United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

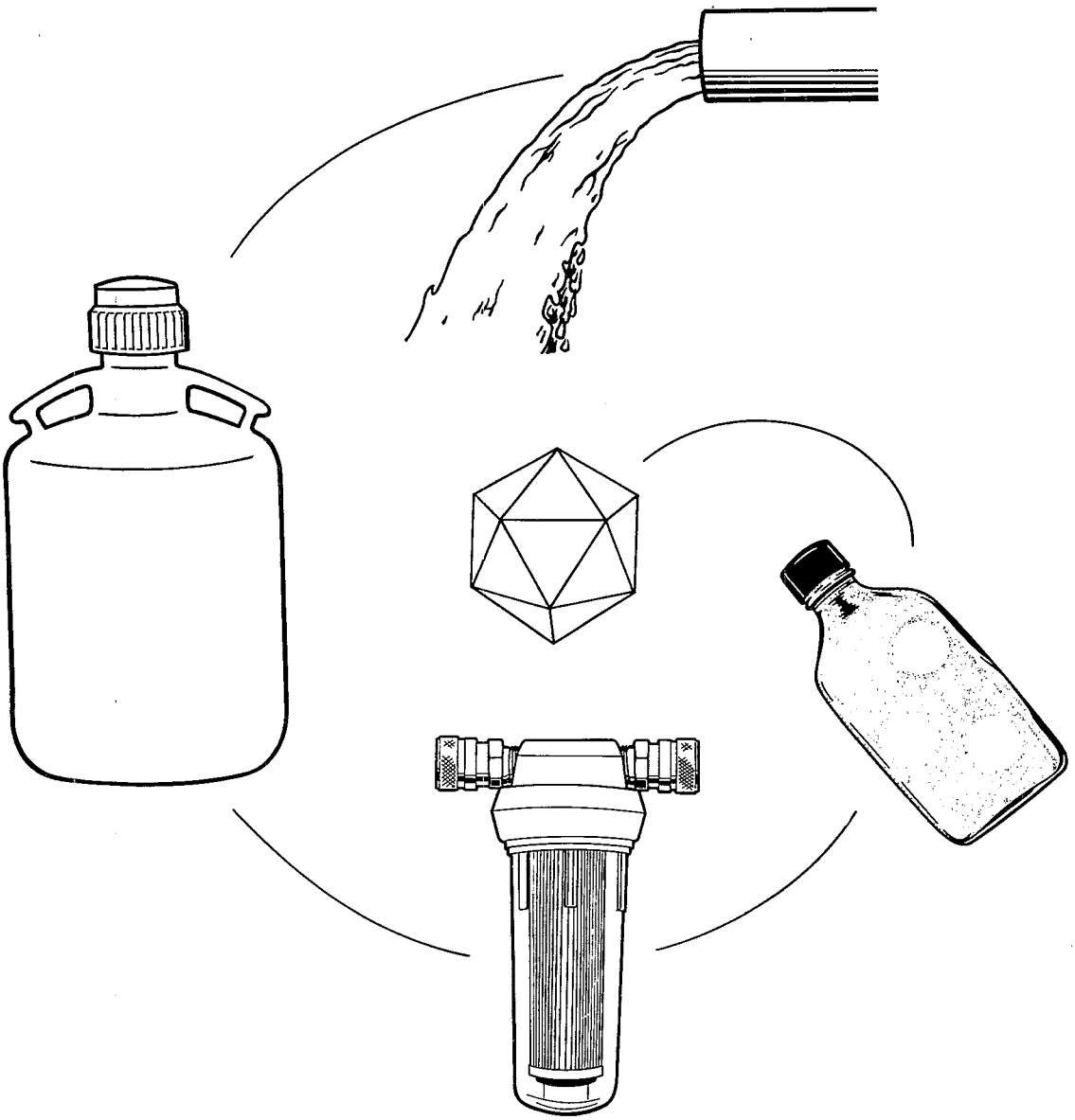
EPA/600/4-84/013(R9)
January 1987
Revision

Research and Development

EPA

USEPA Manual of Methods for Virology

Chapter 9
Revised January 1987



Chapter 9
Cell Culture Preparation and Maintenance

1. Introduction

This chapter outlines procedures and media for culturing Buffalo green monkey (BGM) kidney cells. BGM cells are a continuous line derived from African Green monkey kidney cells. The characteristics of this line were described by A. L. Barron, C. Olshevsky, and M. M. Cohen in 1970. Use of BGM cells for recovering viruses from environmental samples was described by D. R. Dahling, G. Berg, and D. Berman in 1974. The media and methods recommended in Chapters 9 and 10 are the results of the BGM cell line optimization studies by D. R. Dahling and B. A. Wright, published in 1986. The BGM cells can be obtained by qualified laboratories from the Virology Section, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

Chapter 9 is intended for the individual who is experienced in cell culture preparation. Although the procedures and media outlined in this chapter are for use with the BGM cell line, the method may be applied to the Madin and Darby bovine kidney (MDBK) cell line. The MDBK cell line has been effectively used for the plaque assay of reoviruses. The cytopathic effect of reoviruses is slow to appear; thus, human enteroviruses present in a sample may interfere with their detection. Enterovirus interference is avoided however with use of the MDBK cells, since they do not support the growth of these viruses. Cells of the MDBK line may be obtained from the American Type Culture Collection (ATCC product no. CCL 22). This method for cell culture preparation and maintenance may also be used as is or with minor modification for cells other than BGM and MDBK. BGM cells are highly susceptible to many enteric viruses (Dahling *et al.*, 1984; Dahling and Wright, 1986); however, these cells are not sensitive for detecting all enteroviruses or certain other viruses that may occur in environmental samples. Thus, to maximize the number of viruses recovered from environmental samples, several cell lines may need to be used. Moreover, viruses such as reovirus require modification to the agar overlay procedure. These modifications are presented in Chapter 10.

2. Medium Preparation

2.1 Apparatus and Materials

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

Storage vessels must be equipped with airtight closures.

2.1.2 Magnetic stirrer and stir bars.

2.1.3 Autoclavable inner-braided tubing with metal quick-disconnect connectors or with thumb-screw-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.

2.1.4 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.

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

2.1.6 Disc filter holders—142-mm or 293-mm diameter (Millipore Corp., or equivalent).

Use only pressure type filter holders.

2.1.7 Sterilizing filters—0.22-μm pore size (Millipore Corp., GS series, or equivalent).

2.1.8 Fiberglass prefilters (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.

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

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

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

2.1.12 Cell culture vessels, Pyrex borosilicate glass (Corning Glass Works, or equivalent), soda or flint glass prescription (Rx) bottles (Brockway, Inc., or equivalent), plastic flasks (Falcon Tissue Culture Labware, Becton, Dickinson and Co., or equivalent), disposable glass roller bottles (Bellco Glass, Inc., or equivalent), or disposable plastic roller bottles (Corning Glass Works, or equivalent).

Vessels (tubes, flasks, bottles) for growth of cell cultures must be clear glass or plastic to allow observation of the cultures. Plastic vessels must be treated by the manufacturer to allow cells to adhere properly. Vessels for cell cultures must be equipped with airtight closures.

2.1.13 Screw caps, black with rubber liners (thread finish no. 24-414 for 6-oz* prescription (Rx) bottles, Brockway, Inc., or equivalent).

Caps for larger culture bottles usually supplied with bottles.

2.1.14 Roller apparatus (7730-Series, Bellco Glass, Inc., or equivalent).

2.1.15 pH meter measuring to an accuracy of at least 0.1 pH unit.

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

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

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

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

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

2.1.20 Pipettor syringes, sizes 2 mL, 5 mL and 10 mL (Cornwall-type, Becton, Dickinson and Co., or equivalent).

2.1.21 Pipetting machine (Brewer-type, Curtin Matheson Scientific, or equivalent).

2.1.22 Phase counting chamber (hemocytometer) slide (product no. 158-501, Curtin Matheson Scientific, or equivalent).

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

2.1.24 Rack for tissue culture tubes (product no. 2028, Bellco Glass, Inc., or equivalent).

2.1.25 Bottles, aspirator-type with tubing outlet, size 2,000 mL.
Bottles for use with pipetting machine.

2.1.26 Storage vials, size 2 mL.
Vials must withstand temperatures to –70°C.

2.2 Media and Reagents

2.2.1 Fetal calf serum and GG-free calf serum, filter-sterilized, heat-inactivated at 56°C for 30 min, certified free of viruses, bacteriophage and mycoplasma (GIBCO Laboratories, or equivalent).
Test toxicity of sample of serum on cells before purchasing serum lot.

2.2.2 Trypsin, 1:250 powder (Difco Laboratories, or equivalent) or trypsin, 1:300 powder (BBL, Becton, Dickinson and Co., or equivalent).

2.2.3 Sodium (tetra) ethylenediamine tetraacetate powder (EDTA), technical grade (Fisher Scientific Co., or equivalent).

2.2.4 Thioglycollate medium (Bacto dehydrated fluid thioglycollate medium, Difco Laboratories, or equivalent).

2.2.5 Water, distilled, deionized.
See Chapter 4.

2.2.6 Fungizone (amphotericin B, E. R. Squibb and Sons, or equivalent), Penicillin G and dihydrostreptomycin sulfate (Eli Lilly and Co., or equivalent), tetracycline (Pfizer, Inc., or equivalent).
Use antibiotics of at least tissue culture grade.

2.2.7 Eagle's minimum essential medium (MEM) with Hanks' salts and L-glutamine, without sodium bicarbonate (product no. 410-1200, GIBCO Laboratories, or equivalent).

2.2.8 Leibovitz's L-15 medium with L-glutamine (product no. 430-1300, GIBCO Laboratories, or equivalent).

2.2.9 Trypan blue (Sigma Chemical Co., or equivalent).
Note: This chemical is on the EPA list of proven or suspected carcinogens.

2.2.10 Sodium bicarbonate ($NaHCO_3$).

2.2.11 Hydrochloric acid (HCl)—1 M.

2.2.12 Sodium hydroxide (NaOH)—1 M.

2.2.13 Sodium chloride (NaCl).

2.2.14 Dextrose.

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

2.2.16 Potassium chloride (KCl).

2.2.17 Potassium phosphate monobasic (KH_2PO_4).

2.2.18 Ascorbic acid.

2.2.19 Dimethyl sulfoxide (DMSO).

3. Preparation of Cell Culture Media

3.1 Technique

3.1.1 Equipment care.
Carefully wash and sterilize equipment used for preparing media before each use.

3.1.2 Disinfection of work area.
Thoroughly disinfect surfaces on which medium preparation equipment is to be placed.

3.1.3 Aseptic technique.
Use aseptic technique when preparing and handling media or medium components.

3.1.4 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 hood is unavailable, use an area restricted solely to cell culture manipulations.

3.2 General Procedures

3.2.1 Coding media.
Assign a lot number to each batch of media or medium components prepared.

3.2.2 Sterility test.
Test each lot of medium and medium components to confirm sterility before the lot is used for cell culture (see Section 4).

3.2.3 Storage of media and medium components.
Store media and medium components in clear airtight containers at 4°C or –20°C as appropriate.

3.2.4 Sterilization of $NaHCO_3$-containing solutions.
Sterilize media and other solutions that contain $NaHCO_3$ by positive pressure filtration.
Negative pressure filtration of such solutions increases the pH and reduces the buffering capacity.

3.3 Medium Preparation

3.3.1 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.

3.3.2 Procedure for 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.

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

(b) Add 30 g of trypsin (1:250) and two liters of deionized distilled water to flask.

(c) Place flask on magnetic stirrer and mix trypsin rapidly for a minimum of one hour.
Trypsin remains cloudy.

(d) Add four liters of deionized distilled water to 20-liter clear plastic carboy.

(e) Place three-inch stir bar into carboy.

(f) Place carboy onto magnetic stirrer and stir at a speed sufficient to develop vortex while adding the chemicals listed in steps (g) through (l).
Each chemical does not have to be completely dissolved before adding the next one.

(g) NaCl—80 g.

(h) EDTA—12.5 g.

(i) Dextrose—50 g.

(j) $Na_2HPO_4 \cdot 7H_2O$—11.5 g.

(k) KCl—2.0 g.

(l) KH_2PO_4—2.0 g.

(m) Add four more liters of deionized distilled water to carboy.
Continue mixing after all chemicals are completely dissolved.

(n) Add the two liters of trypsin from step (c) to the prepared solution in step (m) and mix for a minimum of one hour.

(o) Adjust pH of the trypsin reagent to 7.5-7.7.

(p) 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 2.1.8. As an alternative, use the described cartridge prefilter in Section 2.1.9 and the capsule sterilizing filter in Section 2.1.11.

(q) Test sterility of EDTA-trypsin reagent in accordance with directions given in Section 4.

(r) Store reagent in tightly stoppered or capped containers at 4°C.

3.3.3 Procedure for preparation of growth medium.

The procedure described is for preparation of 10 liters of MEM/L-15 growth medium. The medium will be supplemented with 10% fetal calf serum and antibiotics (10 mL of penicillin-streptomycin stock, 5 mL of tetracycline stock and 2 mL of fungizone stock per 10 liters of growth medium) prior to addition of the BGM cells.

(a) Place three-inch stir bar into 20-liter carboy.

(b) Add four liters of deionized distilled water to carboy.

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

(d) Add contents of a five-liter packet of L-15 medium to carboy.

(e) Rinse medium packet with three washes of 200 mL each of deionized distilled water and add to carboy.

(f) Mix until medium is evenly dispersed.
L-15 medium may appear cloudy as it need not be totally dissolved before proceeding to step (g).

(g) Add three liters of deionized distilled water to carboy.

(h) Continue mixing at a speed sufficient to develop vortex.

(i) Add contents of a five-liter packet of MEM medium to carboy.

(j) Rinse medium packet with three washes of 200 mL each of deionized distilled water and add to carboy.

(k) Add 800 mL of deionized distilled water and 7.5 g of $NaHCO_3$ to carboy.

(l) Mix MEM/L-15 for an additional 60 min.

(m) Add medium to pressure can.

(n) Filter under positive pressure through 0.22-μm sterilizing filter and collect in volumes appropriate for the culturing of BGM cells.

(o) Collect sample of medium for sterility testing in accordance with directions given in Section 4.

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

3.3.4 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.

(a) Add 0.5 g of trypan blue to a 250-mL flask.

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

(c) Swirl flask until the trypan blue is completely dissolved.

(d) Sterilize solution in accordance with instructions given in Chapter 3, Section 2.1.

(e) Store in a screw-capped container at room temperature.

3.3.5 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, to avoid repeated freezing and thawing of these stock solutions distribute them in quantities that are sufficient to support a week's cell culture work.

(a) Preparation of penicillin-streptomycin stock solution.
The procedure described is for preparation of ten 10-mL volume penicillin-streptomycin stock solu-

tions 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 (a.1) may not correspond to the concentrations obtained from other lots or from a different source.

(a.1) Add appropriate amounts 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 in 10-mL volumes into screw-capped containers.

(b) Preparation of tetracycline stock solution.

(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 in 5-mL volumes into screw-capped containers.

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

(c.1) Add 0.125 g of amphotericin B 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 2.5-mL volumes into screw-capped containers.

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 antibiotics, media and medium components has been demonstrated.

4.1 Procedure for Verifying Sterility of Small Volumes of Liquids

4.1.1 Inoculate 5 mL of the material to be tested for sterility into 5 mL of the thioglycollate broth.

4.1.2 Shake the mixture and incubate at 36.5°± 1°C.

4.1.3 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.

4.2 Procedure for Verifying Sterility of Large Volumes of Liquids

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

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

Place filter face up on agar.

4.2.3 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.

4.3 Visual Evaluation of Media for Microbial Contaminants

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

4.3.2 Visually examine the clarity of the culture media.

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

4.3.3 Discard any media that lose clarity.

5. Procedures for Preparation of Stock BGM Cell Cultures

The BGM cell line grows readily on the inside surfaces of plastic or glass flat-sided vessels or round vessels. To reduce the risk of contamination, cell cultures should be prepared in controlled facilities used for no other purpose.

5.1 General Procedures

5.1.1 Pass and maintain BGM stock cultures in 16-oz to 32-oz (or equivalent growth area) flat-sided, glass bottles, in 150-cm^2 plastic cell culture flasks, or in 690-cm^2 glass or 850-cm^2 plastic roller bottles.

If available, roller bottles and roller apparatus units are preferable to flat-sided bottles or flasks for growing cells because roller cultures require less medium than flat-sided bottles per unit of cell monolayer surface. For growing cells in roller bottles, adjust roller apparatus rotation speed to one-half revolution per minute.

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 200 25-cm^2 cell culture flasks weekly from a low-level passage of the line would require the preparation of six roller bottles (surface area 690 cm^2 each): two to prepare six roller bottles and four to prepare the 25-cm^2 flasks.

5.1.2 Except during handling operations, maintain BGM cells at 36.5° ± 1°C in airtight cell culture vessels.

(a) Maintain rotation of roller bottles so that cells are constantly bathed in the growth medium.

(b) Maintain flat-sided cell culture bottles or flasks that contain cells in a stationary position with the flat side (cell monolayer side) down.

5.1.3 To reduce shock to cells, warm growth media and maintenance media

to 36.5° ± 1°C before placing them on cell monolayers.

5.1.4 Change medium on stock BGM cell cultures on day three or four.

Use growth medium described in Section 3.3.3. Supplement the medium with antibiotics and either 2% or 5% fetal calf serum. Use the lower percentage (maintenance medium) if the cell monolayer is at least 95% confluent. Add a sufficient amount of deionized distilled water to the change medium so as to compensate for the volume difference between the 10% fetal calf serum to be used initially in the medium and the 2% or 5% serum concentration which is to be used in the change medium.

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

Table 9-1. *Guide for Preparation of BGM Stock Cultures*

Vessel Type	*Volume of EDTA-Trypsin Used to Remove Cells (mL)*	*Volume of Medium (mL)**	*Final Cell Count per Bottle*
*16-oz** glass flat bottles*	*10*	*25*	*2.5×10^6*
32-oz glass flat bottles	*20*	*50*	*5.0×10^6*
150-cm^2 plastic flat flask	*25*	*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*

**Serum requirements: growth medium contains 10% fetal calf serum; maintenance medium contains 2-5% fetal calf 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.

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

6. Procedure for Passage of BGM Cells

Pass stock BGM cell cultures at approximately seven-day intervals.

6.1 General Procedure

If at all feasible use a laminar flow hood while processing cell cultures. Otherwise, use an area restricted solely to cell culture manipulations. Viruses or other microorganisms must not be transported, handled, or stored in cell culture transfer facilities.

6.1.1 Add serum and antibiotics to the stored growth medium (see Section 3.3.3) on day BGM cells will be subcultured.

6.1.2 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.

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

See Table 9-1.

To reduce shock to cells, warm EDTA-trypsin reagent to 36.5° ± 1°C before placing it on cell monolayers.

Dispense the EDTA-trypsin reagent directly onto the cell monolayer.

6.1.4 Allow EDTA-trypsin reagent to remain in contact with the cells at either room temperature or incubation temperature of 36.5° ± 1°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.

6.1.5 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.

6.1.6 Centrifuge cell suspension at 1,000 x *g* for 10 min to pellet cells.

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

6.1.7 Pour off and discard the supernatant.

6.1.8 Suspend the pelleted cells in growth medium.

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.

6.1.9 Perform a viable count on the cell suspension according to procedures in Section 6.2.

6.1.10 Dilute the cell suspension to the appropriate cell concentration with growth medium (see Section 3.3.3).

Calculate the dilution factor requirement using the cell count established in Section 6.2.6 and the cell concentration parameters given in Table 9-1.

6.1.11 Dispense the cell suspension into cell culture vessels with either a Cornwall-type syringe or Brewer-type pipetting machine dispenser.

The volume of cell suspension that must be used for a particular stock culture vessel is listed in Table 9-1.

6.2 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.

6.2.1 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 hem-

ocytometer section should be between 20 and 50. This range is equivalent to between 6.0 x 10^5 and 1.5 x 10^6 cells per mL of cell suspension. Thus, a dilution of 1:10 is usually required for an accurate count of a cell suspension.

6.2.2 Disperse cells by repeated pipetting.

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

6.2.3 With a capillary pipette, carefully fill hemocytometer chambers on one side of a slip-covered hemocytometer slide.

Do not under or over fill the chambers.

6.2.4 Rest slide on flat surface for about one min to allow trypan blue to penetrate cell membranes of nonviable cells.

6.2.5 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.

6.2.6 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.

6.3 Procedure for Changing Medium on Cultured Cells

Three to four days after seeding with an appropriate number of cells, monolayers normally become 95% to 100% confluent, and growth medium becomes acidic. Growth medium on confluent stock cultures should then be replaced with maintenance medium. If stock culture cell monolayers have not reached 95% to 100% confluency by this time and the medium on these cultures has not become acidic, then the medium should not be changed until the monolayers reach 95% to 100% confluency. If, three to four days after passage, monolayers are not yet 95% to 100% confluent and the medium in which they are immersed has become acidic, then the medium must be replaced with fresh growth medium supplemented with 5% fetal calf serum.

6.3.1 Pour spent medium from cell culture vessels and discard the spent medium.

6.3.2 Add to the cell culture vessels a volume of fresh maintenance or growth medium equal to the volume of spent medium discarded.

7. Procedure for Preparation of BGM Cell Cultures for Virus Assay

This section is an extension of the procedure described in Section 5. BGM cell cultures planted for virus assay are generally found to be at their most sensitive level between the third and sixth days, and cultures older than seven days should not be used. The maintenance medium change recommended for the stock cultures is not necessary in the preparation of these cells. However, prior to inoculation with virus the cultures should be washed with Earle's balanced salts with lactalbumin hydrolysate (ELAH) maintenance medium that contains no serum (see Chapter 10). Care must be taken to ensure that all caps on bottles, flasks or tubes are tight; otherwise, the gas seal will not be complete and poor growth will result.

7.1 Preparation of Cell Culture Bottles or Flasks

7.1.1 Use cell suspension from Section 6.1.8 to prepare cell cultures for virus plaque assay.

7.1.2 Dilute the cell suspension to the appropriate cell concentration with MEM/L-15 growth medium supplemented with 10% fetal calf serum and antibiotics (see Section 3.3.3).

Calculate the dilution factor requirement using the cell count established in Section 6.2 and the cell concentration parameters given in Table 9-2.

7.1.3 Dispense the cell suspension into cell culture vessels with either a Cornwall-type syringe or a Brewer-type pipetting machine dispenser.

The volume of cell suspension that must be used for a particular virus assay culture vessel is listed in Table 9-2.

7.1.4 Place tightly-capped cell culture vessels that contain cells in a stationary position at 36.5° ± 1°C with the flat side down so that cell monolayers develop on the proper surface.

7.1.5 Conduct plaque assay for viruses in accordance with instructions given in Chaper 10.

7.2 Preparation of Cell Culture Tubes

7.2.1 Use cell suspension from Section 6.1.8 to prepare cell cultures for plaque confirmation procedure (Chapter 11) and virus identification (Chapter 12).

7.2.2 Dilute the cell suspension to the appropriate cell concentration with growth medium (see Section 3.3.3).

Calculate the dilution factor requirement using the cell count established in Section 6.2 and the cell concentration parameters given in Table 9-2.

7.2.3 Dispense the cell suspension into cell culture tubes with a 2.0-mL Cornwall-type syringe.

7.2.4 Place tightly-capped tubes in tissue culture rack and incubate at 36° ± 1°C statically for three days before inoculating with viruses.

If tubes are to be held longer than 5 days, replace growth medium with maintenance medium as directed in Section 5.1.4. Cells may be held in maintenance medium for an additional five days.

7.2.5 Use the cell culture tubes to pass viruses recovered from plaques according to the technique described in Chapter 11.

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 15 years with a minimum loss in cell viability.

8.1 Preparation of Cells for Storage

8.1.1 Prepare 80 mL of growth medium as directed in Section 3.3.3.

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

Base the actual number of vials to be prepared on usage of the line and

Table 9-2. Guide for Preparation of Virus Assay Cell Cultures

Vessel Type	*Volume of Medium* (mL)*	*Final Cell Count per Bottle*
*1-oz** glass bottle*	*4*	*9.0×10^5*
25-cm² plastic flask	*10*	*3.5×10^6*
6-oz glass bottle	*15*	*5.6×10^6*
75-cm² plastic flask	*30*	*1.0×10^7*
16-mm X 150-mm tubes	*2*	*4.0×10^4*

**Serum requirements: growth medium contains 10% fetal calf serum or 10% GG-free calf 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.
***Size is given in oz only when it is commercially designated in that unit.*

the anticipated time interval requirement between cell culture start-up and full culture production. At least two vials are needed to start-up a new working culture.

8.1.2 Add 10 mL of fetal calf serum and 10 mL of DMSO to growth medium.

8.1.3 Sterilize cell storage medium by passage through an 0.22 μm sterilizing filter.

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

8.1.4 Harvest BGM cells from cell culture vessels as directed in Sections 6.1.2 thru 6.1.7.

8.1.5 Suspend cells in the storage medium prepared in Section 8.1.3.

Cell concentration per mL must be at least 1×10^6.

8.1.6 Place flask containing suspended cells on magnetic stirrer.

8.1.7 Mix contents of flask for 30 min.

8.1.8 Perform viable cell count according to procedure given in Section 6.2.

If cell culture is below 1×10^6 cells per mL, centrifuge cell suspension at 1,000 x g for 10 min to pellet cells, resuspend cells in lesser volume of storage medium and determine new cell concentration.

8.1.9 Dispense 1-mL volumes of cell suspension into 2-mL vials.

8.2 Procedure for Freezing Cells

The freezing procedure requires slow cooling of the cells with the optimum rate of 1°C per min. Because a controlled temperature freezer is generally unavailable, the following method is recommended as an alternative system.

8.2.1 Place vials in rack.

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

8.2.2 Place rack in refrigerator at 4°C.

8.2.3 Remove rack from refrigerator after 30 min and immediately place in –20°C freezer.

8.2.4 Remove rack from –20°C freezer after 30 min and immediately place in –70°C freezer.

8.2.5 Hold overnight at –70°C before placing 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.

8.3 Procedure for Thawing Cells

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

8.3.1 Add appropriate volume of MEM/L-15 growth medium supplemented with 10% fetal calf serum and antibiotics to either a 6-oz tissue culture bottle or 75-cm² tissue culture flask.

See Table 9-2 and Section 3.3.3.

8.3.2 Place vial into 36°C water bath and agitate vigorously by hand until all ice has melted.

8.3.3 Wipe vials with disinfectant solution of 0.5% I_2 in 70% ethanol (Chapter 3, Section 3.1).

8.3.4 Add BGM cells to growth medium prepared in Section 8.3.1.

8.3.5 Incubate BGM cells at 36.5° ± 1°C.

8.3.6 Pour off growth medium after incubation period of 18 h to 24 h.

8.3.7 Add fresh MEM/L-15 growth medium supplemented with 10% fetal calf serum and antibiotics.

8.3.8 Re-incubate BGM cells at 36.5° ± 1°C for an additional five days.

8.3.9 Pass and maintain stock BGM cell cultures as directed in Section 5.

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