

(a) ELAH--Earle's base, with 0.5 percent lactalbumin hydrolysate and without NaHCO_3 (Hazleton/Kansas City Biological, product no. DM-303, or equivalent). See Section 2.1.4, Step (h) for options in sterilization of the ELAH--Earle's base solution.

(b) Sodium bicarbonate (NaHCO_3).

(c) Antibiotics--penicillin G, dihydrostreptomycin sulfate, tetracycline and amphotericin B (Sigma Chemical Co., or equivalent). Use antibiotics of at least tissue culture grade. See Section 2.1.3 for preparation of stock antibiotic solutions.

(d) Ascorbic acid.

(e) Water, deionized, distilled. See Chapter 4.

(f) Nutrient agar (Difco Laboratories, or equivalent).

2.1.3 Procedure for preparation of stock antibiotic solutions. If not purchased in sterile form, stock antibiotic solutions must be filter-sterilized by the use of 0.22-micrometer membrane filters. Antibiotic stock solutions should be placed in screw-capped containers and stored at -20 degrees C until needed. Once thawed they may be refrozen; however, to avoid repeated freezing and thawing of these stock solutions distribute them in quantities that are sufficient to support no more than a week's virus assay work. Stock solutions can be stored for up to 4 months. Quantities prepared in steps (a), (b) and (c) are sufficient for at least 100 liters of media.

(a) Preparation of penicillin-streptomycin stock solution. The procedure described is for preparation of ten 10-mL volume penicillin-streptomycin stock solutions at concentrations of 1,000,000 units of penicillin and 1,000 mg of streptomycin per 10-mL unit. The antibiotic concentrations listed in step (a.1) may not correspond to the concentrations obtained from other lots or from a different source.

(a.1) Add appropriate amounts (within 5 percent) of penicillin G and dihydrostreptomycin sulfate to a 250-mL flask containing 100 mL of deionized distilled water. For penicillin supplied at 1435 units per mg, add 7 g of the antibiotic. For streptomycin supplied at 740 mg per g, add 14 g of the antibiotic.

(a.2) Mix contents of flask on magnetic stirrer until antibiotics are dissolved.

(a.3) Sterilize antibiotics by filtration through 0.22-micrometer membrane filter.

(a.4) Dispense the penicillin-streptomycin stock in 10-mL volumes into screw-capped containers.

(b) Preparation of tetracycline stock solution.

The procedure described is for preparation of ten 5-mL tetracycline stock solutions at concentrations of 0.125 g per 5 mL unit.

(b.1) Add 1.25 g of tetracycline hydrochloride powder and 3.75 g of ascorbic acid to a 125-mL flask containing 50 mL of deionized distilled water.

(b.2) Mix contents of flask on magnetic stirrer until antibiotic is dissolved.

(b.3) Sterilize antibiotic by filtration through 0.22-micrometer membrane filter.

(b.4) Dispense the tetracycline stock in 5-mL volumes into screw-capped containers.

(c) Preparation of amphotericin B (fungizone) stock solution.

The procedure described is for preparation of ten 2.5-mL fungizone stock solutions at concentrations of 0.0125 g per 2.5 mL unit.

(c.1) Add 0.125 g of fungizone to a 50-mL flask containing 25 mL of deionized distilled water.

(c.2) Mix contents of flask on magnetic stirrer until antibiotic is dissolved.

(c.3) Sterilize antibiotic by filtration through 0.22-micrometer membrane filter.

(c.4) Dispense the fungizone stock in 2.5-mL volumes into screw-capped containers.

2.1.4 Preparation of maintenance medium.

(a) Determine the volume of ELAH--Earle's base solution required. The volume of ELAH--Earle's base solution needed will be equal to the volume of growth medium used in propagation of cell cultures for the plaque assay procedure. Thus, see Table 2 in Chapter 9 to determine the volume required. The procedure described is for preparation of 1 liter of ELAH--Earle's base solution in a 1X formulation and

likelihood that a test sample will be toxic to cell cultures or may be so darkly colored as to result in inaccurate plaque counts, the cell monolayer should be treated in accordance with the method described in Chapter 8 (April, 1986 revision).

2.2.1 Apparatus and materials.

(a) Glassware, Pyrex glass, clear (Corning Glass Works, or equivalent).

(b) Magnetic stirrer and stir bars.

(c) Autoclavable inner-braided tubing with metal quick-disconnect connectors or with thumbscrew-drive clamps for connecting tubing to equipment to be used under pressure. Quick-disconnect connectors can be used only after equipment has been properly adapted.

(d) Positive pressure air or nitrogen source equipped with pressure gauge. Pressure source, if laboratory air-line or pump, must be equipped with oil filter. Deliver to pressure vessel and filter holder no more pressure than recommended by manufacturer.

(e) Dispensing pressure vessel--5- or 20-liter capacity (Millipore Corp., or equivalent).

(f) Waterbath set at 36 plus or minus 1 degree C. Used for maintaining the temperature of the overlay medium. See Section 2.2.5, Step (c).

(g) Waterbath set at 50 plus or minus 1 degree C. Used for maintaining the agar temperature. See Section 2.2.6, Step (d).

(h) Incubator capable of maintaining the temperatures of cell cultures at 36.5 plus or minus 1 degree C.

(i) Sterilizing filter--0.22-micrometer pore size with a 142-mm diameter to sterilize Medium 199 from Section 2.2.3 and reagents in excess of 1 liter volumes (Millipore Corp., GS series, or equivalent). Where the volumes of media prepared are for large-scale viral analyses (20 liters or more), the use of a 293-mm diameter sterilizing filter may be more appropriate. Use a 47-mm diameter filter for sterilizing volumes of less than 1 liter.

(j) Fiberglass prefilters for use with sterilizing filters (Millipore Corp. AP15 and AP20, or equivalent). Stack AP20 and AP15 prefilters and 0.22-micrometer membrane filter into disc

(a) Calculate virus titer in plaque forming units (PFU) for each virus-containing sample concentrate. To determine the numbers of PFU per mL in water, sewage sludge, soil, or dredge spoil sample concentrate, multiply the number of PFU by the reciprocal of the inoculum volume. If the inoculum volume was diluted, also multiply the number of PFU by the reciprocal of the dilution made.

(b) Calculate virus content of original sample. To obtain virus content of the original sample in terms of PFU per mL, multiply the product from Section 2.3.2, Step (a) by the concentration factor which is calculated by dividing the volume of the original sample by the volume of the sample concentrate. For soil, digested dewatered sludge and dredge spoil samples, correct for water content and report in PFU per gram of dry weight. Dry weight is determined by evaporating a given sample in a weighed dish, drying in an oven at 104 plus or minus 1 degree C to a constant weight and then determining the increase in weight over that of the empty dish.

2.4 Procedure for Verifying Sterility of Liquids. There are many techniques available for verifying the sterility of liquids such as cell culture media and medium components. Three techniques, described below, are standard in many laboratories. The capabilities of these techniques, however, are limited to detecting microorganisms that grow unaided in the test medium utilized. Viruses, mycoplasma, and microorganisms that possess fastidious growth requirements or that require living host systems will not be detected. Nonetheless, with the exception of a few special contamination problems, the test procedures and microbiological media listed below should prove adequate. Do not add antibiotics to media or medium components until after sterility of the reagents, media and medium components has been demonstrated.

2.4.1 Procedure for verifying sterility of small volumes of liquids.

(a) Inoculate 5 mL of the material to be tested for sterility into 5 mL of thioglycollate broth.

(b) Shake the mixture and incubate at 36.5 plus or minus 1 degree C.

(c) Examine the inoculated broth daily for seven days to determine whether growth of contaminating organisms has occurred. Vessels that contain thioglycollate medium must be tightly sealed before and after medium is inoculated. A clouded condition that develops in the media indicates the occurrence of contaminating organisms.

(g) Petri dish--50-mm diameter (Falcon Labware Division, or equivalent).

3.2.2 Media and reagents.

(a) Medium 199 prepared at a 2X concentration with Earle's salts, 0.05 percent lactalbumin and L-glutamine and without phenol red and NaHCO_3 (Grand Island Biological Co., product no. 400-1100, or equivalent).

(b) HEPES--1 M (Sigma Chemical Co., product no. H-3375, or equivalent). Prepare 50 mL of a 1 M solution of HEPES.

(c) GG-free newborn calf serum--heat inactivated at 56 degrees C for 30 min, certified free of viruses, bacteriophage and mycoplasma (Grand Island Biological Co., product no. 210-6400, or equivalent). Procure at least one 100-mL size bottle.

(d) Sodium bicarbonate (NaHCO_3)--7.5 percent solution. Prepare 50 mL of a 7.5 percent solution of sodium bicarbonate. Sterilized by filtration through 0.22-micrometer filter.

(e) Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)--1.0 percent solution. Prepare 50 mL of a 1.0 percent solution of magnesium chloride.

(f) Water, deionized distilled. See Chapter 4.

(g) Hydrochloric acid (HCl)--1 M. Prepare 100 mL of a 1M solution of hydrochloric acid.

(h) Sodium hydroxide (NaOH)--1 M. Prepare 100 mL of a 1M solution of sodium hydroxide.

(i) Neutral red solution--0.1 percent (Grand Island Biological Co., product no. 630-5330, or equivalent). Procure one 100-mL bottle. Sterilize by filtration through 0.22-micrometer filter.

(j) Bacto skim milk (Difco Laboratories, product no. 0032-01, or equivalent). Prepare 100 mL of Bacto skim milk in accordance with directions given by manufacturer.

(k) GIBCO bacteriological agar (Grand Island Biological Co., product no. M00010B, or equivalent).

(l) Antibiotics--penicillin G, dihydrostreptomycin sulfate, tetracycline and amphotericin B (Sigma Chemical Co., or equivalent). Use antibiotics of at least tissue culture grade. See Section 3.2.3 for preparation of stock antibiotic solutions.

(m) Ascorbic acid.

(n) Thioglycollate medium (Bacto dehydrated fluid thioglycollate medium, Difco Laboratories, or equivalent). Prepare 100 mL of thioglycollate medium in accordance with directions given by manufacturer.

(o) Nutrient agar (Difco Laboratories, or equivalent). Prepare 100 mL of nutrient agar and pour 5 mL aliquots into petri dishes for use in Section 3.4.2.

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(a.4) Dispense the penicillin-streptomycin stock in 10-mL volumes into screw-capped containers.

(b) Preparation of tetracycline stock solution. The procedure described is for preparation of ten 5-mL tetracycline stock solutions at concentrations of 0.125 g per 5 mL unit.

occurrence of contaminating organisms.

(c) Discard any media that lose clarity.

4. BIBLIOGRAPHY

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