United States Department of Agriculture

Food Safety and Inspection Service
Office of Public Health Science
Laboratory Quality Assurance,
Response and Coordination Staff
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Laboratory Guidebook
Notice of Change

New, **revised**, or archived: MLG Appendix 1.14

Title: Media and Reagents

Effective Date: 12/16/24

Description and purpose of change(s):

Two media were added to modernize laboratory technology in the *Listeria* method.

- LPT broth has replaced Modified University of Vermont Broth (UVM) and Morpholinepropanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB) for a single step enrichment process versus a two-step primary and secondary enrichment process. Both UVM and MOPS-BLEB have been deleted from this document.
- Harlequin® Listeria Chromogenic Agar (HLCA), a media that is both selective and differential for L. monocytogenes, has replaced both Modified Oxford (MOX) agar, a selective media, and Horse Blood Overlay (HBO), a differential media. HBO has been deleted from this document.

United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science

SOP No: MLG Appendix 1.14		Page **1** of **43**
Title: Media and Reagents		
Revision: .14	Replaces: MLG Appendix 1.13	Effective: 12/16/24

Scope

This document identifies commercially available dehydrated media and reagents used for pathogen analyses, and these MLG method chapters can be found on the public FSIS website. FSIS acknowledges that equivalent products are available for laboratory use and does not specifically endorse any of the manufacturer's recipes and instructions.

APPENDIX (APP) 1 Specific Procedure(s)

APP 1.1 Introduction

- All media and reagents needed to perform each analysis are listed in every chapter. This appendix presents the formulations and procedures for preparing media and reagents in alphabetical order.

- Formulations and preparations for media that will be used for general microbiological procedures, which are not listed in this appendix, will be obtained by consulting readily available reference materials such as:
  - general microbiology textbooks,
  - commercially available media formulation handbooks,
  - the Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam), and
  - the America Public Health Association (APHA)'s Compendium of Methods for the Microbiological Examination of Foods (https://ajph.aphapublications.org/doi/book/10.2105/MBEF.0222).

- The carbohydrates (sugars) should be chemically pure and suitable for biological use; inorganic chemicals used as reagents should be American Chemical Society (ACS) grade or better; dyes must be certified by the "Biological Stain Commission" for use in media.

- Any manufacturer may supply the ingredients and chemical used to prepare the media and reagents if comparative tests show satisfactory results. For convenience, any brand of dehydrated media with equivalent formulation will be used unless instructions indicate otherwise. Pre-mixed, dehydrated media are to be examined for indications of separation or deterioration before being used. Each batch of medium are to be tested for sterility and growth promotion/inhibition characteristics, as appropriate following the Quality Control (QC) procedures described by the manufacturer.

- If commercial dehydrated medium is used, follow the manufacturer's instructions for preparation, specified pH, time, and temperature of sterilization, etc.

- Determine the hydrogen ion concentration (pH) of media using a calibrated electronic pH meter against known buffers. These buffers are prepared according to the Official Methods of Analysis of the Association of Official Analytical Chemists (16th Edition; https://www.cabidigitallibrary.org/doi/full/10.5555/19951414840). If necessary to obtain the desired pH range, adjust the pH of a medium 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. If a recipe is made from individual components instead of a commercially available dehydrated media, it is recommended that the pH be checked prior to sterilization.

- Follow all manufacturers' precautions. Personnel who handle the materials will read the product's Safety Data Sheets.

- Media and reagents can be filter sterilized.

- Any departures from manufactures' standard media preparation practices/techniques (e.g, preparation volumes, sterilization/heating requirements, formulations, etc.) will require equivalency data to support the change(s). The laboratory will retain all equivalency study records.

- Agars must be heated with frequent agitation before dispensing into bottles or tubes prior to the sterilization step. Do not overheat. This can be an essential step in obtaining the correct pH for the final medium. Heating prior to sterilization can be disregarded when using an autoclave with stirring capabilities and may not be necessary with smaller volumes.

- Depending on the type and quantity of media needed, tubed media may be 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, are to be dispensed after autoclaving only.

- Media are not to be used if there are signs of deterioration, hemolysis, or contamination prior to the expiration date.

- Expiration dates and storage times are applied to all media prepared within the laboratory from dehydrated media. The expiration date of the prepared medium will be based on the prepared product and not the expiration dates of the components added to the medium. However, all components added to the medium are to be within their date of expiration. If a change in color, a precipitate, or drying is observed, then the product is discarded regardless of the expiration date. Recommendations of

expiration dates/storage times can be obtained from manufacturers of dehydrated product(s). A study is recommended to determine the shelf-life of a medium or to extend it past the recommended guidelines.

- For Microbiologically Suitable (MS) water requirements, use only water that has been treated to be free from traces of dissolved metal, bactericidal, and inhibitory compounds will 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 will be kept.

Weekly testing (or prior to use):
- > 1.0 megaohms-cm resistance at 25°C or
- < 1.0 microSiemens-cm conductivity at 25°C.

Monthly testing:
- Total Residual Chlorine are to be < 0.1 mg/L
- Aerobic Plate Count are to be < 1,000 colony forming unit (cfu) /mL

Annual testing:
- Heavy Metals (Cd, Cr, Cu, Ni, Pb, and Zn-single) are to be < 0.05 mg/L
- Heavy Metals (total) are to be < 0.10 mg/L
- Water toxicity is to receive passing results to be considered suitable for microbiological analyses.

APP 1.2 MEDIA PREPARATION INSTRUCTIONS

ANTIBIOTIC MEDIUM #4

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

Dissolve ingredients in MS water. Autoclave at 121°C for 15 minutes.
Final pH 6.55 ± 0.05 at 25°C.

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

Dissolve ingredients in MS water. Autoclave at 121°C for 15 minutes.
Final pH 7.9 ± 0.1 at 25°C.

ANTIBIOTIC MEDIUM #8

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

Dissolve ingredients in MS water. Autoclave at 121°C for 15 minutes.
Final pH 5.85 ± 0.05 at 25°C.

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

Dissolve ingredients in MS water. Autoclave at 121°C for 15 minutes.
Final pH 7.95 ± 0.05 at 25°C.

ALL PURPOSE MEDIUM WITH TWEEN® APT AGAR

Pancreatic digest of casein	12.5 g
Dextrose	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
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 – 121°C at 13 psi for 15 minutes. Avoid excessive heating. Dispense into sterile Petri dishes or leave in tubes.

Final pH 6.7 ± 0.2 at 25°C.

BACILLUS CEREUS (BC) MOTILITY MEDIUM

Trypticase	10.0 g
Yeast Extract	2.5 g
Dextrose	5.0 g
Disodium Hydrogen Phosphate	2.5 g
Agar	3.0 g
MS water	1.0 L

Dissolve the ingredients in MS water and heat to boiling to completely dissolve the agar. Mix thoroughly and dispense 2.0 mL into 13 x 100 mm tubes. Autoclave at 121°C for 15 minutes. Allow medium to solidify and store at room temperature for up to 2 or 3 days for best results.

Final pH 7.4 ± 0.2 at 25°C.

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 $6H_2O$	5.0 g
Agar	20.0 g
MS water	950.0 mL

Dissolve ingredients in MS water. Autoclave at 121°C for 15 minutes.

Final pH 6.9 ± 0.1 at 25°C.

Complete medium

a. Add 50 mL pre-warmed (to at least room temperature) Bacto EY tellurite enrichment to 950 mL base medium which has been tempered 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. Ensure that the surface of the plate is dry before use.

BLOOD-FREE BOLTON ENRICHMENT BROTH (2X BF-BEB)

Basal Ingredients	
Meat peptone	20 g
Lactalbumin hydrolysate	10g
Yeast extract	10 g
Sodium chloride	10 g
Sodium pyruvate	1.0 g
α Ketoglutamic acid	2.0 g
Sodium metabisulfite	1.0 g
Sodium carbonate	1.2 g
Haemin	0.02 g
MS Water	1.0 L
Commercial Supplements:	
Cefoperazone	40 mg
Vancomycin	40 mg
Trimethoprim lactate	40 mg
Amphotericin B or Cycloheximide	20 mg 100 mg

Laboratory Made Supplements:

Amphotericin B

Amphotericin B	1.0 g
MS water	100.0 mL

Dissolve 1.0 g of Amphotericin B in 100 mL MS water in a volumetric flask and filter sterilize. Store up to 3 weeks at 2 – 8°C. Add 2 mL/L to yield a final concentration of 20 mg/L.

Cefoperazone

Cefoperazone sodium salt	2.0 g
MS water	100.0 mL

Dissolve 2.0 g of cefoperazone in 100 mL MS water in a volumetric flask and filter sterilize. Store up to 5 days at 2 – 8°C; 14 days at ≤ -20°C; or 5 months at ≤ -70°C. Freeze 2 mL aliquots in sterile plastic tubes. Add 2 mL/L to yield a final concentration of 40 mg/L.

Trimethoprim Lactate

Trimethoprim lactate	0.66 g
MS water	100.0 mL

Dissolve 0.66 g of trimethoprim lactate in 100 mL MS water in a volumetric flask and filter sterilize. Store up to 1 year at 2 – 8°C. Add 8 mL/L to yield a final concentration of 40 mg/L.

Vancomycin

Vancomycin	2.0 g
MS water	100.0 mL

Dissolve 2.0 g of vancomycin in 100 mL MS water in a volumetric flask and filter sterilize. Store up to 2 months at 2 – 8°C. Add 2 mL/L to yield a final concentration of 40 mg/L.

All the above-mentioned ingredients/supplements are for the preparation of 1 L of double strength blood-free Bolton's enrichment broth (2X BF-BEB).

When rehydrating the supplements, follow the manufacturer's instructions. Use ethyl alcohol (USP grade only). Denatured ethanol must not be used because the additives could possibly be toxic to *Campylobacter*.

Add all basal ingredients to water for a 2X BF-BEB solution and mix until ingredients dissolve completely. Autoclave for 15 minutes at 121°C. Cool to at least 50°C if adding supplements at time of preparation. The 2X BF-BEB without supplements is stable for two weeks after preparation stored at 2 – 8°C. To make 500 mL of 2X BF-BEB, add two vials containing all the above-mentioned supplements. After supplement addition, the medium is stable up to 48 hours at 2 – 8°C.
Final pH 7.4 ± 0.2 at 25°C.

NOTE: This 2X BF-BEB formulation is twice as concentrated to meet the specific needs for diluting 1:2 with a 30 mL test portion; i.e., 30 mL of 2X BF-BEB plus 30 mL sample makes 60 mL of 1X BF-BEB sample enrichment for incubation.

BRAIN HEART INFUSION (BHI) AGAR

Calf Brain (infusion from 200 g)	7.7 g
Beef Heart (infusion from 250 g)	9.8 g
Proteose peptone or gelysate	10.0 g
NaCl	5.0 g
Na_2HPO_4	2.5 g
Dextrose	2.0 g
Agar	15.0 g
MS water	1.0 L

Dissolve ingredients in MS water. Dispense as desired and autoclave at 121°C for 15 minutes.

This may also be prepared by adding 15 g of agar to each liter of BHI broth.

Final pH 7.4 ± 0.2 at 25°C.

BRAIN HEART INFUSION (BHI) BROTH

Calf Brain (infusion from 200 g)	7.7 g
Beef Heart (infusion from 250 g)	9.8 g
Proteose peptone or gelysate	10.0 g
NaCl	5.0 g
Na_2HPO_4	2.5 g
Dextrose	2.0 g
MS water	1.0 L

Add components to MS water. Mix thoroughly. Dispense and autoclave at 121°C for 15 minutes.

Final pH 7.4 ± 0.2 at 25°C.

BRILLIANT GREEN SULFA AGAR (BGS)

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
MS water	1.0 L

Dissolve ingredients in MS water. Autoclave at 121°C for 15 minutes. Cool to approximately 50°C and dispense approximately 20 mL into sterile Petri dishes.

Final pH 6.9 ± 0.2 at 25°C.

BROMCRESOL PURPLE (BCP) DEXTROSE BROTH

Peptone	10.0 g
Beef Extract	3.0 g
Sodium Chloride	5.0 g
Bromo Cresol Purple	0.04 g
Dextrose	10 g
MS water	1.0 L

Suspend all ingredients in MS water. Mix thoroughly with frequent agitation to dissolve the powder. Dispense 8.0 mL aliquots into 16 x 150 mm tubes. Autoclave for 10 minutes at 121°C.

Final pH 7.0 ± 0.2 at 25°C.

BRUCELLA BROTH

Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Dextrose	1.0 g
Yeast Extract	2.0 g
Sodium Chloride	5.0 g
Sodium Bisulfite	0.10 g
MS water	1.0 L

Dissolve the dehydrated ingredients in MS water. Dispense as desired and autoclave at 121°C for 15 minutes.

Final pH 7.0 ± 0.2 at 25°C.

BUFFERED PEPTONE WATER (BPW)

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.

CAMPY-CEFEX AGAR

Basal Ingredients	
Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Dextrose	1.0 g
Yeast Extract	2.0 g
Sodium Chloride	5.0 g
Ferrous Sulfate	0.5 g
Sodium Bisulfite	0.3 g
Pyruvic Acid (Sodium Pyruvate)	0.5 g
Cycloheximide	0.2 g
Agar	15.0 g
MS Water	1.0 L
Supplement	
Lysed Horse Blood	50 mL
Cefoperazone	33 mg

Suspend all ingredients in l L of MS water and heat with frequent agitation to dissolve. Autoclave for 15 minutes at 121°C and cool to 50°C. Add supplements. Dispense into petri dishes (20 mL/plate).

Final pH 7.0 ± 0.2 at 25°C.
After media plate preparation, plates may either be held up to 90 days in 2 – 8°C away from direct light or held in the dark at room temperature for 2 – 4 days to allow sufficient time for drying. NOTE: Two milligrams of Amphotericin B may be used in place of 0.2 grams of Cycloheximide. Store lysed horse blood frozen for up to 1 year.

DEY-ENGLEY (DE) NEUTRALIZING BROTH

Tryptone	5.0 g
Yeast Extract	2.5 g
Glucose	10.0 g
Sodium thioglycollate	1.0 g
Sodium thiosulfate	6.0 g
Sodium bisulfite	2.5 g
Polysorbate 80	5.0 g
Lecithin (soybean)	7.0 g
Brom cresol purple	0.02 g
MS water	1.0 L

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

Final pH 7.6 ± 0.2 at 25°C.

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

Sodium Novobiocin Solution

Sodium Novobiocin	1.5 g
MS water	100 mL

Dissolve the sodium novobiocin in the MS water. Filter sterilize. Store at 2 – 8°C in the dark for up to 6 months.

Suspend all ingredients in MS water and heat to boil for 10 minutes. DO NOT HEAT ABOVE 100°C. Cool to approximately 50°C, add 1 mL of Sodium Novobiocin from a filter-sterilized stock solution. Dispense 15 – 20 mL/plate. Store refrigerated for up to 3 weeks. NOTE: If the modified rainbow agar O157 Novobiocin stock is used, add 3.75 mL/L of DMLIA.

Final pH 6.7 ± 0.2 at 25°C.

This medium is also commercially available as a dehydrated powder with a separate novobiocin supplement.

E BUFFER

Bovine Albumin	5.0 g
Tween-20	0.5 mL
Buffered Peptone Water (BPW)	20 g
MS Water	1 L

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.

ENTEROCOCCOSEL AGAR (ECA)

Pancreatic Digest of Casein	17.0 g
Peptic Digest of Animal Tissue	3.0 g
Yeast Extract	5.0 g
Oxgall	10.0 g
Sodium Chloride	5.0 g
Esculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Sodium Azide	0.25 g
Sodium Citrate	1.0 g
Agar	13.5 g
MS Water	1 L

Suspend all ingredients in l L of MS water and heat with frequent agitation to dissolve. Autoclave at 121°C for 15 minutes. Cool to approximately 50ºC and dispense approximately 20 mL into sterile Petri dishes.

Final pH 7.1 ± 0.2 at 25°C.

ENTEROCOCCOSEL BROTH

Pancreatic Digest of Casein	17.0 g
Peptic Digest of Animal Tissue	3.0 g
Yeast Extract	5.0 g
Oxgall	10.0 g
Sodium Chloride	5.0 g
Esculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Sodium Azide	0.25 g
Sodium Citrate	1.0 g
MS Water	1 L

Dissolved powder completely and dispense approximately 3 mL per tube. Autoclave at 121°C for 15 minutes.

Final pH 7.1 ± 0.2 at 25°C.

EOSINE METHYLENE BLUE AGAR (EMB)

Pancreatic Digest of Gelatin	10.0 g
Lactose	10.0 g
Dipotassium Phosphate	2.0 g
Eosin Y	0.4 g
Methylene Blue	0.065 g
Agar	15.0 g
MS Water	1 L

Suspend all ingredients in l L of MS water and heat with frequent agitation to dissolve. Autoclave at 121°C for 15 minutes. Cool to approximately 50°C and dispense approximately 20 mL into sterile Petri dishes.

Final pH 7.1 ± 0.2 at 25°C.

HUNT BROTH

Basal Broth Ingredients	
Beef Extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Yeast Extract	6.0 g
Growth Supplement*	0.75 g
Antibiotic Mix*	0.0725 g
Sterile MS water	950.0 mL

* Ingredients can be found in Hunt Broth alternative preparation.

Commercial Additive:
Sterile lysed horse blood

Sterile lysed horse blood	50.0 mL

Store frozen and discard blood after 12 months. If horse blood is not purchased lysed, lyse horse blood by subjecting it to two freeze/thaw cycles and then store frozen.

All the above-mentioned ingredients/supplements are for the preparation of 1 L of Hunt Broth.

Dissolve the basal broth ingredients in sterile MS water. Mix thoroughly. DO NOT AUTOCLAVE. After supplement addition, the broth is stable up to 24 hours at 2 – 8°C.

Final pH 7.5 ± 0.2 at 25°C. Store at 2 – 8°C.

HUNT BROTH (alternative preparation)

Basal Broth Ingredients	
Beef Extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Yeast Extract	6.0 g
MS water	950.0 mL
Commercial Supplements/Additives	
Campylobacter Growth Supplement – SR0232E	2 vials
Vancomycin Hydrochloride	10.0 mg
Trimethoprim Lactate	12.5 mg
Cefoperazone Sodium	30.0 mg
Amphotericin B	20.0 mg
Sterile Lysed Horse Blood	50.0 mL

Commercial Supplements/Additives:
Sterile lysed horse blood

Sterile lysed horse blood	50.0 mL

Store frozen and discard blood after 12 months. If horse blood is not purchased lysed, lyse horse blood by subjecting it to two freeze/thaw cycles and then store frozen.

Campylobacter Growth Supplement

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

Use: Campylobacter Growth Supplement, e.g., SR0232E. Following manufacturer's instructions, mix well and add to the cooled medium. Add 2 vials for each liter of broth to yield the correct final concentration.

Laboratory Made Supplements:
Vancomycin Stock Solution

Vancomycin Hydrochloride	0.25 g
MS water	100.0 mL

In a 100 mL volumetric flask, dissolve 0.25 g vancomycin in 100 mL of MS water, mix well, and filter sterilize. Store up to 2 months at 2 – 8°C. Add 4 mL/L to yield a final concentration of 10.0 mg/L.

Trimethoprim Lactate Stock Solution

Trimethoprim Lactate	0.3125 g
MS water	100.0 mL

In a 100 mL volumetric flask, dissolve 0.3125 g trimethoprim lactate in 100 mL of MS water, mix well, and filter sterilize. Store up to 12 months at 2 – 8°C. Add 4 mL/L to yield a final concentration of 12.5 mg/L.

Cefoperazone Stock Solution

Cefoperazone Sodium	0.375 g
MS water	100.0 mL

In a 100 mL volumetric flask, dissolve 0.375 g cefoperazone sodium in 100 mL of MS water, mix well, and filter sterilize. Store up to 5 months at -70°C. Add 8 mL/L to yield a final concentration of 30.0 mg/L.

Amphotericin B Stock Solution

Amphotericin B	1.0 g
MS water	100.0 mL

In a sterile 100 mL volumetric container, dissolve 1.0 g Amphotericin B in 100 mL of sterile MS water and mix well. Do not filter sterilize. Store up to 3 weeks at 2 – 8°C. Add 2 mL/L to yield a final concentration of 20.0 mg/L. All the above-mentioned ingredients/supplements are for the preparation of 1 L of Hunt Broth.

When rehydrating commercial supplements, follow the manufacturer's instructions. If alcohol is recommended, use ethyl alcohol (USP grade only). Denatured ethanol must not be used because the additives could possibly be toxic to *Campylobacter*.

Dissolve the Basal components in MS water. While mixing, heat with frequent agitation to completely dissolve components. Autoclave at 121°C for 15 minutes. Cool broth to at least 50°C if adding supplements at time of preparation. The broth without supplements is stable for three weeks after preparation, stored at 2 – 8°C. After supplement addition, the broth is stable up to 48 hours at 2 – 8°C.

Final pH 7.0 ± 0.2 at 25°C after addition of supplements.

KENNER FECAL (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
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 a boil. 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.

LIMEWATER

Calcium Hydroxide	5.7 g
MS water	1.0 L

In a clean glass jar, add 5.7 grams of calcium hydroxide to 1 L of MS water. (Limewater is a saturated solution, which means there will be some extra calcium hydroxide that doesn't dissolve. The 5.7 grams of calcium hydroxide in 1 L of MS water will result in a fully saturated solution.) Shake the jar vigorously for 1 – 2 minutes, then let it stand for 24 h. Be careful not to stir up the sediment. Pour the clearer solution off the top of the jar through clean filter paper. Repeat the filtering step, if necessary, to obtain a clear limewater solution. Store in a clean jar or bottle.

HARLEQUIN® LISTERIA CHROMOGENIC LISTERIA AGAR (OTTAVIANI & AGOSTI)

Basal Ingredients	
Harlequin® Listeria Chromogenic Agar Base*	69.5 g
MS Water	950 mL

*Harlequin® Listeria Chromogenic Agar Base (approximate Formula per liter)	
Enzymatic Digest of Animal Tissue	18.0 g
Enzymatic Digest of Casein	6.0 g
Yeast Extract	10.0 g
Lithium Chloride	10.0 g
Sodium Chloride	5.0 g
Disodium Hydrogen Phosphate (anhydrous)	2.5 g
Sodium Pyruvate	2.0 g
Glucose	2.0 g
Magnesium Glycerophosphate	1.0 g
Magnesium Sulfate (anhydrous)	0.5 g
5-Bromo-4-Chloro-3-Indolyl-β-D-Glucopyranoside	0.05 g
Agar	12.5 g

Mix 69.5 g of powder base in 950 mL MS water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave for 15 minutes at 121°C. Cool to 45 – 50°C.

Supplements	
Listeria Chromogenic Selective Supplement – NCM4002-0.5	2 vials
Listeria Chromogenic Diagnostic Supplement – NCM4001-0.5	2 vials

Reconstitute each vial Listeria chromogenic selective supplement (NCM4002-0.5) with 5 mL of sterile water. Swirl to mix. Add Listeria chromogenic selective supplement to cooled base. Swirl to mix.

Preheat Listeria chromogenic diagnostic supplement to 48 – 50 °C. Add supplement to base and mix well using gentle end-over-end mixing.

Dispense into petri dishes (20 mL/plate). Final pH 7.2 ± 0.2 at 25°C.

LPT BROTH

LPT Broth *	50.0 g
Polysorbate 80	0.5 g
MS Water	1 L

*LPT Broth (approximate Formula Per liter)	
Enzymatic digest of animal tissue (bovine or porcine)	5 g
Enzymatic digest of casein (bovine)	5 g
Meat extract (bovine or porcine)	5 g
Yeast extract	5 g
Buffer system	16.755 g
Lithium chloride	6 g
Mixture of salts	5.2 g
Glucose	2 g
Acriflavine	0.005 g
Nalidixic acid	0.04 g

Suspend 50 g of LPT Broth into 1 L of MS water. Add 0.5 g of polysorbate 80 per liter of medium. Mix for approximately 10 minutes to obtain a homogenous mixture. Adjust the pH to 7.7 ± 0.1. Dispense as desired and autoclave at 121°C for 15 minutes.

Final pH 7.7 ± 0.2 at 25°C. LPT broth can be stored for up to 1 month at 2 – 8°C.

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
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 (Sigma-Aldrich, St. Louis, Missouri, or equivalent product) 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 15 minutes, cool to 50ºC in a water bath, and add 50 mL of Preparation B and 10 mL of Preparation C. Mix well. Dispense 18 – 20 mL into Petri dishes, allow to solidify, and dry for 24 h at room temperature. Plates may be stored at 2 – 8°C for 30 days.

Final pH 7.2 ± 0.1 at 25°C.

MODIFIED CHARCOAL CEFOPERAZONE DEOXYCHOLATE AGAR 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 g of Cook Meat Medium (a) and 16 mL of diluent (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.

NOTE: The diluent may be heated to dissolve starch if necessary. Steam tubes of Cook Meat Medium for 10 minutes and cool just prior to use.

Final pH 6.8 ± 0.2 at 25°C.

MODIFIED OXFORD MEDIUM (MOX)

MOX Agar

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

Suspend all ingredients in l L of MS water and heat with frequent agitation to dissolve. Autoclave 121°C for 15 minutes, mix again, and cool to 45 – 50°C in a water bath. Add 2 mL of 1% filter sterilized Moxalactam solution to make the complete MOX medium, mix well, and dispense 12 – 14 mL per plate.

Final pH 7.0 ± 0.2 at 25°C.

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

Sodium (or Ammonium) Moxalactam	1.0 g
0.1 M Phosphate Buffer, pH 6.0	100.0 mL

Dissolve, filter sterilize, dispense in small quantities for use and store in freezer at -10°C or below. Refreezing may decrease potency.

CAUTION: DO NOT use the Modified Oxford Antibiotic Supplement since it contains both moxalactam and colistin.

MODIFIED RAINBOW® AGAR O157 (mRBA)

Rainbow agar base	60.0 g
Potassium Tellurite solution	0.15 mL
Sodium Novobiocin solution	1.25 mL
Cefixime solution (concentration of 0.5mg/mL)	0.1 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 2 – 8°C for up to 8 days.

Sodium Novobiocin Solution

Sodium novobiocin	0.4 g
MS water	100 mL

Dissolve the sodium novobiocin in the MS water. Filter sterilize. Store in the dark up to 6 months at 2 – 8°C.

Cefixime Solution

Cefixime Trihydrate	0.050 g
Methanol	10 mL

Prepare dilution by adding 1 mL of Cefixime Solution to 9 mL of MS water for a working concentration of 0.5 mg/mL. The Cefixime solution can be stored at -20°C for six months. The 1:10 dilution should be filter sterilized and prepared the day of use.

Add 60 g of Rainbow agar base to 1 L of MS water. Autoclave for 10 minutes at 121°C. Cool to 50°C. Add 1.25 mL of sodium novobiocin solution, 0.15 mL of potassium tellurite solution, and 0.1 mL of cefixime solution and mix well. Dispense approximately 20 mL per plate into petri plates. Store in a closed container in the dark. Shelf life of the prepared medium is 21 days if stored under refrigeration in sealed container such as sealed plastic bags.

Final pH 8.1 ± 0.2 at 25°C.

MODIFIED TRYPTONE SOYA BROTH (mTSB)

Base Ingredients	
Modified Tryptone Soya Broth (Oxoid Product # CM0989B or equivalent	33.0 g
Casaminoacids (casein acid hydrolysate)	10.0 g
MS water	1.0 L

Suspend all ingredients in MS Water and autoclave for 15 minutes at 121°C. If refrigerated, media must be pre-warmed to 18 – 35°C prior to use.

Final pH 7.3 ± 0.2 at 25°C.

MODIFIED TRYPTONE SOYA BROTH WITH NOVOBIOCIN (mTSB+n) with 8 mg/L of SODIUM NOVOBIOCIN

Base Ingredients	
Modified Tryptone Soya Broth	33.0 g
Casaminoacids (casein acid hydrolysate)	10.0 g
MS water	1.0 L
Supplement	
Sodium novobiocin solution (concentration of 4 mg/mL)	2 mL

The use of other manufacturer's modified Tryptone Soya broth or Trypticase™ (Tryptic) Soy Broth base (other than Oxoid) is permitted if the formula is equivalent. Suspend all ingredients in MS water and autoclave for 15 minutes at 121°C.

Let media cool to at least 50°C. Add 2 mL of filter sterilized, aqueous sodium novobiocin solution prepared at a concentration of 4 mg/mL (adjusted for potency; Sigma N1628) for each liter of medium. If refrigerated, media must be pre-warmed to 18 – 35°C prior to use. Store Novobiocin stock solution at 2 – 8°C for up to 6 months

Final pH 7.3 ± 0.2 at 25°C.

MOTILITY TEST MEDIUM

Beef Extract	3.0 g
Pancreatic Digest of Casein	10.0 g
Sodium Chloride	5.0 g
Agar	4.0 g
MS water	1.0 L

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

Final pH 7.3 ± 0.2 at 25°C.

NEUTRALIZING BUFFER PEPTONE WATER (nBPW)

Buffered Peptone (Difco or equivalent)	20.0 g
Refined Soy Lecithin (MP Biomedicals, item # 102147, store at -20^{0}C or Alfa Aesar #36486, CAS 8002-43-5) or equivalent)	7 g
Sodium Thiosulfate	1 g
Microbiology Suitable (MS) water	1 L
Sodium Bicarbonate	12.5g

Mix Buffered Peptone, soy lecithin, and sodium thiosulfate in 833 mL of MS water. Autoclave at 121°C for 15 minutes. Dissolve the sodium bicarbonate in 167 mL MS water, filter sterilize and add to autoclaved broth after it cools to at least 55°C. Constant agitation while sterilely dispensing this medium is recommended. Volumes may be adjusted for larger or smaller batches.

Final pH 7.7 ± 0.5 at 25°C after the addition of sodium bicarbonate.

NUTRIENT AGAR

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

Dispense into tubes, plates, 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

Dispense and autoclave 15 minutes at 121°C.

Final pH 6.8 ± 0.2 at 25°C.

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. Sterilize at 121°C for 15 minutes.

Final pH 7.0 ± 0.1 at 25°C.

RAPPAPORT-VASSILIADIS R10 BROTH (Available from Difco)

Pancreatic Digest of Casein	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

Suspend the ingredients in MS water. 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 EMD Science)

	EMD Science	Oxoid
Magnesium Chloride	29 g (hexahydrate)	13.58 g (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

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

Final pH 5.2 ± 0.2 at 25°C.

RAPPAPORT-VASSILIADIS BROTH, modified (Available from Fluka)

Papaic digest of soybean meal	5.0 g
Sodium Chloride	8.0 g
Monopotassium Phosphate	1.6 g
Magnesium Chloride hexahydrate	18.7 g
Malachite Green	0.04 g
MS water	1.1 L

Add ingredients to MS water. Heat gently, if necessary, to dissolve the medium completely. Sterilize at 115°C for 15 minutes.

Final pH 5.2 ± 0.2 at 25°C.

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

Suspend ingredients in MS water. Mix thoroughly. Autoclave at 121°C for 15 minutes. Dispense as desired.

Final pH 7.3 ± 0.2 at 25°C.

TRYPTICASE™ SOY AGAR with 5% SHEEP BLOOD (SHEEP BLOOD AGAR or SBA)

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. Sterilize at 121°C for 15 minutes. Cool to approximately 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. For *Listeria monocytogenes* CAMP test usage, pour 9 ± 1 mL quantities into sterile 100 x 15 mm Petri dishes for ease of plate interpretation.

Final pH 7.3 ± 0.2 at 25°C.

TRYPTICASE™ SOY BROTH

Trypticase™ (Tryptic)	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

Suspend ingredients in MS water. Mix thoroughly. Dispense into tubes and sterilize at 121°C for 15 minutes.

Final pH 7.3 ± 0.2 at 25°C.

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 Enrichment (50%)	50.0 mL
Cycloserine Solution	10.0 mL
MS water	940.0 mL

First 7 ingredients available commercially as Shahadi-Ferguson Perfringens (SFP) 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 10 mL of sterile 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).

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.

Final pH 7.6 ± 0.2 at 25°C.

TT BROTH (HAJNA AND DAMON, 1956)

TT Broth Base

Yeast Extract	2.0 g
Tryptose	18.0 g
Dextrose	0.5 g
d-Mannitol	2.5 g
Sodium Deoxycholate	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.

Final pH of base broth 7.6 ± 0.2 at 25°C.

Iodine Solution

Potassium Iodide	8 g
Iodine crystals	5 g
MS water	20 mL

Dissolve potassium iodide in 20 mL MS water. Under a vent hood, add iodine crystals and stir until completely dissolved. Add MS water to volume of 40 mL. Mix thoroughly. Store in the dark at 2 – 30°C.

Just prior to inoculation, add 4% iodine solution per final volume. 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 base may be stored at 2 – 8°C for up to six months prior to the addition of iodine.

WANG'S FREEZING/STORAGE MEDIUM

Base Ingredients	
Brucella broth powder	28 g
Glycerol	200 mL
MS water	750 mL
Supplement	
Lysed horse blood	5 mL/95 mL

Add Brucella broth powder to MS water and heat the mixture until visual examination shows that it is well dissolved. Add 200 mL of glycerol to the homogenous mixture and mix well. Dispense 95 mL of the mixture into individual bottles. Autoclave for 15 minutes at 121°C and cool it to 50°C. Add 5 mL lysed horse blood per 95 mL bottle and mix thoroughly. Dispense 1 mL of Wang's storage medium into a 2 mL cryovial.

Once prepared, expiration date for the base medium prior to addition of lysed horse blood is 90 days. Lyse horse blood by subjecting it to two freeze/thaw cycles. Once the lysed horse blood has been added to the prepared base medium, Wang's storage medium can be stored up to 3 weeks at 2 – 8°C in a flask or dispensed into cryovials.

Final pH 7.0 ± 0.2 at 25°C before addition of supplement.

WANG'S TRANSPORT MEDIUM (SEMISOLID)

Base Ingredients	
Purified grade agar	4 g
Brucella broth powder	28 g
MS water	950 mL
Supplement	
Lysed horse blood	5 mL/95 mL

Add Brucella broth powder and purified grade agar to water and bring to a boil to dissolve completely. Dispense 95 mL of the mixture into individual bottles. Autoclave for 15 minutes at 121°C and cool it to 50°C. Add 5 mL lysed horse blood to each 95 mL bottle and mix thoroughly. Dispense 1 mL Wang's transport medium into at least a 2 mL size cryovial.

Once prepared, expiration date for the base medium prior to addition of lysed horse blood is 90 days. Lyse horse blood by subjecting it to two freeze/thaw cycles. Once the lysed horse blood has been added to the prepared base medium, Wang's transport medium can be stored up to 3 weeks at 2 – 8°C in cryovials. Final pH 7.0 ± 0.2 at 25°C before addition of supplement.

XLD AGAR

Yeast Extract	3.0 g
L-Lysine HCl	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sodium Thiosulfate	6.8 g
Sodium Chloride	5.0 g
Agar	12.5 g
Phenol Red	0.08 g
Ferric ammonium citrate	0.8 g
Sucrose	7.5 g
Sodium desoxycholate	1.0 g

Suspend 53 g in 1 liter of distilled water. Heat with frequent agitation until the medium boils. **DO NOT OVERHEAT**. Transfer immediately to a water bath at 50°C. Pour into sterile Petri dishes as soon as the medium has cooled.

Final pH 7.4 ± 0.2 at 25°C.

XLT4 AGAR

XLT4 Agar Base* (BD# 223420)	59.0 g
XLT4 Agar Supplement (A 27% solution (approximate) of the surfactant Tergitol™ 4)	4.6 mL
MS water	1.0 L

*XLT4 Agar Base (approximate Formula Per liter)	
Proteose Peptone No. 3	1.6 g
Yeast Extract	3.0 g
L-Lysine	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Saccharose	7.5 g
Ferric Ammonium Citrate	0.8 g
Sodium Thiosulfate	6.8 g
Sodium Chloride	5.0 g
Agar	18.0 g
Phenol Red	0.08 g

Dissolve 59.0 g of XLT4 Agar Base in 1.0 L MS water and mix with a magnetic stir-bar. Add 4.6 mL of XLT 4 agar supplement. Heat to boiling to dissolve completely. Cool to 45 – 50°C in a water bath and mix again gently. Dispense approximately 20 mL into sterile 100 x 15 mm Petri dishes. Allow plates to dry at room temperature overnight and then refrigerate (in plastic bags or containers) at 2 – 8°C. Final pH 7.5 ± 0.2 at 25°C.

XLT4 plates have a shelf life of 10 weeks when stored refrigerated in a closed plastic bag or other container.

APP 1.3 REAGENTS

BROMTHYMOL BLUE

Dissolved 0.1 g dye in 18.5 mL 0.01 N NaOH. Dilute to 250 mL with MS water.

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 BUFFER

a. Stock solution

Dissolve 34 g KH_2PO_4 in 500 mL MS water, adjust to pH 7.2 with ca. 175 mL 1 N NaOH, and dilute to 1 L. Store under refrigeration.

b. Diluent

Dilute 1.25 mL stock solution (a) to 1 L with MS water. Re-adjust 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.

CALCIUM CARBONATE

Transfer the needed aliquot into an autoclavable container. Sterilize the aliquot for 15 min at 121°C using dry cycle parameters. Use the expiration date specified by manufacturer.

CRYSTAL VIOLET (1% AQUEOUS)

For purchased solutions, steam at 100°C for 15 minutes or filter sterilize.

ENDOSPORE STAIN

a. Solution A

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

b. Solution B

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

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.

NOTE: Gram stain kits and reagents can be easily purchased and are recommended for laboratory usage instead of preparing the individual solutions as described above.

NEUTRAL RED (1% AQUEOUS)

Dissolved 0.1 gm in 60 mL of ethanol and diluted to 100 mL of MS water as required.

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.

NITRATE REDUCTION REAGENTS

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. Autoclave at 121°C for 15 minutes. Dispense as desired into sterile containers.

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

0.15 M PHOSPHATE BUFFERED SALINE at pH 7.2 (PBS)

"Acid" solution

Anhydrous Na_2HPO_4	10.36 g
NaCl	4.38 g
MS water	1.0 L

"Base" solution

$NaH_2PO_4.H_2O$	10.65 g
NaCl	4.38 g
MS water	1.0 L

Prepare 'acid' and 'base' solutions by added ingredients to MS 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 (KH_2PO_4) in about 800 mL of MS water. Check the pH of the solution. Adjust, if necessary, by the dropwise addition 0.1 N HCl or NaOH. Dilute to l L. Autoclave for 15 minutes at 121°C.

0.1 M phosphate buffer, pH 6.0 (± 0.1)

Potassium dihydrogen phosphate (KH_2PO_4)	11.2 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.8 g

Dissolve in about 800 mL of MS water. Check the pH of the solution. Adjust, if necessary, by the dropwise addition of 0.1 N HCl or NaOH. Dilute to l L. Autoclave for 15 minutes at 121°C.

0.1 M phosphate buffer, pH 8.0 (± 0.1)

Potassium dihydrogen phosphate (KH_2PO_4)	0.523 g
Dipotassium hydrogen phosphate (K_2HPO_4)	16.73 g

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

0.2 M phosphate buffer, pH 8.0 (± 0.1)

Potassium dihydrogen phosphate (KH_2PO_4)	1.046 g
Dipotassium hydrogen phosphate (K_2HPO_4)	33.46 g

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

HYPOTONIC SALINE SOLUTION 0.45% (STERILE)

Sodium Chloride	4.5 g
MS water	1.0 L

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

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.

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.

APP 1.4 Reference(s):

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