
Appendix H

Method for the Recovery and Assay of Total Culturable Viruses from Sludge

1. Introduction

1.1. Scope

This chapter describes the method that must be followed to produce Class A sludge when virus monitoring under 40 CFR Part 503 is required. The method is designed to demonstrate that sludges meet the requirement that human enteric viruses (i.e., viruses that are transmitted via the fecal-oral route) are less than one plaque-forming unit (PFU) per 4 g of total dry solids.

1.2. Significance

More than 100 different species of pathogenic human enteric viruses may be present in raw sludge. The presence of these viruses can cause hepatitis, gastroenteritis and numerous other diseases. Hepatitis A virus and noroviruses are the primary human viral pathogens of concern, but standard methods for their isolation and detection have not been developed. The method¹ detailed in this chapter detects total culturable viruses, which primarily include the human enteroviruses (e.g., polioviruses, coxsackieviruses, echoviruses) and reoviruses.

1.3. Safety

The sludges to be monitored may contain pathogenic human enteric viruses. Laboratories performing virus analyses are responsible for establishing an adequate safety plan and must decontaminate and dispose of wastes according to their safety plan and all applicable regulations. Aseptic techniques and sterile materials and apparatus must be used throughout the method.

2. Sample Collection

For each batch of sludge that must be tested for viruses, prepare a composite sample by collecting ten representative samples of 100 mL each (1,000 mL total) from different locations of a sludge pile or at different times from batch or continuous flow processes. Combine and mix thoroughly all representative samples for a composite. Batch samples that cannot be assayed within 24 hours of collection must be frozen at -70°C; otherwise, they should be held at 4°C until processed. If representative samples must be frozen before they can be combined, then thaw, combine and mix them thoroughly just prior to assay. Then remove a 50 mL portion from each composite sample for

solids determination as described in section 3. The remaining portion is held at 4°C while the solids determination is being performed or frozen for later processing if the assay cannot be initiated within 8 hours.

Freeze/thawing biosolids may result in some virus loss.

3. Determination of Total Dry Solids²

3.1. Weigh a dry weighing pan that has been held in a desiccator and is at a constant weight. Place the 50 mL sludge portion for solids determination into the pan and weigh again.

3.2. Place the pan and its contents into an oven maintained at 103-105°C for at least one hour.

3.3. Cool the sample to room temperature in a desiccator and weigh again.

3.4. Repeat the drying (1 h each), cooling and weighing steps until the loss in weight is no more than 4% of the previous weight.

3.5. Calculate the fraction of total dry solids (T) using the formula:

$$T = \frac{(A - C)}{(B - C)}$$

where A is the weight of the sample and dish after drying, B is the weight of the sample and dish before drying, and C is the weight of the dish. Record the fraction of dry solids (T) as a decimal (e.g., 0.04).

4. Total Culturable Virus Recovery from Sludge

4.1. Introduction

Total culturable viruses in sludge will primarily be associated with solids. Although the fraction of virus associated with the liquid portion will usually be small, this fraction may vary considerably with different sludge types. To correct for this variation, samples will first be treated to

¹Method D4994-89, ASTM (1992)

²Modified from EPA/600/4-84/013(R7), September 1989 Revision (section 3). This and other cited EPA publications may be requested from the Biohazard Assessment Research Branch, National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, USA 45268.

bind free virus to solids. Virus is then eluted from the solids and concentrated prior to assay.

4.2. Conditioning of Suspended Solids

Conditioning of sludge binds unadsorbed total culturable viruses present in the liquid matrix to the sludge solids.

Each analyzed composite sample (from the portion remaining after solids determination) must have an initial total dry solids content of at least 16 g. This amount is needed for positive controls and for storage of a portion of the sample at -70°C as a backup in case of procedural mistakes or sample cytotoxicity.

4.2.1 Preparation

(a) Apparatus and Materials

(a.1) Refrigerated centrifuge capable of attaining 10,000 ×g and screw-capped centrifuge bottles with 100 to 1000 mL capacity.

Each bottle must be rated for the relevant centrifugal force.

(a.2) A pH meter with an accuracy of at least 0.1 pH unit, equipped with a combination-type electrode.

(a.3) Magnetic stirrer and stir bars.

(b) Media and Reagents

Analytical Reagent or ACS grade chemicals (unless specified otherwise) and deionized, distilled water (dH₂O) should be used to prepare all reagents. All water used must have a resistance of greater than 0.5 megohms-cm, but water with a resistance of 18 megohms-cm is preferred.

(b.1) Hydrochloric acid (HCl) — 1 and 5 M.

Mix 10 or 50 mL of concentrated HCl with 90 or 50 mL of dH₂O, respectively.

(b.2) Aluminum chloride (AlCl₃ · 6H₂O) — 0.05 M.

Dissolve 12.07 g of aluminum chloride in a final volume of 1000 mL of dH₂O. Autoclave at 121°C for 15 minutes.

(b.3) Sodium hydroxide (NaOH) — 1 and 5 M.

Dissolve 4 or 20 g of sodium hydroxide in a final volume of 100 mL of dH₂O, respectively.

(b.4) Beef extract (Difco Product No. 0115-17-3 or equivalent).

Prepare buffered 10% beef extract by dissolving 10 g beef extract, 1.34 g Na₂HPO₄ · 7H₂O and 0.12 g citric acid in 100 mL of dH₂O. The pH should be about 7.0. Dissolve by stirring on a magnetic stirrer. Autoclave for 15 minutes at 121°C.

Do not use paste beef extract (Difco Laboratories Product No. 0126) for virus elution. This beef extract tends to elute cytotoxic materials from sludges.

(b.5) HOCl — 0.1%

Add 19 mL of household bleach (Clorox, The Clorox Co., or equivalent) to 981 mL of dH₂O and adjust the pH of the solution to 6-7 with 1 M HCl.

(b.6) Thiosulfate — 2% and 0.02%

Prepare a stock solution of 2% thiosulfate by dissolving 20 g of thiosulfate in a total of 1 liter of dH₂O. Sterilize the solution by autoclaving at 121°C for 15 minutes. Prepare a working solution of 0.02% thiosulfate just prior to use by mixing 1 mL of 2% thiosulfate with 99 mL of sterile dH₂O.

4.2.2 Conditioning Procedure

Figure 1 gives a flow diagram for the procedure to condition suspended solids.

(a) Calculate the amount of sample to condition.

Use a graduated cylinder to measure the volume. If the volumes needed are not multiples of 100 mL (100, 200, 300 mL, etc.), add sterile water to bring the volume to the next multiple of 100 mL. Each sample should then be aliquoted into 100 mL portions before proceeding. Samples must be mixed vigorously just before aliquoting because solids begin to settle out as soon as the mixing stops. Each aliquot should be placed into a 250 mL beaker containing a stir bar.

CAUTION: Always avoid the formation of aerosols by slowly pouring samples down the sides of vessels.

(a.1) Calculate the amount needed to measure the endogenous total culturable virus in a composite sludge sample using the formula:

$$X_{ts} = \frac{12}{T}$$

where X_{ts} equals the milliliters of sample required to obtain 12 g of total solids and T equals the fraction of total dry solids (from section 3).³

(a.2) Calculate the amount needed for a recovery control for each sludge composite from the formula:

$$X_{pc} = \frac{4}{T}$$

where X_{pc} equals the milliliters of sample required to obtain 4 g of total solids.

Add 400 plaque forming units (PFU) of a Sabin poliovirus stock to the recovery control sample. Use a virus stock that has been filtered through a 0.2 μm filter (see Section 4.3.1) prior to assay to remove clumped virus particles.

(a.3) Place 30 mL of 10% buffered beef extract and 70 mL of dH₂O into a 250 mL beaker with stir bar to serve as a negative process control.

(a.4) Freeze any remaining composite sample at -70°C for backup purposes.

³This formula is based upon the assumption that the density of the liquid in sludge is 1 g/mL. If the fraction of total dry solids is too low (e.g., less than 0.02), then the volume of sludge collected must be increased.

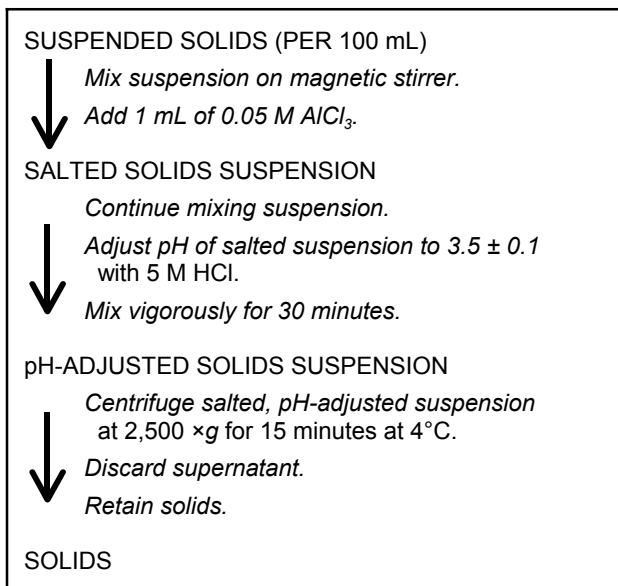


Figure 1. Flow diagram of method for conditioning suspended solids

(b) Perform the following steps on each 100 mL aliquot from steps 4.2.2a.1 to 4.2.2a.3.

(b.1) Place the beaker on a magnetic stirrer, cover loosely with aluminum foil, and stir at a speed sufficient to develop vortex. Add 1 mL of 0.05 M AlCl_3 to the mixing aliquot.

The final concentration of AlCl_3 in each aliquot is approximately 0.0005 M.

(b.2) Place a combination-type pH electrode into the mixing aliquot. Adjust the pH of the aliquot to 3.5 ± 0.1 with 5 M HCl. Continue mixing for 30 minutes.

The pH meter must be standardized at pH 7 and 4. When solids adhere to an electrode, clean it by moving up and down gently in the mixing aliquot.

After adjusting the pH of each sample, rinse the electrode with dH_2O and sterilize it with 0.1% HOCl for five minutes. Neutralize the HOCl by submerging the electrode in sterile 0.02% thiosulfate for one to five minutes.

The pH of the aliquot should be checked at frequent intervals. If the pH drifts up, readjust it to 3.5 ± 0.1 with 5 M HCl. If the pH drifts down, readjust it with 5 M NaOH. Use 1 M acid or base for small adjustments. Do not allow the pH to drop below 3.4.

(b.3) Pour the conditioned aliquot into a centrifuge bottle and centrifuge at 2,500 $\times g$ for 15 minutes at 4°C.

To prevent the transfer of the stir bar into the centrifuge bottle when decanting the aliquot, hold another stir bar or magnet against the bottom of the beaker. Solids that adhere to the stir bar in the beaker may be removed by manipulation with a pipette. It may be necessary to pour the aliquot back and forth several times from the centrifuge

bottle to the beaker to obtain all the solids in the bottle. If a large enough centrifuge bottle is available, the test sample aliquots may be combined into a single bottle at this step. If there is more than one recovery control aliquot, they may also be combined into another centrifuge bottle.

(b.4) Decant the supernatant into a beaker and discard. Replace the cap onto the centrifuge bottle. Elute the solids by following the procedure described in section 4.3.

4.3. Elution of Viruses from Solids

4.3.1 Apparatus and Materials

In this and following sections only apparatus and materials which have not been described in previous sections are listed.

(a) Membrane filter apparatus for sterilization — 47 mm diameter Swinnex filter holder and 60 mL slip-tip syringe (Millipore Corp. Product No. SX00 047 00 and Becton Dickinson Product No. 1627 or equivalent).

(b) Disc filters, 47 mm diameter — 3.0, 0.45, and 0.2 μm pore size filters (Mentec America, Filterite Div., Duo- Fine series, Product No. 8025-030, 8025-034 and 8025-037 or equivalent). Filters may be cut to the proper diameter from sheet filters.

Disassemble a Swinnex filter holder. Place the filter with a 0.2 μm pore size on the support screen of the filter holder and stack the remaining filters on top in order of increasing pore size. Reassemble and tighten filter holder. Wrap filter stack in foil and sterilize by autoclaving at 121°C for 15 min.

Filters stacked in tandem as described tend to clog more slowly when turbid material is filtered through them. Prepare several filter stacks.

4.3.2 Elution Procedure

A flow diagram of the virus elution procedure is given in Figure 2.

(a) Place a stir bar and 100 mL of buffered 10% beef extract into the centrifuge bottle containing the solids (from section 4.2.2b.4).

If the test and control samples are divided into more than one centrifuge bottles, the solids should be combined at this step.

Place the centrifuge bottle on a magnetic stirrer, and stir at a speed sufficient to develop a vortex for 30 min at room temperature.

To minimize foaming (which may inactivate viruses), do not mix faster than necessary to develop vortex.

(b) Remove the stir bar from each bottle with a long sterile forceps or a magnet retriever and centrifuge the solids-eluate mixture at 10,000 $\times g$ for 30 minutes at 4°C. Decant supernatant fluid (eluate) into a beaker and discard the solids.

Determine if the centrifuge bottle is appropriate for the centrifugal force that will be applied.

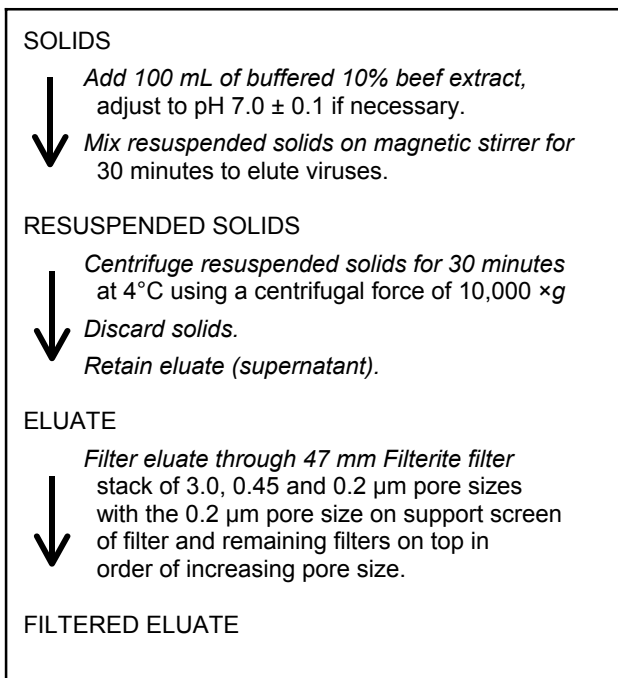


Figure 2. Flow diagram of method for elution of virus from solids.

Centrifugation at 10,000 ×g is normally required to clarify the sludge samples sufficiently to force the resulting supernatant through the filter stacks.

(c) Place a filter holder that contains filter stacks (from section 4.3.1b) onto a 250 mL Erlenmeyer receiving flask. Load 50 mL syringes with the supernatants from step 4.3.2c. Place the tip of the syringe into the filter holder and force the supernatant through the filter stacks into 250 mL receiving flasks.

Prior to use, pass 15 mL of 3% beef extract through each filter holder to minimize non-specific adsorption of viruses. Prepare 3% beef extract by mixing 4.5 mL of 10% beef extract and 10.5 mL of dH₂O. Take care not to break off the tip of the syringe and to minimize pressure on the receiving flask because such pressure may crack or topple the flask. If the filter stack begins to clog badly, empty the loaded syringe into the beaker containing unfiltered eluate, fill the syringe with air, and inject air into filter stack to force residual eluate from the filters. Continue the filtration procedure with another filter holder and filter stack. Discard contaminated filter holders and filter stacks. This procedure may be repeated as often as necessary to filter the entire volume of supernatant. Disassemble each filter holder and examine the bottom 0.2 μm filters to be certain they have not ruptured. If a bottom filter has ruptured, repeat the step with new filter holders and filter stacks.

Proceed immediately to section 4.4.

4.4. Organic Flocculation

This organic flocculation concentration procedure (Katzenelson et al., 1976) is used to reduce the number of cell cultures needed for assays by concentrating total cul-

ture viruses in the eluate. The step significantly reduces costs associated with labor and materials.

Floc formation capacity of the beef extract reagent must be pretested. Because some beef extract lots may not produce sufficient floc, each new lot must be pretested to determine virus recovery. This may be performed by spiking 100 mL of dH₂O with a known amount of poliovirus in the presence of a 47 mm nitrocellulose filter. This sample should be conditioned using section 4.2 above to bind virus to the filter. Virus should then be eluted from the filter using the procedure in section 4.3, and concentrated and assayed using the following procedures. Any lot of beef extract not giving a overall recovery of at least 50% should not be used.

4.4.1 Media and Reagents

In this and following sections only media and reagents which have not been described in previous sections are listed.

(a) Sodium phosphate, dibasic (Na₂HPO₄ · 7H₂O) — 0.15 M.

Dissolve 40.2 g of sodium phosphate in a final volume of 1000 mL. Autoclave at 121°C for 15 minutes.

4.4.2 Virus Concentration Procedure

A flow diagram for the virus concentration procedure is given in Figure 3.

(a) Pour the filtered eluates from the test sample, recovery control and negative process control from section 4.3.2d into graduated cylinders, and record their volumes. Transfer the samples into separate 600 mL beakers and cover them loosely with aluminum foil.

(b) For every 3 mL of beef extract eluate, add 7 mL of dH₂O to the 600 mL beakers. Add stir bars to each beaker.

The concentration of beef extract is now 3%. This dilution is necessary because 10% beef extract often does not process well by the organic flocculation concentration procedure.

(c) Record the total volume of the diluted eluates. Place the beakers onto a magnetic stirrer, cover loosely with aluminum foil, and stir at a speed sufficient to develop vortex.

To minimize foaming (which may inactivate viruses), do not mix faster than necessary to develop vortex.

(d) For each diluted, filtered beef extract, insert a sterile combination-type pH electrode and then add 1 M HCl slowly until the pH of the extract reaches 3.5 ± 0.1. Continue to stir for 30 minutes at room temperature.

The pH meter must be standardized at pH 4 and 7. Sterilize the electrode by treating it with 0.1% HOCl for five minutes. Neutralize the HOCl by treating the electrode with 0.02% sterile thiosulfate for one to five minutes.

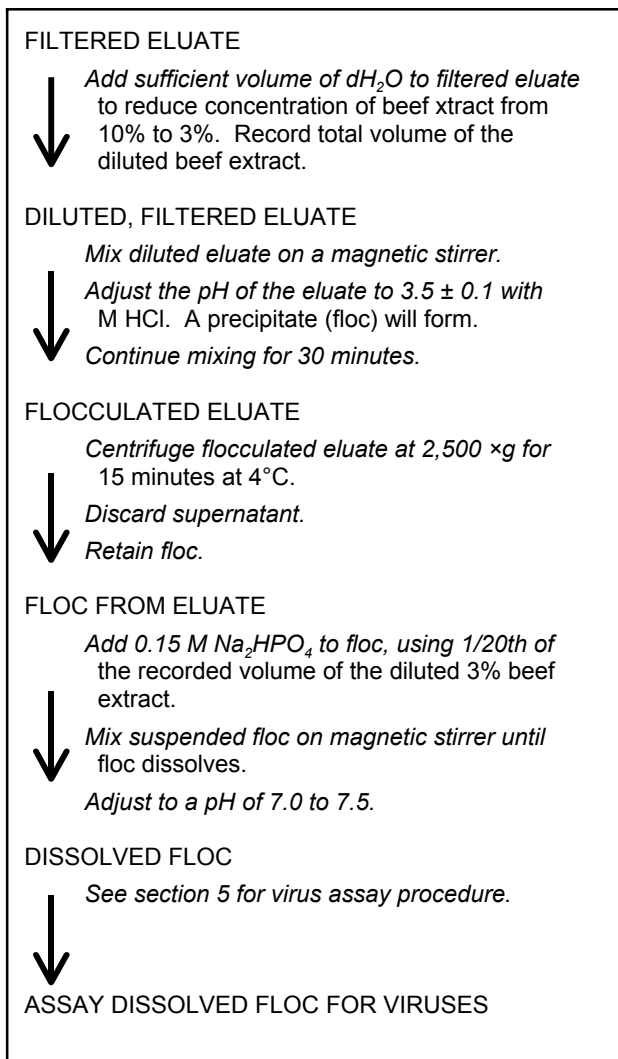


Figure 3. Flow diagram of method for concentration of viruses from beef extract eluate.

A precipitate will form. If the pH is accidentally reduced below 3.4, add 1 M NaOH until it reaches 3.5 ± 0.1. Avoid reducing the pH below 3.4 because some inactivation of virus may occur.

(e) Pour the contents of each beaker into 1,000 mL centrifuge bottles. Centrifuge the precipitated beef extract suspensions at 2,500 ×g for 15 minutes at 4°C. Pour off and discard the supernatants.

To prevent the transfer of the stir bar into a centrifuge bottle, hold another stir bar or magnet against bottom of the beaker when decanting contents.

(f) Place stir bars into the centrifuge bottles that contains the precipitates. To each, add a volume of 0.15 M Na₂HPO₄ · 7H₂O equal to exactly 1/20 of the volume recorded in step 4.4.2c. If the precipitate from a sample is in more than one bottle, divide the 1/20th volume equally among the centrifuge bottles containing that sample. Place the bottles

onto a magnetic stirrer, and stir slowly until the precipitates have dissolved completely.

Support the bottles as necessary to prevent toppling. Avoid foaming which may inactivate or aerosolize viruses. The precipitates may be partially dissipated with sterile spatulas before or during the stirring procedure.

(g) Measure the pH of the dissolved precipitates.

If the pH is above or below 7.0-7.5, adjust to that range with either 1 M HCl or 1 M NaOH.

(h) Freeze exactly one half of the dissolved precipitate test sample (but not the positive and negative controls) at -70°C. This sample will be held as a backup to use should the sample prove to be cytotoxic. Record the remaining test sample volume (this volume represents 6 g of total dry solids). Refrigerate the remaining samples immediately at 4°C until assayed in accordance with the instructions given in section 5 below.

If the virus assay cannot be undertaken within 24 hours, store the remaining samples at -70°C.

5. Assay for Plaque-forming Viruses⁴

5.1. Introduction

This section outlines procedures for the detection of viruses in sludge by use of the plaque assay system. The system uses an agar medium to localize virus growth following attachment of infectious virus particles to a cell monolayer. Localized lesions of dead cells (plaques) developing some days after viral infection are visualized with the vital stain, neutral red, which stains only live cells. The number of circular unstained plaques are counted and reported as plaque forming units, whose number is proportional to the amount of infectious virus particles inoculated.

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

5.2. Plaque Assay Procedure

5.2.1 Apparatus and materials.

(a) Waterbath set at 50 ± 1°C.

Used for maintaining the agar temperature (see section 5.2.2j).

5.2.2 Media and Reagents.

(a) ELAH — 0.65% lactalbumin hydrolysate in Earle's base.

Dissolve 6.5 g of tissue culture, highly soluble grade lactalbumin hydrolysate (Gibco BRL Product No. 11800 or

⁴Modified from EPA/600/4-84/013(R11), March 1988 Revision

equivalent) in 1 L of Earle's base (Gibco BRL Product No. 14015 or equivalent) prewarmed to 50-60°C. Sterilize ELAH through a 0.22 µm filter stack and store for up to two months at 4°C.

(b) Wash medium — Add 1 mL of penicillin-streptomycin stock (see section 6.4.2e.1 for preparation of antibiotic stocks), 0.5 mL of tetracycline stock and 0.2 mL of fungizone stock per liter to ELAH immediately before washing of cells.

(c) HEPES — 1 M (Sigma Chemical Product No. H-3375 or equivalent).

Prepare 50 mL of a 1 M solution by dissolving 11.92 g of HEPES in a final volume of 50 mL dH₂O. Sterilize by autoclaving at 121°C for 15 min.

(d) Sodium bicarbonate (NaHCO₃) — 7.5% solution.

Prepare 50 mL of a 7.5% solution by dissolving 3.75 g of sodium bicarbonate in a final volume of 50 mL dH₂O. Sterilize by filtration through a 0.22 µm filter.

(e) Magnesium chloride (MgCl₂ · 6H₂O) — 1.0% solution.

Prepare 50 mL of a 1.0% solution by dissolving 0.5 g of magnesium chloride in a final volume of 50 mL dH₂O. Sterilize by autoclaving at 121°C for 15 min.

(f) Neutral red solution — 0.333%, 100 mL volume (GIBCO BRL Product No. 630-5330 or equivalent).

Procure one 100 mL bottle.

Some neutral red solutions are cytotoxic. All new solutions should be tested prior to their use for assaying sludge samples. Testing may be performed by assaying a stock of poliovirus with known titer using this plaque assay procedure.

(g) Bacto skim milk (Difco Laboratories Product No. 0032-01 or equivalent).

Prepare 100 mL of 10% skim milk in accordance with directions given by manufacturer.

(h) Preparation of Medium 199.

The procedure described is for preparation of 500 mL of Medium 199 (GIBCO BRL Product No. 400-1100 or equivalent) at a 2X concentration. This procedure will prepare sufficient medium for at least fifty 6 oz glass bottles or eighty 25 cm² plastic flasks.

(h.1) Place a three inch stir bar into a one liter flask. Add the contents of a 1 liter packet of Medium 199 into the flask. Add 355 mL of dH₂O. Rinse medium packet with three washes of 20 mL each of dH₂O and add the washes to the flask.

Note that the amount of dH₂O is 5% less than desired final volume of medium.

(h.2) Mix on a magnetic stirrer until the medium is completely dissolved. Filter the reagent under pressure through a filter stack (see section 6.2.6).

Test each lot of medium to confirm sterility before the lot is used (see section 6.5). Each batch may be stored for two months at 4°C.

(i) 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 5.2.2j.

(i.1) Add 79 mL of Medium 199 (2X concentration) and 4 mL of serum to a 250 mL flask.

(i.2) Add the following to the flask in the order listed, with swirling after each addition: 6 mL of 7.5% NaHCO₃, 2 mL of 1% MgCl₂, 3 mL of 0.333% neutral red solution, 4 mL of 1 M HEPES, 0.2 mL of penicillin-streptomycin stock (see section 6.4.2e for a description of antibiotic stocks), 0.1 mL of tetracycline stock, and 0.04 mL of fungizone stock.

(i.3) Place flask with overlay medium in waterbath set at 36 ± 1°C.

(j) Preparation of overlay agar for plaque assay.

(j.1) Add 3 g of agar (Sigma Chemical Product No. A-9915 or equivalent) and 100 mL of dH₂O to a 250 mL flask. Melt by sterilizing the agar solution in an autoclave at 121°C for 15 min.

(j.2) Cool the agar to 50°C in waterbath set at 50 ± 1°C.

(k) Preparation of agar overlay medium.

(k.1) Add 2 mL of 10% skim milk to overlay medium prepared in section 5.2.2i.

(k.2) 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.

5.2.3 Procedure for Inoculating Test Samples.

Section 6.6 provides the procedures for the preparation of cell cultures used for the virus assay in this section.

BGM 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 or which are not 100% confluent should not be used.

(a) Decant and discard the 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) Replace discarded medium with an equal volume of wash medium (from section 5.2.2b) on the day the cultures are to be inoculated.

Table 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 Number of Cells
1 oz glass bottle ¹	0.1	5	1×10^7
25 cm ² plastic flask	0.1-0.5	10	2×10^7
6 oz glass bottle	0.5-1.0	20	4×10^7
75 cm ² plastic flask	1.0-2.0	30	6×10^7

¹Size is given in oz only when it is commercially designated in that unit.

To reduce shock to cells, prewarm the wash medium to $36.5 \pm 1^\circ\text{C}$ before placing it onto the cell monolayer.

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

(c) Identify cell culture test vessels by coding them with an indelible marker. Return the cell culture test vessels to a $36.5 \pm 1^\circ\text{C}$ incubator and hold at that temperature until the cell monolayers are to be inoculated.

(d) Decant and discard the wash medium from cell culture test vessels.

Do not disturb the cell monolayer.

(e) Inoculate BGM cultures with the test sample and positive and negative process control samples from section 4.4.2h. Divide each sample onto a sufficient number of BGM cultures to ensure that the inoculum volume is no greater than 1 mL for each 40 cm² of surface area. Use Table 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.

If the samples are frozen, thaw them rapidly by placing them in warm water. Samples should be shaken during the thawing process and removed from the warm water as soon as the last ice crystals have dissolved.

(e.1) Inoculate BGM cultures with the entire negative process control sample using an inoculum volume per vessel that is appropriate for the vessel size used.

(e.2) Inoculate two BGM cultures with an appropriate volume of 0.15 M Na₂HPO₄ · 7H₂O preadjusted to pH 7.0-7.5 and seeded with 20-40 PFU of poliovirus. These cultures will serve as a culture sensitivity control.

(e.3) Remove a volume of the test sample concentrate exactly equal to 1/6th (i.e., 1 g of total dry solids) of the volume recorded in section 4.4.2h. Seed this subsample with 20-40 PFU of poliovirus. Inoculate the subsample onto one or more BGM cultures using a inoculum volume per vessel that is appropriate for the vessel size used. These cultures will serve as controls for cytotoxicity (see section 5.2.5b).

(e.4) Inoculate BGM cultures with the entire recovery control sample using an inoculum volume per vessel that is appropriate for the vessel size used.

(e.5) Record the volume of the remaining 5/6th portion of the test sample. This remaining portion represents a total dry solids content of 5 g. Inoculate the entire remaining portion (even if diluted to reduce cytotoxicity) onto BGM cultures using an inoculum volume per vessel that is appropriate for the vessel size used. Inoculation of the entire volume is necessary to demonstrate a virus density level of less than 1 PFU per 4 g total dry solids.

(f) Rock the inoculated cell culture test vessels gently to achieve uniform distribution of inoculum over the surface of the cell monolayers. Place the 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.

(g) Incubate the inoculated cell cultures at room temperature for 80 min to permit viruses to adsorb onto and infect cells and then proceed immediately to section 5.2.4.

It may be necessary to rock the vessels every 15-20 min during the 80 min incubation to prevent cell death in the middle of the vessels from dehydration.

5.2.4 Procedure for Overlaying Inoculated Cultures with Agar.

If there is a likelihood that a test sample will be toxic to cell cultures, the cell monolayer should be treated in accordance with the method described in section 5.2.5b.

(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 (see Table 1).

The preparation of the overlay agar and the agar overlay medium must be made far enough in advance so that they will be at the right temperature for mixing at the end of the 80 min inoculation period.

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

(b) Place cell culture test vessels, monolayer side down, on a level stationary surface at room temperature (22-25°C) so that the agar will remain evenly distributed as it solidifies. Cover the vessels with a sheet of aluminum foil, a tightly woven cloth, or some other suitable cover to reduce the light intensity during solidification and incubation. Neutral red can damage or kill tissue culture cells by light-induced crosslinking of nucleic acids.

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.

Agar will fully solidify within 30 min.

- (c) After 30 min, invert the cell culture test vessels and incubate them covered in the dark at $36.5 \pm 1^\circ\text{C}$.

5.2.5 Plaque Counting Technique.

- (a) Count, mark and record plaques in cell culture test vessels on days one, two, three, four after adding the agar overlay medium. Plaques should be counted quickly using a lightbox (Baxter Product No. B5080-1 or equivalent) in a darkened room. Most plaques should appear within 1 week.

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 necessary. Virus titers are calculated from the total plaque count. Note that not all plaques will be caused by viruses.

- (b) Determine if samples are cytotoxic by macroscopic examination of the appearance of the cell culture monolayer (compare negative, positive and recovery controls from section 5.2.3e with seeded and unseeded test samples) after one to four days of incubation at $36.5 \pm 1^\circ\text{C}$. Samples show cytotoxicity if cell death is observed on test and recovery control samples prior to its development on positive controls. Cytotoxicity should be suspected when the agar color is more subdued, generally yellow to yellow-brown. This change in color results in a mottled or blotchy appearance instead of the evenly diffused "reddish" color observed in "healthy" cell monolayers. Cytotoxicity may also cause viral plaques to be reduced in number or to be difficult to distinguish from the surrounding monolayer. To determine if this type of cytotoxicity is occurring, compare the two types of positive controls (section 5.2.3e). If samples are cytotoxic, do not proceed to the next steps. Re-assay a small amount of the remaining sample using 1:2, 1:4 and 1:8 dilutions. Then re-assay the remaining sample as specified in section 5.2.3 using the dilution which removes cytotoxicity and the specified number of flasks times the reciprocal of the dilution.

A small amount of sample may be tested for cytotoxicity prior to a full assay.

- (c) Examine cell culture test vessels as in step 5.2.5a on days six, eight, twelve and sixteen.

If no new plaques appear at 16 days, proceed with step 5.2.6; otherwise continue to count, mark and record plaques every two days until no new plaques appear between counts and then proceed with step 5.2.6.

Inoculated cultures should always be compared to uninoculated control cultures so that the deterioration of the cell monolayers is not recorded as plaques. If experience shows that cultures start to deteriorate prior to 16 days, a second layer of agar can be added after 7 days as described in section 5.2.4.

If negative process controls develop plaques or if positive controls fail to develop plaques, stop all assays until the source of the problem is corrected.

Samples giving plaque counts that are greater than 2 plaques per cm^2 should be diluted and replated.

5.2.6 Virus Plaque Confirmation Procedure

The presence of virus in plaques must be confirmed for all plaques obtained from sludge samples. Where more than ten plaques are observed, it is allowable to confirm at least ten well-separated plaques per sample or 10% of the plaques in a sample, whichever is greater. Flasks may be discarded after samples are taken for plaque confirmation.

- (a) Apparatus, Materials and Reagents

- (a.1) Pasteur pipettes, disposable, cotton plugged — 229 mm (9 inches) tube length and rubber bulb — 1 mL capacity.

Flame each pipette gently about 2 cm from end of the tip until the tip bends to an approximate angle of 45° . Place the pipettes into a 4 liter beaker covered with aluminum foil and sterilize by autoclaving or by dry heat.

- (a.2) 16 x 150 mm cell culture tubes containing BGM cells.

See section 6.6 for the preparation of cell culture tubes.

- (a.3) Tissue culture roller apparatus — 1/5 rpm speed (New Brunswick Scientific Product No. TC-1 or equivalent) with culture tube drum for use with roller apparatus (New Brunswick Scientific Product No. ATC-TT16 or equivalent).

- (a.4) Freezer vial, screw-capped (with rubber insert) or cryogenic vial — 0.5-1 dram capacity.

- (b) Procedure for obtaining viruses from plaque.

In addition to plaques from sludge samples, perform the procedure on at least three negative regions of negative process control flasks and at least three plaques from positive control flasks.

- (b.1) Place a rubber bulb onto the upper end of a cotton-plugged Pasteur pipette and then remove the screw-cap or stopper from a plaque bottle.

- (b.2) Squeeze the rubber bulb on the Pasteur pipette to expel the air and penetrate the agar directly over the edge of a plaque with the tip of the pipette. Gently force the tip of the pipette through the agar to the surface of the vessel, and scrape some of the cells from the edge of the plaque.

Repeatedly scratch the surface and use gentle suction to insure that virus-cell-agar plug enters the pipette.

- (b.3) Remove the pipette from the plaque bottle and tightly replace the cap or stopper.

- (c) Procedure for inoculating cell cultures with agar plugs from negative control samples and from plaques.

- (c.1) Prepare plaque conformation maintenance medium by adding 5 mL of serum and 5 mL of dH_2O per 90 mL of wash medium (section 5.2.2b) on day samples are to be tested.

- (c.2) Pour the spent medium from cell culture tubes and discard the medium. Replace the discarded medium with 2

mL of the plaque conformation maintenance medium. Label the tubes with sample and plaque isolation identification information.

To prevent splatter, a gauze-covered beaker may be used to collect spent medium.

To reduce shock to cells, warm the maintenance medium to $36.5 \pm 1^\circ\text{C}$ before placing on cell monolayer.

To prevent disturbing cells with the force of the liquid against the cell monolayer, add the maintenance medium to the side of cell culture test tube opposite the cell monolayer. Note that cells will be only on the bottom inner surface of the culture tube relative to their position during incubation.

(c.3) Remove the cap from a cell culture tube and place the tip of a Pasteur pipette containing the agar plug from section 5.2.6b.3 into the maintenance medium in the cell culture tube. Force the agar plug from the Pasteur pipette by gently squeezing the rubber bulb. Withdraw and discard the pipette, and replace and tighten down the screw-cap on the culture tube.

Tilt cell culture tube as necessary to facilitate the procedure and to avoid scratching the cell sheet with the pipette.

Squeeze bulb repeatedly to wash contents of pipette into the maintenance medium.

(c.4) Place the cell culture tubes in the drum used with the tissue culture roller apparatus. Incubate the cell cultures at $36.5 \pm 1^\circ\text{C}$ while rotating at a speed of 1/5 rpm. Examine the cells daily microscopically for 1 week for evidence of cytopathic effects (CPE).

CPE may be identified as cell disintegration or as changes in cell morphology. Rounding-up of infected cells is a typical effect seen with enteric virus infections. However, uninfected cells round up during mitosis and a sample should not be considered positive unless there are significant clusters of rounded-up cells over and beyond what is observed in the uninfected controls. If there is any doubt about the presence of CPE or if CPE appears late (i.e., on day 6 or 7), the conformation process should be repeated by transferring 0.2 mL of the medium in the culture tube to a freshly prepared tube.

Incubation of BGM cells in roller apparatus for periods greater than 1 week is not recommended as cells under these conditions tend to die-off if held longer.

If tubes receiving agar plugs from negative controls develop CPE or tubes receiving agar plugs from positive controls fail to develop CPE, stop all assays until the source of the failure is identified and corrected.

Tubes developing CPE may be stored in a -70°C freezer for additional optional tests (e.g., the Lim Benyesh-Melnick identification procedure).⁵

(c.5) Determine the fraction of confirmed plaques (C) for each sludge sample tested. Calculate "C" by dividing the number of tubes inoculated with agar plugs from plaques

that developed CPE by the total number of tubes inoculated (i.e., if CPE was obtained from 17 of 20 plaques, $C = 0.85$).

5.2.7 Calculation of virus titer.

If more than one composite sample was assayed, average the titer of all composite samples and report the average titer and the standard deviation for each lot of sludge tested.

(a) If the entire remaining portion of a test sample was inoculated onto BGM cultures as described in section 5.2.3e.5, calculate the virus titer (V) in PFU per 4 g of total dry solids according to the formula:

$$V = 0.8 \times P \times C$$

where P is the total number of plaques in all test vessels for that sample and C equals the fraction of confirmed plaques.

(b) If the sample was diluted due to high virus levels (e.g., when the virus density of the input to a process is being determined; see section 5.2.5c), calculate the virus titer (V) in PFU per 4 g total dry solids with the formula:

$$V = 0.8 \times \frac{P}{I} \times D \times S \times C$$

where P is the total number of plaques in all test vessels for dilution series, I is the volume (in mL) of the dilution inoculated, D is reciprocal of the dilution made on the inoculum before plating, S is the volume of the remaining portion of the test sample (as recorded in section 5.2.3e.5) and C is the fraction of confirmed plaques.

5.2.8 Calculate the percent of virus recovery (R) using the formula:

$$R = \frac{P}{400} \times 100$$

where P is the total number of plaques on all test vessels inoculated with the recovery control.

6. Cell Culture Preparation and Maintenance⁶

6.1. Introduction

This section outlines procedures and media for culturing the Buffalo Green monkey (BGM) kidney cell line and is intended for the individual who is experienced in cell culture preparation. BGM cells are a continuous cell line derived from African Green monkey kidney cells. The characteristics of this line were described by Barron et al. (1970). Use of BGM cells for recovering viruses from environmental samples was described by Dahling et al. (1974). The media and methods recommended are the results of the BGM cell line optimization studies by Dahling and Wright (1986). The BGM cell line can be obtained by qualified laboratories from the Biohazard Assessment Research Branch, National Exposure Research Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, USA 45268. Although BGM

⁵For more information see EPA/600/4-84/013(R12), May 1988 Revision

⁶Modified from EPA/600/4-84/013(R9), January 1987 Revision

cells will not detect all enteric viruses that may be present in sludges, the use of this cell line alone is sufficient to meet the requirements of 40 CFR Part 503.

6.2. Apparatus and Materials

6.2.1 Glassware, Pyrex (Corning Product No. 1395 or equivalent).

Storage vessels must be equipped with airtight closures.

6.2.2 Autoclavable inner-braided tubing with metal quick-connect connectors or with screw clamps for connecting tubing to equipment to be used under pressure.

Quick-connect connectors can be used only after equipment has been properly adapted.

6.2.3 Positive pressure air, nitrogen or 5% CO₂ source equipped with pressure gauge.

Pressure sources from laboratory air lines and pumps must be equipped with an oil filter. The source must not deliver more pressure to the pressure vessel than is recommended by manufacturer.

6.2.4 Dispensing pressure vessel — 5 or 20 liter capacity (Millipore Corp. Product No. XX67 00P 05 and XX67 00P 20 or equivalent).

6.2.5 Disc filter holders — 142 mm or 293 mm diameter (Millipore Corp. Product No. YY30 142 36 and YY30 293 16 or equivalent).

Use only pressure type filter holders.

6.2.6 Sterilizing filter stacks — 0.22 µm pore size (Millipore Corp. Product No. GSWP 142 50 and GSWP 293 25 or equivalent). Fiberglass prefilters (Millipore Corp. Product No. AP15 142 50 or AP15 293 25 and AP20 142 50 or AP20 293 25 or equivalent).

Stack AP20 and AP15 prefilters and 0.22 µm membrane filter into a disc filter holder with AP20 prefilter on top and 0.22 µm membrane filter on bottom.

Always disassemble the filter stack after use to check the integrity of the 0.22 µm filter. Refilter any media filtered with a damaged stack.

6.2.7 Positively-charged cartridge filter — 10 inch (Zeta plus TSM, Cuno Product No. 45134-01-600P or equivalent). Holder for cartridge filter with adaptor for 10 inch cartridge (Millipore Corp. Product No. YY16 012 00 or equivalent).

6.2.8 Culture capsule filter (Gelman Sciences Product No. 12140 or equivalent).

6.2.9 Cell culture vessels — Pyrex, soda or flint glass or plastic bottles and flasks or roller bottles (e.g.,

Brockway Product No. 1076-09A, 1925-02, Corning Product No. 25100-25, 25110-75, 25120-150, 25150-1750 or equivalent).

Vessels must be made from clear glass or plastic to allow observation of the cultures and be equipped with airtight closures. Plastic vessels must be treated by the manufacturer to allow cells to adhere properly.

6.2.10 Screw caps, black with rubber liners (Brockway Product No. 24-414 for 6 oz bottles⁷ or equivalent).

Caps for larger culture bottles usually supplied with bottles.

6.2.11 Roller apparatus (Bellco Product No. 7730 or equivalent).

6.2.12 Incubator capable of maintaining the temperature of cell cultures at 36.5 ± 1°C.

6.2.13 Waterbath, equipped with circulating device to assure even heating at 36.5 ± 1°C.

6.2.14 Light microscope, with conventional light source, equipped with lenses to provide 40X, 100X, and 400X total magnification.

6.2.15 Inverted light microscope equipped with lenses to provide 40X, 100X, and 400X total magnification.

6.2.16 Cornwall syringe pipettors, 2, 5 and 10 mL sizes (Curtin Matheson Scientific Product No. 221-861, 221-879, and 221-887 or equivalent).

6.2.17 Brewer-type pipetting machine (Curtin Matheson Scientific Product No. 138-107 or equivalent).

6.2.18 Phase contrast counting chamber (hemocytometer) (Curtin Matheson Scientific Product No. 158-501 or equivalent).

6.2.19 Conical centrifuge tubes, sizes 50 mL and 250 mL.

6.2.20 Rack for tissue culture tubes (Bellco Product No. 2028 or equivalent).

6.2.21 Bottles, aspirator-type with tubing outlet, size 2,000 mL.

Bottles for use with pipetting machine.

6.2.22 Storage vials, size 2 mL.

Vials must withstand temperatures to -70°C.

6.3. Media and Reagents

6.3.1 Sterile fetal calf, gammagobulin-free newborn calf or iron-supplemented calf serum, certified free of

⁷Size is given in oz only when it is commercially designated in that unit.

viruses, bacteriophage and mycoplasma (GIBCO BRL or equivalent).

Test each lot of serum for cell growth and toxicity before purchasing. Serum should be stored at -20°C for long-term storage. Upon thawing, each bottle should be heat-inactivated at 56°C for 30 min and stored at 4°C for short term use.

6.3.2 Trypsin, 1:250 powder (Difco Laboratories Product No. 0152-15-9 or equivalent) or trypsin, 1:300 powder (BBL Microbiology Systems Product No. 12098 or equivalent).

6.3.3 Sodium (tetra) ethylenediamine tetraacetate powder (EDTA), technical grade, (Fisher Scientific Product No. S657-500 or equivalent).

6.3.4 Thioglycollate medium (Difco Laboratories Product No. 0257-01-9 or equivalent).

6.3.5 Fungizone (amphotericin B, Sigma Chemical Product No. A-9528 or equivalent), Penicillin G (Sigma Chemical Product No. P-3032 or equivalent), dihydrostreptomycin sulfate (ICN Biomedicals Product No. 100556 or equivalent), and tetracycline (ICN Biomedicals Product No. 103011 or equivalent).

Use antibiotics of at least tissue culture grade.

6.3.6 Eagle's minimum essential medium (MEM) with Hanks' salts and L-glutamine, without sodium bicarbonate (GIBCO BRL Product No. 410-1200 or equivalent).

6.3.7 Leibovitz's L-15 medium with L-glutamine (GIBCO BRL Product No. 430-1300 or equivalent).

6.3.8 Trypan blue (Sigma Chemical Product No. T-6146 or equivalent).

Note: This chemical is on the EPA list of proven or suspected carcinogens.

6.3.9 Dimethyl sulfoxide (DMSO; Sigma Chemical Product No. D-2650 or equivalent).

6.3.10 Mycoplasma testing kit (Irvine Scientific Product No. T500-000 or equivalent).

6.4. Preparation of Cell Culture Media

6.4.1 General Principles

(a) Equipment care — Carefully wash and sterilize equipment used for preparing media before each use.

(b) Disinfection of work area — Thoroughly disinfect surfaces on which the medium preparation equipment is to be placed. Many commercial disinfectants do not adequately kill total culturable viruses. To ensure thorough disinfection, disinfect all surfaces and spills with either a solution of 0.5% (5 g per liter) iodine in 70% ethanol or 0.1% HOCl.

(c) Aseptic technique — Use aseptic technique when preparing and handling media or medium components.

(d) Dispensing filter-sterilized media — To avoid post-filtration contamination, dispense filter-sterilized media into storage containers through clear glass filling bells in a microbiological laminar flow hood. If a hood is unavailable, use an area restricted solely to cell culture manipulations.

(e) Coding media — Assign a lot number to and keep a record of each batch of medium or medium components prepared. Place the lot number, the date of preparation, the expiration date, and the initials of the person preparing the medium on each bottle.

(f) Sterility test — Test each lot of medium and medium components to confirm sterility as described in section 6.5 before the lot is used for cell culture.

(g) Storage of media and medium components — Store media and medium components in clear airtight containers at 4°C or -20°C as appropriate.

(h) Sterilization of NaHCO₃-containing solutions — Sterilize media and other solutions that contain NaHCO₃ by positive pressure filtration.

Negative pressure filtration of such solutions increases the pH and reduces the buffering capacity.

6.4.2 Media Preparation Recipes

(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 dH₂O and sterilization. Media from commercial sources are quality-controlled. The conditions specified by the supplier for storage and expiration dates should be strictly observed. However, media can also be prepared in the laboratory directly from chemicals. Such preparations are labor intensive, but allow quality control of the process at the level of the preparing laboratory.

(b) Procedure for the preparation of EDTA-trypsin.

The procedure described is for the preparation of 10 liters of EDTA-trypsin reagent. It is used to dislodge cells attached to the surface of culture bottles and flasks. This reagent, when stored at 4°C, retains its working strength for at least four months. The amount of reagent prepared should be based on projected usage over a four-month period.

(b.1) Add 30 g of trypsin (1:250) or 25 g of trypsin (1:300) and two liters of dH₂O to a six liter flask containing a three inch stir bar. Place the flask onto a magnetic stirrer and mix the trypsin solution rapidly for a minimum of one hour.

Trypsin remains cloudy.

(b.2) Add four liters of dH₂O and a three-inch stir bar into 20 liter clear plastic carboy. Place the carboy onto a magnetic stirrer and stir at a speed sufficient to develop a vortex while adding the following chemicals: 80 g NaCl, 12.5 g

EDTA, 50 g dextrose, 11.5 g Na₂HPO₄ · 7H₂O, 2.0 g KCl, and 2.0 g KH₂PO₄.

Each chemical does not have to be completely dissolved before adding the next one.

(b.3) Add four more liters of dH₂O to carboy.

Continue mixing until all chemicals are completely dissolved.

(b.4) Add the two liters of trypsin from step 6.4.2b.1 to the prepared solution in step 6.4.2b.3 and mix for a minimum of one hour. Adjust the pH of the EDTA-trypsin reagent to 7.5 - 7.7.

(b.5) Filter reagent under pressure through a disc filter stack and store the filtered reagent in tightly stoppered or capped containers at 4°C.

The cartridge prefilter (section 6.2.7) can be used in line with the culture capsule sterilizing filter (section 6.2.8) as an alternative to a filter stack (section 6.2.6).

(c) Procedure for the preparation of MEM/L-15 medium.

The procedure described is for preparation of 10 liters of MEM/L-15 medium.

(c.1) Place a three inch stir bar and four liters of dH₂O into 20 liter carboy.

(c.2) Place the carboy onto a magnetic stirrer. Stir at a speed sufficient to develop a vortex and then add the contents of a five liter packet of L-15 medium to the carboy. Rinse the medium packet with three washes of 200 mL each of dH₂O and add the rinses to the carboy.

(c.3) Mix until the medium is evenly dispersed.

L-15 medium may appear cloudy as it need not be totally dissolved before proceeding to step 6.4.2c.4.

(c.4) Add three liters of dH₂O to the carboy and the contents of a five liter packet of MEM medium to the carboy. Rinse the MEM medium packet with three washes of 200 mL each of dH₂O and add the rinses to the carboy. Add 800 mL of dH₂O and 7.5 g of NaHCO₃ and continue mixing for an additional 60 min.

(c.5) Transfer the MEM/L-15 medium to a pressure can and filter under positive pressure through a 0.22 µm sterilizing filter. Collect the medium in volumes appropriate for the culturing of BGM cells (e.g., 900 mL in a 1 liter bottle) and store in tightly stoppered or capped containers at 4°C.

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

(d) Procedure for preparation of trypan blue solution.

The procedure described is for the preparation of 100 mL of trypan blue solution. It is used in the direct determination of the viable cell counts of the BGM stock cultures. As trypan blue is on the EPA suspect carcinogen list, particular care should be taken in its preparation and use so as to avoid skin contact or inhalation. The wearing of rubber gloves during preparation and use is recommended.

(d.1) Add 0.5 g of trypan blue to 100 mL of dH₂O in a 250 mL flask. Swirl the flask until the trypan blue is completely dissolved.

(d.2) Sterilize the solution by autoclaving at 121°C for 15 minutes and store in a screw-capped container at room temperature.

(e) 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. It is important that the recommended antibiotic levels not be exceeded when planting cells as the cultures are particularly sensitive to excessive concentrations at this stage.

Antibiotic stock solutions should be placed in screw-capped containers and stored at -20°C until needed. Once thawed, they may be refrozen; however, repeated freezing and thawing of these stock solutions should be avoided by distributing them in quantities that are sufficient to support a week's cell culture work.

(e.1) Preparation of penicillin-streptomycin stock solution.

The procedure described is for preparation of ten 10 mL aliquots of penicillin-streptomycin stock solution at concentrations of 1,000,000 units of penicillin and 1,000,000 µg of streptomycin per 10 mL unit. The antibiotic concentrations listed in step 6.4.2e.1.1 may not correspond to the concentrations obtained from other lots or from a different source.

(e.1.1) Add appropriate amounts of penicillin G and dihydrostreptomycin sulfate to a 250 mL flask containing 100 mL of dH₂O. Mix the contents of the flasks on magnetic stirrer until the antibiotics are dissolved.

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.

(e.1.2) Sterilize the antibiotics by filtration through 0.22 µm membrane filters and dispense in 10 mL volumes into screw-capped containers.

(e.2) Preparation of tetracycline stock solution. Add 1.25 g of tetracycline hydrochloride powder and 3.75 g of ascorbic acid to a 125 mL flask containing 50 mL of dH₂O. Mix the contents of the flask on a magnetic stirrer until the antibiotic is dissolved. Sterilize the antibiotic by filtration through a 0.22 µm membrane filter and dispense in 5 mL volumes into screw-capped containers.

(e.3) Preparation of amphotericin B (fungizone) stock solution. Add 0.125 g of amphotericin B to a 50 mL flask containing 25 mL of ddH₂O. Mix the contents of the flask on a magnetic stirrer until the antibiotic is dissolved. Sterilize the antibiotic by filtration through 0.22 µm membrane filter and dispense 2.5 mL volumes into screw-capped containers.

6.5. 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. The two techniques described below are standard in many laboratories. The capabilities of these techniques are limited to the detection of 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 antibiotics, media and medium components has been demonstrated. BGM cell lines should be monitored every six months for mycoplasma contamination according to test kit instructions. Cells that are contaminated should be discarded.

6.5.1 Procedure for Verifying Sterility of Small Volumes of Liquids. Inoculate 5 mL of the material to be tested for sterility into 5 mL of thioglycollate broth. Shake the mixture and incubate at $36.5 \pm 1^\circ\text{C}$. 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.

6.5.2 Visual Evaluation of Media for Microbial Contaminants. Incubate media at $36.5 \pm 1^\circ\text{C}$ for at least one week prior to use. Visually examine and discard any media that lose clarity.

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

6.6. Procedures for Preparation and Passage of BGM Cell Cultures

A laminar flow biological safety cabinet should be used to process cell cultures. If a biological safety cabinet is not available, cell cultures should be prepared in controlled facilities used for no other purposes. Viruses or other microorganisms must not be transported, handled, or stored in cell culture transfer facilities.

6.6.1 Vessels and Media for Cell Growth

(a) The BGM cell line grows readily on the inside surfaces of glass or specially treated, tissue culture grade plastic vessels. 16 to 32 oz (or equivalent growth area) flat-sided, glass bottles, 75 or 150 cm² plastic cell culture flasks, and 690 cm² glass or 850 cm² plastic roller bottles are usually used for the maintenance of stock cultures. Flat-sided bottles and flasks that contain cells in a stationary position are incubated with the flat side (cell monolayer side) down. If available, roller bottles and roller apparatus units are preferable to flat-sided bottles and flasks because roller cultures require less medium than flat-sided bottles per unit

of cell monolayer surface. Roller apparatus rotation speed should be adjusted to one-half revolution per minute to ensure that cells are constantly bathed in growth medium.

(b) Growth and maintenance media should be prepared on the day they will be needed. Prepare growth medium by supplementing MEM/L-15 medium with 10% serum and antibiotics (100 mL of serum, 1 mL of penicillin-streptomycin stock, 0.5 mL of tetracycline stock and 0.2 mL of fungizone stock per 900 mL of MEM/L-15). Prepare maintenance medium by supplementing MEM/L-15 with antibiotics and 2% or 5% serum (20 or 50 mL of serum, antibiotics as above for growth medium and 70 or 50 mL of dH₂O, respectively).

6.6.2 General Procedure for Cell Passage

Pass stock BGM cell cultures at approximately seven day intervals using growth medium.

(a) Pour spent medium from cell culture vessels, and discard the medium.

To prevent splatter, a gauze-covered beaker may be used to collect spent medium.

Before discarding, autoclave all media that have been in contact with cells or that contain serum.

(b) Add to the cell cultures a volume of warm EDTA-trypsin reagent equal to 40% of the volume of medium replaced.

See Table 2 for the amount of reagents required for commonly used vessel types.

To reduce shock to cells, warm the EDTA-trypsin reagent to $36.5 \pm 1^\circ\text{C}$ before placing it on cell monolayers. Dispense the EDTA-trypsin reagent directly onto the cell monolayer.

(c) Allow the EDTA-trypsin reagent to remain in contact with the cells at either room temperature or at $36.5 \pm 1^\circ\text{C}$ until cell monolayer can be shaken loose from inner surface of cell culture vessel (about five min).

If necessary, a sterile rubber policeman (or scraper) may be used to physically remove the cell sheet from the bottle. However, this procedure should be used only as a last resort because of the risk of cell culture contamination inherent in such manipulations. The EDTA-trypsin reagent should remain in contact with the cells no longer than necessary as prolonged contact can alter or damage the cells.

(d) Pour the suspended cells into centrifuge tubes or bottles.

To facilitate collection and resuspension of cell pellets, use tubes or bottles with conical bottoms. Centrifuge tubes and bottles used for this purpose must be able to withstand the g-force applied.

(e) Centrifuge cell suspension at 1,000 \times g for 10 min to pellet cells. Pour off and discard the supernatant.

Do not exceed this speed as cells may be damaged or destroyed.

TABLE 2. Guide for Preparation of BGM Stock Cultures

Vessel Type	EDTA-Trypsin Volume (mL) ¹	Media Volume (mL) ²	Total No. Cells to Plate per Vessel
16 oz glass flat bottles ³	10	25	2.5×10^6
32 oz glass flat bottles	20	50	5.0×10^6
75 cm ² plastic flat flask	12	30	3.0×10^6
150 cm ² plastic flat flask	24	60	6.0×10^6
690 cm ² glass roller bottle	40	100	7.0×10^7
850 cm ² plastic roller bottle	50	120	8.0×10^7

¹The volume required to remove cells from vessels.

²Serum requirements: growth medium contains 10% serum; maintenance medium contains 2-5% serum. Antibiotic requirements: penicillin-streptomycin stock solution, 1.0 mL/liter; tetracycline stock solution, 0.5 mL/liter; fungizone stock solution, 0.2 mL/liter.

³Size is given in oz only when it is commercially designated in that unit.

(f) Suspend the pelleted cells in growth medium (see section 6.6.1b) and perform a viable count on the cell suspension according to procedures in section 6.7.

Resuspend pelleted cells in sufficient volumes of medium to allow thorough mixing of the cells (to reduce sampling error) and to minimize the significance of the loss of the 0.5 mL of cell suspension required for the cell counting procedure. The quantity of medium used for resuspending pelleted cells varies from 50 to several hundred mL, depending upon the volume of the individual laboratory's need for cell cultures.

(g) Dilute the cell suspension to the appropriate cell concentration with growth medium and dispense into cell culture vessels with either a Cornwall-type syringe or Brew-er-type pipetting machine dispenser.

Calculate the dilution factor requirement using the cell count established in section 6.7 and the cell and volume parameters given in Table 2 for stock cultures and in Table 3 for virus assay cultures.

As a general rule, the BGM cell line can be split at a 1:3 ratio. However, a more suitable inoculum is obtained if low passages of the line (passages 100-150) are split at a 1:2 ratio and higher passages (generally above passage 250) are split at a 1:4 ratio. To plant two hundred 25 cm² cell culture flasks weekly from a low-level passage of the line would require the preparation of six roller bottles (surface area 690 cm² each): two to prepare the six roller bottles and four to prepare the 25 cm² flasks.

(h) Except during handling operations, maintain BGM cells at $36.5 \pm 1^\circ\text{C}$ in airtight cell culture vessels.

6.6.3 Procedure for Changing Medium on Cultured Cells — Cell monolayers normally become 95 to 100% confluent three to four days after seeding with an appropriate number of cells, and growth medium becomes acidic. Growth medium on confluent stock cultures should then be replaced with maintenance medium containing 2% serum. Maintenance medium with 5% serum should be used when

monolayers are not yet 95% to 100% confluent but the medium in which they are immersed has become acidic. The volume of maintenance medium should equal the volume of discarded growth medium.

6.7. Procedure for Performing Viable Cell Counts

With experience a fairly accurate cell concentration can be made based on the volume of packed cells. However, viable cell counts should be performed periodically as a quality control measure.

6.7.1 Add 0.5 mL of cell suspension (or diluted cell suspension) to 0.5 mL of 0.5% trypan blue solution in a test tube.

To obtain an accurate cell count, the optimal total number of cells per hemocytometer section should be between 20 and 50. This range is equivalent to between 6.0×10^5 and 1.5×10^6 cells per mL of cell suspension. Thus, a dilution of 1:10 (0.5 mL of cells in 4.5 mL of growth medium) is usually required for an accurate count of a cell suspension.

6.7.2 Disperse cells by repeated pipetting.

Avoid introducing air bubbles into the suspension, because air bubbles may interfere with subsequent filling of the hemocytometer chambers.

6.7.3 With a capillary pipette, carefully fill a hemocytometer chamber on one side of a slip-covered hemocytometer slide. Rest the slide on a flat surface for about one min to allow the trypan blue to penetrate the cell membranes of nonviable cells.

Do not under or over fill the chambers.

6.7.4 Under 100X total magnification, count the cells in the four large corner sections and the center section of the hemocytometer chamber.

Include in the count cells lying on the lines marking the top and left margins of the sections, and ignore cells on the lines marking the bottom and right margins. Trypan blue is excluded by living cells. Therefore, to quantify viable cells, count only cells that are clear in color. Do not count cells that are blue.

Table 3. Guide for Preparation of Virus Assay Cell Cultures

Vessel Type	Volume of Medium (mL) ¹	Final Cell Count per Bottle
1 oz glass bottle ²	4	9.0×10^5
25 cm ² plastic flask	10	3.5×10^6
6 oz glass bottle	15	5.6×10^6
75 cm ² plastic flask	30	1.0×10^7
16 mm × 150 mm tubes	2	4.0×10^4

¹Serum requirements: growth medium contains 10% serum; maintenance medium contains 2-5% serum. Antibiotic requirements: penicillin-streptomycin stock solution, 1.0 mL/liter; tetracycline stock solution, 0.5 mL/liter; fungizone stock solution, 0.2 mL/liter.

²Size is given in oz only when it is commercially designated in that unit.

6.7.5 Calculate the average number of viable cells in each mL of cell suspension by totaling the number of viable cells counted in the five sections, multiplying this sum by 4000, and where necessary, multiplying the resulting product by the reciprocal of the dilution.

6.8. Procedure for Preservation of BGM Cell Line

An adequate supply of BGM cells must be available to replace working cultures that are used only periodically or become contaminated or lose virus sensitivity. Cells have been held at -70°C for more than 15 years with a minimum loss in cell viability.

6.8.1 Preparation of Cells for Storage

The procedure described is for the preparation of 100 cell culture vials. Cell concentration per mL must be at least 1×10^6 .

Base the actual number of vials to be prepared on usage of the line and the anticipated time interval requirement between cell culture start-up and full culture production.

(a) Prepare cell storage medium by adding 10 mL of DMSO to 90 mL of growth medium (see section 6.6.1b). Sterilize cell storage medium by passage through an 0.22 μ m sterilizing filter.

Collect sterilized medium in 250 mL flask containing a stir bar.

(b) Harvest BGM cells from cell culture vessels as directed in section 6.6.2. Count the cells according to the procedure in section 6.7 and resuspend them in the cell storage medium at a concentration of 1×10^6 cells per mL.

(c) Place the flask containing suspended cells on a magnetic stirrer and slowly mix for 30 min. Dispense 1 mL volumes of cell suspension into 2 mL vials.

6.8.2 Procedure for Freezing Cells

The freezing procedure requires slow cooling of the cells with the optimum rate of -1°C per min. A slow cooling rate can be achieved using the following method or by using the recently available freezing containers (e.g., Nalge Company Product No. 5100-0001 or equivalent) as recommended by the manufacturers.

(a) Place the vials in a rack and place the rack in refrigerator at 4°C for 30 min, in a -20°C freezer for 30 min, and then in a -70°C freezer overnight. The transfers should be made as rapidly as possible.

To allow for more uniform cooling, wells adjoining each vial should remain empty.

(b) Rapidly transfer vials into boxes or other containers for long-term storage.

To prevent substantial loss of cells during storage, temperature of cells should be kept constant after -70°C has been achieved.

6.8.3 Procedure for Thawing Cells

Cells must be thawed rapidly to decrease loss in cell viability.

(a) Place vials containing frozen cells into a 36°C water bath and agitate vigorously by hand until all ice has melted. Sterilize the outside surface of the vials with 0.5% iodine in 70% ethanol.

(b) Add BGM cells to either 6 oz tissue culture bottles or 25 cm² tissue culture flasks containing an appropriate volume of growth medium (see Table 3). Use two vials of cells for 6 oz bottles and one vial for 25 cm² flasks.

(c) Incubate BGM cells at $36.5 \pm 1^\circ\text{C}$. After 18 to 24 h replace the growth medium with fresh growth medium and then continue the incubation for an additional five days. Pass and maintain the new cultures as directed in section 6.6.

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