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Virus Monitoring Protocol for the Information Collection Requirements Rule

United States Environmental Protection Agency
OGWDW/TSD/WSTB
26 West Martin Luther King Drive
Cincinnati, OH 45268

The use of manufacturer trade names in this document does not constitute endorsement by the United States Environmental Protection Agency.

Contents

About This Document

This bench-top guide is intended for use by laboratory personnel analyzing water samples for the presence of culturable viruses in accordance with the Information Collection Requirements Rule. It provides details of the procedure presented in the accompanying video. To further associate the steps of the procedure with the training video, this manual is illustrated with stills taken directly from the video.

Several graphic conventions are used throughout the manual to differentiate steps or draw attention to important information:

A step icon is used to denote each step in the virus monitoring protocol. These steps also are illustrated in the training video.

Important information, and valuable tips which are not part of the published protocol, are denoted by this icon. In addition, explanatory information is provided in italics throughout the document.

EPA recommends that laboratory analysts and technicians watch the training video and read this manual in order to become familiar with the virus monitoring protocol. The video concludes with a summary of the analytical procedure. Analysts and technicians also can use the manual as a bench-top reference during the execution of the procedure.

Acknowledgments

This bench-top guide and the accompanying training video were developed by the U.S. Environmental Protection Agency (EPA), Office of Water, Office of Ground Water and Drinking Water (OGWDW), Cincinnati, Ohio. Jim Walasek of OGWDW's Technical Support Division (TSD) was the project manager. Shay Fout, Ph.D. of the Office of Research and Development's National Exposure Research Laboratory (NERL) wrote the text of the guide. He was the principal technical advisor in the preparation of the video, and was responsible for the photomicrography used in both the guide and the video. Frank Schaefer, Ph.D. of NERL also served as technical advisor on the project. Ken Mayo of The Cadmus Group, Inc. in Waltham, Massachusetts edited, designed, and coordinated production of the guide.

The photographs used in this guide come from the accompanying training video. The video was taped at the U.S. EPA laboratories in Cincinnati, Ohio. Melissa Godoy of Impact Video Productions in Cincinnati was the producer, and Graham Spencer and Steve Willis were the editors. Donna Jensen of Cadmus acted as analyst during the taping, and Michelle Thomas was the technician. Ms. Thomas is a participant at EPA in a post-graduate research program administered by the Oak Ridge Institute for Science and Education.

Foreword
The Virus Monitoring Protocol

The surface water treatment rule (40 CFR Part 141) established the maximum contaminant level for enteric virus in public water systems by requiring that systems using surface water or ground water under the influence of surface water reduce the amount of virus in source water by 99.99 percent.

The rule requirements are currently met on the basis of treatment alone (e.g., disinfection and/or filtration), and thus the degree of actual protection against waterborne viral disease depends upon the source water quality. Utilities using virus-free source water or source water with low virus levels may be overtreating their water, while utilities using highly contaminated water may not be providing adequate protection.

To determine more adequately the level of protection from virus infection and to reduce the levels of disinfection and disinfection by-products, where appropriate, EPA is requiring all utilities serving a population of at least 100,000 to monitor their source water for viruses monthly for a period of 18 months. Systems finding greater than one infectious enteric virus particle per liter of source water must also monitor their finished water on a monthly basis. The authority for this requirement is Section 1445(a)(1) of the Safe Drinking Water Act, as amended in 1986.

This Virus Monitoring Protocol was developed by virologists at the U.S. Environmental Protection Agency and modified to reflect consensus agreements from the scientific community and comments to the draft rule. The procedures contained herein do not preclude the use of additional tests for research purposes (e.g., polymerase chain reaction-based detection methods for noncytopathic viruses).

The concentrated water samples to be monitored may contain pathogenic human enteric viruses. **Laboratories performing virus analyses are responsible for establishing an adequate safety plan and must rigorously follow the guidelines on sterilization and aseptic techniques given in Appendix B.**

Analytical Reagent or ACS grade chemicals (unless specified otherwise) and reagent-grade water should be used to prepare all media and reagents. The reagent-grade water must have a resistance greater than 0.5 megohms-cm at 25°C, but water with a resistance of 18 megohms-cm is preferred. Water and other reagent solutions may be available commercially. For any given section of this protocol only apparatus, materials, media and reagents which are not described in previous sections are listed, except where deemed necessary. The amount of media prepared for each part of the protocol may be increased proportionally to the number of samples to be analyzed.

Chapter 1 Introduction

Under the U.S. Environmental Protection Agency's (EPA's) Information Collection Requirements Rule (ICR), public water systems that use surface water, or ground water under the influence of surface water, and that serve 100,000 persons or more must monitor their source water for viruses and other pathogens. If more than one pathogen per liter of source water is found, systems also will have to monitor their finished water for pathogens and for other water quality indicators.

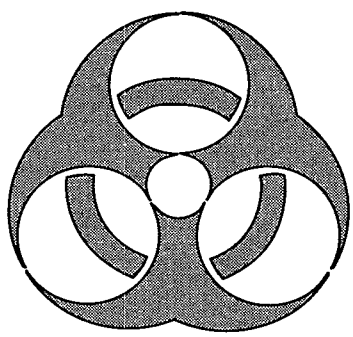
EPA will use the information collected under the ICR when considering possible changes to the Surface Water Treatment Rule (SWTR) and when developing drinking water regulations covering disinfectants and disinfectant by-products. If the SWTR is amended, information collected under the ICR also will help utilities comply with new and updated requirements.

Laboratory Approval Requirements

To make comparing results from different systems easier, EPA requires that all laboratories approved to analyze pathogens monitored under the ICR use the same analytical procedures. To qualify for approval to analyze water samples for viruses, a laboratory must (1) analyze satisfactorily a set of performance evaluation (PE) samples of viruses at various concentrations, (2) meet personnel requirements for at least one principal analyst, and (3) pass an on-site evaluation by EPA.

Cell Culture Preparation

A lab must maintain separate rooms for preparing cell cultures and for processing virus cultures. **If separate rooms are not available, then Class II biological safety cabinets must be used to prevent contamination when preparing cell cultures.**



Personnel Requirements

Principal Analyst/Supervisor

A qualified, experienced microbiologist must be available to act as principal analyst/supervisor. This person oversees the entire analysis of water samples for the presence of viruses. He or she must have a bachelor's degree in life sciences, plus three years of experience in both cell culture and animal virus analyses.

Analyst

The analyst performs at the bench level and is involved in all aspects of the analysis, including sample collection, filter extraction, sample processing, and assay. This person must have two years of full-time college course work in the life sciences, or at least six months' bench experience in cell culturing and animal virus analyses.

Technician

The technician extracts the filter and processes the sample, but does not perform tissue culture work. This person must have at least three month's experience in filter extraction of virus samples and sample processing.

For More Information

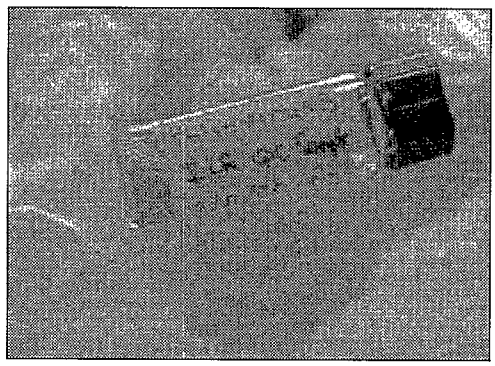
More information about the procedures presented in this document is available from the:

ICR Coordinator
U.S. Environmental Protection Agency
Office of Ground Water and Drinking Water
26 West Martin Luther King Drive
Cincinnati, OH 45268

You can also call EPA's Safe Drinking Water Hotline at 1 800 426-4791.

Chapter 2
Sample Processing

Quality Control and Performance Evaluation Samples



Quality control (QC) samples, containing 1 mL of attenuated poliovirus type 3 of known concentration per vial, and performance evaluation (PE) samples consisting of 1MDS filters containing attenuated poliovirus type 3 at unknown concentrations, will be prepared by an ICR contractor and shipped to analysts seeking approval.

As part of the initial approval process, each analyst participating in the ICR virus monitoring program must successfully analyze PE samples. After initial approval, each analyst must successfully analyze one QC sample set per sample batch and new PE samples every month. A QC sample set is composed of a negative and a positive QC sample. A sample batch consists of all the ICR samples that are analyzed by an analyst during a single week. Each sample batch and its associated QC sample set must be assigned a unique batch number. QC samples do not have to be processed during weekly periods when no ICR samples are processed. QC and PE data should be sent directly to EPA as specified in the final rule.

Gloves and a lab coat should always be worn whenever handling virus and water samples that may contain virus.

QC Samples

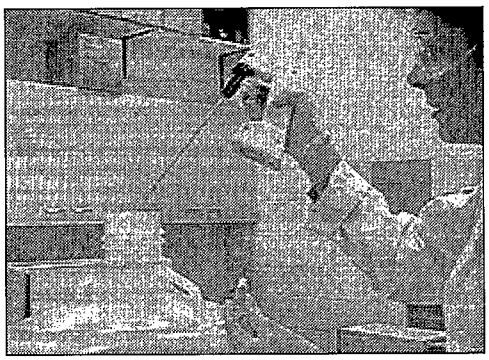
Negative QC Sample

Step 1 Elute a sterile 1 MDS filter in a sterile cartridge housing according to the Elution Procedure given below.

Step 2 Process the eluate using the Organic Flocculation and Total Culturable Assay procedures that follow.

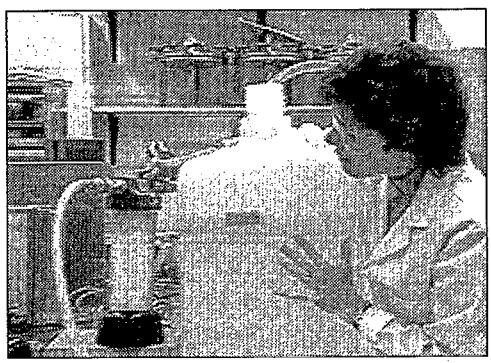
Positive QC Sample

Step 1 Place 40 liters of reagent-grade water in a sterile polypropylene container (Cole-Parmer Product No. G-06063-32) and add 1 mL of QC stock of attenuated poliovirus containing 200 PFU/mL.



! A QC sample with a titer of 200 PFU/mL will be supplied for the QC tests described in this section. The titer of QC sample may be changed before the start or during the testing phase of the ICR. Analysts must use these samples as supplied and not attempt to adjust the titer to 200 PFU/mL. A high titer QC sample will also be shipped to each analyst so that laboratories can develop their own internal QC programs. The high titered sample is not to be used for the QC tests described in this section.

Step 2 Mix and pump the solution through a standard apparatus containing a 1MDS filter.



Step 3 Process and analyze the 1MDS filters containing QC samples using the Elution, Organic Flocculation and Total Culturable Virus Assay procedures given below.

PE Samples

Process and analyze PE samples according to the Elution, Organic Flocculation and Total Culturable Virus Assay procedures of this protocol and according to any additional procedures supplied with the samples.

Sample Arrival

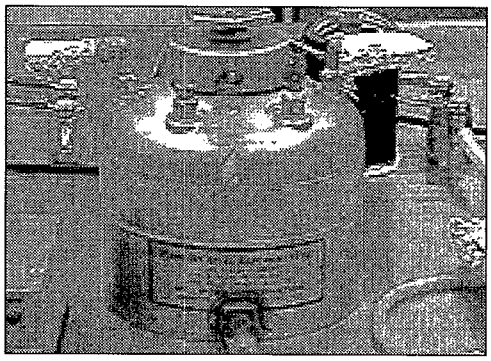
The cartridge filters must arrive from the utility refrigerated, but not frozen. Filters should be refrigerated upon arrival at the laboratory and eluted within 72 hours of the start of the sample collection.

When the sample arrives, record its condition on the Sample Data Sheet. (See Appendix E.)

Also note the sample volumes as recorded on the Sample Data Sheet at the utility. **Between 200 and 300 liters of source water**, or **1,500 - 1,800 liters of finished water**, must have been sampled. Samples that do not fall within these ranges should not be analyzed. More than one 1MDS filter may be submitted for the same sample, however, in order to meet the minimum sample volume requirements.

Elution Procedure

Apparatus and Materials



- Positive pressure air or nitrogen source equipped with a pressure gauge.

  If the pressure source is a laboratory air line or pump, it must be equipped with an oil or air filter.

- Dispensing pressure vessels, 5- or 20-liter capacity (Millipore Corp. Product No. XX67 00P 05 and XX67 00P 20).

- pH meter with combination-type electrode and an accuracy of at least 0.1 pH unit.

- Autoclavable inner-braided tubing with screw clamps or quick connects for connecting tubing to equipment.

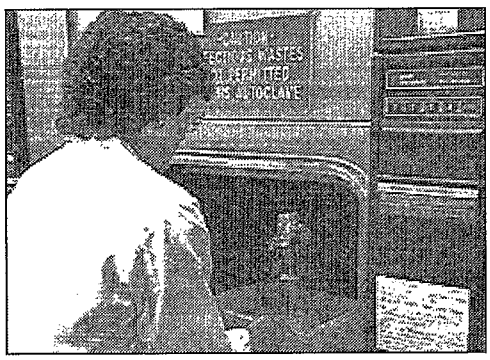
- Magnetic stirrer and stir bars.

Media and Reagents

- Sodium hydroxide (NaOH). Prepare 1 M and 5 M solutions by dissolving 4 g or 20 g of NaOH in a final volume of 100 mL of reagent-grade water, respectively.

  NaOH solutions may be stored for several months at room temperature.

- Beef extract V powder (BBL Microbiology Systems Product No. 97531). Prepare buffered 1.5% beef extract by dissolving 30 g of beef extract powder and 7.5 g of glycine (final glycine concentration = 0.05 M) in 1.9 liters of reagent-grade water. Adjust the pH to 9.5 with 1 or 5 M NaOH and bring the final volume to 2 liters with reagent-grade water. Autoclave at 121°C for 15 minutes and use at room temperature.



When sterilizing the flask of beef extract, be sure to put the flask in a pan of water before placing the flask and the pan in the autoclave. This will ensure even heating of the flask and will help keep the flask from breaking.

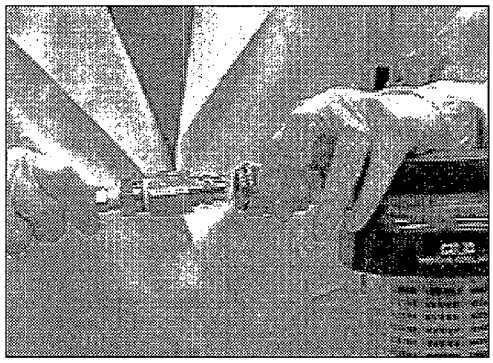
Beef extract solutions may be stored for one week at 4°C or for longer periods at -20°C.

Screen each new lot of beef extract prior to use in the organic flocculation concentration procedure to determine whether virus recoveries are adequate. Perform the screening by spiking 1 liter of beef extract solution with 1 mL of a diluted QC sample containing 200 PFU/mL. Assay the spiked sample according to the Organic Flocculation and Total Culturable Virus Assay procedures given below. Use a single passage with undiluted sample and sample diluted 1:5 and

1:25 along with an equivalent positive control. The mean recovery of poliovirus for 3 trials should be at least 50%.

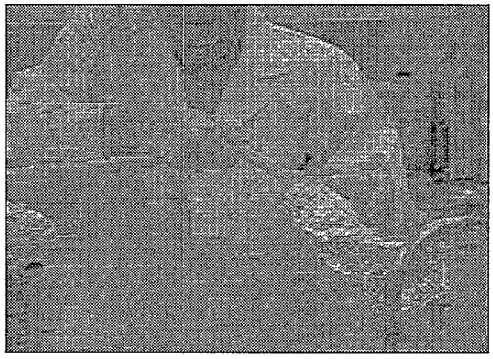
Procedure

Step 1 Attach sections of braided tubing (sterilized on inside and outside surfaces with chlorine and dechlorinated with thiosulfate as described in Appendix B) to the inlet and outlet ports of a cartridge housing module containing a 1MDS filter to be tested for viruses.

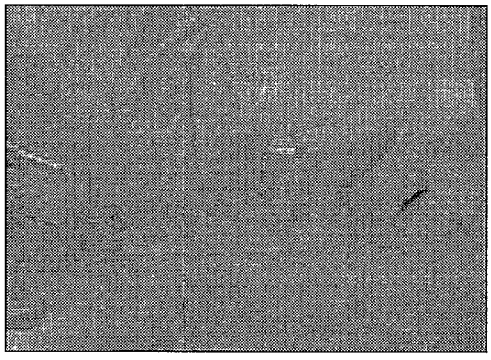


If a prefilter was used, keep the prefilter and cartridge housing modules connected. Attach the tubing to the inlet of the prefilter module and to the outlet of the cartridge housing module.

Step 2 Place the sterile end of the tubing connected to the outlet of the cartridge housing module into a sterile 2-liter glass or polypropylene beaker.



Step 3 Connect the free end of the tubing from the inlet port of the prefilter or cartridge housing modules to the outlet port of a sterile pressure vessel. Connect the inlet port of the pressure vessel to a positive air pressure source.



Add pressure to blow out any residual water from the cartridge housing(s) into a sterile 2-liter glass or polypropylene beaker.

! Discard any residual water that collects in the beaker, and use another sterile beaker for the remainder of this step and for the steps that follow.

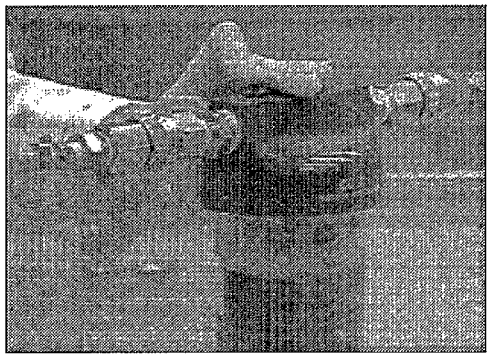
Open the vent/relief valve to release the pressure.

Step 4 Remove the top of the pressure vessel and pour 1,000 mL of buffered 1.5% beef extract (pH 9.5), prewarmed to room temperature, into the vessel.

Replace the top of the pressure vessel and close its vent/relief valve.

Acceptable alternatives to the use of a pressure vessel include: 1) the use of a peristaltic pump and sterile tubing to push the beef extract through the filter, and 2) the addition of beef extract directly to the cartridge housing and the use of positive pressure to push the beef extract through the filter.

Step 5 Open the vent/relief valve(s) on the cartridge housing(s) and apply sufficient pressure to purge trapped air from them.



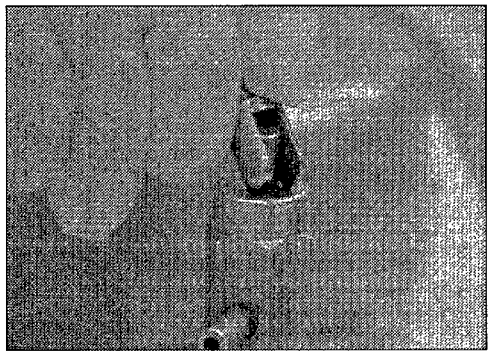
Close the vent/relief valve(s) as soon as the buffered beef extract solution begins to flow from it.

! Make sure to wear gloves and to place a gauze pad treated with iodine over the cartridge housing vent/relief valve for this procedure.

Turn off the pressure and allow the solution to contact the 1MDS filter for one minute.

Wipe up spilled liquid with laboratory disinfectant. Carefully observe alternative housings without vents to ensure that all trapped air has been purged.

Step 6 Increase the pressure to force the buffered beef extract solution through the filter(s).

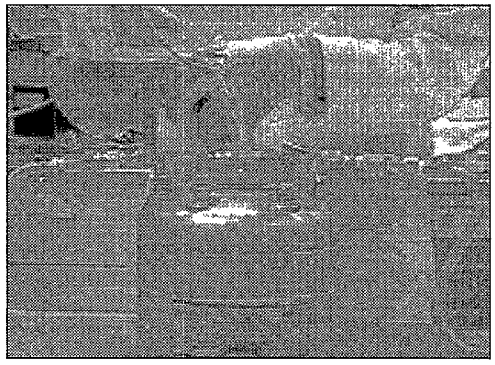


! You can use the pressure vessel's relief valve to control the beef extract's rate of flow. Use of the relief valve provides finer control over the flow rate than does controlling the air pressure at the source.

The solution should pass through the 1MDS filter slowly to maximize the elution contact period. When air enters the line from the pressure vessel, elevate and invert the filter housing to permit complete evacuation of the solution from the filters.

Step 7

Turn off the pressure at the source and open the vent/relief valve on the pressure vessel.



Pour the buffered beef extract from the 2-liter beaker back into the pressure vessel.

Replace the top of the pressure vessel and close its vent/relief valve.

Repeat Steps 5 and 6.

Step 8

Turn off the pressure at the source and open the vent/relief valve on the pressure vessel. Thoroughly mix the eluate.

If archiving is not required, and if the optional coliphage assay is not performed, measure the volume of the eluate and record it on the Virus Data Sheet as the Eluate Volume Recovered. Take the Total Sample Volume from the Sample Data Sheet and record it as the Adjusted Total Sample Volume on the Virus Data Sheet.

If archiving is required, or if the optional coliphage assay will be performed, adjust the pH of the eluate to 7.0-7.5 with 1M HCl. Measure the volume of the adjusted eluate and record it onto the Virus Data Sheet as the Eluate Volume Recovered.

Determine the amount of sample to be removed for archiving by multiplying the Eluate Volume Recovered by 0.1. Record this amount onto the Virus Data Sheet as the Volume of Eluate Archived. Place a volume equal to this amount into a separate container. Freeze the archive sample and ship it to the ICR Laboratory Coordinator, U.S. EPA, Office of Ground Water and Drinking Water, 26 West Martin Luther King Drive, Cincinnati, OH 45268.

Determine the amount of sample to be used in the coliphage assay by multiplying the Eluate Volume Recovered by 0.035. Place a volume equal to this amount into a separate container and store at 4°C. Multiply the recorded Total Sample Volume from the Sample Data Sheet by 0.9 if an archive sample is taken, by 0.965 if a coliphage sample is taken, or by 0.865 if both an archive sample and a coliphage sample are taken. Record the amount on the Virus Data Sheet as the Adjusted Total Sample Volume.

All freezing of sample and cell cultures throughout this protocol should be performed rapidly by placing vessels in a freezer at -70°C or below, or in a dry ice-alcohol bath. Frozen samples and cell cultures should also be thawed rapidly. This may be done by placing vessels in a 37°C water bath, but vessel caps must not be immersed and vessels should be removed from the water bath as soon as, or just before, the last ice crystals melt.

Proceed to the Organic Flocculation Concentration Procedure immediately. If the Organic Flocculation Concentration Procedure cannot be undertaken immediately, store the pH-adjusted eluate at 4°C for up to 24 hours, or for longer periods at -70°C.

Organic Flocculation Concentration Procedure

Apparatus and Materials

- Refrigerated centrifuge capable of attaining 2,500 - 10,000 x g and screw-capped centrifuge bottles with 100 to 1,000 mL capacity.

  Each bottle must be rated for the relevant centrifugal force.

- Sterilizing filter — 0.22 µm Acrodisc filter with prefilter (Gelman Sciences Product No. 4525).

  Samples containing a lot of debris may require a sterilizing filter stack. Prepare sterilizing filter stacks using 0.22 µm

pore size membrane filters (Millipore Corp. Product No. GSWP 47 00) stacked with fiberglass prefilters (Millipore Corp. AP15 47 00 and AP20 47 00).

Stack the prefilters and 0.22 µm membrane in a disc filter holder (Millipore Corp. Product No. SX00 47 00) with the AP20 prefilter on top and 0.22 µm membrane filter on bottom. Disassemble the filter stack after each use to check the integrity of the 0.22 µm filter. Refilter any media filtered with a damaged stack.

Always pass about 10 - 20 mL of beef extract, pH 7.0-7.5 (prepared as above, without pH adjustment), through the filter just prior to use. This step will reduce virus adsorption onto the filter membranes.

Media and Reagents

- Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$) — 0.15 M, pH 9.0-9.5.

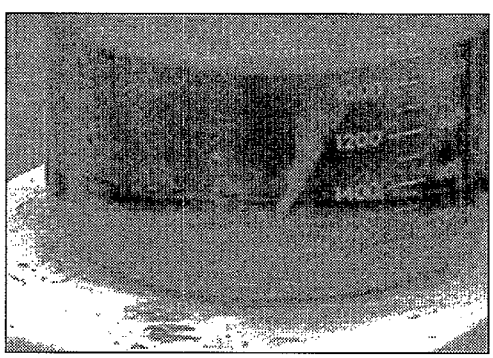
  Dissolve 40.2 g of sodium phosphate in a final volume of 1,000 mL. The pH should be checked to ensure that it is between 9.0 - 9.5 and adjusted with NaOH, if necessary. Autoclave at 121°C for 15 minutes.

- Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$) — 0.15 M, pH 7.0-7.5.

  Dissolve 40.2 g of sodium phosphate in 900 mL. Adjust the pH to 7.0-7.5 with 1M HCl. Bring to 1,000 mL. Autoclave at 121°C for 15 minutes.

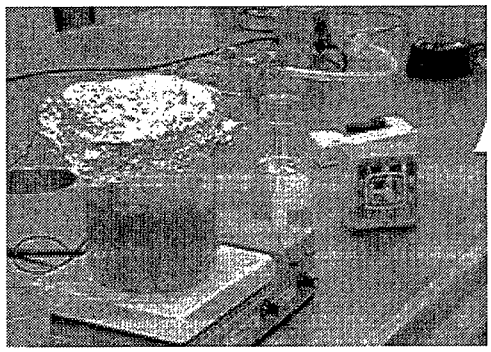
Procedure

Minimize foaming (which may inactivate viruses) throughout the procedure by stirring or mixing no faster than necessary to develop a vortex.



Step 1 Place a sterile stir bar into the beaker containing the buffered beef extract eluate from the cartridge filter(s).

Place the beaker on a magnetic stirrer, and stir at a speed sufficient to develop a vortex.

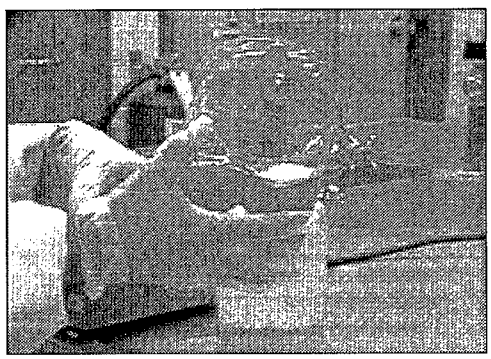


Step 2 Insert a combination-type pH electrode into the beef extract eluate. Slowly add 1 M HCl to the flask, while moving the tip of the pipette in a circular motion away from the vortex to facilitate mixing. Continue adding 1 M HCl until the pH of the beef extract reaches 3.5 ± 0.1.

Continue to stir slowly for 30 minutes at room temperature.

The pH meter must be standardized at pH 4 and 7. Electrodes must be sterilized before and after each use as described in Appendix B.

A precipitate will form. If pH falls below 3.4, add 1 M NaOH to bring it back to 3.5 ± 0.1. Exposure to a pH below 3.4 may result in some virus inactivation.



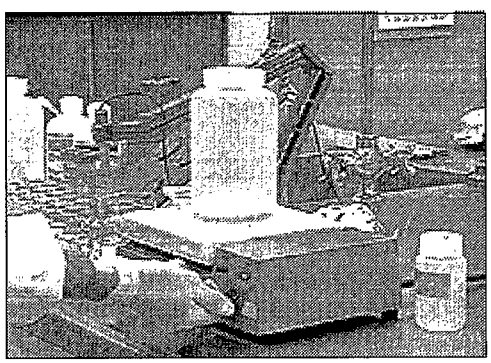
Step 3 Remove the electrode from the beaker, and pour the contents of the beaker into a centrifuge bottle.

Cap the bottle and centrifuge the precipitated beef extract suspension at 2,500 x g for 15 minutes at 4°C.

Remove and discard the supernatant.

To prevent the transfer of the stir bar into a centrifuge bottle, hold another stir bar or magnet against the bottom of the beaker while decanting the contents. The beef extract suspension will usually have to be divided into several centrifuge bottles.

! Balance the centrifuge bottles aseptically with beef extract or normal saline before placing them in the centrifuge whenever centrifugation is called for.

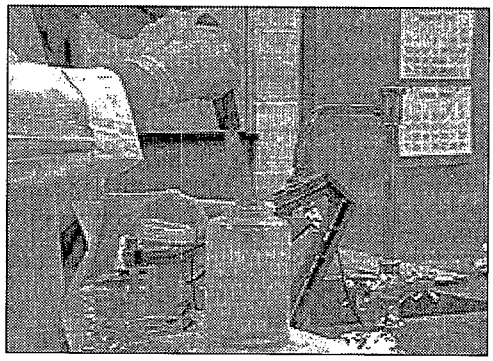


Step 4 Place a stir bar into the centrifuge bottle that contains the precipitate.

Add 30 mL of 0.15 M sodium phosphate.

Place the bottle on a magnetic stirrer, and stir slowly until the precipitate has dissolved completely.

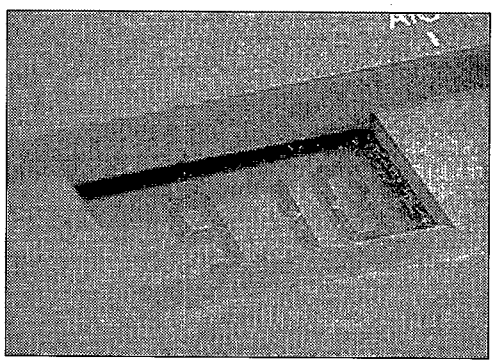
The precipitate may be partially dissipated with a spatula before or during the stirring procedure. It also may be dissolved by repeated pipetting, or by shaking at 160 rpm for 20 minutes on an orbital shaker, in place of stirring. When the centrifugation is performed in more than one bottle, dissolve the precipitates in a total of 30 mL and combine into one bottle. If the precipitate is not completely dissolved before proceeding, significant virus loss may occur in Step 5. Since virus loss may also occur by prolonged exposure to pH 9.0-9.5, laboratories which find it difficult to resuspend the precipitate may dissolve it initially in 0.15 M sodium phosphate that has been adjusted to pH 7.5 with 1 M HCl. If this variation is used, the pH should be readjusted to 9.0-9.5 with 1 M NaOH after the precipitate is completely dissolved and mixed for 10 minutes at room temperature before proceeding to Step 5.



Step 5

Check the pH and readjust to 9.0-9.5 with 1 M NaOH, as necessary.

Remove the stir bar and centrifuge the dissolved precipitate at 4,000 - 10,000 x g for 10 minutes at 4°C.



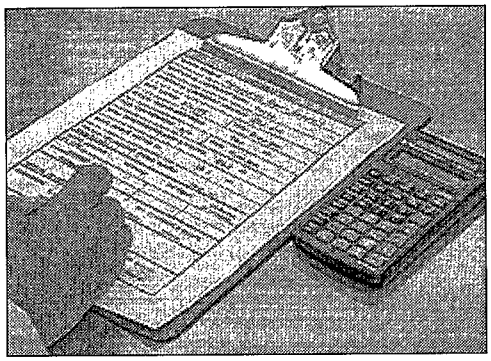
Remove the supernatant and discard the pellet.

Adjust the pH of the supernatant to 7.0-7.5 with 1 M HCl.

To remove microbial contamination, load the supernatant into a 50-mL syringe and force it through a sterilizing filter pretreated with beef extract. (Laboratories may use other approaches to remove contamination, but their effectiveness must be documented.)

Record the final volume of supernatant (designated the Final Concentrated Sample Volume, or FCSV) on the Virus Data Sheet (Appendix E).

If the sterilizing filter begins to clog badly, empty the loaded syringe into the bottle containing the unfiltered precipitate, fill the syringe with air, and inject air into the filter to force any residual sample from it. Continue the filtration procedure with another filter.



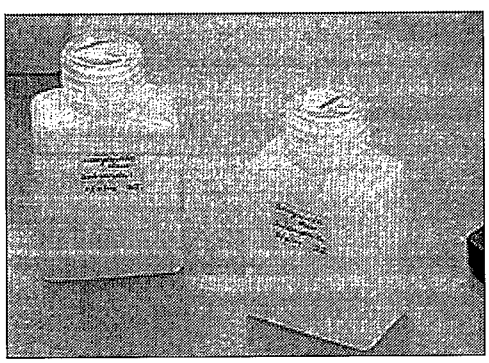
Step 6 Determine the volume of sample that must be assayed. This volume is at least 100 liters for source water or 1,000 liters for finished water, and is designated the Volume of Original Source Water Sample Assayed (D). Record the value of D on the Virus Data Sheet. Calculate the Assay Sample Volume (S) for source and finished water samples using the formula:

$$S = \frac{D}{ATSV} \times FCSV$$

where ATSV is the Adjusted Total Sample Volume from the Virus Data sheet. The Assay Sample Volume is the volume of the Final Concentrated Sample that represents 100 liters of source water or 1,000 liters of finished water.

! Analytical laboratories assaying more than the required volume must use the actual volume to be assayed in the calculation. See Appendix F for examples of the calculations used throughout this protocol.

Record the Assay Sample Volume on the Virus Data Sheet.



Prepare a subsample (subsample 1) containing a volume 0.55 times the Assay Sample volume.

Prepare a second subsample (subsample 2) containing a volume that is 0.67 times the Assay Sample volume. Divide the Final Concentrated Sample from QC and PE samples into two equal subsamples. Calculate the Assay Sample Volumes for these samples by multiplying FCSV by 0.4. Label each subsample with appropriate sampling information for identification.

Hold any portion of the sample that can be assayed within 24 hours at 4°C and freeze all other portions at -70°C.

Final Concentrated Samples, subsamples, PE and QC samples processed to this point by a laboratory not doing the virus assay must be immediately frozen at -70°C and then shipped on dry ice to the laboratory approved for the virus assay within 24 hours. Samples which can be rapidly transported between laboratories can be held at 4°C as long as the samples can be transported and assayed within 24 hours.

Chapter 3
Total Culturable Virus Assay

Quantal Assay

Apparatus and Materials

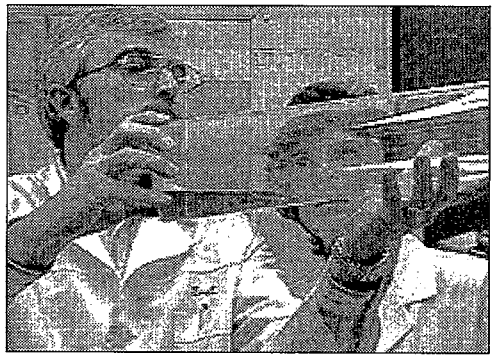
- Incubator capable of maintaining the temperature of cell cultures at 36.5 ± 1°C.

- Sterilizing filter — 0.22 µm (Costar Product No. 140666).

  To minimize virus adsorption to the filter, always pass about 10 - 20 mL of 1.5% beef extract, pH 7.0-7.5, through the filter just prior to use.

Media and Reagents

- Prepare BGM cell culture test vessels using standard procedures.



  BGM cells are a continuous cell line derived from African Green monkey kidney cells and are highly susceptible to many enteric viruses (Dahling et al., 1984; Dahling and Wright, 1986). The characteristics of this line were described by Barron et al. (1970). The use of BGM cells for recovering viruses from environmental samples was described by Dahling et al. (1974). For laboratories with no experience with virus recovery from environmental samples, the media and procedures described by Dahling and Wright (1986) and given in Appendix A are recommended for maximum sensitivity.

  The U.S. Environmental Protection Agency will supply an initial culture of BGM cells to all laboratories seeking approval. Upon receipt, laboratories must prepare an adequate supply of frozen BGM cells using standard procedures to

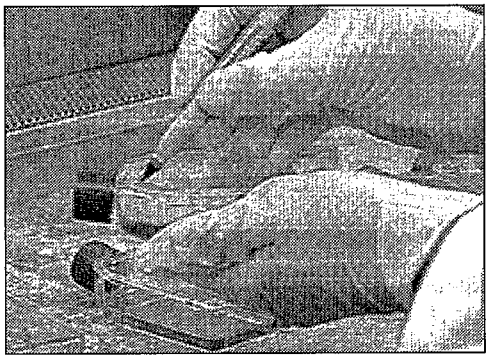
replace working cultures that become contaminated or that lose virus sensitivity. A procedure for Preservation of the BGM Cell Line is given in Appendix A.

Sample Inoculation and CPE Development

Cell cultures used for virus assay are generally found to be at their most sensitive level between the third and sixth days after their most recent passage. Those older than seven days should not be used.

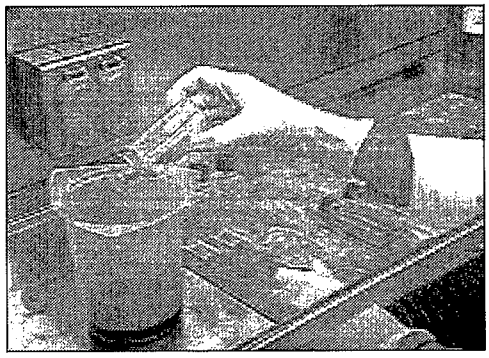
Step 1 Identify cell culture test vessels by coding them with an indelible marker.

Return the cell culture test vessels to a 36.5 ± 1°C incubator and hold at that temperature until the cell monolayer is to be inoculated.



Step 2 Decant and discard the medium from cell culture test vessels. Wash the test vessels with balanced salt solution or maintenance medium without serum using a wash volume of at least 0.06mL/cm^2 of surface area. Rock the wash medium over the surface of each monolayer several times, and then decant and discard the wash medium.

Do not disturb the cell monolayer.



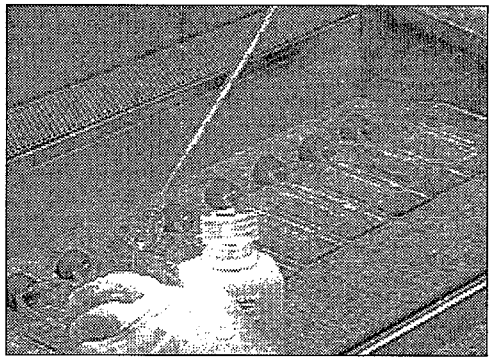
Step 3 Determine the Inoculum Volume by dividing the Assay Sample Volume by 20. Record the Inoculum Volume onto the Virus Data Sheet.

For ease of inoculation, a sufficient quantity of 0.15M sodium phosphate, pH 7.0-7.5, may be added to the Inoculum Volume to give a more usable working Inoculation Volume (e.g., 1.0 mL). For example, if an Inoculum Volume of 0.73 mL is to be placed onto 10 vessels, then 10.5 x (1-0.73) = 2.84 mL of the sodium phosphate could be added to 10.5 x 0.73 = 7.67 mL of

subsample. Each milliliter of the resulting mixture will contain the required Inoculum Volume.

The Inoculum, or Inoculation, Volume should be no greater than 0.04 mL/cm^2 of surface area. If the Inoculum, or Inoculation, Volume is greater than 0.04 mL/cm^2, use larger culture vessels.

Step 4 Inoculate each BGM cell culture test vessel with an amount of assay control or water sample equal to the Inoculum, or Inoculation, Volume and record the date of inoculation on the Sample Data Sheet.



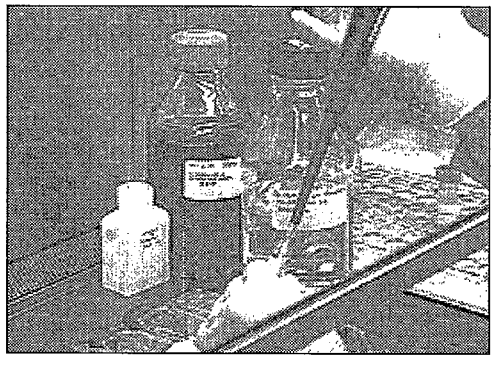
Avoid touching either the cannula or the pipetting device to the inside rim of the cell culture test vessels to avert the possibility of transporting contaminants to the remaining culture vessels.

Negative Controls

Inoculate a BGM culture with a volume of sodium phosphate, pH 7.0-7.5, equal to the Inoculum, or Inoculation, Volume. These cultures will serve as negative controls for the tissue culture quantal assay. If any Negative Assay Control develops cytopathic effects (CPE), all subsequent assays of water samples should be halted until the source of the positive result is determined.

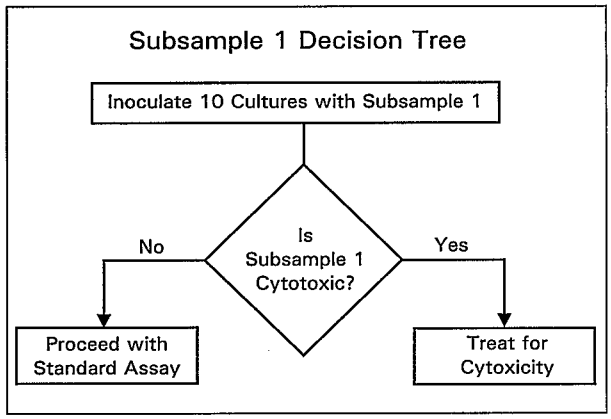
Positive Controls

Dilute attenuated poliovirus type 3 (from the high titered QC stock) in sodium phosphate, pH 7.0-7.5, to give a concentration of 20 PFU per Inoculum or Inoculation Volume. Inoculate a BGM culture with an amount of diluted virus equal to the Inoculum, or Inoculation, Volume. This control will provide a measure for continued sensitivity of the cell cultures to virus infection. Additional positive control samples may be prepared by adding virus to a small portion of the final concentrated sample and/or by using additional virus types. If any Positive Assay Control fails to develop CPE, all subsequent assays of water samples should be halted until the source of the negative result is determined. It may be necessary to thaw and use an earlier passage of the BGM cell line supplied by EPA.



Water Samples

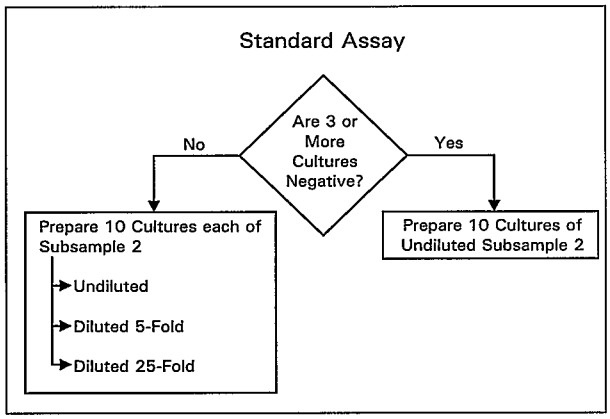
Rapidly thaw subsample 1, if frozen, and inoculate an amount equal to the Inoculum, or Inoculation, Volume onto each of 10 cell cultures. If there is no evidence of cytotoxicity and if at least 3 cell cultures are negative for CPE after 7 days (see below), thaw subsample 2 and inoculate an amount equal to the Inoculum, or Inoculation, Volume onto each of 10 additional cultures.



If dilutions are not required and the Inoculum Volume is not adjusted for surface area, inoculate subsamples 1 and 2 onto a total of 20 cultures (10 replicates per subsample).

Hold a thawed subsample no more than 4 hours at 4°C. Warm the subsample to room temperature just before inoculation.

A small portion of the Final Concentrated Sample may by inoculated onto cultures several days before inoculating subsample 1 as a control for cytotoxicity.



If cytotoxicity is not a problem and more than 7 cultures are positive for CPE after 7 days, prepare 5- and 25-fold dilutions of subsample 2. To prepare a 1:5 dilution, add a volume equal to 0.1334 times the Assay Sample Volume (amount "a") to a volume of 0.15 M sodium phosphate (pH 7.0-7.5) equal to 0.5334 times the Assay Sample volume (amount "b").

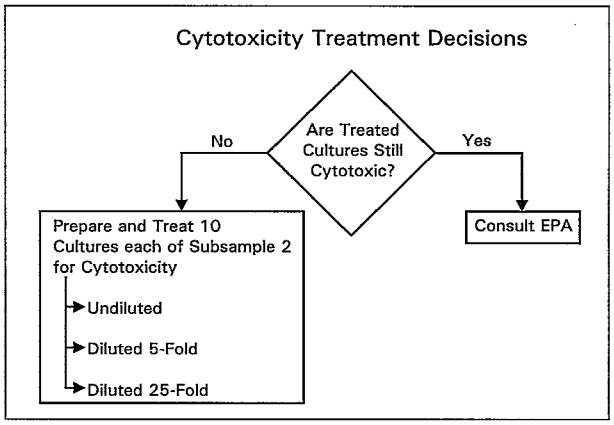
After mixing thoroughly, prepare a 1:25 dilution by adding amount "a" of the 1:5 diluted sample to amount "b" of 0.15 M sodium phosphate (pH 7.0-7.5). A working Inoculation Volume may be prepared from undiluted subsample 2 and from subsample 2 diluted 1:5 and 1:25 as described in Step 3. Using an amount equal to the Inoculum, or Inoculation, Volume, inoculate 10 cell cultures each with undiluted subsample 2, subsample 2 diluted 1:5 and subsample 2 diluted 1:25, respectively. Freeze the remaining portions of the 1:25 dilution at -70°C until the assay results are known.

If all the inoculated cultures are all positive, thaw the remaining 1:25 dilution and prepare 1:125, 1:625, and 1:3125

dilutions by transferring amount "a" of each lower dilution to amount "b" of sodium phosphate as described above. Inoculate 10 cultures each with the additional dilutions and freeze the remaining portion of the 1:3125 dilution.

Continue the process of assaying higher dilutions until at least one test vessel at the highest dilution tested is negative. Higher dilutions can also be assayed along with the initial undiluted to 1:25 dilutions if it is suspected that the water to be tested contains more than 500 most probable number (MPN) of infectious total culturable virus units per 100 liters.

If subsample 1 is cytotoxic, then five cell cultures should be inoculated with Final Concentrated Sample using the same volume required for subsample 1 and the procedures described in the Reduction of Cytotoxicity in Sample Concentrates section below. If these procedures remove cytotoxicity, inoculate subsample 2 using the procedures for removal of cytotoxicity and 10 cultures each with undiluted sample, sample diluted 1:5, and sample diluted 1:25 as described above. If the procedures fail to remove cytotoxicity, write for advice on how to proceed from the ICR Laboratory Coordinator, U.S. EPA, Office of Ground Water and Drinking Water, Technical Support Division, 26 W. Martin Luther King Drive, Cincinnati, OH 45268.



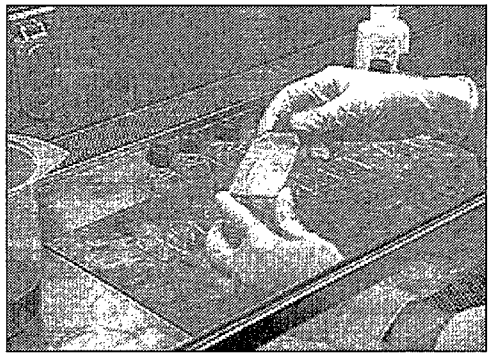
A maximum of 60 and 580 MPN units per 100 liters can be demonstrated by inoculating a total of 20 cultures with the undiluted Assay Sample Volume from source water or a total of 10 cultures each with undiluted sample and sample diluted 1:5 and 1:25, respectively.

QC and PE Samples

Prepare 5- and 25-fold dilutions of subsample 1 for each QC or PE sample, as described above. Inoculate 10 cultures each with undiluted subsample and subsample diluted 1:5 and 1:25 using an amount of inoculum equal to the Inoculum, or Inoculation, Volume.

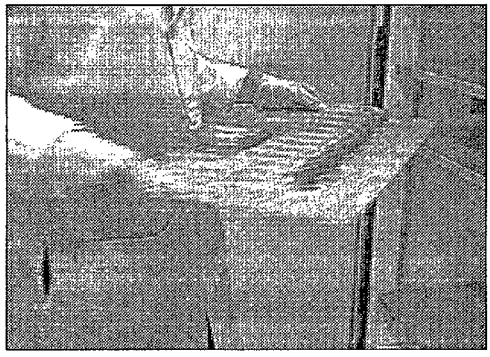
Use subsample 2 only as a backup for problems with the analysis of subsample 1.

Step 5 Rock the inoculated cell culture test vessels gently to achieve uniform distribution of inoculum over the surface of the cell monolayers.



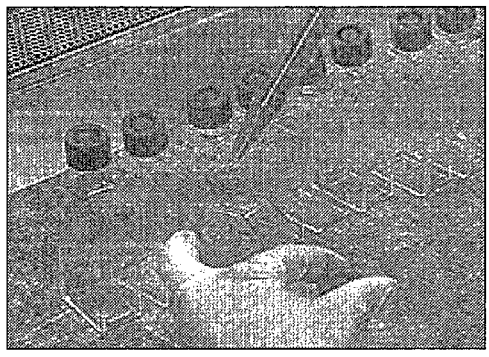
Place the cell culture test vessels on a level stationary surface at room temperature so that the inoculum will remain distributed evenly over the cell monolayer.

Step 6 Continue incubating the inoculated cell cultures for 80 - 120 minutes to permit viruses to adsorb onto and infect cells.



It may be necessary to rock the vessels every 15-20 minutes or to keep them on a mechanical rocking platform during the adsorption period to prevent the death from dehydration of cells in the middle of the vessels.

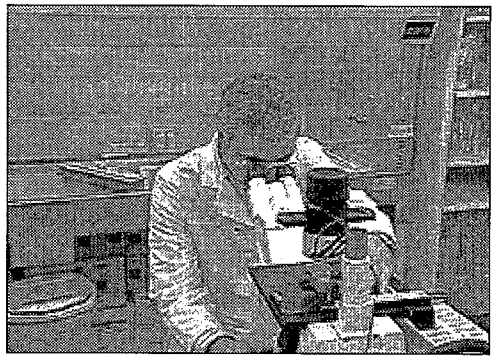
Step 7 After returning the cell cultures to the laboratory, add liquid maintenance medium (see Item 2 of Vessels and Media for Cell Growth in Appendix A for recommended medium) and incubate at 36.5 ± 1°C.



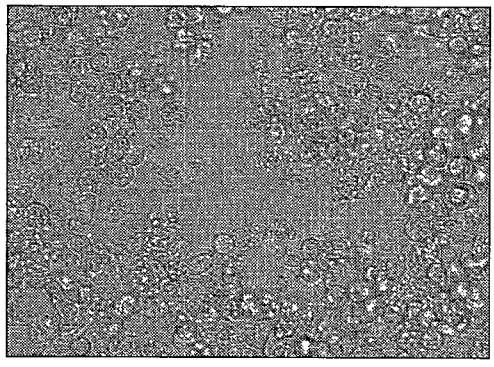
Warm the maintenance medium to 36.5 ± 1°C before placing it onto cell monolayers. Add the medium to the side of the cell culture vessel opposite the cell monolayer. Avoid touching any pipetting devices used to the inside rim of the culture vessels to avert the possibility of transporting contaminants to the remaining vessels.

! A Cornwall syringe or other large pipetting device may be used to add the medium.

Step 8 Examine each culture microscopically for the appearance of cytopathic effects (CPE) daily for the first 3 days and then every couple of days for a total of 14 days.

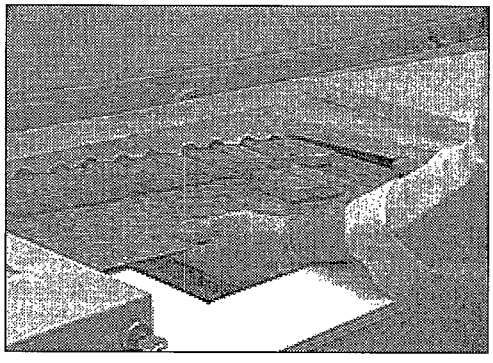


CPE may be identified as cell disintegration or as changes in cell morphology. Rounding-up of infected cells is a typical effect seen with enterovirus infections. However, uninfected cells round up during mitosis, and a sample should not be considered positive unless there are significant clusters of rounded-up cells over and beyond what is observed in the uninfected controls. Photomicrographs demonstrating CPE appear in the reference by Malherbe and Strickland-Cholmley (1980).



Step 9 Freeze cultures at -70°C when more than 75% of the monolayer shows signs of CPE.

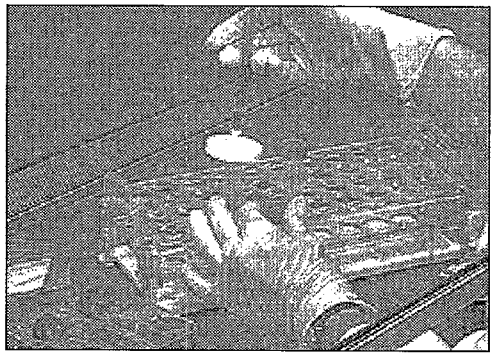
Freeze all remaining negative cultures, including controls, after 14 days.



Step 10 Thaw all the cultures to confirm the results of the previous passage.

Filter through separate 0.22 µm sterilizing filters at least 10% of the medium from each vessel that was positive for CPE or that appeared to be bacterially contaminated.

Then inoculate another BGM culture with 10% of the medium from the previous passage for each vessel, including those that were negative. Repeat Steps 7 and 8.



Confirmation passages may be performed in small vessels or multiwell trays. However, it may be necessary to distribute the inoculum into several vessels or wells to ensure that the volume of inoculum is less than or equal to 0.04 mL/cm² of surface area.

Step 11 Score cultures that developed CPE in both the first and second passages as confirmed positive cultures.

Cultures that show CPE in only the second passage must be passaged a third time along with the negative controls according to Steps 9 and 10.

Score cultures that develop CPE in both the second and third passages as confirmed positive cultures.

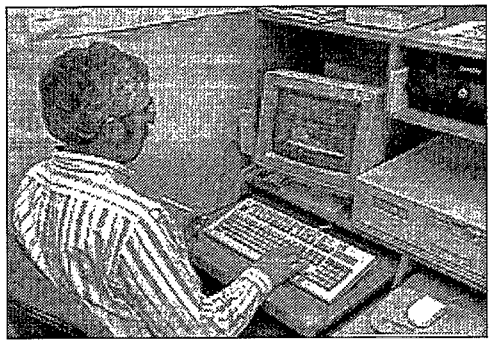
Cultures with confirmed CPE may be stored in a -70°C freezer for research purposes or for optional identification tests.

! For more information, see Chapter 12 (May 1988 revision) of Berg et al. (1984).

Virus Quantitation

Step 1 Record the total number of confirmed positive and negative cultures for each subsample onto the Total Culturable Virus Data Sheet (Appendix E). **Do not include the results of the tests for cytotoxicity!**

Step 2 Transfer the number of cultures inoculated and the confirmed number of positive cultures from the Total Culturable Virus data sheet for each subsample to the Quantitation of Total Culturable Virus data sheet.



If dilutions are not required, add the values to obtain a total undiluted count for each sample.

Calculate the MPN/mL value (M_m) and 95% confidence limits using the total undiluted count and the computer program supplied by the U.S. EPA.

If dilutions are reuired, calculate the MPN/mL value and 95% confidence limits from the subsample 2 values.

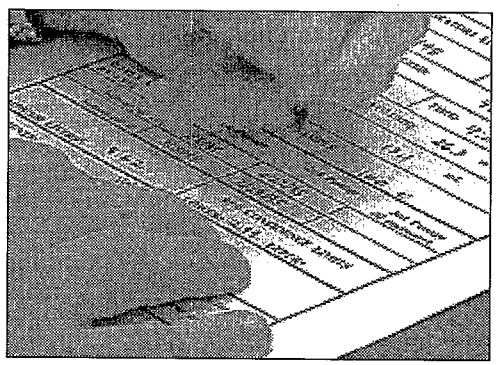
Record these values on the Quantitation of Total Culturable Virus data sheet.

Step 3 Calculate the MPN per 100 liter value (M_L) of the original water sample according to this formula:

$$M_l = \frac{100\ M_m S}{D}$$

where S equals the Assay Sample Volume and D equals the Volume of Original Water Sample assayed. (The values for S and D can be found on the Virus Data Sheet.) Record the value of M_L on the Virus Data Sheet.

Step 4 Calculate the lower 95% confidence limit 100 liter value (CL_l) for each water sample according to the formula:



$$CL_l = \frac{100\ CL_{lm} S}{D}$$

where CL_{lm} is the lower 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus data sheet. Calculate the upper 95% confidence limit per 100 liter value (CL_u) according to the formula:

$$CL_u = \frac{100\ CL_{um} S}{D}$$

where CL_{um} is the upper 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus data sheet. Record the limit per 100 liter values on the Virus Data Sheet.

Step 5 Calculate the total MPN value and the total 95% confidence limit values for each QC and PE sample by multiplying the values per milliliter by S and dividing by 0.4.

Reduction of Cytotoxicity in Sample Concentrates

The procedure described in this section may result in a significant titer reduction and should be applied only to inocula known to be toxic.

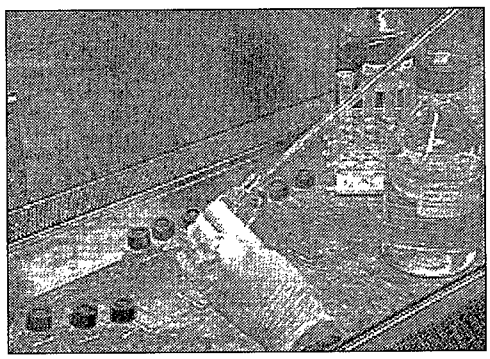
Media and Reagents

- Washing solution.

  Dissolve 8.5 g of NaCl in a final volume of 980 mL of reagent-grade water. Autoclave the solution at 121°C for 15 minutes. Cool to room temperature. Add 20 mL of serum to the sterile salt solution. Mix thoroughly.

  Store the washing solution at 4°C for up to 3 months, or at -20°C.

Procedure for Cytotoxicity Reduction



Step 1 Decant and save the inoculum from inoculated cell culture vessels after the adsorption period (Step 5 of Sample Inoculation and CPE Development).

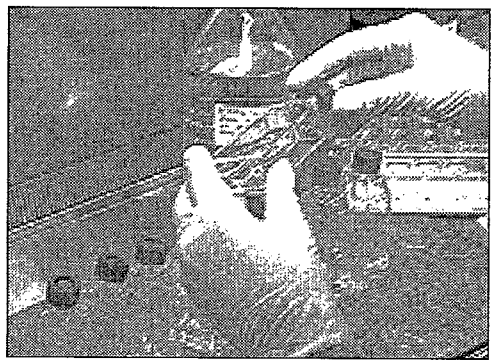
Add 0.25 mL of the washing solution for each cm^2 of cell surface area into each vessel.

Warm the washing solution to 36.5 ± 1°C before placing on the cell monolayer. Add the washing solution to the side of the cell culture vessel opposite the cell monolayer. Avoid touching any pipetting devices used to the inside rim of the culture vessels to avert the possibility of transporting contaminants to the remaining vessels.

The inocula saved after the adsorption period should be stored at -70°C for subsequent treatment and may be discarded when cytotoxicity is successfully reduced.

Step 2 Gently rock the washing solution across the cell monolayer a minimum of two times.

Decant and discard the spent washing solution without disturbing the cell monolayer.



It may be necessary to rock the washing solution across the monolayer more than twice if the sample is oily and difficult to remove from the cell monolayer surface.

Step 3 Continue with Step 7 of the procedure for Sample Inoculation and CPE Development.

If this procedure fails to reduce cytotoxicity with a particular type of water sample, backup samples may be diluted 1:2 to 1:4 before repeating the procedure. This dilution requires that two to four times more culture vessels be used. Dilution alone may sufficiently reduce cytotoxicity of some samples without washing. Alternatively, the changing of liquid maintenance medium at the first signs of cytotoxicity may prevent further development.

Determine cytotoxicity from the initial daily macroscopic examination of the appearance of the cell culture monolayer by comparing the negative and positive controls from Step 4 of the procedure for Sample Inoculation and CPE Development with the test samples from that step). Cytotoxicity should be suspected when the cells in the test sample develop CPE prior the positive control.

Appendix A
Cell Culture Preparation and Maintenance

Preparation of Cell Culture Medium

General Principles

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

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

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

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

5. 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.

6. 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.

7. Antibiotic solutions prepared in-house must be filter sterilized with 0.22 µm membrane filters. It is important that the recommended antibiotic levels not be exceeded during the planting of cells, as 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 freezing them in quantities that are sufficient to support a week's cell culture work.

Apparatus and Materials

1. Glassware, Pyrex (Corning Product No. 1395).

   Storage vessels must be equipped with airtight closures.

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

   Use only positive pressure type filter holders.

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

Stack AP20 and AP15 prefilters and 0.22 µm membrane filter in 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.

4. Positively charged cartridge filter — 10-inch (Zeta plus TSM, Cuno Product No. 45134-01-600P). Cartridge housing with adaptor for 10-inch cartridge (Millipore Product No. YY16 012 00).

5. Culture capsule filter (Gelman Sciences Product No. 12170).

6. 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).

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

7. Screw caps, black with rubber liners (Brockway Product No. 24-414).

   Caps for larger culture bottles usually are supplied with the bottles.

8. Roller apparatus (Bellco Glass Product No. 7730).

   Required only if roller bottles are used for maintenance of stock cultures.

9. Waterbath set at 56 ± 1°C.

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

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

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

13. Conical centrifuge tubes — 50 and 250 mL capacity.

14. Rack for tissue culture tubes (Bellco Product No. 2028).

15. Bottles, aspirator-type with tubing outlet — 2,000 mL capacity.

    Bottles for use with pipetting machine.

16. Storage vials — 2 mL capacity.

    Vials must withstand temperatures to -70°C.

Media and Reagents

1. Sterile fetal calf, gamma globulin-free newborn calf or iron-supplemented calf serum, certified free of viruses, bacteriophage, and mycoplasma.

   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 must be heat-inactivated in a waterbath set at 56 ± 1°C for 30 minutes and stored at 4°C for short-term use.

2. Trypsin, 1:250 powder (Difco Laboratories Product No. 0152-15-9) or trypsin, 1:300 pow-

der (Becton Dickinson Microbiology Systems Product No. 12098).

3. EDTA (Fisher Scientific Product No. S657-500).

4. Fungizone (amphotericin B, Sigma Product No. A-9528), penicillin G (Sigma Product No. P-3032), streptomycin sulfate (ICN Biomedicals Product No. 100556), tetracycline hydrochloride (ICN Biomedicals Product No. 103011).

   Use antibiotics of at least tissue culture grade.

5. Eagle's minimum essential medium (MEM) with Hanks' salts and L-glutamine, without sodium bicarbonate (Life Technologies Product No. 410-1200).

6. Leibovitz's L-15 medium with L-glutamine (Life Technologies Product No. 430-1300).

7. Trypan blue (Sigma Chemical Product No. T-6146).

8. Dimethyl sulfoxide (DMSO; Sigma Chemical Product No. D-2650).

Media Preparation Recipes

The conditions specified by the supplier for storage and expiration dates of commercially available media should be strictly observed.

1. Procedure for the preparation of 10 liters of EDTA-trypsin.

   The procedure described 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 four months.

   Step a. Add 30 g of trypsin (1:250) or 25 g of trypsin (1:300) to 2 liters of reagent-grade water in a 6 liter flask containing a 3-inch stir bar. Place the flask on a magnetic stirrer and mix the trypsin solution rapidly for a minimum of 1 hour.

   Trypsin remains cloudy.

   Step b. Put four liters of reagent-grade water and a 3-inch stir bar into a 20-liter clear plastic carboy. Place the carboy on 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 $Na_2HPO_4 \cdot 7H_2O$, 2.0 g KCl, and 2.0 g KH_2PO_4.

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

   Step c. Add four more liters of reagent-grade water to the carboy and continue mixing until all the chemicals are completely dissolved.

   Step d. Add the two liters of trypsin from Step 2a to the solution from Step 2c and mix for a minimum of one hour. Adjust the pH of the EDTA-trypsin reagent to 7.5 - 7.7.

   Step e. Filter the reagent under pressure through a filter stack and store the filtered reagent in tightly stoppered or capped containers at 4°C.

   The cartridge prefilter (Item 4 of Apparatus and Materials) can be used in line with the culture capsule sterilizing filter (Item 5) as an alternative to a filter stack (Item 3).

2. Procedure for the preparation of 10 liters of MEM/L-15 medium.

Step a. Place a 3-inch stir bar and four liters of reagent-grade water into a 20-liter clear plastic carboy.

Step b. Place the carboy onto a magnetic stirrer. Stir at a speed sufficient to develop a vortex and then add the contents of a 5-liter packet of L-15 medium to the carboy. Rinse the medium packet with three washes of 200 mL each of reagent-grade water and add the rinses to the carboy.

Step c. Mix until the medium is evenly dispersed.

L-15 medium may appear cloudy as it need not be totally dissolved before proceeding to the next step.

Step d. Add 3 liters of reagent-grade water to the carboy and the contents of a 5-liter packet of MEM medium to the carboy. Rinse the MEM medium packet with 3 washes of 200 mL each of reagent-grade water and add the rinses to the carboy. Add 800 mL of reagent-grade water and 7.5 g of $NaHCO_3$ and continue mixing for an additional 60 minutes.

Step e. 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 for up to 2 months.

3. Procedure for preparation of 100 mL of trypan blue solution.

The procedure 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.

Step a. Add 0.5 g of trypan blue to 100 mL of reagent-grade water in a 250 mL flask. Swirl the flask until the trypan blue is completely dissolved.

Step b. Sterilize the solution by autoclaving at 121°C for 15 minutes and store in a screw-capped container at room temperature.

4. Preparation of 100 mL of penicillin-streptomycin stock solution containing 100,000 units/mL of penicillin and 100,000 µg/mL of streptomycin.

Step a. Add 10,000,000 units of penicillin G and 10 g of streptomycin sulfate to a 250 mL flask containing 100 mL of reagent-grade water. Mix the contents of the flasks on a magnetic stirrer until the antibiotics are dissolved.

Step b. Sterilize the antibiotics by filtration through a 0.22 µm membrane filter and dispense in 10 mL volumes into screw-capped containers.

5. Preparation of 50 mL of tetracycline stock solution.

Step a. Add 1.25 g of tetracycline hydrochloride powder and 3.75 g of ascorbic acid to a 125 mL flask containing 50 mL of reagent-grade water. Mix the contents of the flask on a magnetic stirrer until the antibiotic is dissolved.

Step b. Sterilize the antibiotic by filtration through a 0.22 µm membrane filter and dispense in 5 mL volumes into screw-capped containers.

6. Preparation of 25 mL of amphotericin B (fungizone) stock solution.

   Step a. Add 0.125 g of amphotericin B to a 50 mL flask containing 25 mL of reagent-grade water. Mix the contents of the flask on a magnetic stirrer until the antibiotic is dissolved.

   Step b. Sterilize the antibiotic by filtration through a 0.22 µm membrane filter and dispense in 2.5 mL volumes into screw-capped containers.

Preparation and Passage of BGM Cell Cultures

A microbiological biosafety cabinet should be used to process cell cultures. If a hood 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 rooms used for cell culture transfer.

Vessels and Media for Cell Growth

1. The BGM cell line grows readily on the inside surfaces of glass or specially treated, tissue culture grade plastic vessels. Flat-sided, glass bottles (16 to 32 oz. or equivalent growth area), 75 or 150 cm^2 plastic cell culture flasks, and 690 cm^2 glass or 850 cm^2 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 per unit of cell monolayer surface area than do flat-sided bottles. Roller apparatus rotation speed should be adjusted to one-half revolution per minute to ensure that cells are constantly bathed in growth medium.

2. 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 80 or 50 mL of reagent-grade water, respectively). Use maintenance media with 2% serum for CPE development.

General Procedure for Cell Passage

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

Step 1
Pour spent medium from cell culture vessels, and discard the medium.

A gauze-covered beaker may be used to collect spent medium to prevent splatter. Autoclave all media that have been in contact with cells or that contain serum before discarding.

Step 2
Add a volume of warm EDTA-trypsin reagent equal to 40% of the volume of medium replaced to the cell cultures.

See Table 1 for the amount of reagents required for commonly used vessel types. Warm the EDTA-trypsin reagent to 36.5 ± 1 °C before placing it onto cell monolayers.

Step 3
Allow the EDTA-trypsin reagent to remain in contact with the cells at room temperature until the cell monolayer can be shaken loose from the inner

surface of the cell culture vessel (about five minutes).

The EDTA-trypsin reagent should remain in contact with the cells no longer than necessary to prevent cell damage.

Step 4
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.

Step 5
Centrifuge cell suspension at 1,000 x g for 10 minutes to pellet cells. Pour off and discard the supernatant.

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

Step 6
Suspend the pelleted cells in growth medium (see Item 2 of Vessels and Media for Cell Growth) and perform a viable count on the cell suspension according to the Procedure for Performing Viable Cell Counts section below.

Resuspend pelleted cells in a sufficient volume 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 milliliters, depending upon the volume of the individual laboratory's need for cell cultures.

Step 7
Dilute the cell suspension to the appropriate final cell concentration with growth medium and dispense into cell culture vessels with a pipet, a Cornwall-type syringe, or a Brewer-type pipetting machine dispenser.

Calculate the dilution factor requirement using the cell count and the cell and volume parameters given in Table 1 for stock cultures and in Table 2 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 200 25-cm^2 cell culture flasks weekly from a low-level passage of the line would require the preparation of 6 roller bottles (surface area of 690 cm^2 each): the contents of 2 to prepare the next batch of 6 roller bottles, and the contents of the other 4 to prepare the 25-m^2 flasks.

Step 8
Except during handling operations, maintain BGM cells at 36.5 ± 1°C in airtight cell culture vessels.

Step 9
Replace growth medium with maintenance medium containing 2% serum when cell monolayers become 95 to 100% confluent (usually three to four days after seeding with an appropriate number of cells). Replace growth medium which becomes acidic before the monolayers become 95 to 100% confluent with maintenance medium containing 5% serum. The volume of maintenance medium should equal the volume of the discarded growth medium.

Procedure For Performing Viable Cell Counts

Step 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.

Table 1
Guide for Preparation of BGM Stock Cultures

Vessel Type	Volume of EDTA-Trypsin Used to Remove Cells (ml)	Volume of Medium (ml)[a]	Total Number of Cells to Plate per Vessel
16 oz. glass flat bottles	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	7.0×10^6
850 cm^2 plastic roller bottle	48	120	8.0×10^7

[a]Serum 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

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 x 10^5 and 1.5 x 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.

Step 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.

Step 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 minute to allow the trypan blue to penetrate the membranes of nonviable cells.

Do not under- or overfill the chambers.

Step 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 2
Guide for Preparation of Virus Assay Cell Cultures

Vessel Type	Volume of Medium* (ml)	Final Cell Count per Vessel
1 oz. glass bottle	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 x 150 mm tube	2	4.0×10^4

*Serum requirements: growth medium contains 10 % 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

Step 5
Calculate the average number of viable cells in each mL of cell suspension by totaling the number of viable cells counted in the 5 sections, multiplying this sum by 2,000, and where necessary, multiplying the resulting product by the reciprocal of the dilution.

Procedure for Preservation of BGM Cell Line

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

Preparation of Cells for Storage

The procedure described is for the preparation of 100 cell culture vials. Cell concentration must be at least 2 x 10^6 per mL.

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

Step 1
Prepare cell storage medium by adding 10 mL of DMSO to 90 mL of growth medium (see Item 2 of Vessels and Media for Cell Growth). Sterilize the resulting cell storage medium by passage through a 0.22 µm sterilizing filter.

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

Step 2
Harvest BGM cells from cell culture vessels as directed in Steps 1 to 5 of General Procedures for Cell Passage. Count the viable cells as described above and resuspend them in the cell storage medium at a concentration of at least 2 x 10^6 cells per mL.

Step 3
Place the flask containing suspended cells on a magnetic stirrer and slowly mix for 30 minutes. Dispense 1 mL volumes of cell suspension into 2-mL capacity vials.

Procedure for Freezing Cells

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

Step 1
Place the vials in a rack and place the rack in a refrigerator at 4°C for 30 minutes, then in a -20°C freezer for 30 minutes, and finally 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.

Step 2
Rapidly transfer vials into boxes or other containers for long-term storage.

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

Procedure for Thawing Cells

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

Step 1
Place vials containing frozen cells into a 36.5 ± 1°C water bath and agitate vigorously by hand until all ice has melted. Sterilize the outside surface of the vials with 0.5% I_2 in 70% ethanol.

Step 2
Add BGM cells to either 6 oz. tissue culture bottles or 25 cm^2 tissue culture flasks containing an appropriate amount of growth medium (see Table 2). Use two vials of cells for 6 oz. bottles and one vial for 25 cm^2 flasks.

Step 3
Incubate BGM cells at 36.5 ± 1°C. After 18 to 24 hours replace the growth medium with fresh growth medium, then continue the incubation for an additional 5 days. Pass and maintain the new cultures as directed above.

Appendix B Sterilization and Disinfection

General Guidelines

1. Use aseptic techniques for handling test waters, eluates, and cell cultures.

2. Sterilize apparatus and containers that will come into contact with test waters and all solutions that will be added to test waters unless otherwise indicated. Thoroughly clean all items prior to final sterilization using laboratory standard operating procedures.

3. Sterilize all contaminated materials before discarding.

4. Disinfect all spills and splatters.

Sterilization Techniques

Solutions

Sterilize all solutions, except those used for cleansing, standard buffers, hydrochloric acid (HCl), sodium hydroxide (NaOH), and disinfectants by autoclaving them at 121°C for 15 minutes.

The HCl and NaOH solutions and disinfectants used are self-sterilizing. When autoclaving buffered beef extract, use a vessel large enough to accommodate foaming. Place the vessel in a pan of water to ensure even heating and to avoid breakage of the vessel.

Autoclavable Glassware, Plasticware, and Equipment

Water speeds the transfer of heat in larger vessels during autoclaving and thereby speeds the sterilization process. Add reagent-grade water to vessels in quantities indicated in the table below. Lay large vessels on their sides in the autoclave, if possible, to facilitate the displacement of air in the vessels by flowing steam.

1. Cover the openings into autoclavable glassware, plasticware, and equipment loosely with aluminum foil before autoclaving. Autoclave at 121°C for one hour.

   Glassware may also be sterilized in a dry heat oven at a temperature of 170°C for at least one hour.

2. Sterilize stainless steel vessels (dispensing pressure vessel) in an autoclave at 121°C for 30 minutes.

Quantity of Reagent-Grade Water to be Added to Vessels During Autoclaving

Vessel Size (L)	Water (ml)
2 and 3	25
4	50
8	100
24	500
54	1,000

Vent-relief valves on vessels so equipped must be open during autoclaving and closed immediately when vessels are removed from the autoclave.

3. Presterilize 1MDS filter cartridges and prefilter cartridges by wrapping the filters in Kraft paper and autoclaving at 121°C for 30 minutes.

4. Sterilize working instruments, such as scissors and forceps, by immersing them in 95% ethanol and flaming them between uses.

Chlorine Sterilization

Sterilize pumps, plasticware (filter housings), tubing that cannot withstand autoclaving, and vessels that are too large for the autoclave by chlorination. Prefilters, but not 1MDS filters, may be presterilized with chlorine as an alternative to autoclaving. Filter Apparatus modules should be disinfected by sterilization and then cleaned according to laboratory standard operating procedures prior to final sterilization.

Media and Reagents

1. 0.1% chlorine (HOCl) — add 19 mL of household bleach (Clorox, The Clorox Co.) to 981 mL of reagent-grade water and adjust the pH of the solution to 6-7 with 1 M HCl.

Procedures

Ensure that the solutions come in full contact with all surfaces when performing these procedures.

1. Sterilize filter apparatus modules, injector tubing, and plastic bags for transporting injector tubing by recirculating or immersing the items in 0.1% chlorine for 30 minutes. Drain the chlorine solution from objects being sterilized. Dechlorinate using a solution containing 2.5 mL of 2% sterile sodium thiosulfate per liter of sterile reagent-grade water. Rinse with sterile reagent-grade water.

2. Thoroughly rinse pH electrodes after each use to remove particulates. Sterilize before and after each use by immersing the tip of the electrode in 0.1% chlorine for at least one minute. Dechlorinate the electrode as in the step above.

Procedure for Verifying Sterility of Liquids

Do not add antibiotics to media or medium components until after their sterility has been demonstrated. The BGM cell line used should be checked every six months for mycoplasma contamination according to test kit instructions. Cells that are contaminated should be discarded.

Media and Reagents

1. Mycoplasma testing kit (Irvine Scientific Product No. T500-000). Use as directed by the manufacturer.

2. Thioglycollate medium (Difco Laboratories Product No. 0257-01-9). Prepare broth medium as directed by the manufacturer.

Verifying Sterility of Small Volumes of Liquids

Step 1
Inoculate 1 mL portions of the material to be tested for sterility into tubes containing 9 mL of thioglycollate broth by stabbing the inoculum into the broth. Incubate at 36.5 ±1°C.

Step 2
Examine the inoculated broth daily for seven days to determine whether growth of contaminating organisms has occurred.

Containers holding the thioglycollate medium must be tightly sealed before and after the medium is inoculated.

Visual Evaluation of Media for Microbial Contaminants

Step 1
Incubate either the entire stock of prepared media or aliquots taken during preparation which represent at least 5% of the final volume at 36.5 ±1°C for at least one week prior to use.

Step 2
Visually examine and discard any media that lose clarity.

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

Contaminated Materials

1. Autoclave contaminated materials for 30 minutes at 121°C. Be sure that steam can enter contaminated materials freely.

2. Many commercial disinfectants do not adequately kill enteric viruses. To ensure thorough disinfection, disinfect spills and other contamination on surfaces with either a solution of 0.5% iodine in 70% ethanol (5 g I_2 per liter) or 0.1% chlorine. The iodine solution has the advantage of drying more rapidly on surfaces than chlorine, but may stain some surfaces.

Appendix C Bibliography and Suggested Reading

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Appendix D
Vendors

The vendors listed below represent one possible source for required products. Other vendors may supply the same or equivalent products.

American Type Culture Collection
12301 Parklawn Dr.
Rockville, MD 20852
(800) 638-6597

Baxter Diagnostics, Scientific Products Div.
1430 Waukegan Rd.
McGaw Park, IL 60085
(800) 234-5227

BBL Microbiology Systems: products may be ordered through several major scientific supply houses

Becton Dickinson Microbiology Systems
250 Schilling Circle
Cockeysville, MD 21030
(410) 771-0100 (Ask for a local distributor)

Bellco Glass
340 Edrudo Rd.
Vineland, NJ 08360
(800) 257-7043

Brockway: products may be ordered through Continental Glass & Plastics

Cincinnati Valve and Fitting Co.
3710 Southern Ave.
Cincinnati, OH 45227
(513) 272-1212

Cole-Parmer Instrument Co.
7425 N. Oak Park Ave.
Niles, IL 60714
(800) 323-4340

Continental Glass & Plastics
841 W. Cermak Rd.
Chicago, IL 60608
(312) 666-2050

Corning: products may be ordered through most major scientific supply houses

Costar Corp.
7035 Commerce Circle
Pleasanton, CA 94588
(800) 882-7711

Cuno, Inc.
400 Research Parkway
Meriden, CT 06450
(800)243-6894

Curtin Matheson Scientific
P.O. Box 1546
Houston, TX 77251
(713) 820-9898

DEMA Engineering Co.
10014 Big Bend Blvd.
Kirkwood, MO 63122
(800) 325-3362

Difco Laboratories
P.O. Box 331058
Detroit, MI 48232
(800) 521-0851 (Ask for a local distributor)

Fisher Scientific
711 Forbes Ave.
Pittsburgh, PA 15219
(800) 766-7000

Gelman Sciences
600 S. Wagner Rd.
Ann Arbor, MI 48103
(800) 521-1520

ICN Biomedicals
3300 Hyland Ave.
Costa Mesa, CA 92626
(800) 854-0530

Irvine Scientific
2511 Daimler Street
Santa Ana, CA 92705
(800) 437-5706

Life Technologies
P.O. Box 68
Grand Island, NY 14072
(800) 828-6686

Millipore Corp.
397 Williams St.
Marlboro, MA 01752
(800) 225-1380

Nalge Co.
P.O. Box 20365
Rochester, NY 14602
(716) 586-8800 (Ask for a local distributor)

Neptune Equipment Co.
520 W. Sharon Rd.
Forest Park, OH 45240
(800) 624-6975

OMEGA Engineering, Inc.
P.O. Box 4047
Stamford, CT 06907
(800) 826-6342

Plast-o-matic Valves, Inc.
1384 Pompton Ave.
Cedar Grove, NJ 07009
(201) 256-3000 (Ask for a local distributor)

Parker Hannifin Corp.
Commercial Filters Div.
1515 W. South St.
Lebanon, IN 46052
(317) 482-3900

Ryan Herco
2509 N. Naomi St.
Burbank, CA 91504
(800) 848-1141

Sigma Chemical
P.O. Box 14508
St. Louis, MO 63178
(800) 325-3010

United States Plastic Corp.
1390 Neubrecht Rd.
Lima, OH 45801
(800) 537-9724

Watts Regulator
Box 628
Lawrence, MA 01845
(508) 688-1811

Appendix E
Data Sheets

SAMPLE DATA SHEET			
SAMPLE NUMBER:			
UTILITY NAME:			
UTILITY ADDRESS: **CITY:**	**STATE:**		**ZIP:**
SAMPLER'S NAME:			
WATER TEMPERATURE:	**°C**	**TURBIDITY:**	**NTU**
WATER pH:			
ADJUSTED WATER pH:			
THIOSULFATE ADDED:	**(CHECK)**	**__ YES**	**__ NO**
INIT. METER READING: **date:**	**(CHECK UNITS)** **time:**	**__ gallons**	**__ ft^3**
FINAL METER READING: **date:**	**(CHECK UNITS)** **time:**	**__ gallons**	**__ ft^3**
TOTAL SAMPLE VOLUME:		**liters**	
(Final-Initial meter readings x 3.7854 (for readings in gallons) or x 28.316 (for readings in ft^3))			
SHIPMENT DATE:			
CONDITION ON ARRIVAL:			
COMMENTS:			

VIRUS DATA SHEET

SAMPLE NUMBER:			
ANALYTICAL LABORATORY NAME:			
ANALYTICAL LABORATORY ADDRESS: CITY:		STATE:	ZIP:
ADJUSTED TOTAL SAMPLE VOLUME (ATSV)[1]:			L
DATE ELUTED:		TIME:	
ELUATE VOLUME RECOVERED:			mL
VOLUME OF ELUATE ARCHIVED			mL
DATE CONCENTRATED:		TIME:	
FINAL CONCENTRATED SAMPLE VOLUME (FCSV):			mL
ASSAY SAMPLE VOLUME (S):			mL
VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D)			L[2]
INOCULUM VOLUME:			mL

DATES ASSAYED BY CPE:	1st Passage	2nd Passage	3rd Passage (If necessary)
Subsample 1:			
Subsample 2:			

MPN/100 LITERS[3]:	95% CONFIDENCE LIMITS LOWER: UPPER:
COMMENTS:	
ANALYST:	

[1]Enter the Total Sample Volume from the Sample Data Sheet times 0.965 if a coliphage sample is taken, times 0.9 if archiving is required, times 0.865 if a coliphage sample is taken and archiving is required, or times 1.0 if a coliphage sample is not taken and archiving is not required.
[2]This value must be at least 100 liters for source water and 1,000 liters for finished water.
[3]Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section of Part 3.

TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE #:

	Total Number of Replicates					
	Subsample 1			Subsample 2		
Sample	Inoculated	Without CPE	With CPE	Inoculated	Without CPE	With CPE
1st Passage Neg. Cont.						
Pos Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						
2nd Passage[1] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						
3rd Passage[2] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be repassaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the first passage. If higher dilutions are used, record the data from the three highest dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

QUANTITATION OF TOTAL CULTURABLE VIRUS

SAMPLE NUMBER:

Sample	Number Replicates Inoculated	Number with CPE	MPN/mL[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples					
Subsample 1					
Subsample 2					
Total Undiluted					
Subsample 2 results (Dilutions Required)					
Undiluted					
1:5 Dilution					
1:25 Dilution					

[1]Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted, and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column. The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.

Appendix F
Examples of Calculations

Example 1

A source water sample of 211.98 liters was collected at the Sampleville Water Works on May 5, 1995 and shipped by overnight courier to CEPOR Laboratories. CEPOR Laboratories processed the sample on May 2, 1995. After elution, the pH of the beef extract V eluate was adjusted to 7.3 with 1 M HCl. The volume of the preadjusted eluate, 980.0 mL, was recorded. Volumes of 34.3 mL (980 x 0.035) and 98.0 mL (980 x 0.1) were removed for the **Coliphage Assay** (see Section IX of the *ICR Microbial Laboratory Manual*) and for archiving, respectively. An **Adjusted Total Sample Volume (ATSV)** was then calculated by multiplying 211.98 liters x 0.865. An adjusted volume of 183 liters was recorded on the **Virus Data Sheet**.

The sample was immediately processed by the Organic Flocculation Concentration Procedure. Following centrifugation at 4,000 *xg*, the supernatant was adjusted to pH 7.3 and passed through a sterilizing filter. A **Final Concentrated Sample Volume (FCSV)** of 28.0 mL was obtained.

The **Assay Sample Volume** was calculated using the formula:

$$\text{ASSAY SAMPLE VOLUME (S)} = \frac{D}{ATSV} \times FCSV$$

where D is the **Volume of Original Water Sample Assayed** (i.e., 100 liters for source water or 1000 liters for finished water) and ATSV is the **Adjusted Total Sample Volume** from the Virus Data Sheet.

Thus the **Assay Sample Volume** for Sampleville-01 is:

$$S = \frac{100 \text{ liters}}{183 \text{ liters}} \times 28.0 \text{ ml} = 15.3 \text{ ml}$$

The 15.3 mL is the volume of the **Final Concentrated Sample** which must be inoculated onto tissue culture and which represents 100 liters of the source water.

Two subsamples were prepared from the **Final Concentrated Sample**. **Subsample 1** was prepared by placing 0.55 x 15.3 mL = 8.4 mL into a separate container. **Subsample 2** was prepared by placing 0.67 x 15.3 mL = 10.2 mL into a third container. Although only 0.5 x 15.3 = 7.65 mL (representing 50 liters of source water) must be inoculated onto tissue culture flasks for each subsample, the factor "0.55" was used for **subsample 1** to account for unrecoverable losses associated with removing a subsample from its container. The factor "0.67" was used for **subsample 2** to account for losses associated with the container and to provide additional sample for the preparation of dilutions, if required.

Subsample 2 and the remaining portions of the **Final Concentrated Sample** were frozen at -70°C.

The Inoculum Volume was calculated to be 15.3 mL ÷ 20 = 0.76 mL per flask. To make the inoculuation procedure more convenient, it was decided to use an Inoculation Volume of 1.0 mL.

To do this, 10.5 x (1.00-0.76) = 2.52 mL of sodium phosphate, pH 7.3, was added to 10.5 x 0.76 = 7.98 mL of subsample 1. One milliliter of diluted subsample 1 was then inoculated onto each of ten 25-cm^2 flasks of BGM cells at passage 123. A **negative control** was prepared by inoculating a flask with 1.0 mL of sodium phosphate, pH 7.3. A **positive control** was prepared by inoculating a flask with 1.0 mL of sodium phosphate, pH 7.3, containing 20 PFU/mL of attenuated poliovirus type 3. Following adsorption, 9.0 mL of maintenance medium was added and the cultures were incubated at 36.5°C. These cultures and those described below were observed for CPE as described in the protocol, and positive cultures were frozen when 75% of a flask showed signs of CPE.

On May 9, five flasks inoculated with **subsample 1** and the positive control showed signs of CPE. Since fewer than eight flasks inoculated with **subsample 1** showed CPE, 10 additional 25-cm^2 flasks of BGM cells at passage 124 were inoculated with 1.0 mL each of **subsample 2** diluted for inoculation as described above. Another **negative control** and **positive control** were also prepared and inoculated.

By May 16, a total of seven flasks inoculated with **subsample 1** showed signs of CPE. The flasks that had not been previously frozen were now frozen at -70°C, and then all flasks were thawed. Several milliliters of fluid from each of the eight positive flasks (seven samples plus the positive control) were passed through a sterilizing filter. Twelve flasks of BGM cells at passage 125 were inoculated with 1.0 mL of the supernatant from either negative cultures or from filtered positive cultures.

By May 23, a total of five flasks from **subsample 2** showed signs of CPE. All flasks were frozen, thawed, and then passaged as described for **subsample 1** using BGM cells at passage 126.

By May 30, only six flasks from the second passage of **subsample 1** and the positive control showed CPE. Thus one culture from the first passage failed to confirm in the second pass, and a value of 6 was recorded in the **Number of Replicates with CPE** column of the **Total Culturable Virus Data Sheet**. The flasks were then discarded.

On June 6, seven flasks (the five original plus two new flasks) from the second passage of **subsample 2** demonstrated CPE. The two new flasks and controls were frozen at -70°C, thawed, and passaged a third time as described above using BGM cells at passage 127. All other flasks were discarded.

By June 12, the positive control and the two third-passage flasks had developed CPE. All flasks were discarded at this time. (The flasks would have been examined until June 20 if at least one had remained negative.) A value of 7 was recorded into the **Number of Replicates with CPE** column of the **Total Culturable Virus Data Sheet**.

The MPN software program supplied by the U.S. EPA was used to calculate the MPN/mL and 95% confidence limit values. "I. SIZE OF INOCULUM VOLUME (mL)" on the main screen was changed from 1 to 0.76. "A. PROCEED WITH DATA INPUT" was pressed followed by "ENTER" to overwrite the existing output file. Alternatively, "NO" could have been entered and the output file renamed. The number of positive replicates, "13," was then entered. Following the calculation by the program, the MPN and 95% Confidence Limit values were recorded onto the Quantitation of Total Culturable Virus data sheet. The program was exited by pressing "I. EXIT THE PROGRAM."

The MPN per 100 liter value (M_L) was calculated according to the formula:

$$M_l = \frac{100\ M_m S}{D} = \frac{100 \times 1.38 \times 15.3}{100} = 21.1$$

The Lower 95% Confidence Limit per 100 liter (CL_l) was calculated according to the formula:

$$CL_l = \frac{100\ CL_{lm}S}{D} = \frac{100 \times 0.70 \times 15.3}{100} = 10.7$$

where Cl_{lm} is the lower 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus data sheet.

The Upper 95% Confidence Limit per 100 liter (CL_u) was calculated according to the formula:

$$CL_u = \frac{100\ CL_{um}S}{D} = \frac{100 \times 2.27 \times 15.3}{100} = 34.7$$

where CL_{um} is the upper 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus Data Sheet.

SAMPLE DATA SHEET

SAMPLE NUMBER:	**Sampleville-01**		
UTILITY NAME:	**Sampleville Water Works**		
UTILITY ADDRESS:	**1 Water Street**		
CITY: Sampleville		**STATE: OH**	**ZIP: 45999**
SAMPLER'S NAME:	**Mr. Brian Hall**		
WATER TEMPERATURE:	**23.5°C**	**TURBIDITY:**	**3.6 NTU**
WATER pH: 7.8			
ADJUSTED WATER pH: NA			
THIOSULFATE ADDED:	**(CHECK)**	**__ YES**	**X NO**
INIT. METER READING: 6048.10 **date: 5/1/95**	**(CHECK UNITS)** **time: 9 am**	**X gallons**	**__ ft^3**
FINAL METER READING: 6104.10 **date: 5/1/95**	**(CHECK UNITS)** **time: 9:30 am**	**X gallons**	**__ ft^3**
TOTAL SAMPLE VOLUME:	**211.98**	**liters**	
(Final-Initial meter readings x 3.7854 (for readings in gallons) or x 28.316 (for readings in ft^3))			
SHIPMENT DATE: 5/1/95			
CONDITION ON ARRIVAL: Cold/Not frozen			
COMMENTS:			

VIRUS DATA SHEET

SAMPLE NUMBER: SAMPLEVILLE-01			
ANALYTICAL LABORATORY NAME: CEPOR LABORATORIES			
ANALYTICAL LABORATORY ADDRESS: 42 RUECKERT ST. CITY: CINCINNATI STATE: OH ZIP: 45219			
ADJUSTED TOTAL SAMPLE VOLUME (ATSV)[1]: 183 L			
DATE ELUTED: 5/2/95	TIME: 10 am		
ELUATE VOLUME RECOVERED: 980 mL			
VOLUME OF ELUATE ARCHIVED 98.0 mL			
DATE CONCENTRATED: 5/2/95	TIME: 1 pm		
FINAL CONCENTRATED SAMPLE VOLUME (FCSV): 28.0 mL			
ASSAY SAMPLE VOLUME (S): 15.3 mL			
VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D) 100 L[2]			
INOCULUM VOLUME: 0.76 mL			
DATES ASSAYED BY CPE:	1st Passage	2nd Passage	3rd Passage (If necessary)
Subsample 1:	5/2/95	5/16/95	
Subsample 2:	5/9/95	5/23/95	6/6/95
MPN/100 LITERS[3]: 21		95% CONFIDENCE LIMITS LOWER: 11 UPPER: 35	
COMMENTS:			
ANALYST: B.G. Moore			

[1]Enter the Total Sample Volume from the Sample Data Sheet times 0.965 if a coliphage sample is taken, times 0.9 if archiving is required, times 0.865 if a coliphage sample is taken and archiving is required, or times 1.0 if a coliphage sample is not taken and archiving is not required.
[2]This value must be at least 100 liters for source water and 1,000 liters for finished water.
[3]Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section of Part 3.

TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE #: Sampleville-01

	Total Number of Replicates					
	Subsample 1			Subsample 2		
Sample	Inoculated	Without CPE	With CPE	Inoculated	Without CPE	With CPE
1st Passage Neg. Cont.	1	1	0	1	1	0
Pos Cont.	1	0	1	1	0	1
Undiluted	10	3	7	10	5	5
1:5 Dil.						
1:25 Dil.						
2nd Passage[1] Neg. Cont.	1	1	0	1	1	0
Pos. Cont.	1	0	1	1	0	1
Undiluted	10	4	6	10	3	7
1:5 Dil.						
1:25 Dil.						
3rd Passage[2] Neg. Cont.				1	1	0
Pos. Cont.				1	0	1
Undiluted				2	0	2
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be repassaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the first passage. If higher dilutions are used, record the data from the three highest dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

QUANTITATION OF TOTAL CULTURABLE VIRUS

SAMPLE NUMBER: Sampleville-01

Sample	Number Replicates Inoculated	Number with CPE	MPN/mL[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples			1.38	0.70	2.27
Subsample 1	10	6			
Subsample 2	10	7			
Total Undiluted	20	13			
Subsample 2 results (Dilutions Required)					
Undiluted					
1:5 Dilution					
1:25 Dilution					

[1]Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted, and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column. The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.

Example 2

A source water sample of 200.63 liters was collected at the Sampleville Water Works on June 5, 1995 and shipped by overnight courier to CEPOR Laboratories. CEPOR Laboratories processed the sample on June 6, 1995. After elution, the pH was adjusted to 7.3. A volume of 985 mL of pH-adjusted eluate was obtained and 34.5 mL (985 x 0.035) was removed for the **Coliphage Assay** (see Section IX of the *ICR Microbial Laboratory Manual*). Archiving was not required. An **Adjusted Total Sample Volume** of 194 liters (200.63 x 0.965) was recorded on the **Virus Data Sheet.**

The sample was immediately processed by the Organic Flocculation Concentration Procedure. Following centrifugation at 4,000 *xg*, the supernatant was adjusted to pH 7.3 and passed through a sterilizing filter. A **Final Concentrated Sample Volume (FCSV)** of 32.0 mL was obtained, giving an **Assay Sample Volume (S)** for Sampleville-02 of:

$$S = \frac{100.00 \text{ liters}}{194 \text{ liters}} \times 32.0 \text{ ml} = 16.5 \text{ ml}$$

Subsample 1 was prepared by placing 0.55 x 16.5 mL = 9.1 mL into a separate container. **Subsample 2** was prepared by placing 0.67 x 16.5 mL = 11.1 mL into a third container. **Subsample 2** and the remaining portions of the **Final Concentrated Sample** were frozen at -70°C.

Subsample 1 was inoculated onto each of 10 25-cm² flasks of BGM cells at passage 127 using an **Inoculum Volume** of 16.5 mL ÷ 20 = 0.82 mL per flask. A **negative control** was prepared by inoculating a flask with 0.82 mL of sodium phosphate, pH 7.3. A **positive control** was prepared by inoculating a flask with 0.82 mL of sodium phosphate, pH 7.3, containing 25 PFU/mL (20 PFU/0.82 mL) of attenuated poliovirus type 3. Following adsorption, 9.18 mL of maintenance medium was added, and the cultures were incubated at 36.5°C.

On June 13, nine flasks inoculated with **subsample 1** and the positive control showed signs of CPE. After thawing **subsample 2**, a 1:5 dilution was prepared by mixing 0.1334 x 16.5 = 2.20 mL of **subsample 2** with 0.5334 x 16.5 = 8.80 mL of sodium phosphate, pH 7.3. A 1:25 dilution was prepared by mixing 2.20 mL of the 1:5 dilutions with 8.80 mL of sodium phosphate, pH 7.3. Ten 25-cm² flasks of BGM cells at passage 128 were then inoculated with 0.82 mL each of undiluted **subsample 2**. Ten flasks were inoculated with 0.82 mL each of **subsample 2** diluted 1:5, and 10 flasks were inoculated with 0.82 mL each of **subsample 2** diluted 1:25. Another **negative control** and **positive control** were also prepared and inoculated.

By June 20, all 10 flasks inoculated with **subsample 1** showed signs of CPE and were repassaged as described in example 1.

By June 27, all 10 flasks inoculated with undiluted **subsample 2** had developed CPE. Eight flasks inoculated with the 1:5 dilution of **subsample 2** and four flasks inoculated with the 1:25 dilution of **subsample 2** demonstrated CPE. All flasks were passaged again as described for example 1.

By July 5, all 10 flasks from the second passage of **subsample 1** were confirmed as positive and were discarded.

By July 11, all 10 flasks inoculated with the second passage of undiluted **subsample 2** had developed CPE. The eight positive flasks from the first passage of the 1:5 dilution of **subsample 2** were positive in the second passage. Three flasks inoculated with the second passage of the 1:25 dilution of **subsample 2** remained positive.

The MPN software program supplied by the U.S. EPA was used to calculate the MPN/mL and 95% confidence limit values. After the main screen appeared, "G. NUMBER OF DILUTIONS" was changed from 1 to 3. "H. NUMBER OF REPLICATES PER DILUTION" was changed from 20 to 10, and "I. SIZE OF INOCULUM VOLUME (mL)" was changed from 1 to 0.82. "A. PROCEED WITH DATA INPUT" was pressed followed by "ENTER" to overwrite the existing output file. The number of positive replicates per dilution, "10, 8, and 3," was entered with the values separated by spaces. Following program calculations, the MPN/mL and 95% confidence limit values/mL were recorded onto the Quantitation of Total Culturable Virus data sheet. The program was exited by pressing "I. EXIT THE PROGRAM."

The MPN per 100 liter value (M_L) was calculated according to the formula:

$$M_l = \frac{100\ M_m S}{D} = \frac{100.00 \times 10.15 \times 16.5}{100.00} = 167$$

The Lower 95% Confidence Limit per 100 liter (CL_l) was calculated according to the formula:

$$CL_l = \frac{100\ CL_{lm} S}{D} = \frac{100.00 \times 5.04 \times 16.5}{100.00} = 83.2$$

where Cl_{lm} is the lower 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus data sheet.

The Upper 95% Confidence Limit per 100 liter (CL_u) was calculated according to the formula:

$$CL_u = \frac{100\ CL_{um} S}{D} = \frac{100.00 \times 18.25 \times 16.5}{100.00} = 301$$

where CL_{um} is the upper 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus data sheet.

SAMPLE DATA SHEET

SAMPLE NUMBER:	**Sampleville-02**		
UTILITY NAME:	**Sampleville Water Works**		
UTILITY ADDRESS:	**1 Water Street**		
CITY: Sampleville		**STATE: OH**	**ZIP: 45999**
SAMPLER'S NAME:	**Mr. Brian Hall**		
WATER TEMPERATURE:	**26.5°C**	**TURBIDITY:**	**2.3 NTU**
WATER pH: 7.7			
ADJUSTED WATER pH: NA			
THIOSULFATE ADDED:	**(CHECK)**	**__ YES**	**X NO**
INIT. METER READING: 6129.3	**(CHECK UNITS)**	**X gallons**	**__ ft^3**
date: 6/1/95	**time: 8:30 am**		
FINAL METER READING: 6182.3	**(CHECK UNITS)**	**X gallons**	**__ ft^3**
date: 6/5/95	**time: 9:00 am**		
TOTAL SAMPLE VOLUME:	**200.63**	**liters**	

(Final-Initial meter readings x 3.7854 (for readings in gallons) or x 28.316 (for readings in ft^3))

SHIPMENT DATE: 6/5/95

CONDITION ON ARRIVAL: Cold/Not frozen

COMMENTS:

VIRUS DATA SHEET

SAMPLE NUMBER: SAMPLEVILLE-02			
ANALYTICAL LABORATORY NAME: CEPOR LABORATORIES			
ANALYTICAL LABORATORY ADDRESS: 42 RUECKERT ST. CITY: CINCINNATI STATE: OH ZIP: 45219			
ADJUSTED TOTAL SAMPLE VOLUME (ATSV)[1]: 194 L			
DATE ELUTED: 6/6/95	TIME: 9:50 am		
ELUATE VOLUME RECOVERED: 985 mL			
VOLUME OF ELUATE ARCHIVED 0 mL			
DATE CONCENTRATED: 6/6/95	TIME: 1 pm		
FINAL CONCENTRATED SAMPLE VOLUME (FCSV): 32.0 mL			
ASSAY SAMPLE VOLUME (S): 16.5 mL			
VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D) 100.00 L[2]			
INOCULUM VOLUME: 0.82 mL			
DATES ASSAYED BY CPE:	1st Passage	2nd Passage	3rd Passage (If necessary)
Subsample 1:	6/6/95	6/20/95	
Subsample 2:	6/13/95	6/27/95	
MPN/100 LITERS[3]: 167		95% CONFIDENCE LIMITS LOWER: 83 UPPER: 301	
COMMENTS:			
ANALYST: B.G. Moore			

[1]Enter the Total Sample Volume from the Sample Data Sheet times 0.965 if a coliphage sample is taken, times 0.9 if archiving is required, times 0.865 if a coliphage sample is taken and archiving is required, or times 1.0 if a coliphage sample is not taken and archiving is not required.
[2]This value must be at least 100 liters for source water and 1,000 liters for finished water.
[3]Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section of Part 3.

TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE #: Sampleville-02

	Total Number of Replicates					
	Subsample 1			Subsample 2		
Sample	Inoculated	Without CPE	With CPE	Inoculated	Without CPE	With CPE
1st Passage Neg. Cont.	1	1	0	1	1	0
Pos Cont.	1	0	1	1	0	1
Undiluted	10	0	10	10	0	10
1:5 Dil.				10	2	8
1:25 Dil.				10	6	4
2nd Passage[1] Neg. Cont.	1	1	0	1	1	0
Pos. Cont.	1	0	1	10	0	1
Undiluted	10	0	10	10	0	10
1:5 Dil.				10	2	8
1:25 Dil.				10	7	3
3rd Passage[2] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be repassaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the first passage. If higher dilutions are used, record the data from the three highest dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

QUANTITATION OF TOTAL CULTURABLE VIRUS

SAMPLE NUMBER: Sampleville-02

Sample	Number Replicates Inoculated	Number with CPE	MPN/ml[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples			10.15	5.04	18.25
Subsample 1	10	10			
Subsample 2					
Total Undiluted	NA	NA			
Subsample 2 results (Dilutions Required)					
Undiluted	10	10			
1:5 Dilution	10	8			
1:25 Dilution	10	3			

[1]Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted, and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column. The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.