



Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG Appendix 1.02

Title: Media and Reagents

Effective Date: 7/3/03

Description and purpose of change(s):

The Microbiology Laboratory Guidebook method chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory document control system. Obsolete media have been removed. Media used in new methods have been added. Wording for heating and dissolving of media during preparation has been changed. Required final pH testing has been added. Safety Precautions are also now included in the revised chapters.

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APP 1 Specific Procedure(s)

APP 1.1 Introduction

- All media and reagents necessary for each analysis are listed in each chapter. The formulations and procedures for preparing the special media and reagents used throughout this Guidebook are presented in alphabetical order in this appendix.
- 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 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.
- The ingredients and the chemicals used for preparing media and reagents may be the product of any manufacturer if comparative tests show satisfactory results. For convenience, dehydrated media of any brand equivalent to the formulation may be used unless instructions indicate otherwise. Pre-mixed, dehydrated media should be examined before use for indications of separation or deterioration. Each batch of medium should be tested for sterility and growth promotion/inhibition characteristics, as appropriate following the QC procedures described by the manufacturer.
- 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. For testing the pH of agar media, the use of an automatic temperature adjusting pH meter/probe and/or a surface-testing probe are recommended.
- Pre-cautions: All manufacturers' precautions should be followed. The personnel who handle the material should read the product's Material Safety Data Sheets. Chemicals with '†' are of particular concern.

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- Unless otherwise indicated, up to 1 liter of a medium should be sterilized by steam under pressure at 121°C (15-16 psi) for 15 minutes. Alternatively, media may be filter-sterilized.
- Any departures from standard media preparation practices/techniques (i.e. preparation volumes, sterilization/heating requirements, formulations, etc.) will require equivalency data to support the change(s). The laboratory will retain all records. Where the instructions say to dissolve by gently heating, the media shall be checked visually to determine that it is well dissolved. Do not over heat. This can be an essential step in obtaining the correct pH for the final medium.
- Depending on the type and quantity of media needed, tubed media may be either dispensed directly into tubes and sterilized by autoclaving or may be autoclaved in bulk and then aseptically dispensed into pre-sterilized tubes. Dilution tubes, or any tubes where the exact volume is critical, should only be dispensed after autoclaving.
- If commercial dehydrated medium is used, follow the manufacturer's instructions for specified pH, time and temperature of sterilization, etc.
- Microbiology Suitable (MS) water requirements.

Only water that has been treated to be free from traces of dissolved metal, bactericidal, and inhibitory compounds shall be used to prepare culture media, reagents, and dilution blanks. Inhibitor free water is referred to as microbiologically suitable (MS) water. The following tests are performed on the water source to ensure that the water is inhibitor free. Records of the following parameters shall be kept.

Weekly testing (or prior to use):

- >1.0 megohms-cm resistance at 25° C.

Monthly testing:

- Total Residual Chlorine shall be < 0.1 mg/l
- Aerobic Plate Count shall be < 1,000 colony forming unit (cfu) ml

Annual testing:

- Heavy Metals (Cd, Cr, Cu, Ni, Pb, and Zn-single) shall be < 0.05 mg/L
- Heavy Metals (total) shall be < 1.0 mg/L

The suitability of water for microbiological analyses shall pass the test for toxicity annually.

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APP 1.2 Preparation of media

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 ₂ O)	0.3 g
MS water	1.0 L

Suspend above ingredients. Heat and check visually to ensure that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes.

Final pH 6.6 ± 0.2 at 25°C.

ANTIBIOTIC MEDIUM #2

Bacto Peptone	6.0 g
Beef Extract	1.5 g
Yeast Extract	3.0 g
Agar	15.0 g
Dextrose solution, sterile (10g/100 ml)	10.0 ml
MS water	1.0 L

Combine all ingredients except the dextrose solution. Heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes. When cooled but still liquid (60-65°C), add sterile dextrose solution to a final concentration of 1 g/L. Commercially available powdered media may be used with the addition of the dextrose solution after autoclaving.

Final pH 6.6 ± 0.1 at 25°C.

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ANTIBIOTIC MEDIUM #5

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

Heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes.

Final pH 8.0 ± 0.1 at 25°C or as specified by the manufacturer if using commercial dehydrated medium.

ANTIBIOTIC MEDIUM #8

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

Heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes.

Final pH 5.8 ± 0.1 at 25°C or as specified by the manufacturer if using commercial dehydrated medium.

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ANTIBIOTIC MEDIUM #11 (NEOMYCIN ASSAY AGAR)

Gelsate™ Peptone or Bacto Peptone	6.0 g
Trypticase Peptone or Bacto Casitone*	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
MS water	1.0 L

*Pancreatic digest of casein

Heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes. Refrigerate.

Final pH 7.95 ± 0.05 at 25°C or as specified by the manufacturer if using commercial dehydrated medium. Adjust pH if necessary to achieve the correct final pH.

APT AGAR

Pancreatic digest of casein	12.5 g
Dextrose	10.0 g
Yeast Extract	7.5 g
Sodium Chloride	5.0 g
K ₂ HPO ₄	5.0 g
Sodium Citrate	5.0 g
Na ₂ CO ₃	1.25 g
MnCl ₂ ·4H ₂ O	0.14 g
MgSO ₄ ·7H ₂ O	0.8 g
Polysorbate 80	0.2 g
FeSO ₄ ·7H ₂ O	0.04 g
Thiamine Hydrochloride	1.0 mg
Agar	15.0 g
MS water	1.0 L

Add components to MS water, bring volume to 1.0 L, and mix thoroughly. Heat the mixture until visual examination shows that it is well dissolved. 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.

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BAIRD-PARKER MEDIUM

(a) 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 ₂ O	5.0 g
Agar	20.0 g
MS water	950.0 ml

Suspend ingredients in water. Heat the mixture until visual examination shows that it is well dissolved. Dispense portions in screw-capped bottles. Autoclave at 121°C for 15 minutes.

Final pH 7.0 ± 0.2 at 25°C.

Complete medium.

- a. Add 50 ml prewarmed (45-50°C) Bacto EY tellurite enrichment to 950 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 4 weeks 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.

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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 ₂ HP0 ₄	2.5 g
Dextrose	2.0 g
Agar	15.0 g
MS water	1.0 L

Dissolve ingredients in MS water. Heat the mixture until visual examination shows that it is well dissolved. Dispense as desired and autoclave at 121°C for 15 minutes.

Final pH 7.4 ± 0.2 at 25°C. .

BRAIN HEART INFUSION (BHI) BROTH

Prepare same as above except omit the 15.0 g agar.

Dispense and autoclave at 121°C for 15 minutes.

Final pH 7.4 ± 0.2 at 25°C.

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BRILLIANT GREEN SULFA AGAR (OSBORN AND STOKES)

Yeast Extract	3.0g
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
MS water	1.0 L

Mix thoroughly; adjust pH to 6.9 ± 0.2 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.

BROMCRESOL PURPLE (BCP) DEXTROSE BROTH

Peptone	10.0 g
Beef Extract (optional)	3.0 g
Sodium Chloride	5.0 g
Bromcresol Purple (0.16 g/ 10.0 ml of 95% ethanol).	2.0 ml
MS water	1.0 L

Combine the above ingredients with 5 g dextrose 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 6.9 ± 0.1 at 25°C .

NOTE: Dehydrated prepared medium not available commercially.

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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
MS 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 MS water. Heat the mixture until visual examination shows that it is well dissolved. 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.

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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
MS 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 MS water and autoclave at 121°C for 15 minutes. Cool the medium to room temperature and add filter-sterilized FBP solution. (Use Oxoid FBP supplements SR84 or the FBP solution, prepared as described under M 30). Aseptically dispense into screw-capped tubes.

Final pH 7.2 ± 0.2 at 25°C.

BUFFERED PEPTONE WATER

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

Dissolve dry ingredients in MS water, dispense into appropriate containers, and sterilize in the autoclave at 121°C for 15 minutes.

Final pH 7.2 ± 0.2 at 25°C.

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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
MS 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)

Dextrose, 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%. Dextrose, 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 MS 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.

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
MS water	1.0 L
Sodium Novobiocin	0.015 g

Suspend all ingredients except Sodium Novobiocin in 1.0 L MS water and heat to boiling using a hotplate or equivalent (or heat to 100 °C for 10 minutes). **DO NOT AUTOCLAVE.** Cool to 50°C and add Sodium Novobiocin from a filter-sterilized stock solution. Pour 15-20 ml/ plate. 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. Final pH 6.7 ± 0.2 at 25°C.

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E BUFFER

Bovine Albumin (Sigma # 7906 or equivalent)	0.5 g
Tween-20	50 µl
Buffered Peptone Water (BPW).	100 ml

Prepare by mixing Bovine Albumin and Tween-20 into Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8 °C.

Final pH 7.2 ± 0.2 at 25°C .

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
MS 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. Heat the mixture until visual examination shows that it is well dissolved. Autoclave at 121°C for 15 minutes. Cool to 50°C and add the blood.

Final pH 7.0 ± 0.2 at 25°C.

EY-FREE TRYPTOSE SULFITE CYCLOSERINE (TSC) AGAR

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

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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 ₂ PO ₄	1.35 g
Na ₂ HPO ₄	12.0 g
Esculin	1.0 g
Naladixic Acid † (2% in 0.1 M NaOH)	1.0 ml
Lithium Chloride	3.0 g
MS water	1.0 L

*Beef extract

Mix well to resuspend the media and dispense into 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 MS water to each 10 ml tube.

Final pH 7.2 ± 0.2 °C.

The following alternatives may be employed:

- a. Rather than dispense acriflavine solution into individual tubes, 25 mg/L of acriflavine HCL may be incorporated into the medium prior to autoclaving.
- b. FB may be prepared from commercially available modified UVM broth by adding appropriate amounts of lithium chloride, acriflavine and ferric ammonium citrate.

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HORSE BLOOD OVERLAY MEDIUM (HL)

a. Base Layer:

Columbia Blood Agar Base	1.0 L
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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, overlay with blood agar as described below.

b. 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 up to 2 weeks. Discard any plates which become discolored.

Final pH 7.2 ± 0.2 at 25°C.

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
MS water	950.0 ml

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

Final pH 7.5 ± 0.2 at 25 °C

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

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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 MS water in a 100 ml volumetric flask, bring to volume and filter sterilize. Dispense 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 MS water and add to the cooled medium. Add 2 vials for each liter of broth.

c. Antibiotics

Vancomycin Hydrochloride (Sigma)	10.0 mg
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Vancomycin Stock Solution

In a 100 ml volumetric flask, dissolve 0.25 g vancomycin in MS 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
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Trimethoprim Lactate Stock Solution

In a 100 ml volumetric flask, dissolve 0.3125 g trimethoprim lactate in MS 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.

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Cefoperazone Sodium (Sigma)	15.0 mg
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Cefoperazone Stock Solution

In a 100 ml volumetric flask, dissolve 0.375 g cefoperazone in MS 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
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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.

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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 ₂ CO ₃	0.636 g
Sodium Azide†	0.4 g
Phenol Red	0.018 g
MS water	990.0 ml

Stock 2,3,5-triphenyltetrazolium chloride solution:

Place 0.1 g 2,3,5-triphenyltetrazolinum chloride in MS 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 MS 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.

LYSINE IRON AGAR (EDWARDS AND FIFE)

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

Dispense into tubes and autoclave for 12 minutes at 121°C. Slant with deep butt and short slant.

Final pH 6.7 ± 0.2 at 25°C

MANNITOL YOLK POLYMYXIN (MYP) AGAR

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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
MS water	900.0 ml

Preparation B:

Egg yolk Enrichment 50%

Preparation C:

Polymyxin B Sulfate - Dissolve 500,000 units of sterile polymyxin B sulfate (Burroughs Wellcome Co., Research Triangle Park, NC) in 50.0 ml of sterile MS water. Filter sterilize the solution and store in the dark at 4°C. If the solution is prepared under sterile conditions, the filter sterilizing step may be omitted.

Mix the ingredients (Preparation A) in MS water. Heat the mixture until visual examination shows that it is well dissolved. Adjust the pH to 7.2 ± 0.1 , and dispense. Autoclave at 121°C for 20 minutes, cool to 50°C in a waterbath, and add 50 ml of Preparation B 10 ml of Preparation C . 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 30 days.

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MBROTH

M Broth is commercially available. The formula per liter is:

tryptone	12.5 g
yeast extract	5.0 g
D-mannose	2.0 g
sodium citrate	5.0 g
sodium chloride	5.0 g
dipotassium phosphate	5.0 g
manganese chloride	0.14 g
magnesium sulfate	0.8 g
ferrous sulfate	0.04 g
Tween 80 [®]	0.75 g

Dissolve ingredients in 1 liter distilled or deionized water. Heat the mixture until visual examination shows that it is well dissolved. Dispense into appropriate containers and autoclave at 121°C for 15 minutes.

Final pH 7.0 ± 0.2 at 25 °C.

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**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
MS water	1.0 L

*Formula: Lab Lemco Powder (Oxoid: powdered meat extract), 10.0 g;
Peptone, 10.0 g: sodium chloride, 5.0 g

Preparation of cefoperazone solution: Add 0.032 g sodium cefoperazone to MS water and bring volume to 10.0 ml. Mix well. Filter sterilize.

Preparation of medium: Add components, except cefoperazone solution to MS water and bring volume to 990 ml. Mix thoroughly. Heat the mixture until visual examination shows that it is well dissolved. Autoclave at 121°C for 15 minutes. Cool to 45 – 50 C. Aseptically add 10.0 ml sterile cefoperazone solution. Mix thoroughly. Dispense into Petri dishes or tubes.

CCDA (Campylobacter Blood-free Selective Agar) available from Oxoid (CM739) or Remel (Campylobacter Blood Free Selective Agar, 452722) may be used with the addition of the cefoperazone solution.

Dry the agar surfaces prior to streaking by placing the plates on a bench top (protected from light) overnight.

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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
MS water	1.0 L

Adjust to pH 6.8 ± 0.2 . 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. Note: b. maybe heated to dissolve starch.

Final pH 6.8 ± 0.2 at 25 °C.

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 ₂ HPO ₄	4.0 g
KH ₂ PO ₄	1.5 g
NaCl	5.0 g
MS 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).

Final pH 6.9 ± 0.1 at 25 °C.

MODIFIED OXFORD MEDIUM (MOX)

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MOX Agar Base:

Columbia Blood Agar Base (depending on brand)	38-44.0 g
Esculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Lithium Chloride (Sigma L0505)	15.0 g
Colistin	0.01
MS water	1.0 L

Rehydrate commercial Modified Oxford 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.

Final pH 7.2 ± 0.2 at 25°C.

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

1% Moxalactam Solution or use commercially available supplement at same level:

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 small quantities for use and store in freezer at -20°C or below. Refreezing may decrease potency.

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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 ₂ PO ₄	1.35 g
Na ₂ HPO ₄	12.0 g
Esculin	1.0 g
Naladixic Acid (2% in 0.1 M NaOH)	1.0 ml
Acriflavin	12.0 mg
MS water	1.0 L

(Remel catalog # 455254-2/4/6, Difco catalog # 222330 or BBL catalog # 212348, or equivalent, may be used in lieu of the above formulation.)

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.

Final pH 7.2 ± 0.2 at 25°C.

MOPS-BLEB

Listeria Enrichment Broth	36.1 g
MOPS free acid (3-[N-Morpholino]propanesulfonic acid)	6.7 g
MOPS sodium salt (3-[N-Morpholino]propanesulfonic acid sodium salt)	10.5 g
MS water	1.0 L g

Weigh out ingredients as listed above and mix well in a flask. Dispense and sterilize for 15 minutes at 121 °C.

Final pH 7.3 ± 0.2 at 25°C.

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LISTERIA ENRICHMENT BROTH (LEB)

LEB is commercially available-used in MOBS-BLEB

Pancreatic Digest of Casein	17.0g
Soytone	3.0 g
Dextrose	2.5 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5
Yeast Extract	6.0 g
Cycloheximide †	0.05 g
Acriflavine HCL	0.015 g
Nalidixic Acid	0.04

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
MS water	1.0 L

Dissolve the ingredients, except agar, in MS water, and adjust the pH to 7.4. Add the agar, and heat the mixture until visual examination shows that it is well dissolved. Dispense and sterilize 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.

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MOTILITY TEST MEDIUM (EWING)

Meat Extract	3.0 g
Peptone	10.0 g
Sodium Chloride	5.0 g
Agar	4.0 g
MS water	1.0 L

Adjust to pH 7.4. Add agar. Heat the mixture until visual examination shows that it is well dissolved.. Dispense and sterilize at 121°C for 15 minutes.

Final pH 7.4 ± 0.2 at 25°C.

MR-VP MEDIUM (EWING)

Buffered Peptone	7.0 g
Dextrose	5.0 g
K ₂ HPO ₄	5.0 g
MS water	1.0 L

Dispense and sterilize at 121 °C for 15 minutes.

Final pH 6.9 at 25°C.

MUELLER HINTON AGAR

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

Suspend ingredients and heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes.

Final pH 7.3 ± 0.1 at 25°C.

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NITRATE BROTH

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

Suspend above ingredients in MS water and heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave for 15 minutes at 121°C.

Final pH 7.0 ± 0.2 at 25°C.

NUTRIENT AGAR

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

Heat the mixture until visual examination shows that it is well dissolved. Dispense into tubes or flasks. Autoclave 15 minutes at 121°C.

Final pH, 6.8 ± 0.2 at 25°C.

NUTRIENT BROTH, SEMI-SOLID (Holding Media)

Beef Extract	3.0 g
Peptone	5.0 g
Agar	7.5 g
MS water	1.0 L

Heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave 15 minutes at 121°C.

Final pH, 6.8 ± 0.2 at 25°C.

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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
MS water	1.0 L

Suspend ingredients in MS water. Heat the mixture until visual examination shows that it is well dissolved. Sterilize at 121°C for 15 minutes.

Final pH 7.0 ± 0.1 at 25°C.

RAINBOW AGAR O157

Rainbow agar base	60.0 g
Potassium Tellurite solution	0.8 ml
Sodium Novobiocin solution	2.5 ml
MS water	1.0 L

Potassium Tellurite Solution

Potassium tellurite	0.010 g
MS water	10.0 ml

Dissolve the potassium tellurite in the MS water. Filter sterilize. Store in the dark at 4 °C for up to 8 days.

Sodium Novobiocin Solution

Sodium novobiocin	0.40 g
MS water	100 ml

Dissolve the sodium novobiocin in the MS water. Filter sterilize. Store in the dark indefinitely at 4 °C.

Add 60g of Rainbow agar base (Biolog Inc., Hayward California, 94545) to 1 liter of MS water. Boil gently until dissolved. Autoclave for 10 minutes at 121°C. Cool to 50 °C. Add 2.5 ml of sodium novobiocin solution and 0.8 ml of potassium tellurite solution and mix well. Dispense 20 ml per plate into petri plates. Store in a closed container. Shelf life of the prepared medium is two weeks if stored under refrigeration in sealed container such as sealed plastic bags. Final pH 7.9 ± 0.2 at 25°C.

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RAPPAPORT-VASSILIADIS R10 BROTH (Available from Difco)

Bacto Tryptone*	4.54 g
Sodium Chloride	7.20 g
Potassium Dihydrogen Phosphate	1.45 g
Magnesium Chloride, Anhydrous	13.4 g
Malachite Green Oxalate	0.036 g
MS water	1.0 L

* Papaic digest of soybean meal.

Suspend the ingredients in MS water. Heat the mixture until visual examination shows that it is well dissolved. Dispense and sterilize at 115-116°C for 15 minutes.

Final pH 5.1 ± 0.2 at 25°C.

RVS BROTH (Available from Oxoid Unipath or EM Science)

	EM Science	Oxoid
Magnesium Chloride	29 g (hexahydrate)	13.58g (anhydrous)
Sodium Chloride	8.0 g	7.2 g
Peptone from soymeal*	4.5 g	4.5 g
Potassium Dihydrogen Phosphate	0.6 g	1.26 g
Dipotassium Hydrogen Phosphate	0.4 g	0.18 g
Malachite Green	0.036 g	0.036 g
MS water	1.0 L	1.0 L

*Papaic digest of soybean meal

Add ingredients to MS water. Mix thoroughly. Dispense and autoclave for 15 minutes at 10 psi - 115°C.

Final pH 5.2 ± 0.2 at 25°C.

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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
Dextrose	10.0 g
Phenol Red	0.02 g
MS 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 MS water, and adjust pH to 7.4 with 8 N NaOH. Add the agar. Heat the mixture until visual examination shows that it is well dissolved. 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 and autoclave at 121°C for 10 minutes.

Final pH 7.0 ± 0.2 at 25°C.

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. Adjust the pH to 7.0 with 1 N NaOH (less than two ml). Adjust the volume of the solution to 1 liter with MS 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 MgCl₂, 10 ml of a sterile solution of 2M MgSO₄, and a filter sterilized solution of ampicillin (sodium salt) to give a final concentration of 100 µg/ml.

Final pH 7.0 ± 0.2 at 25°C.

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250 mM KCL

KCL	1.86 g
MS Water	100.0 ml

2M MgCL₂

MgCL ₂	19.0 g
MS Water	100.0 ml

Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle.

2M MgSO₄

MgSO ₄	24.1 g
MS Water	90.0 ml

Adjust the volume of the solution to 100 ml with MS water. Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle.

TRIPLE SUGAR IRON (TSI) AGAR

Beef Extract	3.0 g
Yeast Extract	3.0 g
Pancreatic Digest of Casein	15.0 g
Proteose Peptone No. 3	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
MS water	1.0 L

Heat the mixture until visual examination shows that it is well dissolved. Dispense and autoclave at 121°C for 15 minutes. Slant tubes for generous butt. Final pH 7.4 ± 0.2 at 25 °C.

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TRYPTICASE™ SOY AGAR
(TRYPTIC SOY AGAR)

Trypticase™ (Tryptic-pancreatic digest of casein)	15.0 g
Phytone (papaic digest of soybean meal)	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
MS water	1.0 L

Add components to MS water and bring volume to 1.0 liter. Mix thoroughly. Heat the mixture until visual examination shows that it is well dissolved. Distribute into flasks or tubes. Autoclave 121°C for 15 minutes. Do not overheat. Pour into sterile petri dishes or leave in tubes.

Final pH 7.3 ± 0.2 at 25°C.

TRYPTICASE™ SOY AGAR (TS BLOOD AGAR)

Trypticase™ (Tryptic)	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
MS water	1.0 L

Suspend ingredients in water. Heat the mixture until visual examination shows that it is well dissolved. 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.

Final pH 7.3 ± 0.2 at 25 °C.

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TRYPTICASE™ SOY AGAR-YEAST EXTRACT (TSA-YE)

Trypticase™ (Tryptic)	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Yeast Extract	6.0 g
Agar	15.0 g
MS water	1.0 L

Suspend the above ingredients in MS water. Heat the mixture until visual examination shows that it is well dissolved. Autoclave for 15 minutes at 121°C. Temper the medium to 45 - 50°C and pour into sterile Petri dishes.

Final pH 7.3 ± 0.2 at 25 °C.

TRYPTICASE™ SOY BROTH

Trypticase™ (Triptic)	17.0 g
Phytone™	3.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g
MS water	1.0 L

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

Final pH 7.3 ± 0.2 at 25 °C.

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TRYPTICASE™ SOY BROTH (TSB) WITH 10% SODIUM CHLORIDE AND 1% SODIUM PYRUVATE (PTSBS)

Sodium Chloride	100.0 g
Trypticase™ (Tryptic) Pancreatic Digest of Casein)	17.0 g
Phytone (Papaic Digest of Soya Meal)	3.0 g
K ₂ HPO ₄	2.5 g
Dextrose	2.5 g
Sodium Pyruvate	10.0 g
MS 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 MS water. Dispense and sterilize at 121°C for 15 minutes.

Final pH 7.3 ± 0.2 at 25°C.

NOTE: Dehydrated complete medium is not available commercially

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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 ₂ S ₂ O ₅	1.0 g
Egg Yolk Enrichment (50%)	50.0 ml
Cycloserine † Solution	10.0 ml
MS water	940.0 ml

Note: First 7 ingredients available commercially as SFB Base

Cycloserine Solution:

D-Cycloserine †	0.4 g
MS water	10.0 ml

Add cycloserine to MS 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 MS 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 preparation of Egg Yolk Free Tryptose Sulfite Cycloserine Agar (EY-free TSC).

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TT BROTH (HAJNA AND DAMON, 1956)

Yeast Extract	2.0 g
Tryptose	18.0 g
Dextrose	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
MS water	1.0 L

Dissolve and heat to boiling using a hotplate or equivalent. DO NOT AUTOCLAVE. Cool below 50°C. Add 40 ml iodine solution. Store in the refrigerator in the dark. Do not heat after the addition of iodine. Dispense into sterile containers while keeping the solution well mixed and use the day it is prepared. The basal medium without the iodine may be stored indefinitely.

Final pH 7.6 ± 0.2 at 25°C after addition of iodine.

Iodine Solution

Potassium Iodide	8 g
Iodine † crystals	5 g
MS water	20 ml

Dissolve potassium iodide in 20 ml MS water. Add iodine crystals and stir until completely dissolved. Add MS water to volume of 40 ml. Mix thoroughly. Store in the dark at 4°C.

Final pH 7.6 ± 0.2 at 25°C after addition of iodine.

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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
Niaproof 4 (Sigma Chemical Co, formally Tergitol 4)	4.6 ml
MS water	1.0 L

- a. Dissolve Niaproof 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 Heat the mixture until visual examination shows that it is well dissolved.
- 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 ± 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.

CAUTION: 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 sodium azide wastes and dispose of in accordance with the standard chemical waste management procedures for your laboratory.

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APP 1.3 REAGENTS

ANDRADE'S INDICATOR (EWING)

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

The fuchsin is dissolved in the MS 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 (up to ten years). The indicator is used in the amount of 10 ml per liter of medium.

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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
MS 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 MS water.

BUTTERFIELD'S PHOSPHATE DILUENT

a. Stock solution:

Dissolve 34 g KH_2P_0_4 in 500 ml MS 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 MS water. Readjust the pH to 7.2, if necessary, by the drop-wise addition of 0.1 N HCl or 0.1 N NaOH. Autoclave at 121°C for 15 minutes.

ENDOSPORE STAIN

a. Solution A

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

b. Solution B

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

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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
MS 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
MS water	300.0 ml

Dissolve potassium iodide completely in 5 ml MS water, dissolve the iodine crystals, and then bring to volume with MS 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
MS water	100.0 ml

Mix well and store in a glass-stoppered bottle.

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OXIDASE REAGENT

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

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

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.

METHYL RED REAGENT (EWING)

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

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

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NITRATE REDUCTION REAGENTS

Method 1

Solution A:

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

Solution B:

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

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

PEPTONE WATER DILUENT (0.1%)

Peptone	1.0 g
MS water	1.0 L

Dissolve peptone in MS 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.

PHOSPHATE BUFFERED SALINE (PBS)

Anhydrous Na ₂ HPO ₄	12.0 g
NaH ₂ PO ₄ .H ₂ O	2.2 g
NaCl	85.0 g

Dissolve dry ingredients in MS 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 MS water. Check and adjust pH (7.4) if necessary. Sterilize at 121°C for 15 minutes.

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0.15 M PHOSPHATE BUFFERED SALINE at pH 7.2 (PBS)

“Acid” solution

Anhydrous Na ₂ HPO ₄	10.36 g
NaCl	4.38
RO water	1.0 L

“Base” solution

NaH ₂ PO ₄ .H ₂ O	10.65 g
NaCl	4.38 g
RO water	1.0 L

Prepare ‘acid’ and ‘Base’ solutions by added ingredients to RO water. Dissolve completely. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the ‘acid’ solution to the ‘base’ solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers. Autoclave at 121°C for 15 minutes. Store at room temperature.

PHOSPHATE BUFFERS

0.1 M phosphate buffer, pH 4.5 (± 0.1).

Dissolve 13.6 g of potassium dihydrogen phosphate (KH₂PO₄) in about 800 ml of laboratory grade water. Check the pH of the solution. Adjust, if necessary, by the dropwise addition 0.1 N HCl or NaOH. Dilute to 1 liter. Autoclave for 15 minutes at 121°C.

0.1 M phosphate buffer, pH 6.0 (± 0.1).

Potassium dihydrogen phosphate (KH ₂ PO ₄)	11.2 g
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	2.8 g

Dissolve in laboratory grade water. Check the pH of the solution. Adjust, if necessary, by the dropwise addition of 0.1 N HCl or NaOH. Dilute to 1 liter. Autoclave for 15 minutes at 121°C.

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0.1 M phosphate buffer, pH 8.0 (\pm 0.1).

Potassium dihydrogen phosphate (KH ₂ PO ₄)	0.523 g
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	16.73 g

Dissolve in about 800 ml of laboratory grade water. Check the pH of the solution. Adjust if necessary by the dropwise addition of 0.1 N HCl or NaOH. Dilute to 1 liter. Autoclave for 15 minutes at 121°C.

0.2 M phosphate buffer, pH 8.0 (\pm 0.1).

Potassium dihydrogen phosphate (KH ₂ PO ₄)	1.046 g
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	33.46 g

Dissolve in about 800 ml of laboratory grade water. Check the pH of the solution. Adjust if necessary by the dropwise addition of 0.1 N HCl or NaOH. Dilute to 1 liter. Autoclave for 15 minutes at 121°C.

PHYSIOLOGICAL SALINE SOLUTION 0.85% (STERILE)

Sodium Chloride	8.5 g
MS water	1.0 L

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

TRIS BUFFER (0.02 M, pH 7.75)

Trishydroxymethylaminomethane	7.5 g
MS water	3.0 L

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

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

Potassium Hydroxide	40.0 g
Creatine	0.3 g
MS 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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