.

Base	
Urea	20.0 g
Peptone	1.0 g
Sodium Chloride	5.0 g
Glucose	1.0 g
Monobasic Potassium Phosphate	2.0 g
Phenol Red (1:500 solution)	6.0 ml
Distilled water	100.0 ml

Adjust to pH 6.8. Sterilize by filtration.

M 79. XLT4 AGAR

XL Agar Base (Difco #0555-01-8)	47.0 g
Bacto Agar (Difco #0140-01-0)	3.0 g
Ferric Ammonium Citrate	0.8 g
Sodium Thiosulfate (Anhydrous)	6.8 g
Proteose Peptone #3 (Difco #0122-01-2)	1.2 g
Tergitol 4 (Sigma Chemical Co., #T-8256)	4.6 ml
Distilled or deionized water	1.0 L

a. Dissolve Tergitol 4 in distilled or deionized water in a 2 L or larger Erlenmeyer flask and mix with a magnetic stir-bar.
b. Add other ingredients, mix well using stir-bar and bring to a boil while mixing slowly with stir-bar.
c. Cool to 45 - 50°C in a water bath and mix again gently.
d. Pour plates fairly thick (about 5 mm deep). The plates may appear dark at first but should lighten up after cooling overnight.
Allow plates to remain at room temperature overnight to dry, then refrigerate (in plastic bags or containers) at 3-8°C.
e. Remove plates from the refrigerator 24 h prior to use for further drying.
f. pH of XLT4 plates = 7.5 $\pm$ 0.2 (usually no adjustment is necessary).

NOTE: Poured XLT4 plates have a shelf-life of at least 3 months when stored refrigerated in closed plastic bag or other container.

Neither XLD agar nor Tergitol 7 can be used in place of plain XL agar base or Tergitol 4, respectively.

† **Safety Caution:** **KCN is a deadly poison. Avoid inhalation of KCN vapors at all times by preparing media in a chemical fume hood. After autoclaving old inoculated KCN broth tubes, be sure an adequate, external exhaust system is turned on before opening the autoclave door and do not allow body contact with any vapors from the autoclave. Wear appropriate safety gloves.**

Consult a Material Safety Data Sheet (MSDS) before working with KCN.

Do not dispose of hazardous fluids such as sodium azide by pouring down sink drains. Accumulation of sodium azide in lead drains may result in an explosion.

Collect liquid KCN and sodium azide wastes in separate containers and dispose of in accordance with the standard chemical waste management procedures for your laboratory.

II. REAGENTS

R 1. ANDRADE'S INDICATOR (EWING)

Acid fuchsin	0.2 g
Distilled water	100.0 ml
Sodium hydroxide (1.0 N)	16.0 ml

The fuchsin is dissolved in the distilled water, and the sodium hydroxide is added. If, after several hours, the fuchsin is not sufficiently decolorized to a golden color, add an additional 1 or 2 ml of alkali. Sterilize by filtration. The dye content of different samples of acid fuchsin varies quite widely, and the amount of alkali that should be used with any particular sample usually is specified on the label. The reagent improves somewhat on aging and should be prepared in sufficiently large amounts to last for several years. The indicator is used in the amount of 10 ml per liter of medium.

R 2. BUFFERED GLYCEROL SALT SOLUTION

Glycerol (glycerin)	100.0 ml
Dipotassium Phosphate (anhydrous)	12.4 g
Monopotassium Phosphate (anhydrous)	4.0 g
Sodium Chloride	4.2 g
Distilled water	900.0 ml

Dissolve the sodium chloride in part of the water, and make up to 900.0 ml. Add the glycerol and phosphates, and adjust the pH to 7.2. Autoclave for 15 minutes at 121°C. For double strength (20%) glycerol solution, use 200 ml of glycerol and 800.0 ml of distilled water.

R 3. BUTTERFIELD'S PHOSPHATE DILUENT

a. Stock solution:

Dissolve 34 g KH_2P0_4 in 500 ml distilled water, adjust to pH 7.2 with ca. 175 ml 1 N NaOH, and dilute to 1 liter. Store under refrigeration.

b. Diluent:

Dilute 1.25 ml stock solution (a) to 1 liter with distilled water. Readjust pH to 7.2 if necessary. Prepare dilution blanks using this solution,

dispensing a sufficient quantity to allow for losses due to sterilization by autoclaving (121°C for 15 minutes).

R 4. ENDOSPORE STAIN

a. Solution A

Dissolve 5.0 g of Malachite green in 100 ml distilled water. Filter to remove undissolved dyes.

b. Solution B

Dissolve 0.5 g Safranin O in 100 ml of distilled water.

R 5. GRAM STAIN (HUCKER MODIFICATION)

a. Crystal violet solution:

Crystal Violet (90% dye)	2.0 g
Ethanol (95%)	20.0 ml

b. Oxalate solution:

Ammonium Oxalate	0.8 g
Distilled water	80.0 ml

Working crystal violet solution

Mix the above two solutions together and store in a glass-stoppered bottle.

c. Gram's iodine solution:

Iodine crystals	1.0 g
Potassium Iodide	2.0 g
Distilled water	300.0 ml

Dissolve potassium iodide completely in 5 ml distilled water, dissolve the iodine crystals, then bring to volume with distilled water. Mix well and store in an amber glass bottle.

d. Decolorizer:

Ethanol, 95%	500.0 ml

Store in glass-stoppered bottle.

e. Stock safranin (Counterstain):

Safranin O (2.5% solution in 95% ethanol)	10.0 ml
Distilled water	100.0 ml

Mix well and store in a glass-stoppered bottle.

R 6. OXIDASE REAGENT

Tetramethyl-p-phenylenediamine dihydrochloride	1.0 g
Distilled water	100.0 ml

Prepare fresh daily or refrigerate for not longer than 1 week. Alternatively, use commercial oxidase reagents.

R 7. KOVAC'S REAGENT (EWING)

Pure Amyl or Isoamyl Alcohol	150.0 ml
Paradimethylaminobenzaldehyde	10.0 g
Concentrated HCl	50.0 ml

Dissolve aldehyde in alcohol and slowly add acid. The dry aldehyde should be light in color. Prepare reagent in small quantities. Store in refrigerator.

R 8. METHYL RED REAGENT (EWING)

Methyl Red	0.1 g
Ethyl Alcohol (95-96%)	300.0 ml

Dissolve dye in the alcohol and then add distilled water to make 500 ml. Use 5 or 6 drops per 5.0 ml of culture.

R 9. NITRATE REDUCTION REAGENTS

Method 1

Solution A:

Sulfanilic Acid	0.5 g
Glacial Acetic Acid	30.0 ml
Distilled water	120.0 ml

Solution B:

N (1-naphthyl) ethylenediamine dihydrochloride (*Marshal's Reagent)	0.2 g
Glacial Acetic Acid	30.0 ml
Distilled water	120.0 ml

*Cleve's acid (5-amino-2 naphthalene sulfonic acid) may be substituted for Marshal's Reagent.

R 10. PEPTONE WATER DILUENT (0.1%)

Peptone	1.0 g
Distilled water	1.0 L

Dissolve peptone in distilled water and adjust pH to 7.0 ± 0.1. Prepare dilution blanks with this solution, dispensing a sufficient quantity to allow for loss during autoclaving. Autoclave at 121°C for 15 minutes.

R 11. PHOSPHATE BUFFERED SALINE (PBS)

Anhydrous Na_2HPO_4	12.0 g
$NaH_2PO_4.H_2O$	2.2 g
NaCl	85.0 g

Dissolve dry ingredients in distilled water and bring volume to 1 L (10X PBS). Adjust pH to 7.4 with 0.1 N HCl or 0.1 N NaOH. To make 1X PBS, dilute 100 ml 10X PBS in 900 ml distilled water. Check and adjust pH (7.4) if necessary. Sterilize at 121°C for 15 minutes.

R 12. PHYSIOLOGICAL SALINE SOLUTION 0.85% (STERILE)

Sodium Chloride	8.5 g
Distilled water	1.0 L

Dissolve salt completely in distilled water and autoclave at 121°C for 15 minutes.

R 13. TRIS BUFFER (0.02 M, pH 7.75)

Trishydroxymethylaminomethane	7.5 g
Distilled water	3.0 L

Dissolve tris completely in distilled water and adjust pH to 8.5 with 20% HCl. Dispense into 150 ml portions and autoclave at 115°C for 15 minutes.

R 14. V-P REAGENT OF O'MEARA, MODIFIED (EWING)

Potassium Hydroxide	40.0 g
Creatine	0.3 g
Distilled water	100.0 ml

Dissolve alkali in water. Add creatine. Keep refrigerated. Make new reagent every 3 weeks. Use equal parts of reagent and culture. Aerate by shaking. Place test tube at 37°C. Read in 4 hours.

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APPENDIX VOL 1 & 2

MOST PROBABLE NUMBER TABLES

Richard P. Mageau

For the convenience of analysts using the procedures in this Guidebook, tables of Most Probable Numbers (MPN) are provided in this appendix.

MPN is a procedure to estimate the population density of viable microorganisms in a test sample. It is based upon the application of the theory of probability to the numbers of observed positive growth responses to a standard dilution series of sample inoculum placed into a set number of culture media tubes. Positive growth response after incubation may be indicated by such observations as gas production in fermentation tubes or visible cloudiness in broth tubes, depending upon the type of media employed. The sample should be diluted in such a manner that higher dilutions of the sample will result in fewer positive culture tubes in the series. The number of sample dilutions to be prepared is generally based on the expected population contained within the sample. If particularly high microbial populations are expected, the sample must be diluted to a range where the MPN can be obtained. Most reliable results occur when all tubes at the lower dilution are positive and all tubes at the higher dilution are negative. Generally tenfold serial dilutions are used in either a 3, 5 or 10 tube MPN series. When a higher number of tubes is inoculated in the series, the confidence limits of the MPN are narrowed. For particularly high microbial populations, the MPN value obtained is generally considered to be not as precise as population numbers derived from direct plating methods; however, it should be emphasized that MPN values are only estimates while plate counts are direct counts of living organisms expressed in cfu/ml. MPN values are, however, particularly useful when low concentrations of organisms (<100/g) are encountered in such materials as milk, food, water and soil where particulate matter of the matrix may interfere with obtaining accurate colony counts.

In application of probability theory to the determination of MPN values, it should be kept in mind that the following assumptions are generally considered to be accepted: (a) the organisms are randomly and evenly distributed throughout the sample, (b) the organisms exist as single entities, not as chains, pairs or clusters and they do not repel one another, (c) the proper growth medium, temperature and incubation conditions have been selected to allow even a single viable cell in an inoculum to produce

detectable growth and (d) the population does not contain viable, sub-lethally injured organisms that are incapable of growth in the culture medium used.

The following 4 tables present MPN values and corresponding 95% confidence limits for a 3 tube test series using 4 different, commonly used sets of inoculum quantities that should be particularly useful relative to performing the microbiological analyses previously described in various chapters of this Guidebook. These MPN tables may be considered to be abbreviated since not all possible combinations of positive and negative tubes within a series are presented. Those combinations that occur often enough to have statistical significance are included, while those that are improbable have been omitted. If laboratory analyses produce combinations that are not included in the tables, then one should repeat the test on another portion of the original sample (assuming the microbiological integrity of the sample has not been compromised) as a possible performance error or contamination is indicated. If this is not possible and an MPN is imperative, then more complete tables should be consulted from other reference sources or the MPN can be calculated by equation (other reference sources) on the basis of the observed results.

On occasions when more than three dilutions of a sample are used in a decimal series of dilutions of a 3-tube MPN determination, the following guidelines should be followed. Results from only three consecutive dilutions are used to determine the MPN. If one or more dilutions have all tubes positive, select the highest dilution (smallest sample quantity) with positive results in all tubes and the next two higher dilutions, as shown in examples a and b below. When none of the dilutions yield all tubes positive, select the three lowest dilutions for which the middle dilution contains the positive result, as shown in example c below. If a positive result occurs in a higher unselected dilution, add the number of positive tubes in this dilution to the results of the highest dilution of the three selected, as shown in example d below. When all dilutions tested yield all tubes positive, select the three highest dilutions (example e below). For additional information on MPN estimations, consult the APHA's Compendium of Methods for the Microbiological Examination of Foods (3rd edition, 1992, chapter 6).

Example	Sample quantities (g or ml)[a] 10	1	0.1	0.01	0.001	Reported positive values	MPN estimate/ g or ml
a	3/3[b]	3/3	2/3	0/3	0/3	3-2-0	9.33
b	3/3	3/3	3/3	2/3	0/3	3-2-0	93.3
c	0/3	0/3	1/3	0/3	0/3	0-1-0	0.31
d	3/3	3/3	2/3	1/3	1/3	3-2-2	21.5
e	3/3	3/3	3/3	3/3	3/3	3-3-3	>1100

[a] The analyst should make sure that ALL sample dilution factors (including the preparation of any sample homogenate) are correctly applied in calculating the actual sample quantities subjected to MPN analysis.

[b] $\frac{\text{Numerator}}{\text{Denominator}} = \frac{\text{No. positive tubes}}{\text{No. tubes inoculated}}$

The following tables were produced from information and data abstracted from FDA's Bacteriological Analytical Manual (BAM), Appendix 2, 8th Edition, 1995 and also Appendix 2 of the BAM, 7th Edition, 1992.

Table 1. MPN Index and 95% Confidence Limits for Various Combinations of Positive Tubes Most Likely to Occur in a 3 Tube per Dilution Series Using Inoculum Quantities of 10, 1 and 0.1 g (ml).

Combination of Positives (10-1-0.1 g)	MPN Index per g (ml)	95% Confidence Limits	
		Lower	Upper
0-0-0	0.000	0.000	0.095
0-0-1	0.030	0.002	0.096
0-1-0	0.031	0.002	0.107
0-1-1	0.061	0.012	0.181
0-2-0	0.062	0.012	0.181
1-0-0	0.036	0.002	0.181
1-0-1	0.072	0.013	0.182
1-1-0	0.074	0.013	0.203
1-1-1	0.112	0.036	0.380
1-2-0	0.114	0.036	0.420
1-2-1	0.154	0.045	0.420
2-0-0	0.092	0.014	0.375
2-0-1	0.143	0.036	0.420
2-1-0	0.147	0.037	0.420
2-1-1	0.205	0.045	0.420
2-2-0	0.211	0.045	0.425
2-2-1	0.276	0.087	0.945
2-2-2	0.348	0.087	0.945
2-3-0	0.286	0.087	0.945
2-3-1	0.360	0.087	0.945
3-0-0	0.231	0.046	0.945
3-0-1	0.385	0.087	1.050
3-0-2	0.636	0.168	1.830
3-1-0	0.427	0.090	1.830
3-1-1	0.749	0.169	2.000
3-1-2	1.150	0.370	4.250
3-2-0	0.933	0.181	4.250
3-2-1	1.490	0.370	4.250
3-2-2	2.150	0.400	4.270
3-3-0	2.400	0.420	10.000
3-3-1	4.620	0.900	20.000
3-3-2	11.000	1.800	41.000
3-3-3	>11.000	4.250	-

Table 2. MPN Index and 95% Confidence Limits for Various Combinations of Positive Tubes Most Likely to Occur in a 3 Tube per Dilution Series Using Inoculum Quantities of 1, 0.1 and 0.01 g (ml).

Combination of Positives (1-0.1-0.01 g)	MPN Index per g (ml)	95% Confidence Limits	
		Lower	Upper
0-0-0	0.00	0.00	0.95
0-0-1	0.30	0.02	0.96
0-1-0	0.31	0.02	1.07
0-1-1	0.61	0.12	1.81
0-2-0	0.62	0.12	1.81
1-0-0	0.36	0.02	1.81
1-0-1	0.72	0.13	1.82
1-1-0	0.74	0.13	2.03
1-1-1	1.12	0.36	3.80
1-2-0	1.14	0.36	4.20
1-2-1	1.54	0.45	4.20
2-0-0	0.92	0.14	3.75
2-0-1	1.43	0.36	4.20
2-1-0	1.47	0.37	4.20
2-1-1	2.05	0.45	4.20
2-2-0	2.11	0.45	4.25
2-2-1	2.76	0.87	9.45
2-2-2	3.48	0.87	9.45
2-3-0	2.86	0.87	9.45
2-3-1	3.60	0.87	9.45
3-0-0	2.31	0.46	9.45
3-0-1	3.85	0.87	10.50
3-0-2	6.36	1.68	18.30
3-1-0	4.27	0.90	18.30
3-1-1	7.49	1.69	20.00
3-1-2	11.50	3.70	42.50
3-2-0	9.33	1.81	42.50
3-2-1	14.90	3.70	42.50
3-2-2	21.50	4.00	42.70
3-3-0	24.00	4.20	100.00
3-3-1	46.20	9.00	200.00
3-3-2	110.00	18.00	410.00
3-3-3	>110.00	42.50	-

Table 3. MPN Index and 95% Confidence Limits for Various Combinations of Positive Tubes Most Likely to Occur in a 3 Tube per Dilution Series Using Inoculum Quantities of 0.1, 0.01 and 0.001 g (ml).

Combination of Positives (0.1-0.01-0.001 g)	MPN Index per g (ml)	95% Confidence Limits	
		Lower	Upper
0-0-0	0.0	0.0	9.5
0-0-1	3.0	0.2	9.6
0-1-0	3.1	0.2	10.7
0-1-1	6.1	1.2	18.1
0-2-0	6.2	1.2	18.1
1-0-0	3.6	0.2	18.1
1-0-1	7.2	1.3	18.2
1-1-0	7.4	1.3	20.3
1-1-1	11.2	3.6	38.0
1-2-0	11.4	3.6	42.0
1-2-1	15.4	4.5	42.0
2-0-0	9.2	1.4	37.5
2-0-1	14.3	3.6	42.0
2-1-0	14.7	3.7	42.0
2-1-1	20.5	4.5	42.0
2-2-0	21.1	4.5	42.5
2-2-1	27.6	8.7	94.5
2-2-2	34.8	8.7	94.5
2-3-0	28.6	8.7	94.5
2-3-1	36.0	8.7	94.5
3-0-0	23.1	4.6	94.5
3-0-1	38.5	8.7	105.0
3-0-2	63.6	16.8	183.0
3-1-0	42.7	9.0	183.0
3-1-1	74.9	16.9	200.0
3-1-2	115.0	37.0	425.0
3-2-0	93.3	18.1	425.0
3-2-1	149.0	37.0	425.0
3-2-2	215.0	40.0	427.0
3-3-0	240.0	42.0	1000.0
3-3-1	462.0	90.0	2000.0
3-3-2	1100.0	180.0	4100.0
3-3-3	>1100.0	425.0	-

Table 4. MPN Index and 95% Confidence Limits for Various Combinations of Positive Tubes Most Likely to Occur in a 3 Tube per Dilution Series Using Inoculum Quantities of 0.01, 0.001 and 0.0001 g (ml).

Combination of Positives (0.01-0.001-0.0001 g)	MPN Index per g (ml)	95% Confidence Limits	
		Lower	Upper
0-0-0	0.0	0.0	95.0
0-0-1	30.0	2.0	96.0
0-1-0	31.0	2.0	107.0
0-1-1	61.0	12.0	181.0
0-2-0	62.0	12.0	181.0
1-0-0	36.0	2.0	181.0
1-0-1	72.0	13.0	182.0
1-1-0	74.0	13.0	203.0
1-1-1	112.0	36.0	380.0
1-2-0	114.0	36.0	420.0
1-2-1	154.0	45.0	420.0
2-0-0	92.0	14.0	375.0
2-0-1	143.0	36.0	420.0
2-1-0	147.0	37.0	420.0
2-1-1	205.0	45.0	420.0
2-2-0	211.0	45.0	425.0
2-2-1	276.0	87.0	945.0
2-2-2	348.0	87.0	945.0
2-3-0	286.0	87.0	945.0
2-3-1	360.0	87.0	945.0
3-0-0	231.0	46.0	945.0
3-0-1	385.0	87.0	1050.0
3-0-2	636.0	168.0	1830.0
3-1-0	427.0	90.0	1830.0
3-1-1	749.0	169.0	2000.0
3-1-2	1150.0	370.0	4250.0
3-2-0	933.0	181.0	4250.0
3-2-1	1490.0	370.0	4250.0
3-2-2	2150.0	400.0	4270.0
3-3-0	2400.0	420.0	10000.0
3-3-1	4620.0	900.0	20000.0
3-3-2	11000.0	1800.0	41000.0
3-3-3	>11000.0	4250.0	-