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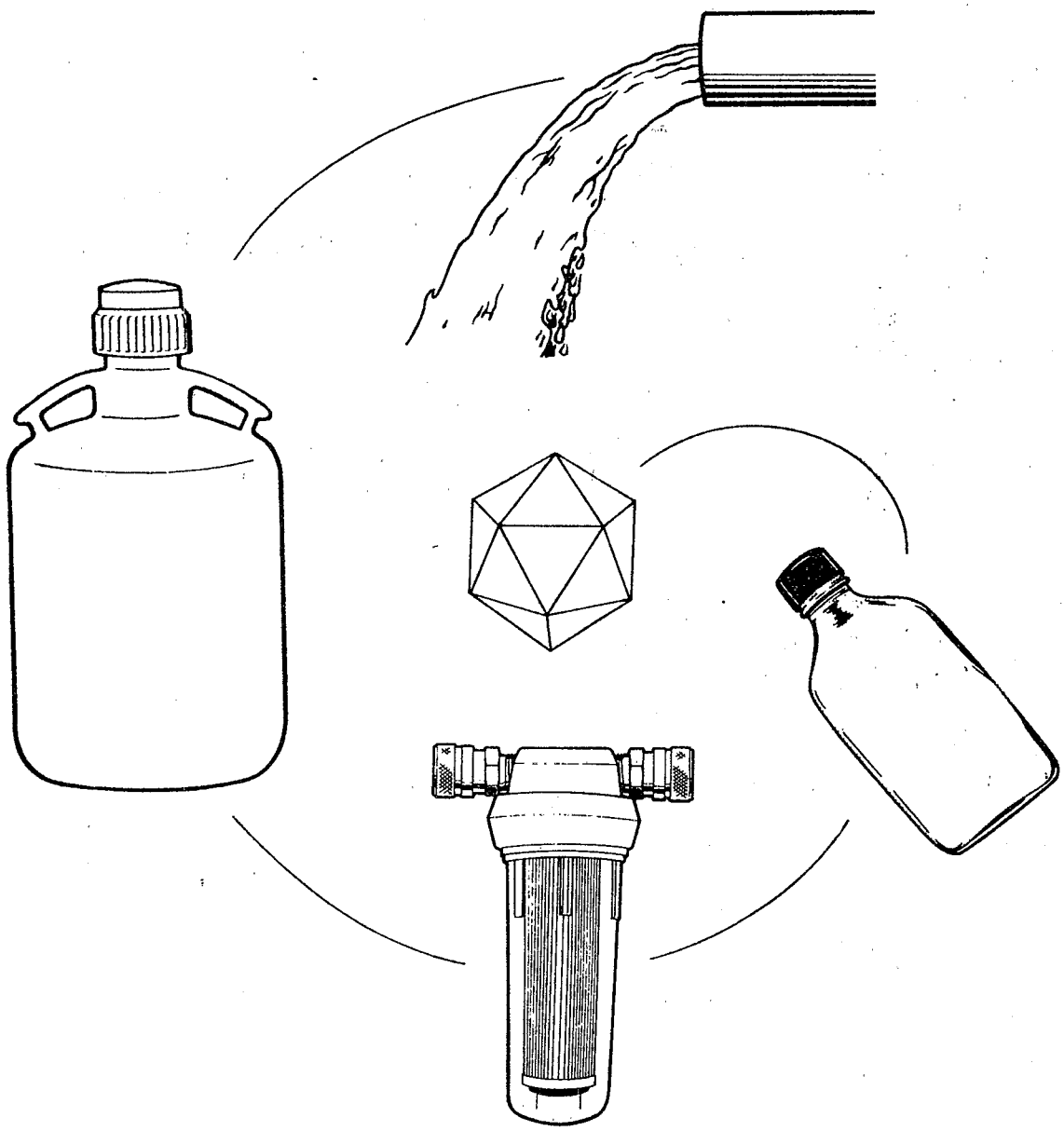

Chapter 10
Cell Culture Procedures for Assaying Plaque-Forming Viruses

1. Introduction

This chapter outlines procedures to detect waterborne viruses by use of the plaque assay system. Use of the method has been described by Dulbecco (1952), Dulbecco and Vogt (1954), Hsiung and Melnick (1955) and Dahling and Wright (1986). The principle of the system is based simply on allowing virus particles to adsorb to the host cells, adding agar medium to localize virus growth and a vital stain such as neutral red to enhance visualization of the cells. Localized lesions (plaques) developing some days after viral infection are then counted. These counts are reported as plaque forming units, whose number is proportionate to the amount of virus inoculated. A detailed method, verifying virus etiology of a plaque by "picking" followed by passage in cell culture monolayer with liquid medium overlay and then observing the monolayer for development of a cytopathic effect, is presented in the next chapter.

Chapter 10 is solely intended as guidance for the investigator who is preparing to isolate and enumerate waterborne viruses utilizing either the cell monolayer or the suspended cell plaque assay technique. The procedures outlined in this chapter are for use with the Buffalo green monkey (BGM) kidney cell line. The method may also be applied to the Madin and Darby bovine kidney (MDBK) cell line, which has been effectively used for the plaque assay of reoviruses, or when deemed preferable to other cell lines that may be used in the plaque assay of these animal viruses. However, it should be noted that waterborne viruses do not all form plaques.

Chapter 9, Section 7 provides the procedures for preparation of cell cultures used for virus assay in this chapter. Cells should be planted three to six days prior to their use in these analyses.

The detection methodology presented here has focused on laboratories with a small-scale virus assay requirement. Where the quantities of cell cultures, media and reagents set forth in this chapter are not sufficient for processing the test sample concentrates, the prescribed measures may be increased proportionately to meet the demands of more expansive test regimes.

2. Cell Monolayer Procedure

2.1 Sample Inoculation of Cell Monolayer for Virus Assay

2.1.1 Apparatus and materials.

(a) Glassware, Pyrex glass, clear (Corning Glass Works, or equivalent).
Storage vessel must be equipped with airtight closures.

(b) Cornwall syringe, or equivalent with cannula or syringe needle.
A pipetting device will expedite virus assay when large volumes of cells, media or reagents must be handled.

(c) Magnetic stirrer and stir bars.

(d) Waterbath set at 36.5 ± 1°C.

(e) Sterilizing filter—0.22-μm pore size with a 47-mm diameter to sterilize stock antibiotic solutions from Section 2.1.3 (Millipore Corp., GS series, or equivalent).
The 47-mm sterilizing filter may also be used for the verification of medium and reagent sterility in Section 2.4.

(f) Sterilizing filter—0.22-μm pore size with a 142-mm diameter to sterilize ELAH—Earle's base solution from Section 2.1.4 (Millipore Corp., GS series, or equivalent).
Where the volumes of media and reagents prepared have been for large-scale viral analyses, the use of a 293-mm diameter sterilizing filter may be more appropriate.

(g) Fiberglass prefilters for use with sterilizing filters (Millipore Corp., AP15 and AP20, or equivalent).
Stack AP20 and AP15 prefilters and 0.22-μm membrane filter into disc filter holder with AP20 prefilter on top and 0.22-μm membrane filter on bottom.

(h) Membrane filter apparatus for sterilization—47-mm diameter filter holder and 50-mL slip tip syringe (Millipore Corp., Swinnex filter, product no. SX0047000, or equivalent for filter holder only).
Disassemble Swinnex filter holder. Place membrane filter and prefilters in holder as instructed in Section 2.1.1, Step (g).

(i) Disc filter holders—142-mm or 293-mm diameter (Millipore Corp., or equivalent).
Use only pressure type filter holders. Place membrane filter and prefilters in holder as instructed in Section 2.1.1, Step (g).

(j) 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.

(k) Positively-charged cartridge filter—10-inch (Zeta plus TSM, product no. 45134-01-600P, AMF Cuno Division, or equivalent).

(l) Holder for cartridge filter with adaptor for 10-inch cartridge (type PL-1, product no. YY1601200, Millipore Corp., or equivalent).

(m) Culture capsule filter (product no. 12140, Gelman Sciences Inc., or equivalent).

(n) 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.

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

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

(q) Incubator capable of maintaining temperature of cell cultures at 36.5° ± 1°C.

2.1.2 Media and reagents.

(a) ELAH—Earle's base, with 0.5% 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-μm membrane filters.
Antibiotic stock solutions should be placed in screw-capped containers and stored at -20°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%) of penicilllin 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-μm membrane filter.

(a.4) Dispense the penicilllin-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-μm 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-μm 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 9-2 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 will provide sufficient media for at least sixty 6-oz glass bottles or ninety 25-cm^2 plastic flasks.

(b) Place a three-inch stir bar into a two-liter flask.

(c) Add contents of a 1-liter packet of ELAH—Earle's base reagent into flask.

(d) Add 890 mL of warmed deionized distilled water (50-60°C) to reagent in flask.

(e) Rinse medium packet with three washes of 20 mL each of warmed deionized distilled water and add to flask.
Note measure of deionized distilled water is 5% less than desired total volume of reagent.

(f) Mix on magnetic stirrer until reagent is completely dissolved.

(g) Add 2.25 g of $NaHCO_3$ to the dissolved reagent and continue mixing for an additional 15 min.

(h) Filter reagent under pressure through a disc filter stack.
This sterilizing step requires the use of prefilters in line before the final sterilizing filter. Prepare filter stack according to instructions in Section 2.1.1, Step (g).
As an alternative, use the cartridge prefilter described in Section 2.1.1, Step (k) and the capsule sterilizing filter in Section 2.1.1, Step (m).

(i) Sterility testing.
Test each lot of reagent to confirm sterility (see Section 2.4).

(j) Store sterilized reagent in tightly stoppered or capped container at 4°C.
Reagent may be stored for periods up to two months.

(k) Add 1 mL of penicillin-streptomycin stock, 0.5 mL of tetracycline stock and 0.2 mL of fungizone stock (Section 2.1.3) to ELAH—Earle's base solution immediately before washing of cells.

2.1.5 Procedure for inoculating test sample.
Cell cultures used for virus assay are generally found to be at their most sensitive level between the third and sixth days after initiation. Those older than seven days should not be used.

(a) Decant growth medium from previously prepared cell culture test vessels.
To prevent splatter, a gauze-covered beaker may be used to collect spent medium.
The medium is changed from one to four hours before cultures are to be inoculated and carefully decanted so as not to disturb the cell monolayer.

(b) Discard growth medium.

(c) Replace discarded medium with an equal volume of maintenance medium on day cultures are to be inoculated.
For BGM and MDBK cells use antibiotic supplemented ELAH—Earle's base solution from Section 2.1.4, Step (k). To reduce shock to cells, warm maintenance medium to 36.5 ± 1°C before placing on cell monolayer.
To prevent disturbing cells with the force of the liquid against the cell monolayer, add maintenance medium to the side of the cell culture test vessel opposite the cell monolayer.

(d) Identify cell culture test vessels by coding them with an indelible marker.

(e) Return cell culture test vessels to 36.5 ± 1°C incubator and hold vessel at the temperature until the cell monolayer is to be inoculated.

(f) Decant maintenance medium from cell culture test vessels.
Do not disturb the cell monolayer.

(g) Discard maintenance medium.

(h) Inoculate onto each cell monolayer a volume of test sample concentrate appropriate for the cell surface area of the cell culture test vessels used.
Inoculum volume should be no greater than 1 mL for each 40 cm² of surface area. Use Table 10-1 as a guide for inoculation size.
Avoid touching either the cannula or the pipetting device to the inside rim of the cell culture test vessels to avert the possibility of transporting contaminants to the remaining culture vessels.

(i) Rock inoculated cell culture test vessels gently to achieve uniform distribution of inoculum on surface of cell monolayers.

(j) Place cell culture test vessels on a level stationary surface at room temperature (22-25°C) so that the inoculum will remain distributed evenly over the cell monolayer.

(k) Incubate inoculated cell cultures at room temperature for 80 min to permit viruses to adsorb onto and infect cells.

(l) Proceed immediately to Section 2.2.

2.2 Agar Overlay Procedure for Plaque Assay
If there is a 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° ± 1°C.
Used for maintaining the temperature of the overlay medium.
See Section 2.2.5, Step (c).

Table 10-1. *Guide for Virus Inoculation, Suspended Cell Concentration and Overlay Volume of Agar Medium*

Vessel Type	*Volume of Virus Inoculum (mL)*	*Volume of Agar Overlay Medium (mL)*	*Total Numbers of Cells*
1-oz glass bottle*	*0.1*	*5*	*1×10^7*
25-cm² plastic flask	*0.1-0.5*	*10*	*2×10^7*
6-oz glass bottle	*0.5-1.0*	*20*	*4×10^7*
75-cm² plastic flask	*1.0-2.0*	*30*	*6×10^7*

**Size is given in oz only when it is commercially designated in that unit.*

(g) Waterbath set at 50° ± 1°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° ± 1°C.

(i) Sterilizing filter—0.22-μm 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-μm membrane filter into disc filter holder with AP20 prefilter on top and 0.22-μm membrane filter on bottom.

(k) Disc filter holders—47-mm, 142-mm or 293-mm diameter (Millipore Corp., or equivalent).
Use only pressure type filter holders. Place membrane filter and prefilters in holder as instructed in Section 2.2.1, Step (j).

(l) Positively-charged cartridge filter—10-inch (Zeta plus TSM, product no. 45134-01-600P, AMF Cuno Division, or equivalent).

(m) Holder for cartridge filter with adaptor for 10-inch cartridge (type PL-1), product no. YY1601200, Millipore Corp., or equivalent).

(n) Culture capsule filter (product no. 12140, Gelman Sciences, Inc., or equivalent).

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

2.2.2 Media and reagents.

(a) Medium 199 prepared at a 2X concentration with Earle's salts, 0.05% 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. H3375, or equivalent).
Prepare 50 mL of a 1 M solution of HEPES.

(c) GG-free newborn calf serum—heat inactivated at 56°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% solution.
Prepare 50 mL of a 7.5% solution of sodium bicarbonate. Sterilized by filtration through 0.22-μm filter.

(e) Magnesium chloride ($MgCl_2 \cdot 6H_2O$)—1.0% solution.
Prepare 50 mL of a 1.0% solution of magnesium chloride.

(f) Sodium chloride (NaCl).

(g) Sodium phosphate dibasic ($Na_2HPO_4 \cdot 7H_2O$).

(h) Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$).

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

(j) Hydrochloric acid (HCl)—1 M.
Prepare 100 mL of a 1M solution of hydrochloric acid.

(k) Sodium hydroxide (NaOH)—1 M.
Prepare 100 mL of a 1M solution of sodium hydroxide.

(l) Pancreatin 1X powder (ICN Nutritional Biochemicals, product no. 102557, or equivalent).

(m) Neutral red solution—0.1%, 100 mL volume (Grand Island Biological Co., product no. 630-5330, or equivalent).
Procure one 100-mL bottle.

(n) 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.

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

(p) Antibiotics—penicillin G, dihydrostreptomycin sulfate, tetracycline and amphotericin B (Sigma Chemical Co., or equivalent).
Use stock antibiotic solutions previously prepared in Section 2.1.3.

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

(r) Nutrient agar (Difco Laboratories, or equivalent).
Prepare 100 mL of nutrient agar and pour 5 mL aliquots into petri dishes for use in Section 2.4.2.

2.2.3 Preparation of Medium 199.

(a) Sources of cell culture media.
Commercially-prepared liquid cell culture media and medium components are available from several sources. Cell culture media can also be purchased in powder form that requires only dissolution in deionized distilled water and sterilization.
Media from commerical sources are quality controlled. However, media can also be prepared in the laboratory from chemicals. Such preparations are labor intensive and may be expensive but allow quality control of the process by the preparing laboratory.
The procedure described is for preparation of 500 mL of Medium 199 at a 2X concentration. This procedure will prepare sufficient medium for at least fifty 6-oz glass bottles or eighty 25-cm^2 plastic flasks.

(b) Place a three-inch stir bar into a one-liter flask.

(c) Add contents of a 1-liter packet of Medium 199 into flask.

(d) Add 355 mL of deionized distilled water to medium in flask.

(e) Rinse medium packet with three washes of 20 mL each of deionized distilled water and add to flask.
Note measure of deionized distilled water is 5% less than desired final volume of medium.

(f) Mix on magnetic stirrer until medium is completely dissolved.

(g) Filter reagent under pressure through a disc filter stack.
This sterilizing step requires the use of prefilters in line before the final sterilizing filter. Prepare filter stack according to instructions in Section 2.2.1, Step (j).
As an alternative, use cartridge prefilter described in Section 2.2.1, Step (l) and the capsule sterilizing filter in Section 2.2.1, Step (n).

(h) Sterility testing.
Test each lot of medium to confirm sterility before the lot is used for plaque assay procedure (see Section 2.4).

(i) Store sterilized medium in tightly stoppered or capped container at 4°C.
Medium may be stored for periods of up to two months.

2.2.4 Preparation of pancreatin solution for use in detecting reovirus.

(a) Add 0.03 g of $NaH_2PO_4 \cdot H_2O$ to a 250-mL flask.

(b) Add 100 mL of deionized distilled water to the flask.

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

(d) Add 0.05 g of $Na_2HPO_4 \cdot 7H_2O$ to a 250-mL flask.

(e) Add 100 mL of deionized distilled water to the flask.

(f) Mix contents of flask until reagent is dissolved.

(g) Sterilize NaH_2PO_4 and Na_2HPO_4 stock solutions in accordance with instructions given in Chapter 3, Section 2.1.

(h) Cool to room temperature (22°-25°C).

(i) Prepare phosphate buffer from the stock solutions by mixing 16 mL of NaH_2PO_4 with 84 mL of Na_2HPO_4 in a 250-mL flask.

(j) Place three-inch stir bar into a 2000-mL flask.

(k) Add 984 mL of deionized distilled water to flask.

(l) Place flask on magnetic stirrer and stir at a speed sufficient to develop vortex.

(m) Add 16 mL of phosphate buffer from Step (i) to the water.
Remaining phosphate buffer solution may be stored at 4°C until needed.

(n) Add 10 g of pancreatin from Section 2.2.2, Step (l) and 8.5 g of NaCl to the phosphate buffered water.

(o) Continue mixing contents of flask on magnetic stirrer until reagents are dissolved.

(p) Check pH of pancreatin solution and adjust to pH 7.5 if necessary.

(q) Filter solution under pressure through a disc filter stack.
This sterilizing step requires the use of prefilters in line before the final sterilizing filter. Prepare filter stack according to instructions in Section 2.2.1, Step (j).
As an alternative, use the cartridge prefilter described in Section 2.2.1, Step (l) and the capsule sterilizing filter in Section 2.2.1, Step (n).

(r) Sterility testing.
Test each lot to confirm sterility before the lot is used for reovirus recovery (see Section 2.4).

(s) Store sterilized pancreatin solution in tightly stoppered or capped container at -20°C.
Recommend solution be divided into 50-mL aliquots. Quantities prepared in Steps (a) through (s) are sufficient for at least fifty 6-oz glass bottles or eighty 25-cm^2 plastic flasks.
Solution may be stored for periods of up to six months.

2.2.5 Preparation of overlay medium for plaque assay.
The procedure described is for preparation of 100 mL of overlay medium and will prepare sufficient media for at least ten 6-oz glass bottles or twenty 25-oz plastic flasks when mixed with the agar prepared in Section 2.2.6.

(a) Add 79 mL of Medium 199 (2X concentration from Section 2.2.3, Step (i) and 4 mL of GG-free newborn calf serum from Section 2.2.2, Step (c) to a 250-mL flask.

(b) Add the stock solutions listed in Steps (b.1) through (b.7) to flask.
Start with Step (b.1) and add in the order listed, swirling to mix after each addition.

(b.1) 6 mL of $NaHCO_3$ from Section 2.2.2, Step (d).

(b.2) 2 mL of $MgCl_2$ from Section 2.2.2, Step (e).

(b.3) 3 mL of neutral solution from Section 2.2.2, Step (m).

(b.4) 4 mL of 1 M HEPES from Section 2.2.2, Step (b).

(b.5) 0.2 mL of penicillin-streptomycin stock from Section 2.1.3, Step (a).

(b.6) 0.1 mL of tetracycline stock from Section 2.1.3, Step (b).

(b.7) 0.04 mL of fungizone stock from Section 2.1.3, Step (c).
For preparation of overlay medium for reovirus recovery, the serum and the skim milk from Section 2.2.7, Step (a) are replaced with 2.8 mL of pancreatin solution and 3.2 mL of deionized distilled water.

(c) Place flask with overlay medium in waterbath set at 36° ± 1°C.

2.2.6 Preparation of overlay agar for plaque assay.

(a) Add 3 g of agar from Section 2.2.2, Step (o) to a 250-mL flask.

(b) Add 100 mL of deionized distilled water to flask.

(c) Sterilize agar in accordance with instructions given in Chapter 3, Section 2.1.

(d) Hold agar in waterbath set at 50° ± 1°C.

2.2.7 Preparation of agar overlay medium.

(a) Add 2 mL of skim milk from Section 2.2.2, Step (n) to overlay medium prepared in Section 2.2.5.

(b) Mix equal portions of overlay medium and agar by adding the medium to the agar flask.

To prevent solidification of the liquified agar, limit the portion of agar overlay medium mixed to that volume which can be dispensed in 10 min.

2.2.8 Addition of overlay agar to cell culture test vessels.

(a) To each cell culture test vessel, add the volume of warm (42-46°C) agar overlay medium appropriate for the cell surface area of the vessels used.

The volume of agar overlay medium that is appropriate for a particular virus assay culture vessel is listed in Table 10-1.

To prevent disturbing cells with the force of the liquid against the cell monolayer, add agar overlay medium to the side of the cell culture test vessel opposite the cell monolayer.

(b) Place cell culture test vessel, monolayer side down, on a level stationary surface at room temperature (22-25°C) so that the agar will remain distributed evenly before it solidifies.

Care must be taken to ensure that all caps on bottles and flasks are tight; otherwise, the gas seal will not be complete and an erroneous virus assay will result.

(c) Cover cell culture test vessels with a sheet of aluminum foil, a tightly woven cloth, or some other suitable cover to reduce light intensity and thus, prevent damage to the cell monolayer.

Agar is fully solidified within 30 min.

(d) After 30 min invert cell culture test vessels and incubate in the dark at 36.5° ± 1°C.

2.3 Counting Viral Plaques

2.3.1 Counting technique.

(a) Count, mark and record plaques in cell culture test vessels on days two, three, four, six, eight and twelve after adding the agar overlay medium.

Depending on the virus density and virus types present in the inoculated sample, rescheduling of virus counts at plus or minus one day may be deemed preferable. Virus titers are calculated from total count.

(b) Examine cell culture test vessels on day sixteen.

If no new plaques appear at 16 days, discard vessels; otherwise continue to count, mark and record plaques every two days until no new plaques appear between counts.

Note that most plaques will appear within 1 week.

2.3.2 Calculation of virus titer.

(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 ± 1°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° ± 1°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.

2.4.2 Procedure for verifying sterility of large volumes of liquids.

(a) Filter 50-100 mL of the liquid tested for sterility through a 47-mm diameter, 0.22-μm pore size membrane filter.

(b) Remove filter from its holder, and place filter on surface of solidified nutrient agar in a Petri dish.

Place filter face up on agar.

(c) Incubate Petri dish at 36.5° ± 1°C and examine filter surface daily for seven days to determine whether growth of contaminating organisms has occurred.

2.4.3 Visual evaluation of media for microbial contaminants.

(a) Incubate cell culture media that contain $NaHCO_3$ at 36.5° ± 1°C for at least one week prior to use.

(b) Visually examine the clarity of the culture media.

A clouded condition that develops in the media indicates the occurrence of contaminating organisms.

(c) Discard any media that lose clarity.

3. Suspended Cell Procedure

The suspended cell technique for enumeration of viruses from environmental samples has the advantage of detecting more viruses at a lower concentration than the cell monolayer procedure. The suspended cell technique also requires less time because plaque assay involves no prior planting of the cells or medium changes. Plaques are easier to pick because the cells are suspended within the agar layer and not on the surface of the bottle or flask where they must be scraped off, and the system is independent of the surface area of the plaque assay vessel. On the other hand, the suspended cell technique requires ten times more cells initially than the cell monolayer procedure. Suspended cells last only about one week in the agar overlay as compared to three weeks for cells used in the monolayer technique. Moreover, plaques in the suspended cell procedure are not as well defined and thus more difficult to count and because the addition of pancreatin in the overlay would inhibit or retard cell growth in this procedure, it cannot be used for reovirus detection.

Where low numbers of indigenous viruses are anticipated in a test sample (5 PFU or less per mL), the suspended cell procedure should be used by itself or together with the cell monolayer procedure. For assaying samples with high viral concentrations, the recommended method would be the cell monolayer procedure.

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

3.1 Sample Inoculation of Suspended Cells for Virus Assay

3.1.1 Apparatus and materials.

(a) Glassware, Pyrex glass, clear (Corning Glass Works, or equivalent).
Storage vessel must be equipped with airtight closures.

(b) Culture tubes, disposable, borosilicate glass—18 x 150 mm (Curtin Matheson Scientific, product no. 339-333, or equivalent).

(c) Culture tube caps, slip-on-type—18 mm diameter (VWR Scientific, product no. 60879-162, or equivalent).

(d) Test tube rack for culture tubes.

(e) Cornwall syringe, or equivalent with cannula or syringe needle.
A pipetting device will expedite virus assay when large volumes of cells, media or reagents must be handled.

(f) Magnetic stirrer and stir bars.

(g) Shaker, rocker platform style (Belco Glass, Inc., product no. 7740-20020, or equivalent).

(h) Incubator capable of maintaining temperature of cells at 36.5° ± 1°C.

3.1.2 Procedure for inoculating test sample.

(a) Arrange culture tubes in series.
Number of culture tubes required will be equal to the mL of sample to be tested.

(b) Place stir bar in flask.

(c) Add cell suspension from Chapter 9, Section 6.1.8 to flask.

(d) Dilute cell suspension to the appropriate cell concentration, while mixing on magnetic stirrer.
See Table 10-1 for cell concentration parameters. Dilute cell suspension with growth medium prepared in Chapter 9, Section 3.3.3.
Base cell dilution on results of cell counts performed in Chapter 9, Section 6.2

(e) Add 1 mL of diluted cell suspension to each tube.
A pipetting device will expedite processing when large cell volumes must be dispensed.

(f) Add into each tube a 1-mL volume of test sample concentrate.

(g) Swirl each tube gently to achieve a uniform mixture.

(h) Place inoculated tubes in rack.

(i) Place test tube rack on rocker platform shaker housed in a 36° ± 1°C incubator.

(j) Rock tubes at a rate of 16 oscillations per min for 60 min to permit viruses to adsorb onto and infect cells.
Complete preparation for agar overlay procedure during 60 min incubation period.

(k) Before cell culture test vessels are inoculated, identify vessels by coding them with an indelible marker.

3.2 Agar Overlay Procedure for Plaque Assay

3.2.1 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 disconnectors 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° ±1°C.
Used for maintaining the temperature of the overlay medium.
See Section 3.2.5, Step (c).

(g) Waterbath set at 50° ±1°C.
Used for maintaining agar temperature.
See Section 3.2.6, Step (d).

(h) Incubator capable of maintaining the temperatures of cell cultures at 36.5° ±1°C.

(i) Sterilizing filter—0.22-μm pore size with a 47-mm diameter to sterilize stock antibiotic solutions from Section 3.2.3 (Millipore

Corp., GS series, or equivalent).
The 47-mm sterilizing filter may also be used for the verification of medium and reagent sterility in Section 3.4.

(j) Sterilizing filter—0.22-μm pore size with a 142-mm diameter to sterilize Medium 199 from Section 3.2.4 (Millipore Corp., GS series, or equivalent).
Where the volumes of media prepared are for large-scale viral analyses, the use of a 293-mm diameter sterilizing filter may be more appropriate.

(k) Fiberglass prefilters for use with sterilizing filters (Millipore Corp., AP15 and AP20, or equivalent).
Stack AP20 and AP15 prefilters and 0.22-μm membrane filter into disc filter holder with AP20 prefilter on top and 0.22-μm membrane filter on bottom.

(l) Membrane filter apparatus for sterilization—47-mm diameter filter holder and 50-mL slip tip syringe (Millipore Corp., Swinnex filter, product no. SX0047000, or equivalent for filter holder only).
Disassemble Swinnex filter holder. Place membrane filter and prefilters in holder as instructed in Section 3.2.1, Step (k).

(m) Disc filter holders—142-mm or 293-mm diameter (Millipore Corp., or equivalent).
Use only pressure type filter holders. Place membrane filter and prefilters in holder as instructed in Section 3.2.1, Step (k).

(n) Positively-charged cartridge filter—10-inch (Zeta plus TSM, product no. 45134-01-600P, AMF Cuno Division, or equivalent).

(o) Holder for cartridge filter with adaptor for 10-inch cartridge (type PL-1, product no. YY1601200, Millipore Corp., or equivalent).

(p) Culture capsule filter (product no. 12140, Gelman Sciences, Inc., or equivalent).

(q) 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% 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°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% solution.
Prepare 50 mL of a 7.5% solution of sodium bicarbonate. Sterilized by filtration through 0.22-μm filter.

(e) Magnesium chloride ($MgCl_2 \cdot 6H_2O$)—1.0% solution.
Prepare 50 mL of a 1.0% 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% (Grand Island Biological Co., product no. 630-5330, or equivalent).
Procure one 100-mL bottle. Sterilize by filtration through 0.22-μm 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.

3.2.3 Procedure for preparaton of stock antibiotic solutions.
If not purchased in sterile form, stock antibiotic solutions must be filter-sterilized by the use of 0.22-μm membrane filters.
Antibiotic stock solutions should be placed in screw-capped containers and stored at –20°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%) 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-μm 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-μm 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-μm membrane filter.

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

3.2.4 Preparation of Medium 199.

(a) Sources of cell culture media.

Commercially-prepared liquid cell culture media and medium components are available from several sources. Cell culture media can also be purchased in powder form that requires only dissolution in deionized distilled water and sterilization.

Media from commercial sources are quality controlled. However, media can also be prepared in the laboratory from chemicals. Such preparations are labor intensive and may be expensive but allow quality control of the process at the level of the preparing laboratory. The procedure described is for preparation of 500 mL of Medium 199 at a 2X concentration. This procedure will prepare sufficient medium for at least fifty 6-oz glass bottles or eighty 25-cm² plastic flasks.

(b) Place a three-inch stir bar into a one-liter flask.

(c) Add contents of a 1-liter packet of Medium 199 into flask.

(d) Add 355 mL of deionized distilled water to medium in flask.

Note measure of deionized distilled water is 5% less than desired total volume of medium.

(e) Rinse medium packet with three washes of 20 mL each of deionized distilled water and add to flask.

(f) Mix on magnetic stirrer until medium is completely dissolved.

(g) Filter reagent under pressure through a disc filter stack.

This sterilizing step requires the use of prefilters in line before the final sterilizing filter. Prepare filter stack according to instructions in Section 3.2.1, Step (k).

As an alternative, use the cartridge prefilter described in Section 3.2.1, Step (n) and the capsule sterilizing filter in Section 3.2.1, Step (p).

(h) Sterility testing.

Test each lot of medium to confirm sterility before the lot is used for plaque assay procedure (see Section 3.4).

(i) Store sterilized medium in tightly stoppered or capped container at 4°C.

Medium may be stored for periods of up to two months.

3.2.5 Preparation of overlay medium for plaque assay.

The procedure described is for preparation of 100 mL of overlay medium and will provide sufficient medium for at least ten 6-oz glass bottles or twenty 25-cm² plastic flasks when mixed with the agar prepared in Section 3.2.6.

(a) Add 79 mL of Medium 199 (2X concentration) from Section 3.2.4, Step (i) and 4 mL of GG-free newborn calf serum from Section 3.2.2, Step (c) to a 250-mL flask.

(b) Add the stock solutions listed in Steps (b.1) through (b.7) to flask.

Start with Step (b.1) and add in the order listed, swirling to mix after each addition.

(b.1) 6 mL of $NaHCO_3$ from Section 3.2.2, Step (d).

(b.2) 2 mL of $MgCl_2$ from Section 3.2.2, Step (e).

(b.3) 3 mL of neutral red solution from Section 3.2.2, Step (i).

(b.4) 4 mL of 1 M HEPES from Section 3.2.2., Step (b).

(b.5) 0.2 mL of penicillin-streptomycin stock from Section 3.2.3, Step (a).

(b.6) 0.1 mL of tetracycline stock from Section 3.2.3, Step (b).

(b.7) 0.04 mL of fungizone stock from Section 3:2.3, Step (c).

(c) Place flask with overlay medium in waterbath set at 36° ± 1°C.

3.2.6 Preparation of overlay agar for plaque assay.

(a) Add 3 g of agar from Section 3.2.2, Step (k) to a 250-mL flask.

(b) Add 100 mL of deionized distilled water to flask.

(c) Sterilize agar in accordance with instructions given in Chapter 3, Section 2.1.

(d) Hold agar in waterbath set at 50° ± 1°C.

3.2.7 Preparation of agar overlay medium.

(a) Add 2 mL of skim milk from Section 3.2.2, Step (j) to overlay medium prepared in Section 3.2.5.

(b) Mix equal portions of overlay medium and agar by adding the medium to the agar flask.
To prevent solidification of the liquified agar, limit the portion of agar overlay medium mixed to that volume which can be dispensed in 10 min.

3.2.8 Procedure.

(a) Following incubation of test tubes in Section 3.1.2, Step (j), add to one tube at a time the volume of warm (42-44°C) agar overlay medium appropriate for the cell surface area of the cell culture test vessels to be used.
The volume of agar overlay medium that is appropriate for a particular virus assay culture vessel is listed in Table 10-1.

(b) Immediately after adding agar to tube, pour contents into cell culture test vessel.

(c) Tightly cap vessel.

(d) Swirl to mix contents of vessel.

(e) Place cell culture test vessel on a level stationary surface at room temperature (22-25°C) so that the contents will distribute evenly before it solidifies.
Care must be taken to ensure that all caps on bottles and flasks are tight; otherwise, the gas seal will not be complete and an erroneous virus assay will result.

(f) Cover cell culture test vessels with a sheet of aluminum foil, a tightly woven cloth, or some other suitable cover to reduce light intensity and thus, prevent damage to the cell monolayer.
Agar is fully solidified within 30 min.

(g) Invert cell culture test vessels and incubate in the dark at 36.5° ± 1°C.

3.3 Counting Viral Plaques

3.3.1 Technique.

(a) Mark and record plaque numbers at each counting period.
Virus titers are calculated from total count.

(b) Count on days two, three, four, five and seven after adding the agar overlay medium.
Cells that are suspended in the agar overlay degenerate more rapidly that those from an overlayed monolayer culture. Thus, scheduling plaque counts for longer than seven days is not recommended.
Depending on the virus density and virus types present in the inoculate sample, a rescheduling of viral counts at plus or minus one day may be deemed preferable within the seven-day counting period.

3.3.2 Virus enumeration.

(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.

3.4 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 on 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.

3.4.1 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°± 1°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.

3.4.2 Procedure for verifying sterility of large volumes of liquids.

(a) Filter 50-100 mL of the liquid tested for sterility through a 47-mm diameter, 0.22-μm pore size membrane filter.

(b) Remove filter from its holder, and place filter on surface of solidified nutrient agar in a Petri dish.
Place filter face up on agar.

(c) Incubate Petri dish at 36.5° ± 1°C and examine filter surface daily for seven days to determine whether growth of contaminating organisms has occurred.

3.4.3 Visual evaluation of media for microbial contaminants.

(a) Incubate cell culture media that contain $NaHCO_3$ at 36.5° ± 1°C for at least one week prior to use.

(b) Visually examine the clarity of the culture media.
A clouded condition that develops in the media indicates the

occurrence of contaminating organisms.

(c) Discard any media that lose clarity.

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