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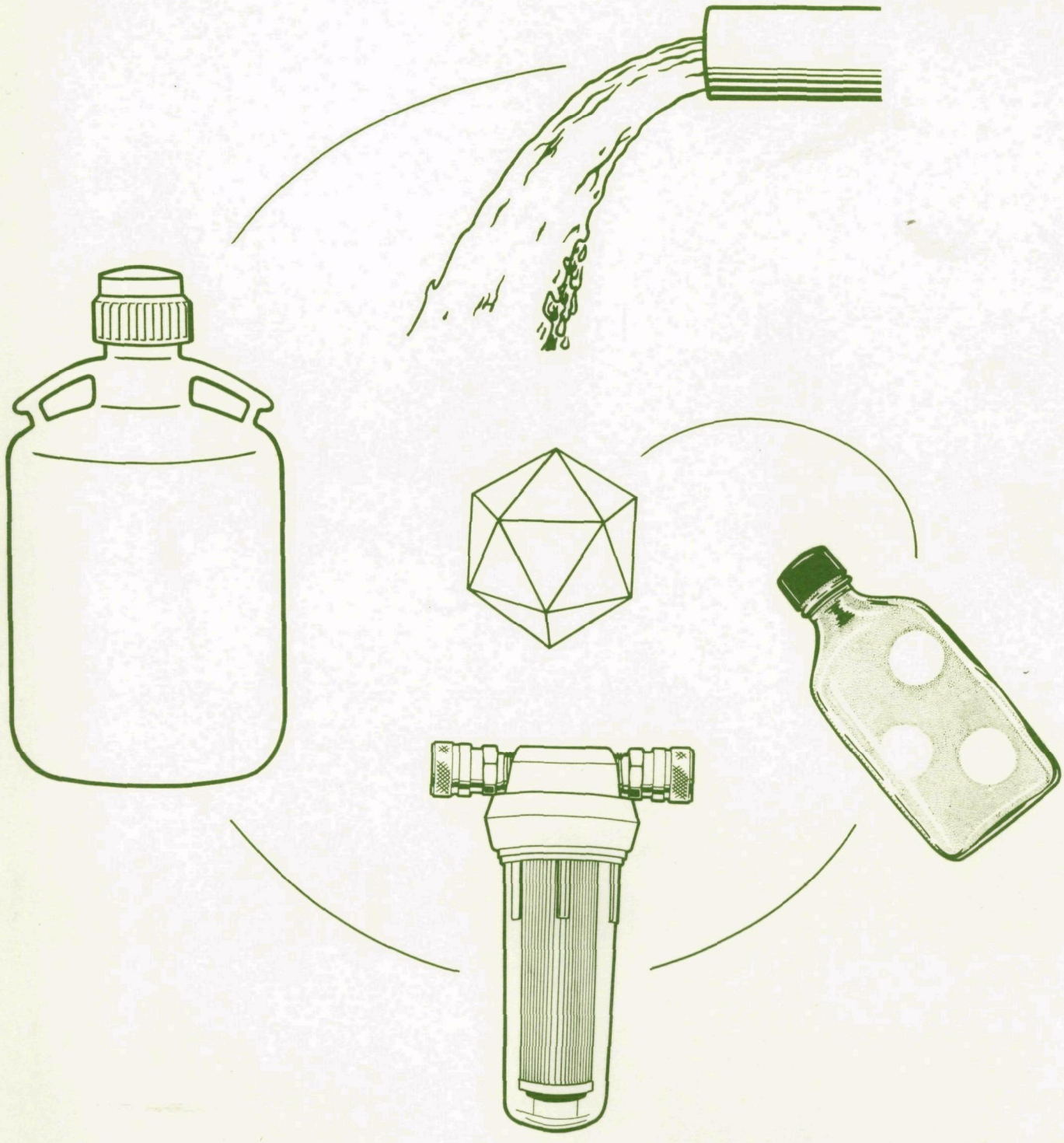
USEPA Manual of Methods for Virology



U S E P A M A N U A L O F M E T H O D S F O R V I R O L O G Y

by

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NOTICE

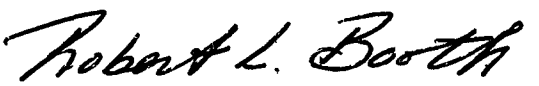
This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring and Support Laboratory - Cincinnati conducts research to:

- Develop and evaluate methods to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid wastes.
- Investigate methods for the concentration, recovery, and identification of viruses, bacteria, and other microbiological organisms in water and to determine the responses of aquatic organisms to water quality.
- Develop and operate an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.
- Develop and operate a computerized system for instrument automation leading to improved data collection, analysis, and quality control.

This manual was prepared in order to meet mandates of the Congress of the United States of America as directed in the Clean Water Act (PL 95-217), the Safe Drinking Water Act (PL 93-523), the Marine Protection, Research, and Sanctuaries Act (PL 92-532), and the Resource Conservation and Recovery Act (PL 94-580). The manual presents a standardized, step-by-step procedure for recovering viruses from most environmental samples other than air.



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PURPOSE

"This manual makes it possible for any competent water bacteriology laboratory that can arrange for viral assays (and identifications) by a competent virology laboratory to concentrate and recover viruses from waters and from sludges and other solids." (See Chapter 1, Section 5.)

TABLE OF CONTENTS

FIGURES

TABLES

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1. PERSPECTIVES IN ENVIRONMENTAL VIROLOGY

The ability to multiply, to direct processes in the cells they infect, and the ability to mutate are the only characteristics of life that the virus is capable of manifesting. In essence, the virus is alive only when it infects. Outside of living cells, the virus is inert. Its essential viability in the hostile environment outside the cell is time-marked. Yet, among those viruses excreted by infected people into sewage discharged into rivers, streams, and lakes, many often survive to reach the water intakes and recreational areas of downstream communities. If that sewage or its treated effluents or sludges are discharged to the land instead, sufficient numbers of viruses may survive to contaminate crops or ground waters in the aquifers below. If the discharge is to the oceans, viruses may contaminate recreational beach waters or approved shellfish-harvest waters. Over the years, cases of such contamination have been documented repeatedly even in the apparent absence of indicator bacteria.

The smallest numbers of viruses detectable in cell cultures, the most sensitive hosts for many viruses, may be sufficient to infect susceptible individuals who consume them. Thus, any number of viruses that reaches a water intake or that is consumed by a recreationalist is a potential hazard. To detect such small numbers of viruses in water requires concentrating viruses from large volumes of water.

In the past several years, a growing awareness of the waterborne virus problem has developed within the scientific community. This awareness has resulted in the development of a number of techniques for recovering viruses from waters of various qualities. These waters range from sewage to tap water. The techniques that have been developed include filter adsorption-elution, glass powder adsorption-elution, ultrafiltration, polyelectrolyte adsorption, aluminum hydroxide adsorption, protamine precipitation, hydroextraction, two-phase separation, organic flocculation, and alginate membrane filtration. Some of these methods are modestly efficient in limited circumstances. None of them has universal potential at present. There is endless change in the chemical quality of waste and receiving waters, and the unpredictable effects of such change on the efficiencies of the methods for quantitatively concentrating viruses from waters is a problem that may long be with us. Thus, methods may always require selection and flexibility to meet the needs of changing situations. Guidance for such selection and flexibility is given herein.

2. THE VIRUSES IN ENVIRONMENTAL WATERS

Enteroviruses (polioviruses, coxsackieviruses [groups A and B], echoviruses, and hepatitis A virus), rotaviruses and other reoviruses (Reoviridae), adenoviruses, and Norwalk-type agents -- a total of more than 100 different serological types -- constitute the major enteric virus complement of human origin. Most of these viruses have been detected in sewage and in receiving waters over the years. Members of other virus groups have been recovered from human feces and urine, but none has been reported with great frequency or in large numbers in sewage or in receiving waters.

Viruses of non-human sources abound in environmental waters. Some of these viruses, such as reoviruses, may infect man; the significance of certain other viruses from non-human sources is as yet undetermined.

The numbers of viruses detected per liter of sewage range from less than 100 infective units to more than 100,000 infective units. In temperate climates, the numbers generally increase in the warmer months and decrease in the colder months, reflecting overall infection and excretion patterns in the community. In the tropics, the numbers of viruses in sewage are highest during the rainy season. Since viruses do not multiply outside of susceptible living cells, dilution in hostile receiving waters and the toll of time eventually reduce the numbers of viruses to levels often barely detectable by the best techniques available, even when 1,000-L quantities of water are tested. In receiving streams, however, such numbers of viruses, in terms of the daily water intake requirements of even small communities, are not small.

When one considers the low efficiencies of the methods that we have for concentrating these viruses, that the cell culture systems used for detecting viruses are usually sensitive to less than half of the virus types excreted by man, that the plaque procedure usually used for detecting and quantifying viruses is itself relatively inefficient, and that there are undoubtedly viruses in sewage that have not yet been detected and identified, it seems reasonable to surmise that the numbers of viruses we now detect in environmental waters are probably an order of magnitude or more below the quantities actually present there. The numbers of viruses that reach recreational waters and intakes downstream of outfalls may thus be very large indeed.

3. CONCLUSIONS AND RECOMMENDATIONS OF THE WORLD HEALTH ORGANIZATION (WHO) SCIENTIFIC GROUP ON HUMAN VIRUSES IN WATER, WASTEWATER AND SOIL*

In 1979, the World Health Organization (WHO) published the report of a WHO Scientific Group on Human Viruses in Water, Wastewater and Soil. The Group included USEPA participation. The Conclusions and Recommendations of the Group follow and are quoted directly:

3.1 [Conclusions]

> While bacterial contamination of water and soils and the associated health risks have been thoroughly studied, attention is now increasingly being focused on the hazards associated with virus contamination of water. The Scientific Group reviewed the current state of knowledge on the subject and concluded that the contamination of water and soil by wastewater and human faeces containing enteric viruses may pose real public health problems. This is also applicable to areas of the world in which the major waterborne bacterial diseases have been brought under control.
>
> There are over 100 different types of enteric viruses, all considered pathogenic to man. Their concentration in wastewater may reach 10 000-100 000/l, and they have the ability to survive for months in water and in soil. In some instances, the ingestion of a single infectious unit can lead to infection in a certain proportion of susceptible humans.
>
> On numerous occasions viral hepatitis A epidemics have been waterborne. Many outbreaks of viral hepatitis A have resulted from eating shellfish grown in sewage-contaminated estuarine and coastal waters. It is also probable that a significant proportion of the reported waterborne gastroenteritis outbreaks of nonbacterial etiology have been associated with waterborne viruses (e.g., rotaviruses).
>
> While the Scientific Group recognized that massive water-borne outbreaks of virus-associated diseases have been detected only on limited occasions, it concluded that

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*Human Viruses in Water, Wastewater and Soil, Report of a WHO Scientific Group, Technical Report Series 639. World Health Organization, Geneva, Switzerland, 1979. 50 pp.

the constant exposure of large population groups to even relatively small numbers of enteric viruses in large volumes of water can lead to an endemic state of virus dissemination in the community, which can and should be prevented.

Bacteria used as conventional indicators to evaluate the safety of potable water supplies have been shown to be significantly less resistant than viruses to environmental factors and to water and wastewater treatment processes. As a result, enteric viruses may be present in water that manifests little or no sign of bacterial pollution.

Where surveys have been carried out, viruses have been detected in the drinking-water supply system of a number of cities, despite the fact that these supplies have received conventional water treatment, including filtration and disinfection, which are considered adequate for protection against bacterial pathogens. Plans for the recycling of wastewater for domestic consumption are being considered in some cities, while many others are drawing their water supply from contaminated surface sources carrying a significant proportion of wastewater. In both situations the risk of viruses penetrating the supply system must be carefully evaluated so that adequate monitoring and treatment can be provided.

Methods for the concentration and enumeration of viruses in large volumes of water have been developed but are not yet standardized. Through the use of such methods large water samples can be monitored for viruses on a routine basis.

Water treatment methods capable of accomplishing effective virus removal and inactivation are now available, so that conventional water treatment plants can be suitably modified to deal with this problem. The formation of carcinogenic compounds when water containing organic material is chlorinated may give rise to a potential health problem. However, in situations in which there is a risk of waterborne communicable disease there should be no hesitation in continuing current water disinfection with chlorine until alternate techniques for effective virus inactivation are developed.

Viruses present in wastewater and sludge applied to land for irrigation, fertilization or disposal purposes can survive in soil for periods of weeks or even months. Edible crops, contaminated either by contact with virus-laden soil or by wastewater sprinkler-irrigation, can harbour viruses for sufficient periods of time to survive harvesting and marketing, and thus their eventual consumption constitutes a potential health risk.

Only limited data are available on the health risks resulting from the dispersion of viruses in aerosols created by sewage treatment and land disposal systems.

However, a potential hazard does exist and steps to reduce it may be warranted. Disinfection of effluent prior to land disposal, particularly in the case of sprinkler-irrigation in the vicinity of inhabited areas, could be an effective preventive measure.

3.2 [Recommendations]

(1) Wherever possible, drinking-water should be free from human enteric viruses. To ensure that this goal is being achieved, a 100-l to 1000-l sample should be tested by the most sensitive method available. In all cases of intentional direct wastewater reuse for domestic consumption, this procedure should be considered essential and should be applied at least in large urban areas in which potable supplies are derived from virus-polluted sources, such as surface water containing a significant proportion of wastewater either untreated or insufficiently treated to inactivate viruses. Further consideration should be given to the establishment of recommended virus concentration limits for water for recreational purposes, and wastewater effluent and sludge for agricultural use.

(2) Where virological facilities can be provided, it is desirable to monitor wastewater effluents, raw-water sources and drinking-water for the presence of viruses. This will provide baseline data to evaluate the health risk faced by the population.

(3) In the light of the greater resistance of many enteric viruses to disinfection and other treatment processes compared to that of bacteria utilized as pollution indicators, drinking-water derived from virus-contaminated sources should be treated by methods of proved high efficiency for removing or inactivating viruses and not only bacteria. Particular emphasis should be given in such cases to ensure the effective disinfection of drinking-water with, for example, free available chlorine residuals of 0.5 mg/l maintained for a contact time of 30-60 minutes or an ozone residual of 0.2-0.4 mg/l maintained for 4 minutes.

(4) Because of the ability of viruses to survive for long periods in seawater, it is recommended that coastal bathing and shellfish growing areas should be protected from contamination by wastewater and sludge. Virus monitoring of these areas is a desirable measure.

(5) Control procedures should be instituted in all situations in which wastewater or sludge is used for irrigation or fertilization, to prevent the contamination of vegetables and fruits which are to be eaten raw. (Moreover--even though they may eventually be cooked--contaminated raw vegetables are liable to pollute other food in the kitchen.) Where it is nevertheless planned to irrigate such crops or where sprinkler-irrigation is to be used near populated areas, the effluent should be treated so that it reaches a high microbiological quality approaching that of drinking-water.

(6) Since the factors that influence the movement of viruses in soil are still not fully understood, and since effluent and soil conditions vary so greatly, caution should be exercised if wastewater irrigation or land disposal takes place in the vicinity of wells supplying drinking-water. Careful study of local conditions is required and the cautious siting of such wells and routine virological monitoring of the water are advised as safety measures.

(7) Further research is necessary into the health risks associated with viruses in water and soil. These studies should include the development and evaluation of methods of detecting viruses and alternative indicators of virus pollution (e.g., phages) and the improvement of treatment methods for the inactivation and removal of viruses from water and wastewater. The dissemination and survival of viruses in the natural environment should also be investigated.

(8) A standard method should be developed for the concentration and detection of viruses in large volumes of drinking-water (e.g., 100-1000 l) based on a full evaluation in different laboratories of present techniques. Such an attempt would facilitate the development of virus-monitoring programmes and would ensure a maximum degree of comparability of results. A laboratory quality-control system should be developed to enable participating laboratories to standardize their procedures.

3.3 Summary

Although not a direct response to the efforts of the WHO Scientific Group, this manual should make possible the monitoring operations envisioned by that group.

4. HISTORY OF METHODS SELECTION

In 1965, a symposium on "Transmission of Viruses by the Water Route" included a major segment on methods for recovering viruses from the water environment. The focus on methods, within the context of the water transmission problem, resulted in a growing interest in methods research over the years that followed.

In 1975, a WHO Working Group on Bacteriological and Virological Examination of Water* met in Germany to recommend the promulgation of methods for recovering bacteria and viruses from various environmental waters and sludges. The USEPA participated. Although methods for recovering bacteria are well-advanced, methods for recovering viruses are not. Nonetheless, the Sub-group on Virological Examination, with some reservations, selected several methods for promulgation which it believed were the best methods currently available.** The American Public Health Association, The American Water Works Association and the Water Pollution Control Federation, through their jointly published Standard Methods, and the American Society for Testing Materials have also recommended methods for recovering viruses from the water environment. The methods described in this USEPA manual have seen the benefit of the research and experience of the years that have passed since 1965. Nonetheless, the current state-of-the-art requires that the following caveats are considered:

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*Report of a Working Group on Bacteriological and Virological Examination of Water (World Health Organization in collaboration with the Federal Republic of Germany, Mainz, Germany, April 21-25, 1975). Water Research, 10:177-178, 1976.
Lund, E. 1982. Virological Examination, 3:462-509. In Suess, M. J., ed., Examination of Water for Pollution Control, Pergamon Press, New York.

**The mandate of the sub-group did not include tap and ocean waters, but some of the methods described herein are directly applicable to such waters.

- Changes in the quality of waters sampled may affect markedly the efficiency of each method described. Few studies are available that compare the efficiency of one method with another under the same conditions.

- None of the methods described has been studied with more than a few virus types. Most studies have been laboratory and not field studies. None of the methods is equally efficient for the recovery of all of the types of viruses frequently found in environmental waters.

- Some of the techniques described are labor-intensive. Some require expensive equipment. In a methodology so rapidly evolving, there is a risk of obsolescence and obvious economic consequences.

4.1 Recommendations of the WHO Working Group and the WHO Scientific Group

Both the aforementioned WHO Working Group on Bacteriological and Virological Examination of Water and the WHO Scientific Group on Human Viruses in Water, Wastewater and Soil suggested tentatively for concentrating viruses from 0.2- to 5-L volumes of wastewater and other waters a microporous filter adsorption-elution technique, adsorption-precipitation with various salts, polyethylene glycol hydroextraction, aqueous polymer two-phase separation, and soluble alginate filtration. These Groups tentatively suggested tangential flow ultra-filtration and flow-through adsorption-elution systems for concentrating viruses from 5- to 400-L volumes of relatively clean waters.

The WHO Groups also recommended tentative methods for recovering viruses from solids in waters and from sludges. These methods were based on elution, with beef extract, serum, or other proteinaceous materials, of viruses from the solids.

The tentative methods recommended by the two WHO Groups have not been presented yet as operational procedures that can be followed readily in the laboratory. Several of those methods (but not the subsequent viral assays) are intended for use in bacteriological laboratories that are minimally equipped and staffed. Both Groups recommended that the tentative methods undergo round-robin* testing.

4.2 Recommendations in Standard Methods** for Detecting Viruses in Various Waters

The 15th edition of Standard Methods presents a microporous filter adsorption-elution technique, an aluminum hydroxide adsorption-precipitation technique, and a polyethylene glycol hydroextraction technique, all as tentative standard methods for recovering viruses from waters and wastewaters. The filter adsorption-elution technique is recommended for concentrating viruses from only a few liters of any water (single-stage filter adsorption-elution technique) and from large volumes of purer waters (two-stage filter adsorption-

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*Tests done under identical conditions by several participating laboratories to determine the effectiveness, precision, and accuracy of a method.

**Standard Methods for the Examination of Water and Wastewater, 15th Edition. American Public Health Association, American Water Works Association, Water Pollution Control Federation, Washington, D.C., 1981.

elution technique). The latter technique may be used to concentrate viruses from volumes of 1,000 L and more of finished waters. Standard Methods recommends the aluminum hydroxideadsorption-precipitation technique and the polyethylene glycol hydroextraction technique only for small volumes of waste and other relatively highly contaminated waters.

The Standard Methods procedures have not been round-robin tested.

The 15th edition of Standard Methods does not recommend methods for recovering viruses from solids in water or from sludges, but it does describe virus assay procedures.

Although the methods in Standard Methods have been written in a manner intended as procedural, Standard Methods recommends that testing with these methods "should be done only by competent and specially trained water virologists having adequate facilities."

4.3 Recommendations of the American Society for Testing Materials (ASTM)

Most of the methods described in this USEPA manual have been round-robin tested by the ASTM. A formal acceptance of these methods as ASTM methods is pending.

5. THE USEPA MANUAL

The USEPA manual contained herein is state-of-the-art. The manual comprises the best methodology available today, and it will be revised frequently so that it remains state-of-the-art.

Each method in this manual has been presented as a step-by-step procedure that should be easily followed by technicians trained in bacteriology and familiar with aseptic techniques and safety

procedures. Each method has been subjected to numerous successful laboratory simulations by both experienced and inexperienced technical personnel. Only the assays for viruses, which must be done in cell cultures or in animals, require the skills of trained virologists.

This manual makes it possible for any competent water bacteriology laboratory that can arrange for viral assays (and identifications) by a competent virology laboratory to concentrate and recover viruses from waters and from sludges and other solids.

6. BIBLIOGRAPHY

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CHAPTER 2

CLEANSING LABORATORY WARE AND EQUIPMENT*

Laboratory ware and equipment that are not chemically clean are responsible for considerable losses in personnel time and supplies in many laboratories. These losses may occur as down time when experiments clearly have been adversely affected and as invalid data that are often attributed to experimental error. Chemical contaminants that adversely affect experimental results are not always easily detected. The problem of improper washing is usually worst in large laboratories with common preparation facilities that are staffed with personnel of limited training who often believe that if it's clean enough to eat from it's clean enough to use in the laboratory.

The key to an effective preparation facility lies in the careful training of hands-on personnel who must be made to understand that a preparation facility is not really a kitchen (as the preparation facility is so often referred to, perhaps aggravating the problem). It is, of course, imperative that the supervisor of the preparation facility understands and appreciates the need for chemically clean laboratory ware. Competent supervisors who understand the need, even with personnel who do not understand, can achieve the quality of cleanliness that is necessary.

*Laboratory ware comprises laboratory glassware and plasticware.

1. PRECAUTIONS

1.1 Sterilize contaminated laboratory ware and equipment before cleansing them (see Chapter 3).

1.2 During the washing process, do not allow laboratory ware or equipment to dry until after the final rinse in deionized distilled water. Detergent that has dried on laboratory ware or equipment is difficult to remove.

1.3 Transport strong acids only in appropriate safety carriers.

1.4 Once detergent solution or acid used to clean a vessel has been rinsed away, do not touch lip or inside of vessel with hands. Detergent or acid on hands or gloves and oil even from clean skin are sources of contamination.

1.5 Check cleansed laboratory ware and equipment for residual detergent and acid in accordance with recommendations in Chapter 4.

1.6 Use non-toxic stainless steel, non-toxic glass, non-toxic non-breakable plastic, or other non-toxic materials for plumbing that carries deionized distilled water. Do not use copper plumbing. Do not use plumbing that contains any ions that may be toxic.

1.7 If a washing machine is used, ensure that jets are strong enough to reach all parts of deep vessels. Ensure also that jets are not so powerful they fill narrow-necked vessels and prevent draining during the time that water is being delivered. Ensure that jets and drains are not clogged. Ensure that washing machine operates properly. Check timing of wash and rinse cycles. Descale lime deposits with descaler when necessary.

1.8 Use only cold water for tap water rinsing. Hot water may contain grease or oil removed from plumbing.

1.9 Use only cold water to wash laboratory ware heavily contaminated with proteinaceous material. Hot water may coagulate such material. Laboratory ware contaminated with infectious material, however, must be sterilized before it is cleansed (see Chapter 3, Section 1.3).

1.10 Inspect washed laboratory ware and equipment for cleanliness. Recleanse unclean laboratory ware by appropriate procedures. Check laboratory ware and equipment for damage. Repair or replace damaged laboratory ware and equipment as appropriate.

1.11 In a multi-purpose laboratory in which different levels of cleanliness are required, code all laboratory ware and equipment, cleanse to specifications, and return to owners. Always report cleansing problems, breakage, and damage to owners.

2. ALTERNATE PROCEDURES

2.1 Disposable laboratory ware may be used when available.

2.2 Cleansing procedures described herein are adequate for most laboratory situations. Less rigorous procedures may be used when quality control tests show they are adequate for laboratory's needs.

2.3 Distilled water (see Chapter 4) may be used in place of deionized distilled water for rinsing whenever quality control tests show that distilled water is adequate.

2.4 When contaminants refractory to chromic acid and HNO_3 procedures are encountered on acid-resistant laboratory ware or equipment, aqua regia may be used to cleanse the ware or equipment in the manner described for concentrated chromic acid.

3. PREPARATION OF CLEANSING COMPOUNDS AND REAGENTS

3.1 Liquid detergent compound for machine-washing glassware and equipment (MIR-A-KOL, Du Bois Chemical Co., or equivalent). Use according to manufacturer's instructions.

3.2 Detergent powder for hand-washing glassware and equipment (Buell Cleaner, No. 222, Polychem Corp., or equivalent). Use according to manufacturer's instructions.

3.3 Nitric acid (HNO_3), 10%.

To prepare 10% HNO_3, pour 100 mL of concentrated HNO_3 slowly into 900 mL of cold deionized distilled water. TO AVOID DANGEROUS SPLATTERS, NEVER POUR WATER INTO CONCENTRATED ACID (see also CAUTION, Section 4.2).

3.4 Chromic acid (dichromate solution).

To prepare chromic acid, dissolve 40 g of sodium dichromate ($Na_2Cr_2O_7$) or potassium dichromate ($K_2Cr_2O_7$) in 1 liter of concentrated sulfuric acid. Dissolve $K_2Cr_2O_7$ in the acid on a magnetic stirrer. $Na_2Cr_2O_7$ is more soluble but more expensive than $K_2Cr_2O_7$. TAKE CARE TO AVOID EXPOSURE TO ACID (see CAUTION, Section 4.2). Potassium and sodium dichromate are strong oxidizing agents and must be handled cautiously.

3.5 Aqua regia

Prepare aqua regia in laboratory fume hood only.

To prepare aqua regia, pour 250 mL of fuming (technical grade) HNO_3 into 750 mL of fuming (technical grade) HCl, and mix carefully. Take care to avoid dangerous splatters and exposure to fumes (see CAUTION, Section 4.2).

4. PROCEDURES FOR CLEANSING LABORATORY WARE AND EQUIPMENT

Laboratory ware and equipment may be cleansed in several ways. Those used for cell cultures may require special care.

4.1 Cleansing with Detergent

4.1.1 General laboratory ware and washable equipment.

(a) Washing machine procedure.

Equip washing machine with capability for delivering four deionized distilled water rinses. The water jets in some washing machines are not strong enough to reach all walls in tall vessels. This results in poor washing and rinsing. The water jets in other washing machines are too strong for test tubes and similar vessels and for many other narrow-necked vessels. Jets that are too powerful hold detergent and rinse water in place and do not allow them to drain properly. If washing machine is unable to wash or rinse adequately, use procedure described in Section 4.1.1, Step (b).

(a.1) Immerse washable vessels in detergent solution, and soak them overnight.

If vessels are too large to immerse, fill them to brim with detergent solution, and soak them overnight.

(a.2) Brush-wash vessels with hot (50-60^{o} C) detergent solution.

Hot tap water that exceeds 50^{o} C is adequate for preparing detergent solution.

(a.3) Machine-wash vessels.

Follow manufacturer's instructions carefully. Add four deionized distilled water rinses if not included in manufacturer's instructions.

(a.4) Drain and air dry vessels, or dry vessels in drying chamber.

(a.5) Sterilize vessels by appropriate method (see Chapter 3).

(b) Manual washing procedure.

Use fresh detergent solution daily. Solutions that are saved may become heavily contaminated with bacteria.

(b.1) Immerse vessels in detergent solution, and soak vessels overnight.

(b.2) Brush-wash vessels with hot (50-60^{o} C) detergent solution.

Hot tap water that exceeds 50^{o} C is adequate for preparing detergent solution.

(b.3) Swish-rinse vessels 10 times with cold tap water .

To swish-rinse, pour into the vessel a volume of tap water equal to about 10% of the volume of the vessel, and swish water around entire surface with each rinse.

(b.4) Swish-rinse vessels five times with deionized distilled water.

(b.5) Drain and air dry vessels, or dry vessels in drying chamber.

(b.6) Sterilize by appropriate method (see Chapter 3).

4.1.2 Test Tubes

Test tubes may be washed by procedure described in Section 4.1.1, Step (a) unless a washing machine is unavailable or washing machine jets are so powerful they do not allow adequate evacuation of tubes and thus interfere with washing and rinsing. In either event, the procedure that follows may be used instead of the washing machine procedure.

(a) Place test tubes open end up into covered wire basket, place basket into stainless steel or plastic vessel sufficient in size to allow complete immersion of tubes, and fill vessel with hot detergent solution.

(b) Steam autoclave (100^{o} C) immersed tubes for 30 minutes.

(c) Empty vessel and tubes, and run cold tap water in to flush out detergent solution.
Introduce tap water into bottom of vessel with a hose connnected to tap. Wax pencil and other scum will wash over rim of vessel.

(d) Fill and empty tubes in vessel 10 times with cold tap water.

(e) Fill and empty tubes in vessel five times with deionized distilled water.

(f) Drain and air dry tubes, or dry tubes in drying chamber.

(g) Sterilize screw-cap tubes.

(g.1) Place-screw cap tubes in test tube racks, and cover them with a sheet of aluminum foil.

(g.2) Sterilize tubes in dry heat oven (maintain 170^{o} C [340^{o} F] for one hour).

(h) Sterilize other tubes.

(h.1) Plug tubes with cotton, or protect mouths of tubes with caps or with semi-permeable plastic inserts.

(h.2) Sterilize tubes with cotton plugs in dry heat oven (maintain 170^{o} C [340^{o} F] for one hour).

(h.3) Autoclave tubes with caps or plastic inserts at 121^{o} C for 30 minutes.

4.1.3 Pipettes

(a) Remove cotton plugs from pipettes.
If necessary, remove cotton plugs by forcing a jet of air or water through delivery tips of pipettes.

(b) Place pipettes, with tips up, into pipette holder.

(c) Place pipette holder into a pipette jar, and fill jar with hot (50-60^{o} C) detergent solution.
Hot tap water that exceeds 50^{o} C is adequate for preparing detergent solution. Pipettes must be completely immersed. If air bubbles are present in pipettes, raise and lower pipette holder several times to remove bubbles.

(d) Soak pipettes in detergent solution for 24 hours.
Raise and lower pipette holder five or six times during the 24 hour period to agitate detergent

solution and thus help remove soil and debris from pipettes.

(e) Place pipette holder into automatic pipette washer, and rinse pipettes through 10 cycles of cold tap water.

(f) Rinse pipettes through five cycles of deionized distilled water.

(g) Remove pipettes from automatic pipette washer, and allow pipettes to drain and air dry.

(h) Plug pipettes with cotton.

(i) Sterilize pipettes in dry heat oven (maintain 170° C [340° F] for one hour).

4.1.4 Automatic Pipettor (Brewer-type)

Immediately after pipettor has been used, fill reservoir with tap water and carefully pump sufficient water through the system to remove cellular debris and other materials that might adhere to apparatus. Determine whether syringe delivers properly without cannula connected.

(a) Remove tubing from reservoir, and remove syringe from pipettor; autoclave valve, tubing, reservoir, and syringe at 121° C for 60 minutes.

(b) Disassemble syringe, and remove cannula.

(c) Cleanse syringe, tubing, reservoir, valve, and cannula.

(c.1) Syringe

(c.1.1) Rinse plunger and barrel of syringe with copious quantities of cold tap water.

If plunger and barrel require further cleansing, soak them overnight in 10% HNO_3 or in 10% chromic acid (See Section 4.2.1, Step [b] and Step [a]), and repeat Step (c.1.1). CAUTION: Take care when using acid (see CAUTION, Section 4.2). Do not expose metal to acid.

(c.1.2) Rinse plunger and barrel with copious quantities of deionized distilled water.

(c.1.3) Soak plunger and barrel of syringe overnight in deionized distilled water.

(c.1.4) Allow plunger and barrel of syringe to drain and air dry.

(c.1.5) Proceed to Step (d).

(c.2) Tubing

(c.2.1) Rinse tubing copiously with cold tap water.

If tubing does not come clean, place it in hot (50-60° C) detergent solution, remove air bubbles, and allow tubing to soak for 24 hours. Then, repeat Step (c.2.1).

(c.2.2) Rinse tubing copiously with deionized distilled water.

(c.2.3) Soak tubing overnight in deionized distilled water.

(c.2.4) Allow tubing to drain and air dry.

(c.2.5) Proceed to Step (d).

(c.3) Reservoir

(c.3.1) Fill reservoir with hot (50-60^{o} C) detergent solution, and soak reservoir overnight. Hot tap water that exceeds 50^{o} C is adequate for preparing detergent solution.

(c.3.2) Brush-wash reservoir with hot (50-60^{o} C) detergent solution. If reservoir does not come clean, rinse it with tap water, and soak it overnight in 10% HNO_3 or in 10% chromic acid. See Section 4.2.1, Step (b) and Step (a). CAUTION: Take care when using acid (see CAUTION, Section 4.2).

(c.3.3) Rinse reservoir 10 times with cold tap water.

(c.3.4) Swish-rinse reservoir five times with deionized distilled water.

(c.3.5) Allow reservoir to drain and air dry.

(c.3.6) Proceed to Step (d).

(c.4) Valve

If syringe has been delivering properly with the cannula removed (see Section 4.1.4), no further attention to valve is needed. If syringe has not been delivering properly with the cannula removed, go to Step (c.4.1).

(c.4.1) Remove valve from apparatus.

(c.4.2) Soak valve overnight in 10% HNO_3 or in 10% chromic acid.

CAUTION: Take care when using acid (see CAUTION, Section 4.2).

(c.4.3) Rinse valve copiously with cold tap water.

(c.4.4) Rinse valve copiously with deionized distilled water.

(c.4.5) Allow valve to drain and air dry.

(c.4.6) Return valve to apparatus.

(c.4.7) Proceed to Step (d).

(c.5) Cannula

(c.5.1) Connect cannula to a clean syringe.

(c.5.2) Force 50 mL of deionized distilled water through cannula.

If cannula is unobstructed, go to Step (c.5.3). If cannula is completely or partially obstructed go to Step (c.5.5).

(c.5.3) Allow cannula to drain and air dry.

(c.5.4) Proceed to Step (d).

(c.5.5) If cannula is obstructed, remove cannula from syringe, and soak cannula for 24 hours in 10% HNO_3 or in 10% chromic acid.

Move cannula up and down in acid to remove air bubbles that may have formed.

CAUTION: Take care when using acid (see CAUTION, Section 4.2).

(c.5.6) Rinse cannula copiously in cold tap water.

(c.5.7) Connect cannula to syringe.

(c.5.8) Force 50 mL of cold tap water through cannula.

(c.5.9) Force 50 mL of deionized distilled water through cannula.

(c.5.10) Remove cannula from syringe.

(c.5.11) Allow cannula to drain and air dry.

(c.5.12) Proceed to Step (d).

(d) Reassemble syringe.

(e) Reconnect tubing to reservoir and to syringe.

(f) Connect cannula to appropriate tubing.

(g) Pour 100 mL of deionized distilled water into reservoir, cover opening of reservoir with aluminum foil, protect cannula with glass tube cover, and wrap syringe, interconnecting tubing, and protected cannula in cloth.

(h) Autoclave assembled apparatus at 121° C for 30 minutes.

Use slow exhaust.

4.1.5 Automatic Syringe (Cornwall-type)

Immediately after syringe has been used, fill reservoir with tap water and pump sufficient water through the system to remove cellular debris and other materials that might adhere to apparatus. Determine whether syringe is delivering properly without cannula connected.

(a) Remove tubing from reservoir, and autoclave entire apparatus at 121° C for 60 minutes.

(b) Disassemble syringe.

(c) Cleanse syringe, tubing, and cannula, and replace valves, if necessary.

(c.1) Syringe

(c.1.1) Rinse plunger and barrel of syringe with copious quantities of cold tap water.

If plunger and barrel require further cleansing, soak them overnight in 10% HNO_3 or in 10% chromic acid (see Section 4.2.1, Step [b] and Step [a]), and repeat Step (c.1.1).

CAUTION: Take care when using acid (see CAUTION, Section 4.2). Do not expose metal to acid.

(c.1.2) Rinse plunger and barrel of syringe with copious quantities of deionized distilled water.

(c.1.3) Soak plunger and barrel of syringe overnight in deionized distilled water.

(c.1.4) Allow plunger and barrel of syringe to drain and air dry.

(c.1.5) Proceed to Step (d).

(c.2) Tubing

(c.2.1) Rinse tubing with copious quantities of cold tap water. If tubing does not come clean, place it in hot (50-60° C) detergent solution, remove air bubbles, and allow tubing to soak for 24 hours. Then, repeat Step (c.2.1).

(c.2.2) Rinse tubing with copious quantities of deionized distilled water.

(c.2.3) Soak tubing overnight in deionized distilled water.

(c.2.4) Allow tubing to drain and air dry.

(c.2.5) Proceed to Step (d).

(c.3) Valves

If syringe has been delivering properly with the cannula removed (see Section 4.1.5), no further attention to valves is needed. If

syringe has not been delivering properly with the cannula removed, check inlet and outlet valves. Replace either valve, or both valves, if damaged or hard.

(c.4) Cannula

(c.4.1) Connect cannula to a clean syringe.

(c.4.2) Force 50 mL of deionized distilled water through cannula.
If cannula is completely or partially obstructed, go to Step (c.4.5).

(c.4.3) Allow cannula to drain and air dry.

(c.4.4) Proceed to Step (d).

(c.4.5) If cannula is obstructed, remove cannula from syringe, and soak cannula for 24 hours in 10% HNO_3 or in 10% chromic acid.
Move cannula up and down in acid to remove air bubbles that have formed.
CAUTION: Take care when using acid (see CAUTION, Section 4.2).

(c.4.6) Rinse cannula copiously in cold tap water.

(c.4.7) Connect cannula to syringe.

(c.4.8) Force 50 mL of cold tap water through cannula.

(c.4.9) Force 50 mL of deionized distilled water through cannula.

(c.4.10) Remove cannula from syringe.

(c.4.11) Allow cannula to drain and air dry.

(c.4.12) Proceed to Step (d).

(d) Reassemble syringe.

(e) Connect cannula to syringe.

(f) Protect cannula with glass tube cover, and wrap syringe and protected cannula in cloth.

(g) Autoclave apparatus at 121° C for 30 minutes. Use slow exhaust.

4.1.6 Disc Filter Holder.

(a) Disassemble disc filter holder, and discard membrane.

(b) Rinse filter holder components with copious quantities of cold tap water.

If debris remains after tap water rinse, brush-wash filter holder with hot (50-60° C) detergent solution. Remove refractory debris with non-abrasive scrubber. Use fine grade steel wool only if absolutely necessary. Rinse again with copious quantities of cold tap water.

(c) Rinse filter holder components with copious quantities of deionized distilled water.

(d) Allow filter holder components to drain and air dry.

Check gaskets for distortion (flattened areas), and replace gaskets if necessary.

(e) Attach tubes to inlet and outlet ports of filter holder.

(f) Clamp shut outlet port.

(g) Place filter support on base of filter holder.

(h) Fill base of holder with deionized distilled water.

(i) Place membrane filter on filter support.

(j) Cover filter with deionized distilled water.

(k) Reassemble disc filter holder.

Do not tighten down top of filter holder.

(l) Autoclave filter and filter holder at 121° C for 20 minutes.

Use slow exhaust.

(m) Allow filter holder to cool, and tighten down top of holder.

4.1.7 Dispensing pressure vessel.

(a) Remove lid from dispensing pressure vessel.

(b) Rinse pressure vessel and lid with copious quantities of cold tap water.

If debris remains after tap water rinse, brush-wash vessel and lid with hot (50-60° C) detergent solution. Remove refractory debris with non-abrasive scrubber. Use fine grade steel wool only if absolutely necessary. Rinse again with copious quantities of cold tap water.

(c) Swish-rinse pressure vessel and lid five times with deionized distilled water.

To swish-rinse, pour into the vessel a volume of water equal to about 10% of the volume of the vessel, and swish water around entire surface with each rinse.

(d) Allow vessel and lid to drain and air dry.

(e) Pour 100 mL of deionized distilled water into vessel.

(f) Cover vessel opening with aluminum foil, and wrap lid with aluminum foil.

Be certain vent/relief valve on lid is open.

(g) Autoclave vessel and lid at 121° C for 30 minutes, and dry both in autoclave for 10 minutes.

4.1.8 Plastic Screw-caps.

(a) Place caps in stainless steel or plastic vessel containing detergent solution.

(b) Steam autoclave (100° C) caps in detergent solution for 15 minutes.

(c) Pour water from vessel, and rinse caps with copious quantities of cold tap water.

Run hose from tap to bottom of vessel to achieve thorough rinsing.

(d) Rinse caps with copious quantities of deionized distilled water.

Run hose from deionized distilled water line to bottom of vessel to achieve thorough rinsing.

(e) Place caps in upright position on towel, and allow caps to drain and air dry.

(f) Place caps in upright position in glass petri plates.

(g) Place petri plates in petri plate cannister.

(h) Autoclave caps at 121° C for 30 minutes.

Leave top off cannister during autoclaving to allow penetration of steam.

(i) Allow plates and caps to cool, and secure cover on cannister.

4.2 Cleansing with Acid

Either chromic acid or 10% HNO_3 may be used to cleanse glassware. Ten percent HNO_3 requires longer contact (24 hours) with tubes than chromic acid requires, but residual HNO_3 is not as likely to be toxic to cell cultures as residual chromic acid is.

Do not expose metals or other materials to acids unless certain that those substances are acid-resistant. CAUTION: Chromic acid and other acids may react violently with organics or other oxidizable substances. Take care to avoid such reactions. Cleanse laboratory ware with detergent solutions before cleansing them with acids. Chromic acid and HNO_3 are strong acids capable of producing severe burns even when used in relatively dilute solutions. When working with these or with other strong acids, avoid inhalation of fumes. Protect eyes with safety goggles or with full face mask. Protect clothing with acid-resistant laboratory coat or apron. If eyes are accidentally exposed to acid, immediately wash them with copious quantities of water for at least 15 minutes. Consult a physician immediately thereafter. If other parts of the body are exposed to acid, immediately remove clothing over exposed areas and wash exposed areas with copious quantities of water. Consult a physician immmediately thereafter if affected area is large or if exposure has been lengthy. Subsequently, wash exposed areas of clothing with copious quantities of water.

4.2.1 General Acid-resistant Laboratory Ware.

(a) Chromic acid procedure.

Glassware and other acid-resistant laboratory ware cleansed with chromic acid may retain some chromium ions even after extensive rinsing. For some work, these ions may be undesirable. Chromic acid may be toxic to cells. Glassware and other laboratory ware used for cell culture work, if washed with chromic acid, may subsequently need to be washed with detergent solution to remove chromium ions (see Section 4.1).

(a.1) Thoroughly rinse loose debris and residues from vessel with tap water.

(a.2) Pour into vessel from an acid reservoir a volume of chromic acid equal to about 10% of the capacity of the vessel.

Take care to avoid splatter. Small vessels may be immersed in a vat of acid. Do not allow acid to contact skin (see CAUTION, Section 4.2). When necessary, wear acid-resistant gloves. Gloves must possess good gripping qualities, because acid makes vessels slippery.

(a.3) Rotate vessel so that acid covers entire inside area of vessel.

Allow chromic acid to remain in contact with vessel for about five minutes.

(a.4) Pour acid from vessel into acid reservoir.

Chromic acid is reusable until oxidized (green). If chromic acid is oxidized, dispose of it safely as with other toxic wastes.

(a.5) Fill and empty vessel with cold tap water 10 times.

Be certain that all acid is removed from outside of vessel.

(a.6) Swish-rinse vessel five times with deionized distilled water.

To swish-rinse, pour into the vessel a volume of water equal to about 10% of the volume of the vessel, and swish water around entire surface with each rinse.

(a.7) Drain and air dry vessel, or dry vessel in drying chamber.

(a.8) Sterilize vessel by appropriate method (see Chapter 3).

(b) Nitric acid procedure.

(b.1) Rinse loose debris from vessel with tap water.

(b.2) Fill vessel to brim with 10% HNO_3.

Small vessels may be immersed in a vat of acid. Do not allow acid to contact skin (see CAUTION, Section 4.2). When necessary, wear acid-resistant gloves. Gloves must possess good gripping qualities, because acid makes vessels slippery.

(b.3) Allow 10% HNO_3 to remain in contact with vessel surface for 24 hours.

(b.4) Carefully pour acid down acid-resistant sewer drain, and flush acid away with copious quantities of tap water.

(b.5) Fill and empty vessel 10 times with cold tap water.

Be certain that all acid is removed from outside of vessel.

(b.6) Swish-rinse vessel five times with deionized distilled water.

To swish-rinse, pour into the vessel a volume of water equal to about 10% of the volume of the vessel, and swish water around entire surface with each rinse.

(b.7) Drain and air dry vessel, or dry vessel in drying chamber.

(b.8) Sterilize vessel by appropriate method (see Chapter 3).

4.2.2 Test Tubes

CAUTION: Take care to avoid splatter. Do not allow acid to contact skin (see CAUTION, Section 4.2). When necessary, wear acid-resistant gloves. Gloves must possess good gripping qualities because acid makes tubes slippery.

(a) Rinse loose debris from tubes with tap water.

(b) Place tubes open end up into covered acid-resistant wire basket, and place basket into acid-resistant vessel.

(c) Fill vessel with chromic acid or with 10% HNO_3.

If chromic acid is used, allow acid to remain in contact with tubes for about five minutes. If 10% HNO_3 is used, allow acid to remain in contact with tubes for 24 hours.

(d) Pour acid from tubes.

Chromic acid is reusable until oxidized (green) and may be poured back into reservoir. If chromic acid is oxidized, dispose of it safely as with other toxic wastes. Wash waste HNO_3 down acid-resistant drain with copious quantities of tap water.

(e) Run cold tap water into vessel to flush acid from tubes.

Run tap water through a hose into bottom of vessel. Wax pencil and other scum will wash over rim of vessel.

(f) Fill and empty tubes in vessel 10 times with cold tap water.

(g) Fill and empty tubes in vessel five times with deionized distilled water.

Tubes for cell culture work that have been cleansed with chromic acid must be cleansed with detergent solution. For such tubes, proceed to Section 4.1.2.

(h) Drain and air dry tubes, or dry tubes in drying oven.

(i) Sterilize screw-cap tubes.

(i.1) Place screw-cap tubes in test tube racks, and cover them with a sheet of alumimum foil.

(i.2) Sterilize tubes in dry heat oven (maintain 170^{o} C [340^{o} F] for one hour).

(j) Sterilize other tubes.

(j.1) Plug tubes with cotton, or protect mouths of tubes with semi-permeable plastic inserts.

(j.2) Sterilize tubes with cotton plugs in dry heat oven (maintain 170^{o} C [340^{o} F] for one hour).

(j.3) Autoclave tubes with plastic inserts at 121^{o} C for 30 minutes.

4.2.3 Pipettes

(a) Remove cotton plugs from pipettes.

<u>If necessary, remove cotton plugs by forcing a jet of air or water through delivery tips of the pipettes</u>.

(b) Place pipettes, with tips up, into an acid-resistant plastic pipette holder.

(c) Carefully place pipette holder into an acid-resistant pipette jar filled with 10% HNO_3.

<u>CAUTION: Take care to avoid acid splatter. Do not allow acid to contact eyes or skin (see CAUTION, Section 4.2). Wear eye protection and an acid-resistant laboratory coat or an acid-resistant apron. When necessary, wear</u>

acid-resistant gloves with good gripping qualities. Acid makes pipettes and pipette holders slippery.

(d) Carefully raise and lower pipette holder several times to force air bubbles from pipettes.

(e) Soak pipettes in acid for 24 hours.
Carefully raise and lower pipette holder five or six times during the 24-hour period to agitate acid and thus help remove contaminants and debris from pipettes.

(f) Carefully remove pipette holder from pipette jar, and place holder and pipettes in automatic pipette washer.
Take care to avoid acid splatter.

(g) Immediately rinse pipettes through 10 cycles of cold tap water.
Do not allow acid dripping from pipettes to remain in contact with metal parts of automatic pipette washer. Acid may damage metal.

(h) Rinse pipettes through seven cycles of deionized distilled water.

(i) Remove pipettes from automatic washer, and allow pipettes to drain and air dry.

(j) Plug pipettes with cotton.

(k) Place pipettes in pipette canisters, and sterilize in dry heat oven (maintain 170^{o} C [340^{o} F] for one hour).

4.3 Cleansing with Alkalies

Alkalies such as sodium metasilicate, trisodium phosphate, sodium carbonate, and soft soaps can be used to cleanse laboratory ware and equipment. Alkalies, however, tend much more than acids to etch at least the glassware.

5. BIBLIOGRAPHY

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CHAPTER 3

STERILIZATION AND DISINFECTION

1. GENERAL PROCEDURES

   1.1 Use aseptic techniques for handling test waters, sewages, sludges, and cell cultures.

   1.2 Sterilize apparatus and containers that will come into contact with test waters, sludges, or elutants, all solutions added to test waters unless otherwise indicated, and all elutants.

   1.3 Sterilize all contaminated materials (including all blood and blood products) before discarding.

   1.4 Disinfect all spills and splatters.

2. STERILIZATION TECHNIQUES

   2.1 Solutions

   Unless otherwise indicated, sterilize all solutions except those used for cleansing, standard buffers, hydrochloric acid (HCl), sodium hydroxide (NaOH), Freon and other organic materials, and disinfectants by autoclaving them at 121° C for 15 minutes. HCl, NaOH, Freon, and disinfectants as used herein are self-sterilizing (bactericidal and fungicidal). When autoclaving buffered beef extract, use a vessel large enough to accommodate foaming.

   2.2 Autoclavable Glassware, Autoclavable Plasticware, Dialysis Tubing, and Equipment

Water may speed heat transfer in larger vessels during autoclaving and thereby speed the sterilization process. Add deionized distilled water to vessels in quantities indicated in Table 3-1. Lay large vessels on sides in autoclave, if possible, to facilitate displacement of air in vessels by flowing steam.

2.2.1 Cover openings into glassware, autoclavable plasticware, and equipment with aluminum foil before autoclaving.

2.2.2 Sterilize glassware, unless otherwise noted, in a dry heat oven at a temperature of 170° C for one hour (see Table 3-2 for acceptable alternative time-temperature couplings).

2.2.3 Autoclave at 121° C for one hour plasticware that can withstand autoclaving.

Plasticware requires more time to sterilize than glassware because plastic transfers heat more slowly than glass.

2.2.4 Sterilize stainless steel vessels in an autoclave at 121° C for 30 minutes.

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

2.2.5 Sterilize with ethylene oxide tubing and plasticware that cannot withstand autoclaving.

CAUTION: Avoid exposure to ethylene oxide fumes. Ethylene oxide is toxic.

Sterilize materials in 12% ethylene oxide (20-50% relative humidity). Expose dry materials to ethylene oxide for two hours. Expose materials that are not dry for four hours.

TABLE 3-1

Quantities of deionized distilled water to be added to vessels to facilitate sterilization during autoclaving.*

Vessel Size (Liter)	Quantity of Deionized Distilled Water (mL)
2 and 3	25
4	50
8	100
24	500
54	1000

*Add to vessel the volume of deionized distilled water indicated, cover mouth of vessel with aluminum foil, lay vessel on its side if possible, and autoclave.

TABLE 3-2

Time-temperature couplings for dry sterilization.

Temperature °C	Temperature °F	Hours
140	285	3
150	300	2.5
160	320	2
170	340	1

2.2.6 Autoclave dialysis tubing at 121° C for 15 minutes.
Fill dialysis bag two-thirds full with deionized distilled water before autoclaving.

2.2.7 Autoclave membrane filters in situ in filter holders at 121° C for 20 minutes.
To speed sterilization and to prevent filters from cracking, seat (and cover) filters in a small volume of deionized distilled water.
Open vent/relief valves on filter holders before autoclaving, and close vent/relief valves immediately after autoclaving.

2.2.8 Sterilize cartridge filters according to manufacturer's instructions.

2.2.9 Autoclave apparatus except pumps, cartridge filter holders, and combination-type pH electrodes at 121° C for 30 minutes.

2.2.10 Sterilize drums, other vessels, and other apparatus too large for autoclaves by chlorination.
Fill vessels or apparatus with deionized distilled water containing 10-15 mg of chlorine (NaOCl) per liter, adjusted to pH 6-7 with HCl. Dechlorinate chlorinated water in vessels or apparatus after 30 minutes by adding sufficient sodium thiosulfate ($Na_2S_2O_3$) solution to yield 50 mg per liter. Allow 15 minutes for dechlorination, and drain water from vessels or apparatus.

2.2.11 Sterilize pumps and cartridge filter holders with chlorine or with ethylene oxide.
(a) Chlorine procedure.

(a.1) For 30 minutes, recirculate through pumps and cartridge filter holders 4 liters of deionized distilled water containing 10-15 mg of chlorine (NaOCl) per liter, adjusted to pH 6-7 with HCl.

(a.2) Dechlorinate pumps and cartridge filter holders by passing through them 1 liter of a solution containing 50 mg of sodium thiosulfate ($Na_2S_2O_3$) per liter of deionized distilled water.

(b) Ethylene oxide procedure.

CAUTION: Avoid exposure to ethylene oxide fumes. Ethylene oxide is toxic.

(b.1) Sterilize pumps and cartridge filter holders in a gas sterilizer by exposing them to 12% ethylene oxide (30-50% relative humidity) for four hours at 55-60^{o} C.

(b.2) Aerate pumps and cartridge filter holders in a gas aerator to remove residual ethylene oxide (as recommended by the sterilizer manufacturer), or maintain pumps and cartridge filter holders at 37^{o} C for a minimum of three days before using them.

2.2.12 Sterilize pH electrodes with chlorine or with HCl. Sterilize electrodes before and after each use.

(a) Chlorine procedure.

(a.1) Immerse tip of electrode for one minute into deionized distilled water that contains 10-15 mg of chlorine (NaOCl) per liter, adjusted to pH 6-7 with HCl.

(a.2) Dechlorinate electrode by immersing tip into sterile deionized distilled water that contains 50 mg of $Na_2S_2O_3$ per liter and then rinsing tip with sterile deionized distilled water.

(b) HCl procedure.

(b.1) Immerse tip of pH electrode into 1 M HCl for one minute.

Use only fresh 1 M HCl prepared daily from concentrated HCl.

(b.2) Rinse electrode tip with sterile deionized distilled water.

2.2.12 Sterilize working instruments such as scissors and forceps by autoclaving them at 121° C for 30 minutes. Working instruments such as scissors and forceps may be sterilized between uses by immersing them in 70% ethanol and flaming them.

2.3 Contaminated materials.

Autoclave contaminated materials for one hour at 121° C. Be sure that steam can enter contaminated materials freely. If volume of contaminated materials is unusually large, exposure time at 121° C must be increased appropriately.

3. DISINFECTION TECHNIQUES

3.1 Disinfect spills and other contamination on surfaces that do not stain with a solution of 0.5% I_2 in 70% ethanol.

3.2 Disinfect spills and other contamination on surfaces that stain with a solution of 0.1% HOCl.

0.1% HOCl may be prepared by appropriately diluting an NaOCl solution (Clorox, The Clorox Co., or equivalent) and adjusting its pH to 6-7 with dilute HCl.

4. BIBLIOGRAPHY

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CHAPTER 4

QUALITY ASSURANCE*

1. INTRODUCTION

1.1 Role in Research

1.1.1 In research for any purpose, the quality of data must be protected. Quality assurance plays a key role in the production and protection of scientifically valid data through a variety of planned and systematic activities and procedures. A laboratory quality control program is the orderly application of practices necessary to remove or reduce errors in any laboratory operation that are attributable to personnel, equipment, supplies, sampling procedures, and analytical methods.

1.1.2 A quality control program must be practical, integrated, and require only a reasonable amount of time or it is likely to be bypassed. When properly administered, a balanced, conscientiously applied quality control program assures the production of uniformly high quality data without interfering with the primary analytical functions of the laboratory.

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*Many of the sections of this chapter were adapted from Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-071, 1978.

When possible, this laboratory program should be supplemented by participation of the laboratory in an interlaboratory quality control program.

1.2 Scope of Program

This chapter on Quality Assurance deals with sample collection, facilities, maintenance, personnel, equipment and instruments, supplies, and procedures. See Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-071, U. S. Environmental Protection Agency, Cincinnati, Ohio, 1978, for discussions of statistics applicable to microbiology (p. 225), and for the development of a quality control program (p. 244). For the latter, also see Interim Guidelines and Specifications for Preparing Quality Assurance Project Plans, QAMS-005/80, Office of Monitoring Systems and Quality Assurance, Office of Research and Development, U. S. Environmental Protection Agency, Washington, D. C., 1980. For discussions of safety, see Biosafety in Microbiological and Biomedical Laboratories (Draft), Centers for Disease Control, Atlanta, Georgia, and National Institutes of Health, Bethesda, Maryland, 1983.

2. SAMPLE COLLECTION

2.1 Water and Sewage Samples

2.1.1 Water and sewage samples collected must be representative of the particular environment sampled.

2.1.2 Sample sites and sampling frequency must provide representative characteristics and variabilities.

2.1.3 The number of samples collected must rest within the processing capability of the laboratory.

2.2 Chain of Custody

A strict chain of custody procedure is required for all samples in legal enforcement actions. (For chain of custody procedures, see Handbook for Sampling and Sample Preservation of Water and Wastewater, NTIS, PB83-124503, 1982, pp. 345-355.)

2.3 Sample Handling Procedures

2.3.1 Aseptic technique must be maintained during sampling.

2.3.2 Sterile containers and equipment must be used in all sampling procedures.

2.3.3 When a sample is received in the laboratory, the integrity of the sample container and the condition of the sample must be checked and recorded.

2.3.4 It must be ascertained that the sample has been properly labelled.

2.3.5 In enforcement cases, a check must be made to assure that the chain of custody procedure has been followed.

2.4 Transport of Samples

Proper conditions must be met to maintain viability of viruses during transport.

2.4.1 Samples must be refrigerated or iced immediately upon collection.

2.4.2 All samples that cannot be processed within 24 hours must be frozen and stored at -70^{o} C immediately.

Freezing and thawing of virus samples must be kept to a minimum.

3. LABORATORY FACILITIES

3.1 Air Handling Systems

3.1.1 All laboratories should be maintained under negative air pressure.

3.1.2 Biological safety cabinets should be available for work requiring sterile conditions and protection for personnel and samples.

3.2 Disinfection of Laboratory

Laboratories should be equipped with ultraviolet lights (see Table 4-1) for general decontamination of rooms during periods when personnel are absent.

Take precautions to prevent entry of personnel into laboratories when UV lights are on.

3.3 Space Allocation

3.3.1 Laboratories should provide separate rooms for processing in each of the following categories: potable, surface, and ground waters; sewage and wastewater effluents; sludges, silts, and other solids; cell cultures; and virus identification.

3.3.2 Freezers, incubators, and instruments should be housed in rooms where they can be accessed without disturbing ongoing laboratory effort.

3.3.3 The areas provided for preparation and sterilization of media, glassware, and equipment should be separate from other laboratory work areas but close enough for convenience.

3.4 Traffic

3.4.1 Visitors and through-traffic must be minimized in work areas.

3.4.2 Signs must be posted on doors to limit access to work areas.

3.5 Bench Space Allocation

3.5.1 Sufficient clean bench space must be available for work to be performed efficiently.

For routine studies the minimum area recommended for each worker is six linear feet. Research or other analyses that demand specialized equipment may require significantly more space per worker. These estimates of bench space are exclusive of areas used for preparatory and supportive activities.

3.5.2 Bench tops should be set at heights of 36-38 inches.

This height is usually comfortable for work in a standing or sitting position.

3.5.3 Depth of bench tops should be 28-30 inches.

3.5.4 Desk tops of sit-down benches should be set at a height of 30-31 inches.

This height is required to accommodate microscopy, plaque counting, calculating, and writing.

3.5.5 Bench tops should be stainless steel, epoxy plastic, or other smooth impervious material which is inert and corrosion-resistant.

3.5.6 Bench tops should be seamless or have seams sealed with impervious material.

3.6 Lighting

Laboratory lighting must be even, screened to reduce glare, and provide about 100 footcandles of light intensity on working surfaces.

3.7 Walls and Floors

3.7.1 Walls should be covered with waterproof paint, enamel, or other surface material that provides a smooth finish which is easily cleaned and disinfected.

3.7.2 Floors should be covered with good quality tiles or other heavy duty material which can be maintained with skid-proof wax.

3.8 Monitoring for Cleanliness in Work Areas

3.8.1 High standards of microbiological cleanliness must be maintained in work areas.

3.8.2 Laboratory surfaces and laboratory air should be monitored for microorganisms by one or more procedures.

Tests should be run on a weekly or on some other time basis based on experience to monitor counts in the same work areas over time and to allow comparisons between different work areas. Microbial densities in the air should not exceed 15 colony-forming units per 930 square centimeters (~1 square foot) of agar medium exposed per 15 minutes of exposure.

For a detailed description of these monitoring procedures, see Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-017, 1978, pp. 195-197.

4. LABORATORY MAINTENANCE

4.1 Cleaning

4.1.1 Laboratory benches must be cleaned after each use and at the end of each working day; shelves, floors, and windows must be cleaned on a scheduled basis.

4.1.2 Work benches must be wiped down with disinfectant before and after each use.

Dry-dusting is not permissible in a virology laboratory.

4.1.3 Floors must be wet-mopped and treated with a disinfectant solution to reduce contamination of air in the laboratory.

Sweeping or dry-mopping is not permissible in a virology laboratory.

4.1.4 Spills and leaks must be cleaned up immediately and disinfected when necessary.

4.2 Storage

4.2.1 Laboratory areas must be kept free of clutter.

Clutter can be controlled by cleaning up work areas immediately after each use and by conducting a weekly clean-up of the laboratory.

4.2.2 Equipment and supplies should be stored when not in use.

5. LABORATORY PERSONNEL

Virologists, other microbiologists, technicians, and support personnel in the environmental virology laboratory must have training and experience appropriate for the laboratory's program. The variety and complexity of the tasks and tests performed determine the professional bench and on-the-job-training required.

5.1 Professional Level

5.1.1 Professional staff perform scientific work in connection with identification, culture, study, and control of viruses and other organisms.

Most of the work is performed in a laboratory environment and is generally concerned with research and development, monitoring, regulations, and public health.

5.1.2 Professional responsibilities require the ability to apply and adapt scientific theories and principles of microbiology at a level that allows making limited independent decisions.

5.1.3 The basic educational requirement is a BS/BA degree in virology or microbiology or a BS/BA degree in biology with a minor in virology or microbiology.

5.2 Supervisory and Senior Grade Level

5.2.1 The Supervisory and Senior Grade level staff retain the key positions of responsibility for planning and directing the systematic research on a virus problem area and for organizing, evaluating, and documenting the results pertinent to these activities.

5.2.2 Professional responsibilities include direct leadership in assigned subject matter areas, exercising full and independent responsibility for the development of criteria, methods, and virus data of general applicability for use by others, interpreting the scope and scientific quality of the data, and serving as a specialist in virology.

5.3 Technician Level

5.3.1 The technician level staff typically assist professionals by doing routine tests, performing tasks involving a series of steps, and maintaining records of experiments.

5.3.2 Technician responsibilities include performing repetitive tasks, some understanding of the work done in the laboratory and the relationships of various tasks, recognizing readily observable events or reactions, and making precise measurements.

5.4 Supervision of Personnel in Laboratory

5.4.1 The laboratory should be directed by a Professional Virologist. In a small laboratory where the staff consists of a single non-professional technician, an approved consultant virologist must be available for guidance and counselling.

5.4.2 Work assignments in the laboratory must have readily definable objectives.

5.4.3 The supervisor or consultant must review staff performance at least annually and laboratory procedures used at least quarterly.

Sample collecting and handling, media and glassware preparation, sterilization, routine testing procedures, counting, data handling, quality control techniques, and laboratory safety are areas requiring examination.

6. LABORATORY EQUIPMENT AND INSTRUMENTS

Quality control of laboratory apparatus includes servicing and monitoring the operation of incubators, water baths, hot-air sterilizing ovens, autoclaves, water stills, refrigerators, freezers, and other laboratory equipment. Each item of equipment must be tested to verify that it meets the manufacturer's specifications and the user's needs for accuracy and precision. (See Table 4-1 for check list.)

6.1 Balances

Balances must be kept clean and protected from corrosion, checked monthly with weights meeting class S standards, and serviced annually.

6.2 pH Meters

Before each use, pH meters must be standardized with two standard buffers (pH 4.0, 7.0, 10.0) bracketing pH of sample.

Buffer solutions must not be reused.

6.3 Distilled Water

6.3.1 Conductivity of distilled water must be monitored at least daily, and preferably continuously, with a conductivity meter. For continuous monitoring, an in-line meter should be used.

6.3.2 The water still must be drained and cleaned at least monthly.

6.3.3 The water reservoir must be cleaned at least quarterly.

6.4 Deionized Distilled Water (see Table 4-2)

6.4.1 Conductivity of deionized distilled water must be monitored at least daily and preferably continuously with a conductivity meter. For continuous monitoring, an in-line meter should be used.

6.4.2 Deionized distilled water must be monitored for bacteria monthly.

6.4.3 Chemical analysis of deionized distilled water may be done when necessary by chemists trained in such procedures.

6.4.4 Cartridges of deionizing resins must be replaced as indicated by manufacturer or on the basis of analytical tests.

6.5 Ultraviolet Lights

Ultraviolet lights must be checked quarterly.

When less than 80% of the rated initial output is emitted, the lights must be replaced. Also perform spread plate irradiation test quarterly. For appropriate procedures, see Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-017, 1978, pp. 198-199.

6.6 Centrifuges

6.6.1 Centrifuges must contain a safety interlock.

6.6.2 Centrifuges must be disinfected and cleaned frequently.

6.6.3 On centrifuges not equipped with a built-in tachometer, rheostat controls must be checked against a tachometer at various loadings every six months to insure proper gravitational forces. Rheostats should never be used as final indicators of centrifuge speed when centrifuges are equipped with built-in tachometers.

6.7 Downward Flow Laminar Hoods

6.7.1 Downward flow laminar hoods must be free of clutter, and all surfaces must be cleaned and swabbed with a disinfectant before and after each use.

6.7.2 Hoods must be tested at least once annually to ensure proper operation.

CAUTION: The integrity of the air curtain may be compromised by air currents produced in hoods with clutter.

6.8 Thermometers

Thermometers must be calibrated at least once annually against National Bureau of Standards (NBS) certified thermometers or equivalents.

6.9 Refrigerators

Refrigerator temperatures must be monitored and recorded daily.

6.10 Dispensing Apparatus

Dispensing apparatus must be checked for accuracy of delivery volume at each volume change and periodically throughout extended runs. Apparatus must be recalibrated when necessary.

6.11 Steam Autoclaves

Steam autoclaves must be equipped with steam filters.

Steam autoclaves must be monitored at each use with temperature recording charts and indicator tapes. Autoclave operation must also be checked weekly with maximum-minimum thermometers and spore strips or suspensions. Autoclaves should be checked weekly with a thermocouple inserted into simulated worst case material.

6.12 Gas Sterilizers

Gas sterilizers must be monitored at each use with recording charts and indicator tapes. Such sterilizers must also be checked weekly with spore strips or suspensions.

CAUTION: Ethylene oxide is toxic. Proper precautions must be taken to protect personnel against exposure to ethylene oxide.

6.13 Hot-Air Ovens

Hot-air ovens must be monitored at each use with temperature indicator tapes and thermometers or recording charts calibrated in the 160-180^{o} C range. Hot air ovens must also be checked weekly with spore strips.

6.14 Roller Drum Apparatus

Each roller drum apparatus for cell cultures must have an alarm that signals power failure.

6.15 Freezers

6.15.1 All -70^{o} C freezers must be equipped with temperature-recording charts and alarms to signal excessive temperature changes.

6.15.2 All -20^{o} C freezers must be equipped with temperature-recording charts or with thermometers.

6.15.3 Freezers should be cleaned and defrosted at least every six months.

6.16 Incubators

6.16.1 Walk-in incubators must be equipped with temperature-recording charts and alarms.

6.16.2 Reach-in incubators must contain automatic high temperature cut-offs and must be checked daily with thermometers immersed in water.

6.17 Security

All incubators, freezers, and refrigerators should be secured with locks.

7. LABORATORY SUPPLIES

7.1 Laboratory Ware

7.1.1 Laboratory ware must be thoroughly cleansed (see Chapter 2) and rinsed in deionized distilled water (see Table 4-3).

7.1.2 Unless otherwise indicated, glassware must be sterilized in a dry heat oven at 170° C for one hour; autoclavable plasticware must be sterilized in an autoclave at 121° C for one hour.

Use indicator tape to assure that sterilization temperature has at least been reached; record date of sterilization on tape.

7.1.3 Whenever feasible, cell culture vessels should be discarded after one use.

7.1.4 Laboratory ware should be tested for acid and alkaline residuals and detergents by the procedures described in Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-017, 1978, pp. 199-200. Laboratory ware that has not come clean and laboratory ware with acid, alkaline, or detergent residuals must be recleansed.

7.2 Media and Chemicals

7.2.1 All chemicals and media must be dated upon receipt. Unless otherwise indicated, only the purest grade of commercially available chemicals, usually reagent-grade, may be used. Caked media and media in opened containers for more than six months must be discarded.

7.2.2 When appropriate, media must be pretested for sterility and nutritional quality and lots ordered from approved batches.

7.3 Membrane Filters

Membrane filters must be checked by a bubble test for air leaks and must meet federal government specifications (for bubble test procedure, see Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-017, 1978, p. 205).

7.4 Sintered-Glass Filters

Sintered-glass filters must be checked periodically for retention of bacteria (for testing retention characteristics see 1983 Annual Book of ASTM Standards, Vol. 11.02, p. 856).

8. LABORATORY PROCEDURES

8.1 Cell Cultures

8.1.1 Test for Sterility

Test all cell culture media for sterility before use. To test cell culture media for sterility, incubate all media at 37° C for one week prior to use. If gross contamination is not visible after incubation, media must be tested in thioglycollate broth.

8.1.2 Preparation of Cell Lines

(a) To reduce risk of contaminating one cell line with another, prepare only one cell line in a given room at any one time, and cleanse and disinfect work area thoroughly before introducing another cell line.

(b) To reduce risk of massive microbiological contamination, prepare separately media and reagents for each cell line.

(c) Use only heat-inactivated serum (56° C for 30 minutes) in preparation of media.

(d) Personnel must wear protective clothing, changing after each use.

8.1.3 Preparation of Cell Cultures

(a) Trypsinize and dispense cells into fresh stock media in a downward flow laminar hood.

(b) Check cell density by packed cell volume, or if feasible, by direct cell count before and after distributing cells.

(c) Test all distributed media for sterility in thioglycollate broth to determine whether contamination of media occurred during preparation of cell cultures.

(d) Test cell cultures at least once a month for Mycoplasma contamination (Mycotrim Mycoplasma Detection System, Hana Media, Inc., or equivalent, may be used to test for Mycoplasma).

8.1.4 Record Keeping

A continuous record must be kept of cell line passages.

8.2 Virus Plaque Assays

8.2.1 Preparation for Assay

(a) Cell cultures must be washed by replacement of culture medium in cell culture vessels with serum-free medium four hours or less before cultures are used to assay for viruses.

(b) Cell cultures must be checked microscopically and macroscopically periodically after seeding for growth of cultured cells and for contamination.

(c) The same cell culture batch, and thereby cell cultures of the same age, must be used for any given assay.

(d) Uninoculated overlay controls must be included in each assay to detect endogenous viruses.

(e) Cell line sensitivity must be tested routinely against reference viruses.

8.2.2 Volume Assayed

A minimum of 10% of each processed sample eluate must be assayed; however, the total volume of each processed drinking water sample eluate must be assayed.

8.2.3 Time of Assay

Processed samples must be refrigerated immediately at 4^{o} C and should be assayed as quickly as possible.

If a sample cannot be assayed within 8 hours after processing, it must be frozen quickly and stored at -70^{o} C until assayed.

8.2.4 Controls

Diluents and/or elutants used in processing samples should be assayed as controls.

8.2.5 Counting Plaques

Viral plaques must be counted from first day of appearance; viral plaques must be marked as they are counted.

8.2.6 Disposition of Data

All data must be reviewed for consistency and adequacy and properly documented and stored.

TABLE 4-1

Monitoring Laboratory Equipment

Item	Monitoring Procedure
1. Balance	a. Use an analytical balance with a sensitivity of 1 mg or less at a 10 g load for weighing 2 g or less. For weighing larger quantities, use a balance with a sensitivity of 50 mg at a 150 g load.
	b. Check balance monthly with a set of certified class S weights.
	c. Wipe balance and weights clean after each use.
	d. Protect weights from laboratory humidity and corrosion.
	e. Contract, on an annual basis, with a qualified expert for balance maintenance.
2. pH Meter	a. Compensate pH meter for temperature with each use.
	b. Date standard buffer solution when first opened, and check monthly with another pH meter. Discard buffer solution if the pH is more than + 0.1 pH unit from the manufacturer's stated value or if it is contaminated with microorganisms.
	c. Standardize pH meter with two standard buffers (pH 4.0, 7.0, 10.0) bracketing pH of sample, before each use.
	d. Do not re-use buffer solutions.
	e. Contract, on an annual basis, with a qualified expert for pH meter maintenance.
3. Water Still	a. Drain and clean still at least monthly, according to instructions from the manufacturer.
	b. Drain and clean distilled water reservoir at least quarterly.
	c. Monitor distilled water daily for conductance. Conductivity should not exceed 2 mho/cm at 25° C.

TABLE 4-1
(Continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
4. Water Deionizer	a. Monitor deionized distilled water for conductance at least daily and continuously when possible. Conductivity may not exceed 0.1 µmho/cm at 25° C. Monitor for trace metals and other toxic compounds when necessary (see Table 4-2). b. Replace cartridges of deionizing resins as indicated by manufacturer or as indicated by analytical results. c. Monitor bacterial counts at exit point of deionizer unit. Replace cartridges when standard plate count exceeds 1,000 CFU/mL.
5. Ultraviolet Lamps	a. Clean ultraviolet lamps monthly by wiping them with a soft cloth moistened with ethanol. b. Test ultraviolet lamps with a light meter quarterly; if lamps emit less than 80% of their rated initial output, replace them. c. Perform spread plate irradiation test quarterly. For procedure, see Microbiological Methods for Monitoring the Environment. I. Water and Wastes, EPA-600/8-78-017, pp. 198-199.
6. Centrifuges	a. Disinfect and clean centrifuges frequently. b. Check brushes and bearings for wear every six months. c. For centrifuges not equipped with built-in tachometers, check rheostat control against a tachometer at various loadings every six months to ensure proper gravitational fields.

TABLE 4-1
(Continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
7. Microscope	a. Allow only trained technicians to use microscopes. b. Appoint one laboratory worker to be responsible for the care of the microscopes. c. Clean optics and stage of microscope after every use. Use only lens paper for cleaning. d. Keep microscopes covered when not in use. e. Establish annual maintenance on contract.
8. Downward Flow Laminar Hood	a. With an appropriate instrument, check filters in hood monthly for plugging or obvious dirt accumulation. Clean or replace filters as needed. b. Check hood for leaks and for appropriate rate of air flow every three months. c. Expose blood agar plates to air flow in hood for one hour once per month to measure contamination. d. Every two weeks, remove plug from outlet of hood, and clean ultra-violet lamps with a soft cloth moistened with ethanol. e. Test ultraviolet lamps quarterly with a light meter. If lamp emits less than 80% of its rated output, replace lamp. f. Perform maintenance as directed by the manufacturer. g. Once a week, measure efficiency of air flow at hood face with a pressure monitor control device.

TABLE 4-1
(Continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
9. Thermometers and Recording Devices	a. Check the accuracy of thermometers and temperature recording instruments, in the monitoring range, at least annually against an NBS certified thermometer or equivalent. Thermometer graduations should not exceed the deviation permitted in the analytical method. Check mercury columns for breaks. b. Record calibration checks in a quality control record. Mark NBS calibration correction on each thermometer or on the outside of the incubator, refrigerator, or freezer containing the thermometer. c. Record daily temperature checks on charts, and retain records for at least six months.
10. Refrigerator	a. Check and record refrigerator temperature daily. b. Clean refrigerator monthly. c. Identify and date all material in refrigerator. d. Defrost unit, and discard outdated materials in refrigerator and freezer compartments every six months.
11. Dispensing Apparatus	a. Check accuracy of delivery from dispensing apparatus with an NBS class A, graduated cylinder at the start of each volume change and periodically throughout extended runs. b. Lubricate moving parts of apparatus according to manufacturer's instructions and at least once per month. c. Correct immediately any leaks, loose connections, or malfunctions in apparatus. d. After dispensing agar or medium, pass a large volume of hot deionized distilled water through dispenser to remove traces of agar or medium. e. At the end of the work day, disassemble parts that have come in contact with disposed fluid, wash well, rinse with deionized distilled water, and dry.

TABLE 4-1
(Continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
12. Steam Autoclave	a. Equip autoclave with steam filter.
	b. Record temperature in autoclave continuously with recording thermometer.
	c. Verify that autoclave maintains uniform operating temperature.
	d. Test performance of autoclave at each use with indicator tape and weekly with maximum-minimum thermometer and with spore strips or suspensions. If evidence of contamination occurs, identify and eliminate cause.
	e. Test performance of autoclave weekly with a thermocouple inserted into simulated worst case material.
	f. Procure semi-annual preventive maintenance inspections.
13. Hot Air Oven	a. Equip oven with a thermometer accurate in 160-180^{o} C range.
	b. Each time a hot air oven is used, monitor performance of oven with temperature indicator tape and thermometer or temperature recording chart.
	c. Monitor sterilization weekly with spore strips.
14. Freezers	a. Check temperatures in freezers continuously with a recording thermometer.
	b. Equip each freezer with a temperature/power alarm system.
	c. Identify and date all materials in freezers.
	d. Clean and defrost freezers every six months. Discard outdated materials.

TABLE 4-1
(Continued)

Monitoring Laboratory Equipment

Item		Monitoring Procedure
15.	Incubators (Air/Water-Jacket)	a. If partially-submersible glass thermometer is used to monitor incubator temperature, bulb and stem must be immersed in water to the mark on stem. b. Monitor temperatures in incubators continuously with recording thermometers. Measure temperatures daily on top and bottom shelves of incubators. Periodically measure temperatures on all shelves in use. (For walk-in incubators, expand test points proportionately.) c. Equip each incubator with a temperature/power alarm system. d. Whenever possible, locate incubators where room temperature is in the 16-27^{o} C range.

TABLE 4-2

Standards for Deionized Distilled Water

Parameter	Ideal Monitoring Frequency	Limit
Chemical Tests		
Conductivity	With each use	0.1 mho/cm at 25° C
pH	Optional	5.5-7.5
Total Organic Carbon	Optional	1.0 mg/liter
Trace Metal, Single	Optional	0.05 mg/liter
Trace Metals, Total (Cd, Cr, Cu, Ni, Pb, Zn)	Optional	1.0 mg/liter
Ammonia/Amines	Optional	0.1 mg/liter
Free chlorine	Optional	None detectable by amperometric titration
Bacteriological Test		
Standard Plate Count for Freshly Dispensed Water	Monthly	1,000 CFU/mL

TABLE 4-3

Laboratory Ware Maintenance

1. Utensils and Vessels	Use utensils and vessels of non-corrodible and non-contaminating materials such as Pyrex glass, stainless steel, and appropriate plastics.
2. Laboratory Ware (Reusable)	a. With each use, examine laboratory ware especially screw-capped dilution vessels and flasks, for chipped or broken edges and etched surfaces. Discard chipped or badly-etched laboratory ware. b. Inspect laboratory ware after cleansing. Water should sheet without beading significantly. If water beads excessively on the cleansed surfaces, recleanse the laboratory ware. c. Test laboratory ware for acid or alkaline residues by adding bromthymol blue indicator to representative laboratory ware items (see Section 7.1.4). d. Test laboratory ware for residual detergent (see Section 7.1.4).

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CHAPTER 5

VIRUS ADSORPTION-ELUTION (VIRADEL) DISC FILTER PROCEDURES FOR RECOVERING VIRUSES FROM SEWAGES, EFFLUENTS, AND WATERS

Waters that contain chlorine and cannot be processed immediately must be dechlorinated immediately upon collection. Immediate dechlorination may be accomplished by placing into the collection vessel 0.8 mL of a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) for each liter of water to be collected. That quantity of $Na_2S_2O_3$ is sufficient for neutralizing 15 mg of chlorine per liter.

Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (see Chapters 2 and 3).

1. ADSORPTION -- METHOD ONE

This procedure may be used for volumes of 100 mL to 20 liters for all sewages and for all heavily polluted waters.

1.1 Preparation

1.1.1 Apparatus and Materials

Unless thumb-screw-drive-clamps are to be used to connect tubing to equipment, install quick-disconnect connectors on the ports of all apparatus.

(a) Disc filter holders -- 47, 90, 142, or 293 mm diameters (Millipore Corp., or equivalent).

Use only pressure type filter holders. The diameter of the holder used depends upon the volume and turbidity of the water tested. Experience with the clogging

potential of the volumes of sewage, effluents, or other waters under study dictates the diameter of the filter holders used. See Sections 1.2 and 2 for further guidance.

(b) Virus-adsorbing disc filters -- 0.45-μm pore size (Millipore Corp. HA series, or equivalent).
Select diameter of filter appropriate for the disc filter holder that is used.

(c) Fiberglass prefilters (Millipore Corp., AP15 and AP20, or equivalents).

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

(e) Positive air or nitrogen pressure source equipped with pressure gauge.
Pressure source, if laboratory air line or pump, must be equipped with oil filter. If source is capable of producing high pressure, deliver to pressure vessel and filter holder no more pressure than recommended by the filter manufacturer.

(f) Carboy, autoclavable plastic, or flask of a size sufficient to collect total volume of sample.

(g) pH meter, measuring to an accuracy of at least 0.1 pH unit, equipped with combination-type electrode (Van London Co., or equivalent, for electrode only).

(h) Autoclavable inner-braided tubing with metal quick-disconnect connectors or with thumb-screw-drive-clamps for connecting tubing to equipment to be used under pressure.

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

(i) Magnetic stirrer and stir bars.

(j) Filling bell attached to inner-braided tubing.

1.1.2 Media and Reagents

(a) Hydrochloric acid (HCl) -- 1 M.

Prepare 1 liter of 1 M hydrochloric acid solution. This solution may be stored at room temperature for several months.

(b) Sodium hydroxide (NaOH) -- 1 M.

Prepare 100 mL of 1 M NaOH. This solution may be stored at room temperature for several months.

(c) Magnesium chloride ($MgCl_2 \cdot 6H_2O$) -- 1 M.

Prepare 50 mL of 1 M $MgCl_2$ for each liter of sample.

1.2 Procedure (see Figure 5-1 for flow diagram of procedure)

Usually virus-adsorbing filters with diameters of 47 or 90 mm, coupled with prefilters of appropriate size, are adequate for raw sewage and primary effluents where volumes of 200 mL or less need to be filtered. Filters of larger diameter are required for the larger volumes of secondary and tertiary effluents that must be processed.

1.2.1 Assembly of Apparatus (see Figures 5-2 and 5-3)

Use inner-braided tubing to make all connections between apparatus to be used under pressure.

(a) Remove top of filter holder C.

(b) With two sets of forceps, place 0.45-μm virus-adsorbing filter onto support screen of holder.

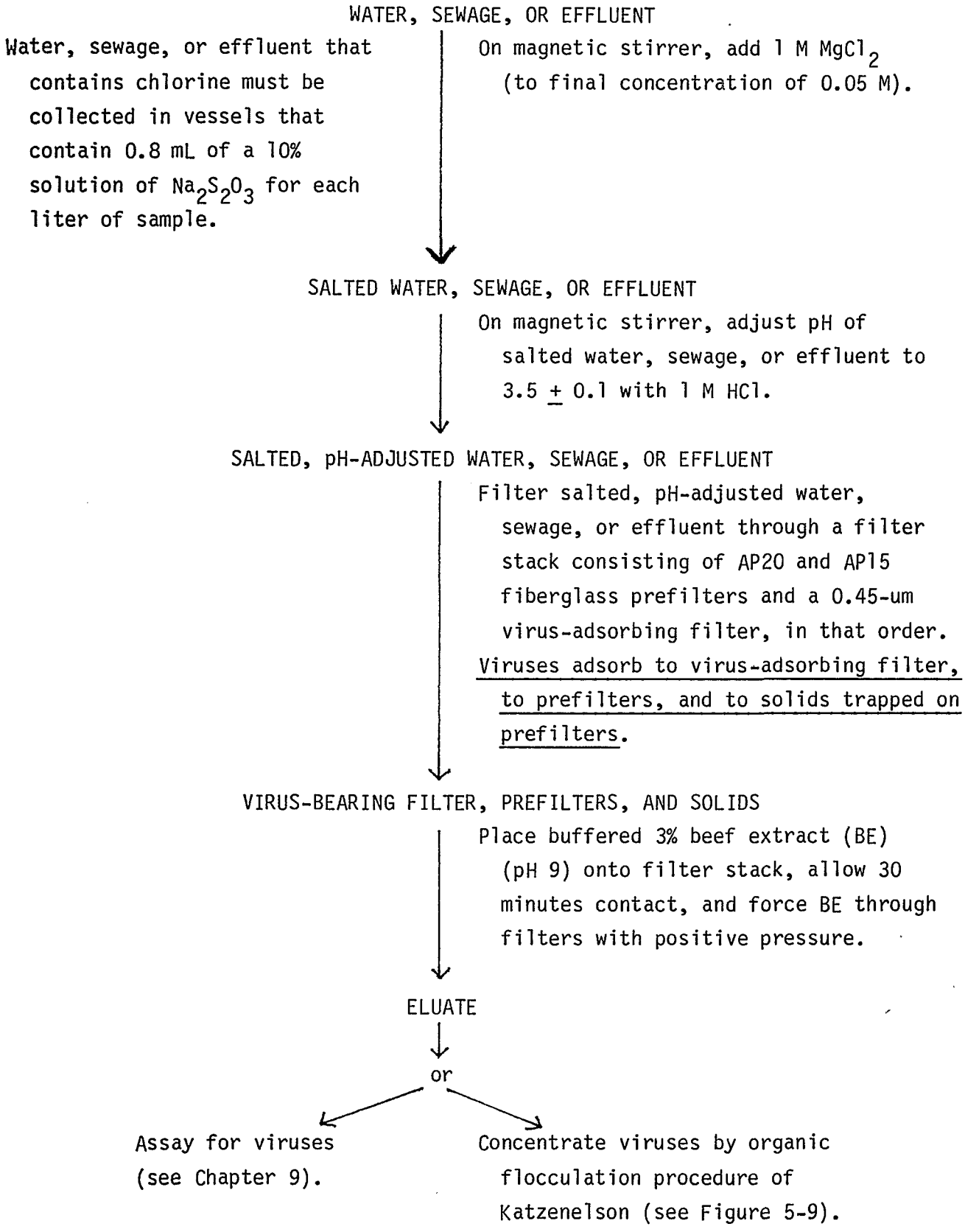

Figure 5-1. Flow Diagram of Method for Recovering Viruses from Small Volumes (100 mL to 20 Liters) of Water, Sewage, or Effluent.

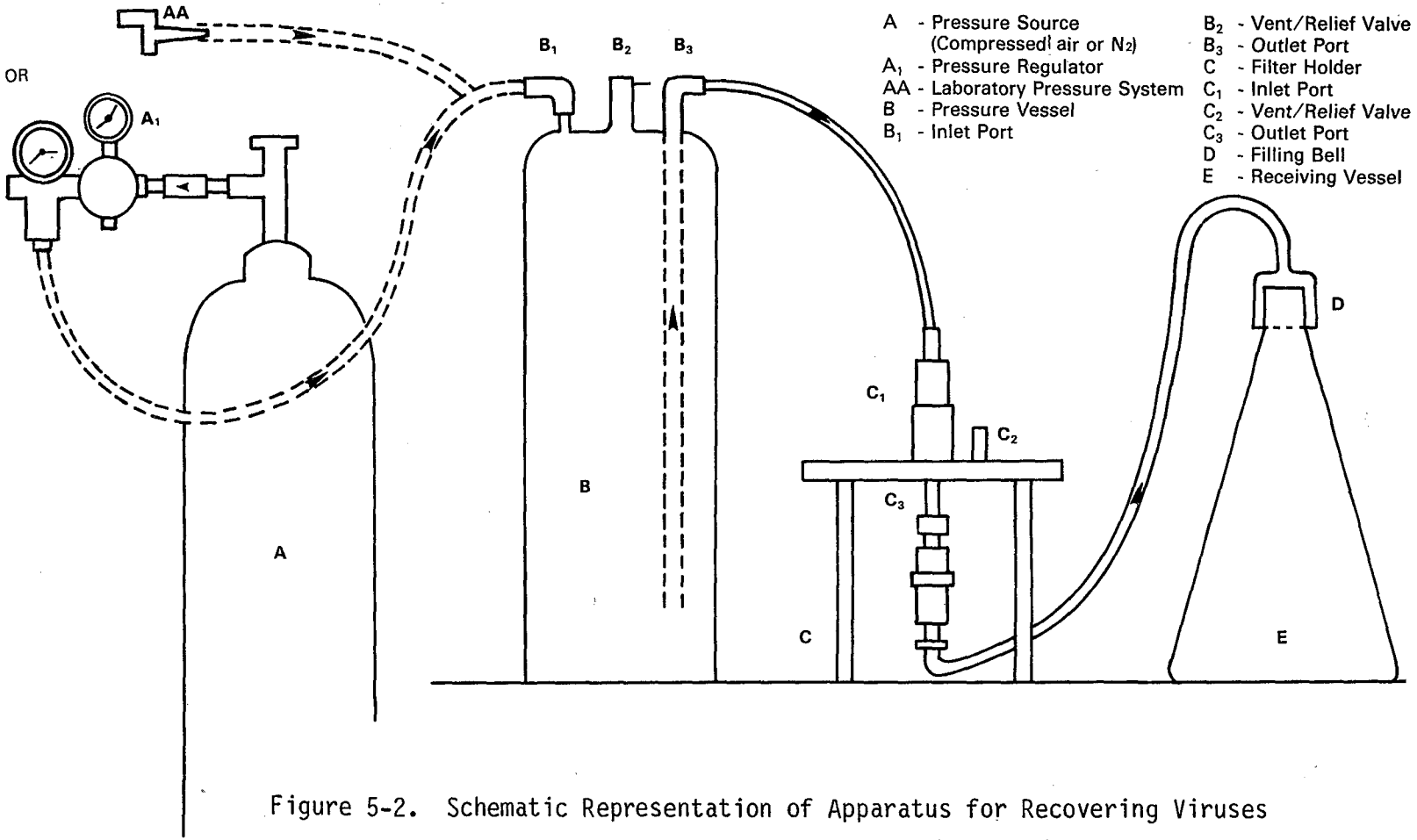

Figure 5-2. Schematic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Disc Filter Procedure for Small Volume Filtrations (see Figure 5-3 for Photographic Representation of Apparatus).

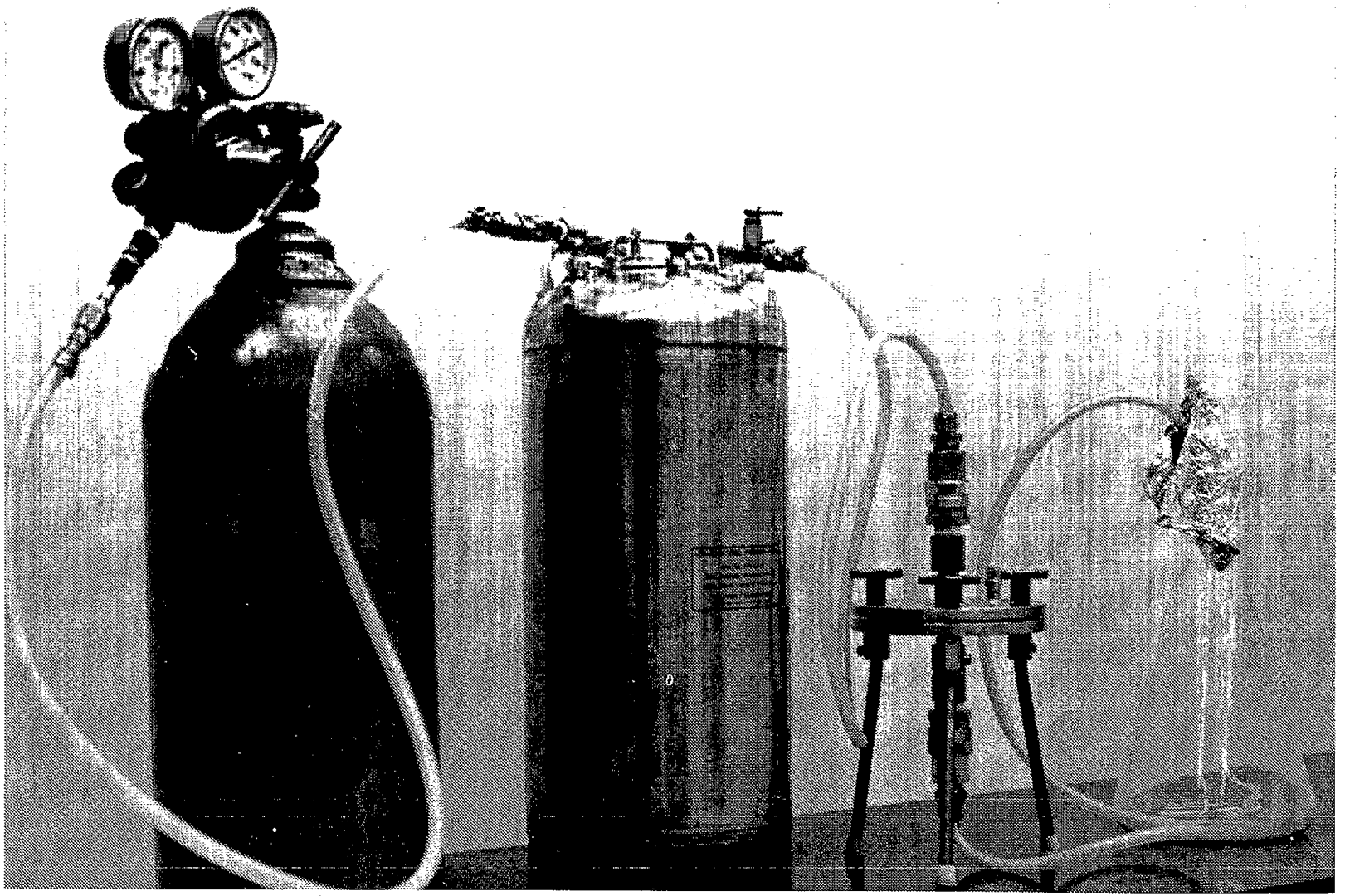

Figure 5-3. Photographic Representation of Apparatus for Recovering Viruses by the Virus-Adsorption-Elution (VIRADEL) Disc Filter Procedure for Small Volume Filtrations (see Figure 5-2 for Annotated Schematic Representation of Apparatus).

(c) With two pairs of forceps, place AP 15 prefilter on top of 0.45-µm filter.

(d) With two pairs of forceps, place AP 20 prefilter on top of AP 15 prefilter.

(e) Replace and tighten down top of filter holder C.

(f) Connect positive pressure source A or AA to inlet port B_1 of 20-liter pressure vessel B.

(g) Connect outlet port B_3 of pressure vessel B to inlet port C_1 of filter holder C.

(h) Place filling bell D, with inner-braided tubing attached, over opening of flask or carboy (E) of a size sufficient to collect the total volume of sample.

(i) Connect free end of tube on the filling bell to outlet port C_3 of filter holder C.

1.2.2 Salt Supplementation of Sample.

(a) Place stir bar into container holding sample.

(b) Place sample container on magnetic stirrer, and stir at speed sufficient to develop vortex.

(c) Add sufficient quantity of 1 M $MgCl_2$ to bring the concentration of $MgCl_2$ in the sample to 0.05 M.

1.2.3 Adjustment of pH of Sample.

Optimal conditions of pH vary for concentrating different viruses, especially viruses from different taxonomic groups. Conditions that favor recovery of enteroviruses are described below.

(a) Place pH electrode into salted water sample.

(b) Add sufficient 1 M HCl to bring pH of salted sample to 3.5 ± 0.1.

Rapid mixing of acid into sample is important because slow mixing may result in pH levels sufficiently low in parts of the sample to inactivate viruses.

(c) Turn off magnetic stirrer.

(d) Remove pH electrode from sample.

1.2.4 Filtration of Salted, pH-adjusted Sample (from Section 1.2.3, Step [b]).

(a) Remove top from pressure vessel B.

(b) Pour salted pH-adjusted sample into pressure vessel B. To prevent transfer of stir bar into pressure vessel, hold another stir bar or magnet underneath flask when decanting sample.

(c) Replace top on pressure vessel B and tighten down.

(d) Wrap vent/relief valve C_2 on top of filter holder C with disinfectant-soaked gauze, and open valve about one-half turn.

(e) Apply pressure sufficient to purge trapped air from filter holder C.

(f) Close vent/relief valve C_2 as soon as sample begins to flow from valve.

(g) Wipe up spilled sample with laboratory disinfectant.

(h) Increase pressure sufficiently to force sample through the filter (usually 0.4-1.5 kg/cm^2).

(i) When all of sample has passed through filters, turn off pressure source A or AA.

(j) Wrap vent/relief valve B_2 with disinfectant-soaked gauze, and open valve to relieve pressure in pressure vessel B.

(k) When pressure is relieved, close vent/relief valve B_2.

(l) Discard filtrate.

(m) Elute viruses from filters immediately as described in Section 3.1.

2. ADSORPTION -- METHOD TWO

This method is recommended for volumes larger than 20 liters but not larger than 400 liters (e.g., tertiary effluents, surface waters, ground waters, and tap waters). The usefulness of this method is limited by the clarity of the water that is filtered. Prefilters must be replaced as they clog. More than ten changes of prefilters are generally impractical. Usually, 20 liters or less of river or ocean water clog a prefilter with a diameter of 293 mm. For chlorinated waters that contain sufficient solids to require elution, do not use this method. Instead, use the Viradel Cartridge Filter Procedure Method Two in Chapter 6.

2.1 Preparation

2.1.1 Apparatus and Materials

Unless thumb-screw-drive-clamps are to be used to connect tubing to equipment, install quick-disconnect connectors on the ports of all apparatus except on the additive pumps. Provide physical support as necessary for equipment that is not free-standing.

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

(b) Virus-adsorbing disc filters for 142 mm filter holder -- 0.45-μm pore size (Millipore Corp., HA series, or equivalent).

(c) Fiberglass prefilters for 293 mm filter holder (Millipore AP15 and AP20, or equivalents).

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

(e) Positive pressure source equipped with pressure gauge. Pressure source, if laboratory air line or pump, must be equipped with oil filter. If source is capable of producing high pressure, deliver to pressure vessel and filter holder no more pressure than recommended by the filter manufacturer.

(f) Plastic-coated drum(s) -- 200-liter capacity, or other container of size suitable to hold sample if sample is not pumped directly from source.

(g) Sterilizable self-priming water pump that delivers approximately 25-50 liters per minute.
Pump is not needed if sampled water is under pressure, e.g., tap water.

(h) Carboy, autoclavable plastic with nipple on bottom fitted with tube clamped to a dispensing Y (clamp tube closed between nipple and Y) -- 20-liter capacity.
If the water at the sampling site is to be drawn directly from a pressurized source and is to be dechlorinated, then two similarly fitted carboys are needed. Otherwise only one carboy is needed.

(i) Fluid proportioner consisting of fluid-driven motor with four additive pumps (Johanson and Son Machine Corp., Model M 14 Q with one P-562 and one P-750 additive pump affixed to each side of the fluid-driven motor, or equivalent).

Assemble fluid proportioner, and connect tubing in accordance with manufacturer's instructions.

(j) Mixing chamber (Johanson and Son Machine Corp., C-SS, or equivalent).

(k) pH meter, measuring to an accuracy of at least 0.1 pH unit, equipped with a combination-type electrode (Van London Co., or equivalent, for electrode only).

(l) Tee, stainless steel, with three female NPT* ports. Equip center port with pH electrode in-line adapter (Van London Co., or equivalent, for adapter only).

(m) Autoclavable inner-braided tubing with metal quick-disconnect connectors or with thumb-screw-drive-clamps for connecting tubing to equipment to be used under pressure.

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

(n) Filling bell attached to inner-braided tubing.

(o) Magnetic stirrer and stir bars.

(p) Sterile aluminum foil.

(q) Water meter (Badger Meter Inc., or equivalent).

2.1.2 Media and Reagents

(a) Hydrochloric acid (HCl) -- 0.12 M and 12 M (concentrated) solutions.

Prepare 100 mL of 0.12 M HCl.

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* National Pipe Thread

(b) Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) -- 40% stock solution (with respect to $Na_2S_2O_3 \cdot 5H_2O$).

Prepare 50 mL of 40% (w/v) stock solution for each 100 liters of water to be processed. Prepare one liter of $Na_2S_2O_3$ solution by dissolving 400 g of $Na_2S_2O_3 \cdot 5H_2O$ in 500 mL of deionized distilled water and bringing final volume of solution to one liter with deionized distilled water. If lesser quantities of $Na_2S_2O_3$ are needed, lesser quantities may be prepared. Sodium thiosulfate is used for dechlorinating waters that cannot be dechlorinated except immediately prior to test procedure (e.g., tap water tested directly at source). For dechlorinating all other waters, see page 5-1.

(c) Magnesium chloride ($MgCl_2 \cdot 6H_2O$) -- 5 M stock solution.

Prepare 1 liter of solution for each 100 liters of water to be processed.

(d) Tween 80 -- 0.1% (v/v) prepared in deionized distilled water.

Prepare 6 liters of 0.1 % Tween 80.

2.2 Procedure (see Figure 5-4 for flow diagram of procedure)

Usually, prefilters with diameters of 293 mm and virus-adsorbing filters with diameters of 142 mm are appropriate for volumes greater than 20 liters.

2.2.1 Preparation and Implementation

It is usually convenient to sterilize each piece of apparatus and equipment one or more days before it is used (see Chapter 3). It is convenient to sterilize apparatus in small units

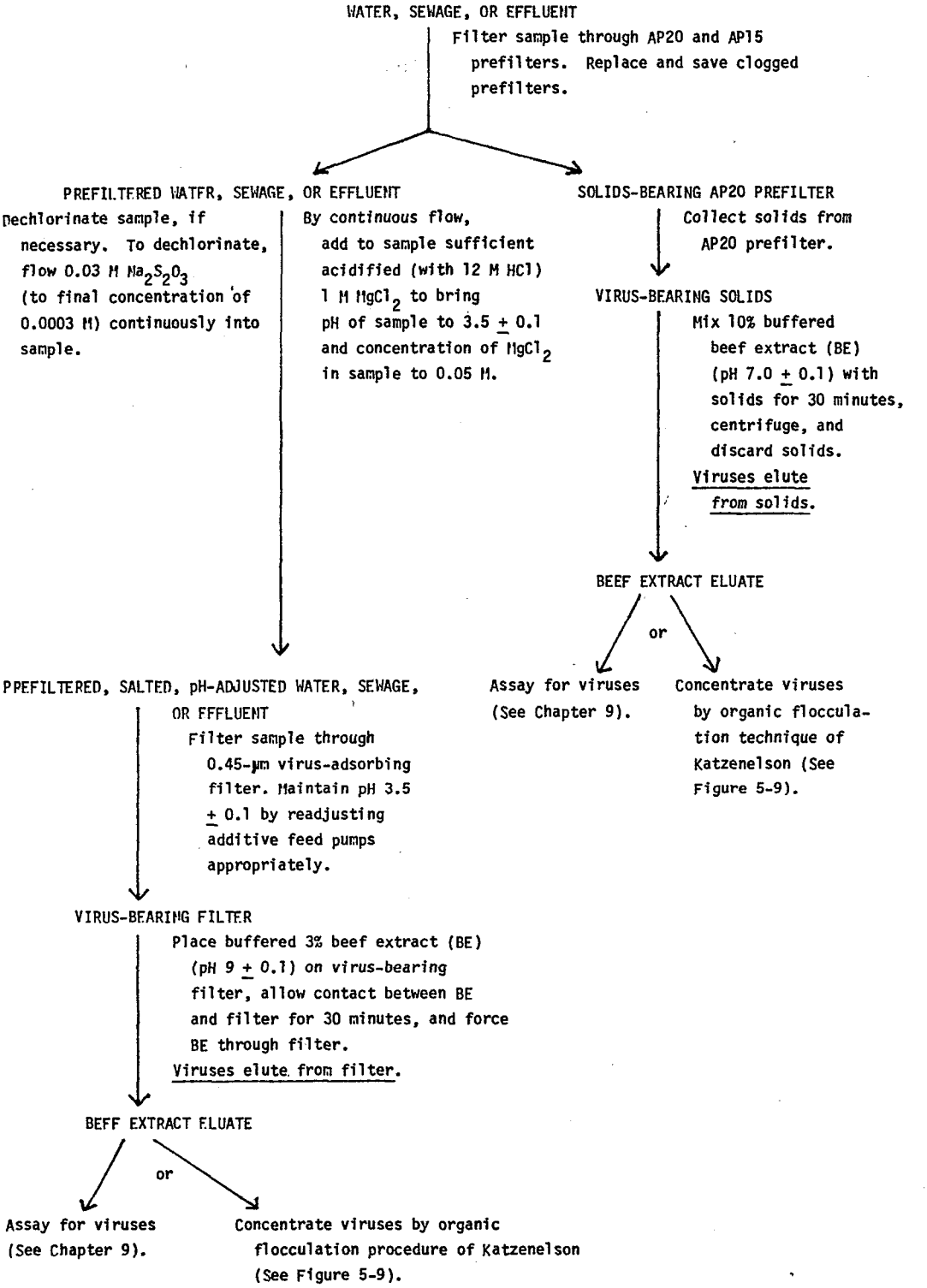

Figure 5-4. Flow Diagram of Method for Recovering Viruses from Large Volumes (More than 20 Liters) of Water, Sewage, or Effluents.

when sterilization is accomplished by steam or ethylene oxide. However, it is advisable to assemble and connect units of apparatus that are to be sterilized by chlorination. The interconnected apparatus can be disassembled after the chlorination procedure is completed, the ports covered with aluminum foil, and the units stored until used.

(a) Assembly of apparatus (see Figures 5-5 and 5-6)

Use inner-braided tubing to make all connections for apparatus to be used under pressure. To simplify procedures and maintain sterility, the apparatus is totally assembled at this time although sections of the apparatus will need to be disassembled and reassembled later.

(a.1) If sample is under pressure (e.g., tap water), connect water source H to inlet port I_1 of filter holder I (293 mm). If sample is not under pressure, connect sample source to inlet port HH_1 of self-priming water pump HH, and connect outlet port HH_2 of pump HH to inlet port I_1 of filter holder I.

(a.2) Connect outlet port I_3 of filter holder I to inlet port J_2 of fluid proportioner J.

(a.3) Connect outlet port J_4 of fluid proportioner J to inlet port M_1 of mixing chamber M.

Mixing chamber must be supported to prevent it from falling.

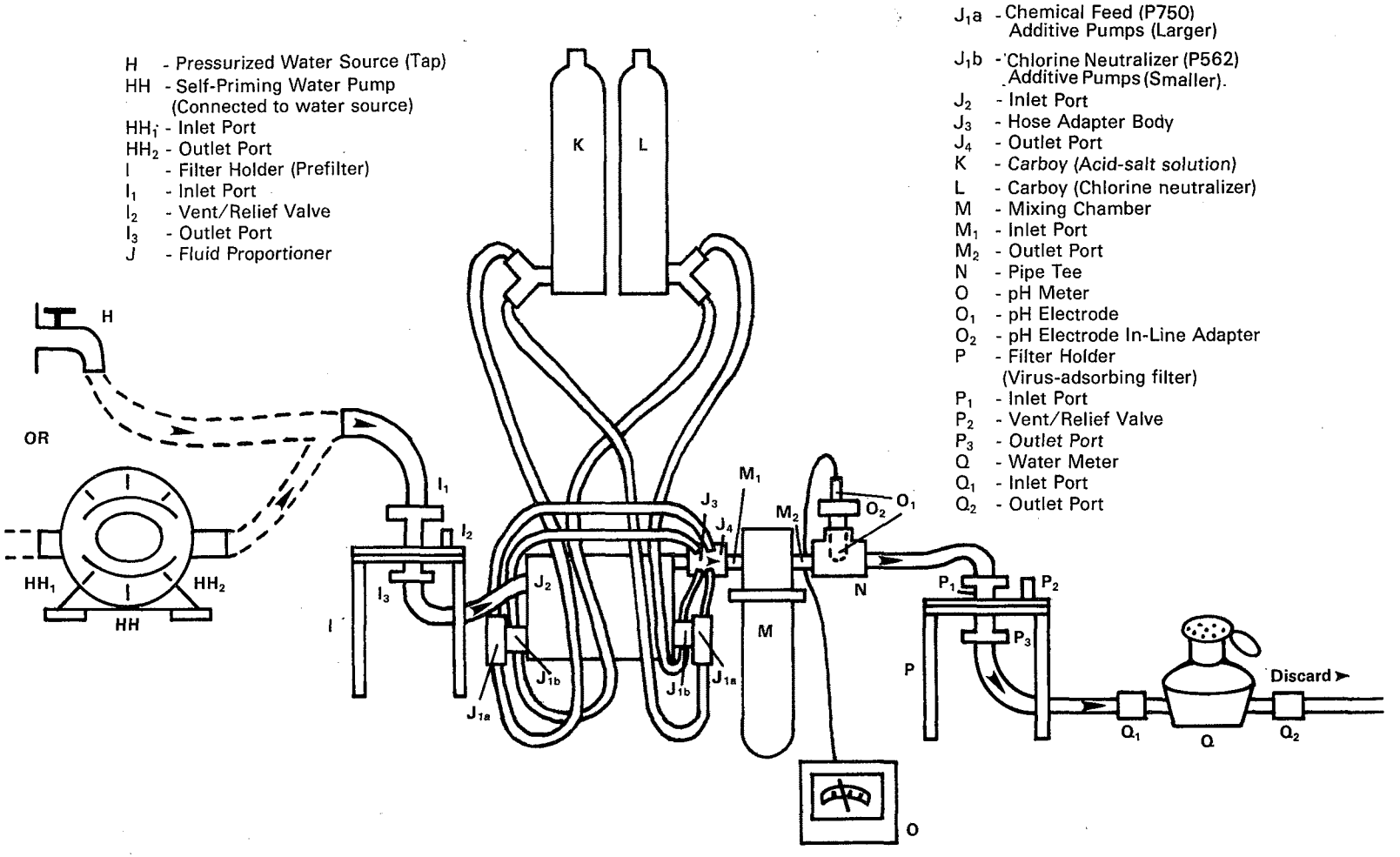

Figure 5-5. Schematic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Disc Filter Procedure for Large Volume Filtrations (See Figure 5-6 for Photographic Representation of Apparatus).

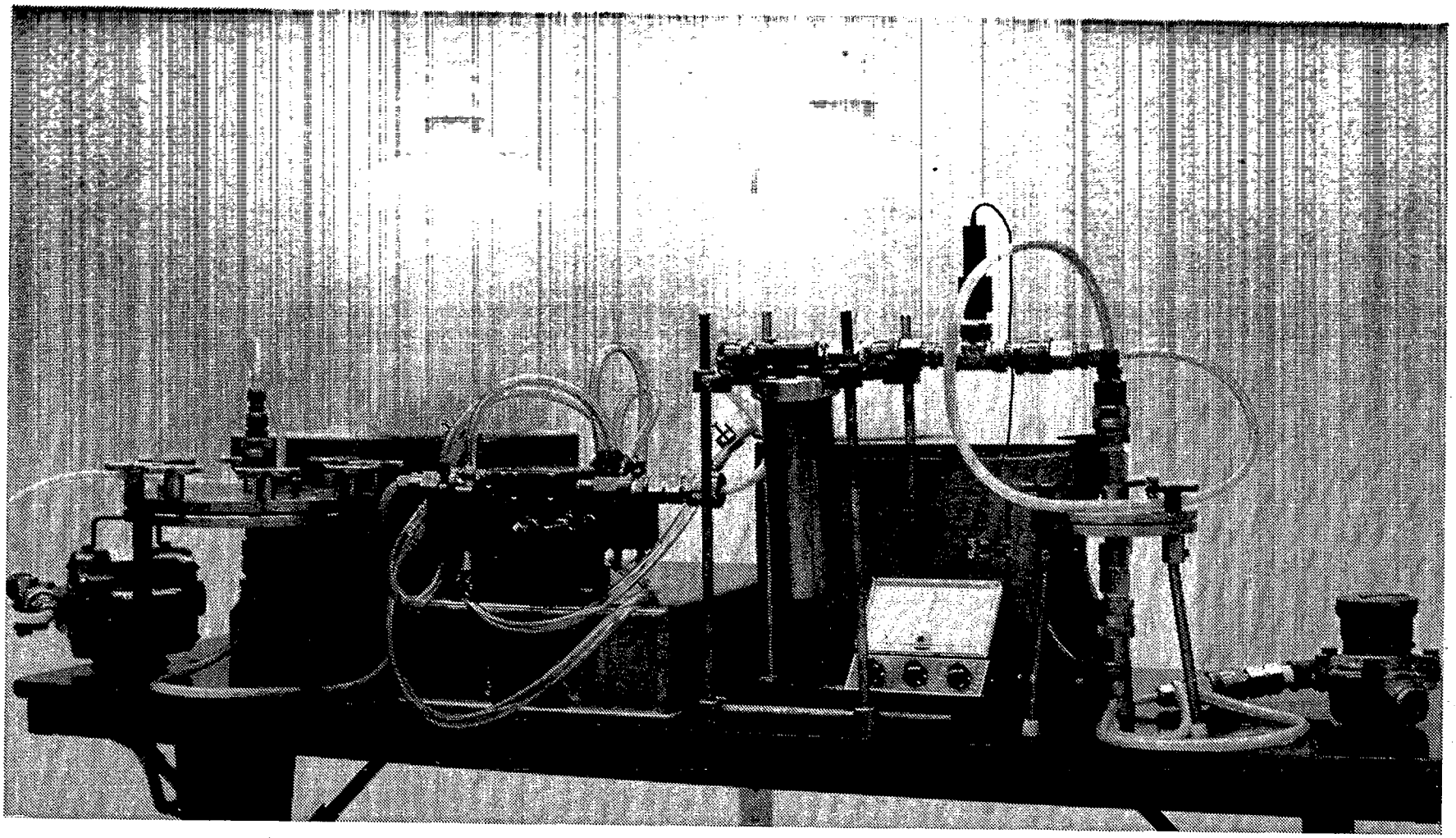

Figure 5-6. Photographic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Disc Filter Procedure for Large Volume Filtrations (See Figure 5-5 for Annotated Schematic Representation of Apparatus).

(a.4) Connect outlet port M_2 of mixing chamber M to one arm of pipe tee N.

Support pipe tee N to protect electrode, if necessary.

(a.5) Lock pH electrode O_1 into pH electrode in-line adapter O_2 in center post of pipe tee N.

Same pH electrode (after sterilization) that is used to adjust pH in Step (d.4) may be used.

(a.6) Connect other arm of pipe tee N to inlet port P_1 of filter holder P.

(a.7) Connect outlet port P_3 of filter holder P to inlet port Q_1 of water meter Q.

(a.8) Connect outlet port Q_2 of water meter Q to discard.

(b) Treatment of prefilters with Tween 80 to prevent adsorption of viruses (see Figures 5-7 and 5-8; also see Figures 5-5 and 5-6).

Treat AP15 and AP20 prefilters separately. AP15 and AP20 prefilters cannot be readily distinguished one from the other.

(b.1) Remove top of filter holder I.

(b.2) With two sets of forceps, place AP15 prefilters onto support screen of filter holder I.

Up to 10 prefilters may be stacked in filter holder I for treatment. The number of prefilters stacked is the number that experience suggests will be needed to filter the waters to be tested. In the absence of experience, treat

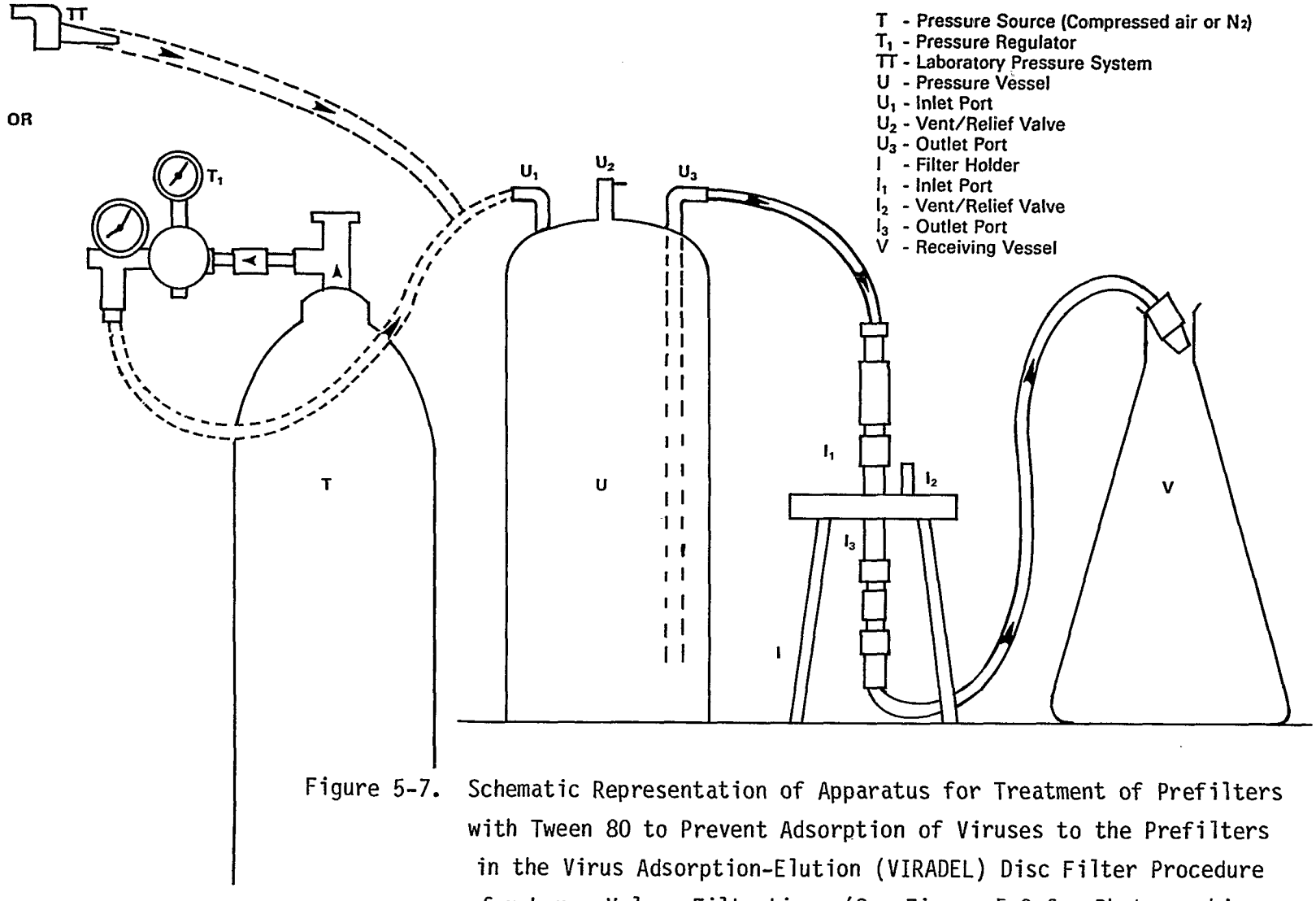

Figure 5-7. Schematic Representation of Apparatus for Treatment of Prefilters with Tween 80 to Prevent Adsorption of Viruses to the Prefilters in the Virus Adsorption-Elution (VIRADEL) Disc Filter Procedure for Large Volume Filtrations (See Figure 5-8 for Photographic Representation of Apparatus).

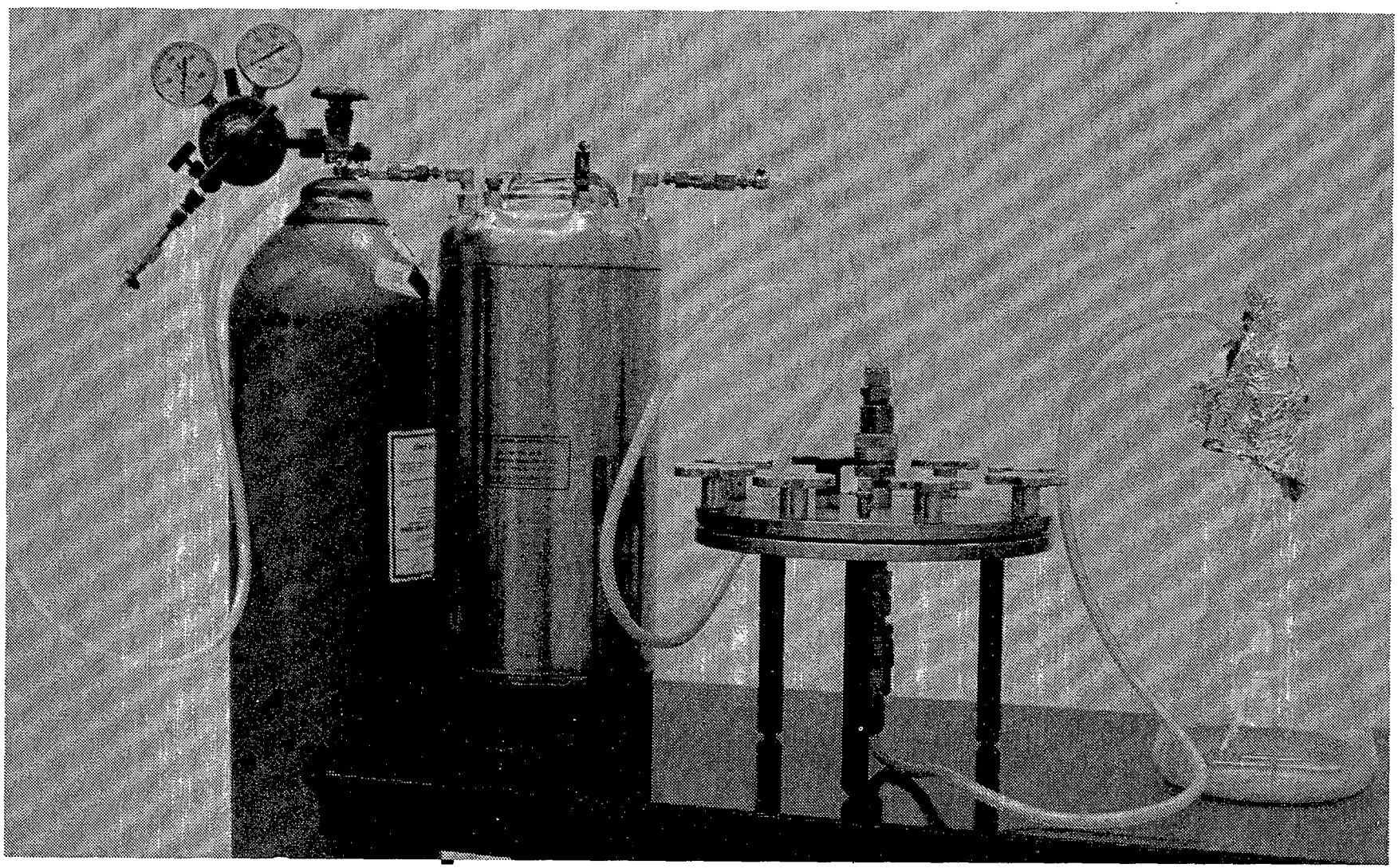

Figure 5-8. Photographic Representation of Apparatus for Treatment of Prefilters with Tween 80 to Prevent Adsorption of Viruses to the Prefilters in the Virus Adsorption-Elution (VIRADEL) Disc Filter Procedure for Large Volume Filtrations (See Figure 5-7 for Annotated Schematic Representation

five prefilters of each type for relatively clear waters and 10 prefilters of each type for more turbid waters. Unused Tween-treated prefilters may be stored aseptically at 4^{o} C for up to two weeks.

(b.3) Replace and tighten down top of filter holder I.

(b.4) Open vent/relief valve I_2.

(b.5) Disconnect tube from inlet port I_1 of filter holder I.

Protect sterility of exposed tube.

(b.6) With a new length of tubing, connect inlet port I_1 of filter holder I to outlet port U_3 of 20-liter pressure vessel U.

(b.7) Connect pressure source T or TT to inlet port U_1 of pressure vessel U.

(b.8) Remove top of pressure vessel U.

(b.9) Pour 2 liters of 0.1% Tween 80 into pressure vessel U.

(b.10) Replace top on pressure vessel U and tighten down.

Check vent/relief valve U_2 on pressure vessel U to be certain it is closed.

(b.11) Disconnect tube at inlet port J_2 of fluid proportioner J, and place end of tube into 6-liter flask V.

(b.12) Cover inlet port J_2 of fluid proportioner J with sterile aluminum foil.

(b.13) Apply pressure (T or TT) (about 0.4 kg/cm^2) sufficient to force Tween 80 through prefilters.

(b.14) Close vent/relief valve I_2 on filter holder I as soon as Tween 80 flows through vent, and allow all of the Tween 80 to flow through the prefilters.

(b.15) Turn off pressure source (T or TT)

(b.16) Relieve pressure in pressure vessel U by opening vent/relief valve U_2.

(b.17) Remove tube from flask V, discard Tween 80, and return tube to flask V.

(b.18) Remove top of pressure vessel U.

(b.19) Pour 4 liters of deionized distilled water into pressure vessel U.

(b.20) Replace and tighten down top of pressure vessel U.

(b.21) Close vent/relief valve U_2.

(b.22) Open vent/relief valve I_2 on filter holder I.

(b.23) Apply pressure (about 0.4 kg/cm^2) sufficient to force water through prefilters (prefilter rinse).

(b.24) Close vent/relief valve I_2 on filter holder I as soon as water flows through vent, and allow all of the deionized distilled water to flow through the prefilters.

(b.25) Turn off pressure source (T or TT).

(b.26) Relieve pressure in pressure vessel U by opening vent/relief valve U_2.

(b.27) Discard rinse water, and replace tube from outlet port I_3 of filter holder I into same flask.

(b.28) Remove top of filter holder I.

(b.29) With two sets of forceps, remove the AP15 prefilters from filter holder I, and place the prefilters on aluminum foil.

(b.30) Cover the stack of prefilters with another piece of foil.

(b.31) Repeat steps (b.2) through (b.10) and (b.13) through (b.30) with AP20 prefilters.

(b.32) Remove aluminum foil from inlet port J_2 of fluid proportioner J.

(b.33) Remove tube from 6-liter flask, and connect to inlet port J_2 of fluid proportioner J.

(b.34) With two sets of forceps, remove top AP15 prefilter from stack.

(b.35) Place the AP15 prefilter onto support screen of filter holder I.

(b.36) With two sets of forceps, remove top AP20 prefilter from stack, and lay the AP20 prefilter on top of AP15 prefilter.

(b.37) Replace and tighten down top of filter holder I.

(b.38) Disconnect tube from outlet port U_3 of pressure vessel U, and cover tube end with aluminum foil.

(b.39) Disconnect tube from inlet port I_1 on filter holder I.

(b.40) Reconnect tube from pressure source H or HH_2 to inlet port I_1 on filter holder I.

(c) Preparation of salt supplement

Preparation of sufficient salt supplement for 400 liters of processed water is described below. If less water is to be processed, proportionately less salt supplement needs to be prepared.

(c.1) Remove cover from 20-liter carboy K.

(c.2) Pour 8 liters of deionized distilled water into carboy K.

(c.3) Add 4 liters of 5 M $MgCl_2$ solution to the deionized distilled water in carboy K.

(c.4) Replace cover loosely on carboy K.

(d) Preparation of acid for adjustment of pH

(d.1) Pour 380 mL of test water into a 600-mL beaker.

(d.2) Place stir bar into test water.

(d.3) Place beaker on magnetic stirrer, and stir at speed sufficient to develop vortex in test water.

(d.4) Place pH electrode into test water.
pH meter must be standardized before it is used.

(d.5) Add sufficient 0.12 M HCl to test water to obtain pH 3.5 $\pm$ 0.1.

(d.6) Record volume of 0.12 M HCl used.

(d.7) Add to salt solution from Step (c.3) above a volume of 12 M HCl equal to 11 times the quantity of 0.12 M HCl needed to produce the required pH in the 380-mL volume of test water.

(d.8) Bring acid-salt solution to 20 liters with deionized distilled water, and mix solution well.

(e) Preparation of $Na_2S_2O_3$ solution for dechlorination

Step (e) applies only to chlorinated waters processed directly from a source (e.g., tap water). All chlorinated test waters obtained from sources outside of the processing facility must be dechlorinated immediately when the samples are obtained (see page 5-1). Preparation of sufficient $Na_2S_2O_3$ for dechlorinating 400 liters of processed water is described below. If less water is to be processed, proportionately less $Na_2S_2O_3$ needs to be prepared.

(e.1) Remove cover from 20-liter carboy L.

(e.2) Pour 10 liters of deionized distilled water into carboy L.

(e.3) Add 186 mL of 40% $Na_2S_2O_3$ solution to the deionized distilled water in carboy L to give a final molarity of 0.03, and mix solution well.

(e.4) Replace cover loosely on carboy L.

(f) Fluid proportioner

(f.1) Connect a long length of tubing to each end of dispensing Y on 20-liter carboy K that contains the acid-salt solution prepared in Step (d.7) above.

Tubing is already in place if additive pumps are sterilized with chlorine (see Section 2.2.1). In this instance, disconnect tubing from bottom of additive pump J_{1a}, and continue with Step (f.2).

(f.2) Remove cover from top of carboy K.

(f.3) Place free end of each tube into mouth of carboy K.

(f.4) Release pinch clamp, and allow acid-salt solution to flow into tubes.

(f.5) Remove tubes from mouth of carboy K, and insert tubes into the inlet (bottom) ports of larger additive pumps J_{1a}.

Allow acid-salt solution to flow freely into tubing, but manipulate tubes to prevent overflow.

(f.6) Replace cover loosely on carboy K.

(f.7) Adjust the calibration on the metering rod for each pump J_{1a} to a setting of 3.2.

This calibration equals delivery rate of 1 part of acid-salt solution to each 19 parts of test water. If dechlorination is not necessary, leave the ports of the two remaining additive pumps J_{1b} covered (see Section 2.2.1), and go to Step (f.15).

If pressurized source is used, water should first be run for a length of time sufficient to cleanse spigot.

(f.8) Connect a long length of tubing to each end of dispensing Y on 20-liter carboy L that contains the 0.03 M $Na_2S_2O_3$ solution prepared in Step (e) above.

Tubing may already be in place if pumps are sterilized with chlorine (see Section 2.2.1). In this instance, disconnect tubing from bottom of additive pumps, and continue with Step (f.9).

(f.9) Remove cover from top of carboy L.

(f.10) Place free end of each tube into mouth of carboy L.

(f.11) Release pinch clamp, and allow $Na_2S_2O_3$ solution to flow into tubes.

(f.12) Remove the tubes from mouth of carboy L, and insert tubes into the inlet (bottom) ports of smaller additive pumps J_{1b}.
Allow $Na_2S_2O_3$ solution to flow freely into tubes, but manipulate tubes to prevent overflow.

(f.13) Replace cover loosely on carboy L.

(f.14) Adjust the calibration on the metering rod for each additive pump J_{1b} to a 1.3 setting.
This calibration equals delivery rate of 1 part of 0.03 M $Na_2S_2O_3$ solution to each 99 parts of test water.

(f.15) Disconnect tube from inlet port M_1 of mixing chamber M, and connect tube to discard.

(f.16) To remove air from tubes, prime pumps by hand-operating metering rods in a reciprocating motion.

(f.17) Reconnect tube from outlet port J_4 of fluid proportioner J to inlet port M_1 of mixing chamber M.

2.2.2 Filtration of Sample

(a) Make initial reading on water meter Q, and record reading.

(b) Remove top of filter holder P.

(c) With two sets of forceps, place 0.45 μm virus-adsorbing filter onto support screen of holder.

(d) Replace and tighten down top of filter holder P.

(e) Open vent/relief valves I_2 and P_2 on filter holders I and P.

(f) Open pressurized water source H or start water pump HH and purge trapped air from filter holders I and P.

(g) Close vent/relief valves I_2 and P_2 on filter holders I and P as soon as sample begins to flow from valves.

(h) Wipe up spilled sample with laboratory disinfectant.

(i) Read pH meter O to ascertain that proper pH is achieved. Check meter periodically to be certain that proper pH is maintained. If pH readjustment is necessary, appropriately alter settings on metering rods for additive pumps P-750.

(j) When appropriate volume has been filtered, or if flow rate becomes significantly reduced, turn off pressure either at pressurized source H or at water pump HH.

(k) Open vent/relief valves I_2 and P_2 on filter holders I and P.

(l) Disconnect tube from pressurized source H or water pump HH, and connect free end of tube to positive air or nitrogen pressure source.

(m) Close vent/relief valve I_2 on filter holder I.

(n) Apply pressure sufficient to force remaining sample water from filter holder I.

(o) Turn off pressure at positive air or nitrogen pressure source.

(p) Open vent/relief valve I_2 on filter holder I.

(q) Disconnect hose from positive pressure source, and reconnect to pressurized source H or water pump HH.

(r) Remove top of filter holder I.

(s) Replace clogged prefilters with new prefilters as described in Steps (b.34) through (b.37).

If appropriate volume of sample has been filtered, do not insert new filter into filter holder.

(t) Place each set of clogged prefilters on aluminum foil, and cover.

See Section 3.2 for processing solids on clogged prefilters.

If appropriate volume of sample has been filtered, proceed to Step (cc).

(u) Close vent/relief valve I_2 on filter holder I.

(v) Continue filtration procedure.

Bleed air from both filter holders I and P at vent/relief valves I_2 and P_2 each time apparatus is opened to replace prefilters. As many changes of prefilters should be made as are necessary to process entire sample.

Steps (q) through (t) may be completed for each set of prefilters as filtration procedure continues.

(w) Uncover one set of prefilters.

(x) With spatula, scrape solids from top prefilter (AP20).

(y) Place solids in a tared beaker, and cover mouth of beaker with aluminum foil.

(z) Maintain beaker at 4^{o} C.

See Section 3.2 for processing solids.

(aa) After required volume of water has been filtered, turn off pressure either at pressurized source H or at water pump HH.

(bb) Open vent/relief valves I_2 and P_2 on filter holders I and P.

(cc) Disconnect at pipe tee N the tube leading to inlet port P_1 of filter holder P, and connect free end of tube to positive pressure source.

(dd) Close vent/relief valve P_2 on filter holder P.

(ee) Apply pressure sufficient to force remaining sample water from filter holder P.

(ff) Make final reading on water meter.

Subtract initial reading from final reading to determine total volume filtered. Subtract volume of acid-salt solution and, if used, volume of $Na_2S_2O_3$ solution from total volume filtered to determine volume of water sampled.

(gg) Turn off pressure at positive pressure source.

(hh) Open vent/relief valve p_2 on filter holder P.

(ii) Disconnect tube from outlet port P_3 of filter holder P, and replace with tube connected to filling bell.

(jj) Elute viruses from virus-adsorbing filter as described in Section 3.

3. ELUTION AND RECONCENTRATION

3.1 Procedure for Eluting Viruses from Filters (see Figures 5-2 and 5-3, and Figures 5-5 and 5-6)

3.1.1 Apparatus and Materials

(a) Positive pressure source equipped with pressure gauge. Gauge necessary only if pressure source is capable of producing pressures exceeding tolerances of equipment. Pressure source, if laboratory air line or pump, must be equipped with an oil filter. If source is capable of producing high pressure, deliver to pressure vessel and filter holder no more pressure than recommended by manufacturer.

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

(c) Autoclavable inner-braided tubing fitted with metal quick-disconnect connectors or with thumb-screw-drive-clamps for connecting tubing to equipment.

(d) Magnetic stirrer and stir bars.

3.1.2 Media and Reagents

(a) Sodium hydroxide (NaOH) -- 1 M.
Prepare 500 mL of 1 M NaOH. This solution may be stored for several months at room temperature.

(b) Glycine.

(c) Beef extract powder (Grand Island Biological Co., or equivalent).
Prepare buffered 3% beef extract by dissolving 60 gm of beef extract powder and 7.5 g of glycine (final glycine concentration = 0.05 M) in 2 liters of deionized

distilled water. Autoclave beef extract solution, and adjust pH to 9 with 1 M NaOH.

3.1.3 Procedure

(a) Place filling bell attached to outlet port of filter holder C (Method 1, Figures 5-2 and 5-3) or P (Method 2, Figures 5-5 and 5-6) on receiving flask. To prevent toppling, it may be necessary to support flask.

(b) Disconnect tube from inlet port of filter holder C (Method 1, Figures 5-2 and 5-3) or P (Method 2, Figures 5-5 and 5-6).

(c) Open vent/relief valve on filter holder.

(d) Pour into inlet port of filter holder 0.45 mL of beef extract (pH 9) for each square cm of effective filter area.

Determine total effective filter area from manufacturer's specifications. Volume of beef extract thus needed for 142 mm filter is 44 mL.

(e) Close vent/relief valve on top of filter holder.

(f) Connect tube to inlet port of filter holder.

(g) Allow beef extract to remain in contact with filter(s) for 30 minutes.

(h) Apply pressure sufficient to force beef extract through filter(s).

Lower receiving flask and tilt filter holder to permit complete evacuation of buffered 3% beef extract from filter(s).

(i) Turn off pressure at source.

(j) Open vent/relief valve on filter holder.

(k) Unless beef extract eluate is reconcentrated or assayed for viruses immediately, refrigerate eluate immediately at 4^{o} C, and maintain at that temperature until eluate is reconcentrated or is assayed for viruses. If reconcentration or assay for viruses cannot be undertaken within eight hours, store eluate immediately at -70^{o} C. The number of cell cultures necessary for the viral assay may be reduced by reconcentrating the viruses in the beef extract by the organic flocculation procedure of Katzenelson (see Section 3.3).

3.2 Procedure for Processing Solids

Often more viruses are recovered from the solids in waters than from the waters from which the solids are obtained.

3.2.1 Apparatus and Materials

(a) Magnetic stirrer and stir bars.

(b) Membrane filter apparatus for sterilization -- 47 mm diameter filter holder with 30-mL slip tip syringe (Millipore Corp., Swinnex filter No. SX0004700, or equivalent for filter holder only).

(c) Membrane filters, 47 mm diameter -- 5-, 1.2-, 0.65-, and 0.45-µm pore sizes (Millipore Corp., HA series, or equivalent).

Place filter with 0.45-µm pore size on support screen of Swinnex filter holder, and stack the remaining filters on top in order of increasing pore size.

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

(d) Refrigerated centrifuge capable of attaining 2,500 x g.

3.2.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$).

(b) Citric acid.

(c) Beef extract powder (Gibco, or equivalent).

Prepare buffered (pH 7.0) 10% beef extract by dissolving 10 g beef extract powder, 1.34 g $Na_2HPO_4 \cdot 7H_2O$, and 0.12 g citric acid in 100 mL of deionized distilled water.

3.2.3 Procedure

(a) Weigh beaker that contains solids scraped from prefilters (from Section 2.2.2, Step [z]).

Calculate weight of solids by subtracting tare weight of beaker from weight of beaker with solids.

(b) Place stir bar into beaker.

(c) Measure into beaker 3 mL of 10% buffered beef extract for every gram of solids.

(d) Place beaker on magnetic stirrer, and stir for 30 minutes.

Viruses elute from solids.

(e) Pour suspension of solids and buffered beef extract eluate into 250-mL centrifuge bottle.

Glass centrifuge bottles may not be able to withstand g force that will be applied. To prevent transfer of stir bar into centrifuge bottle, hold another stir bar or magnet underneath beaker when decanting solids.

(f) Centrifuge suspension for 30 minutes at approximately 2,500 x g.

(g) Decant buffered beef extract eluate into beaker of appropriate size, and discard solids.

The number of cell cultures necessary for the viral assay may be reduced by reconcentrating the viruses in the beef extract by the organic flocculation procedure of Katzenelson. If viruses in eluate are to be reconcentrated, proceed to Step (h). If reconcentration is not required, proceed to Step (i).

(h) Add 7 mL of deionized distilled water to each 3 mL of eluate if reconcentration is required, and proceed according to Section 3.3.

(i) Load eluate into 30-mL syringe.

(j) Place tip of syringe into filter holder, and place filter holder on a 125-mL receiving flask.

(k) Force eluate through filters into 125-mL receiving flask.

Take care not to put pressure on receiving flask. If filter clogs, invert filter, draw remaining fluid from top of clogged filter into syringe, and replace filter holder and filters.

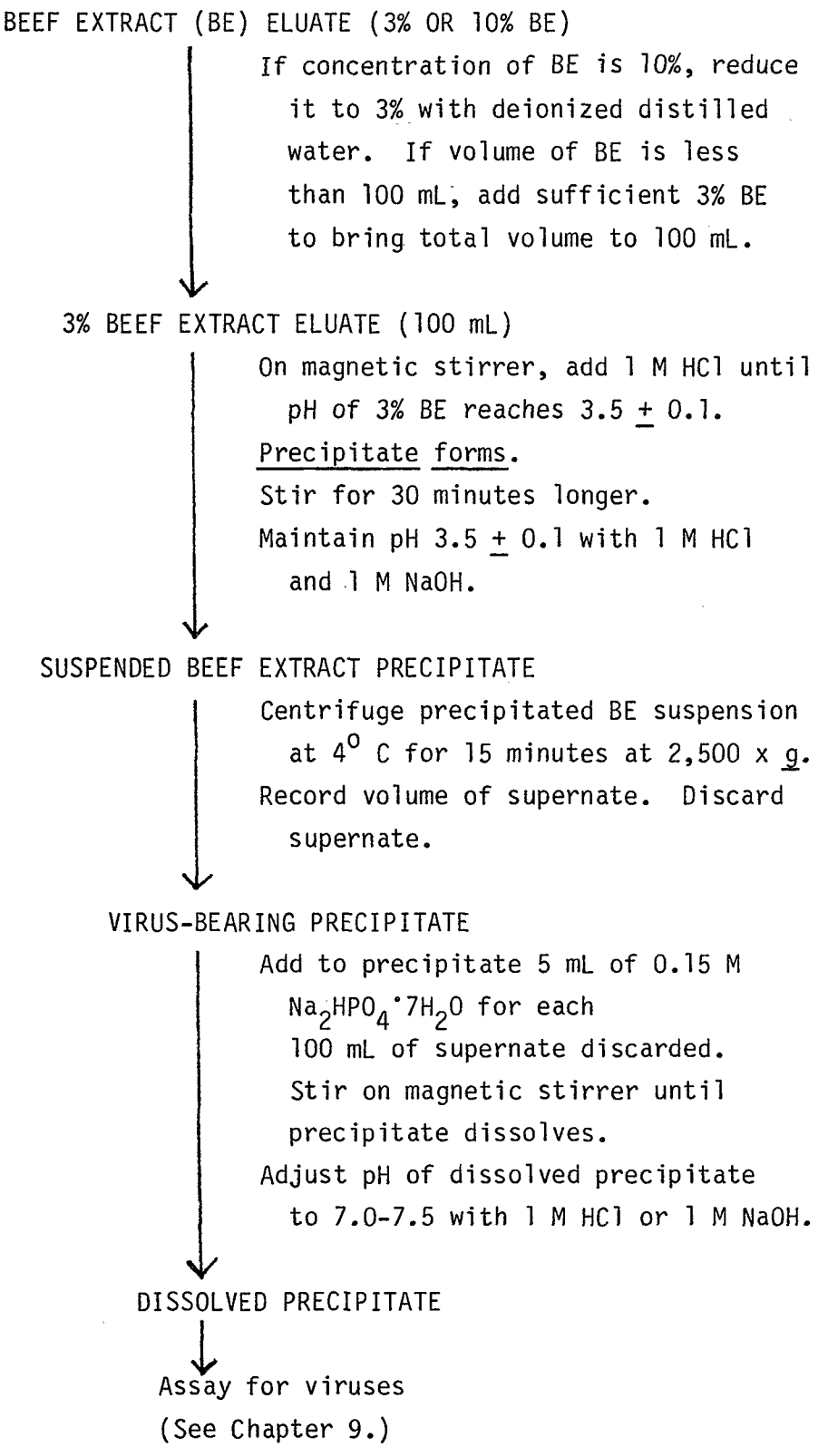

Figure 5-9. Flow Diagram of Reconcentration Procedure (Organic Flocculation Procedure of Katzenelson).

Steps (i) thru (k) may be repeated as often as necessary to filter entire volume of eluate.

(l) Refrigerate eluate immediately at 4^{o} C and maintain at that temperature until eluate is assayed for viruses.

If assay for viruses cannot be undertaken within eight hours, store eluate immediately at -70^{o} C.

3.3 Organic Flocculation Concentration Procedure of Katzenelson (see Figure 5-9 for flow diagram of procedure).

It is preferable to assay eluted viruses in the beef extract eluate without further concentrating them, because some loss of viruses may occur in concentration. However, the numbers of cell cultures needed for assays may be reduced by further concentrating the viruses.

3.3.1 Apparatus and Materials

(a) Magnetic stirrer and stir bars.

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

(c) Refrigerated centrifuge capable of attaining 2,500 x g.

3.3.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) -- 0.15 M.

(b) Hydrochloric acid (HCl) -- 1 M.

(c) Sodium hydroxide (NaOH) -- 1 M.

3.3.3 Procedure

(a) If concentration of beef extract in eluate is 10%, reduce it to 3% with deionized distilled water; if volume of beef extract eluate is less than 100 mL, add

sufficient 3% beef extract to bring total volume to 100 mL.

(b) Place stir bar in flask that contains beef extract eluate.

(c) Place flask that contains beef extract eluate on magnetic stirrer, and stir at a speed sufficient to develop vortex.

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

(d) Insert pH electrode into beef extract eluate.

(e) Add 1 M HCl to flask slowly until pH of beef extract reaches 3.5 $\pm$ 0.1.

A precipitate will form. If pH is accidentally reduced below 3.4, add 1 M NaOH until pH is 3.5 $\pm$ 0.1. Avoid, if possible, reducing pH below 3.4 because some inactivation of viruses may occur.

(f) Continue to stir for 30 minutes more, and maintain pH at 3.5 $\pm$ 0.1.

(g) Remove pH electrode from beef extract.

(h) Remove cap from 250-mL screw-capped centrifuge bottle.

Glass centrifuge bottles may not be able to withstand g force that will be applied.

(i) Pour contents of flask into 250-mL screw-capped centrifuge bottle.

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

(j) Replace and tighten down cap on screw-capped centrifuge bottle.

(k) Centrifuge precipitated beef extract suspension in refrigerated centrifuge (4^{o} C) for 15 minutes at 2,500 x g.

(l) Remove cap from screw-capped centrifuge bottle.

(m) Pour supernate into graduated cylinder, and record volume.

(n) Discard supernate.

(o) Place a stir bar into centrifuge bottle containing the precipitate.

(p) Add to the precipitate 5 mL of 0.15 M Na_2HPO_4 for each 100 mL of supernate decanted.

(q) Replace and tighten down cap on screw-capped centrifuge bottle.

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

Support bottles as necessary to prevent toppling. Avoid foaming which may inactivate or aerosolize viruses. Precipitate may be partially dissipated with spatula before or during stirring procedure.

(s) Remove cap from screw-capped centrifuge bottle.

(t) Measure pH of concentrate (dissolved precipitate). If pH is above or below 7.0-7.5, adjust to that range with either 1 M HCl or 1 M NaOH.

(u) Replace and tighten down cap on screw-capped centrifuge bottle.

(v) Refrigerate concentrate immediately at 4^{o} C, and maintain at that temperature until assay for viruses is undertaken.

If assay for viruses cannot be undertaken within eight hours, store concentrate immediately at -70^{o} C.

(w) Assay for viruses in accordance with instructions given in Chapter 9.

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CHAPTER 6

VIRUS ADSORPTION-ELUTION (VIRADEL) CARTRIDGE FILTER PROCEDURES FOR RECOVERING VIRUSES FROM SEWAGES, EFFLUENTS, AND WATERS

In principle, the Virus Adsorption-Elution (VIRADEL) Cartridge Filter Procedures described in this chapter are the same as Method 2 described in Chapter 5. The VIRADEL cartridge filter procedures require much greater volumes of elutant than Method 2 in Chapter 5 requires, but the cartridge filter procedures may be used for sample volumes greater than 200 liters and perhaps for volumes greater than 2000 liters.

Waters that contain chlorine and cannot be processed immediately must be dechlorinated immediately upon collection. Immediate dechlorination may be accomplished by placing into the collection vessel 0.8 mL of a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) for each liter of water to be collected. That quantity of $Na_2S_2O_3$ is sufficient for neutralizing 15 mg of chlorine per liter.

Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (see Chapters 2 and 3).

Provide physical support as necessary for equipment that is not free-standing.

1. ADSORPTION -- METHOD ONE

   This procedure may be used for all waters that do not require prefiltration.

   1.1 Preparation

1.1.1 Apparatus and Materials

Install quick-disconnect connectors on ports of all apparatus except on additive pumps.

(a) Holder for 10-inch* cartridge filter (Fulflo, Model No. F15-10, Commercial Filter Division, Carborundum Co., or equivalent).

(b) Cartridge filter, pleated epoxy-fiberglass -- 10-inch, 0.45-μm pore size (DUO-FN 10-E-0.45 A ECIS, Filterite Corp., or equivalent).

(c) Plastic-coated drums -- 200-liter capacity, or other containers of size suitable to hold sample, if sample is not pumped directly from source.

(d) Sterilizable self-priming water pump that delivers approximately 25-50 liters per minute.
Pump is not needed if sampled water is under pressure, e.g., tap water.

(e) Carboy, autoclavable plastic with nipple on bottom fitted with tubing clamped to a dispensing Y (clamp tubing closed between nipple and Y) -- 20-liter capacity.
If the water at the sampling site is to be drawn directly from a pressurized source and is to be dechlorinated, then two similarly fitted carboys are needed. Otherwise, only one carboy is needed.
Twice the number of carboys is needed under these conditions if water volumes greater than 400 liters are to be processed.

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

(f) Fluid proportioner consisting of fluid-driven motor with four additive pumps (Johanson and Son Machine Corp., Model M14Q with one P-562 and one P-750 additive pump affixed to each side of the fluid-driven motor, or equivalents).
Assemble fluid proportioner, and connect tubing in accordance with manufacturer's instructions.

(g) Mixing chamber (Johanson and Son Machine Corp., C-SS, or equivalent).

(h) pH meter, measuring to an accuracy of at least 0.1 pH unit, equipped with a combination-type electrode (Van London Co., or equivalent, for electrode only).

(i) Tee, stainless steel, with three female NPT* ports. Center port equipped with pH electrode in-line adapter (Van London Co., or equivalent, for adapter only).

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

(k) Magnetic stirrer and stir bars.

(l) Water meter (Badger Meter Inc., or equivalent).

(m) Positive pressure source equipped with pressure gauge.

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*National Pipe Thread

Pressure source, if laboratory air line or pump, must be equipped with oil filter. If source is capable of producing high pressure, deliver to filter holder no more pressure than recommended by manufacturer.

1.1.2 Media and Reagents

(a) Hydrochloric acid (HCl) -- 0.12 and 12 M (concentrated) solutions.

Prepare 100 to 500 mL of 0.12 M HCl. This solution may be stored for several months at room temperature.

(b) Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) -- 40% (w/v) stock solution (with respect to $Na_2S_2O_3 \cdot 5H_2O$).

Prepare one liter of $Na_2S_2O_3$ solution by dissolving 400 g of $Na_2S_2O_3 \cdot 5H_2O$ in 500 mL of deionized distilled water and bringing final volume of solution to one liter with deionized distilled water. This solution may be stored in dark, rubber-stoppered bottle for up to one month at room temperature. This solution is to be used to dechlorinate water that cannot be dechlorinated except immediately prior to test procedure (e.g., tap water tested directly at source). For dechlorinating all other waters, see Page 6-1.

(c) Aluminum chloride ($AlCl_3 \cdot 6H_2O$) -- 3 M stock solution.

Prepare 100 mL of 3 M $AlCl_3$ for each 400 liters of water to be processed.

1.2 Procedure (see Figure 6-1 for flow diagram of procedure)

In this procedure an apparatus is described that can be used with clean waters, such as tap waters, where only a 0.45 μm pleated epoxy-fiberglass cartridge filter is needed. For waters that are sufficiently turbid so that the volume filtered will clog this filter, prefilters are required and Method 1 cannot be used. For turbid waters, use Method 2 described in Section 2. Experience usually dictates the method of choice. (CAUTION: Turbid water may clog the fluid proportioner and, if abrasive, may damage it).

1.2.1 Preparation and Implementation

It is usually convenient to sterilize each piece of apparatus and equipment one or more days before it is used (see Chapter 3). It is convenient to sterilize apparatus in small units when sterilization is accomplished by steam or ethylene oxide. However, it is advisable to assemble and connect units of apparatus that are to be sterilized by chlorination. The interconnected apparatus can be disassembled after the chlorination procedure is completed, the ports covered with aluminum foil, and the units stored until used.

(a) Assembly of apparatus (see Figures 6-2 and 6-3).

Use inner-braided tubing fitted with quick-disconnect connectors to make all connections for apparatus to be used under pressure.

(a.1) If sample is under pressure, connect water source A (e.g., tap water), to inlet port B_2 of fluid proportioner B. If sample is not under pressure, connect water source to inlet port AA_1 of self-priming water pump AA.

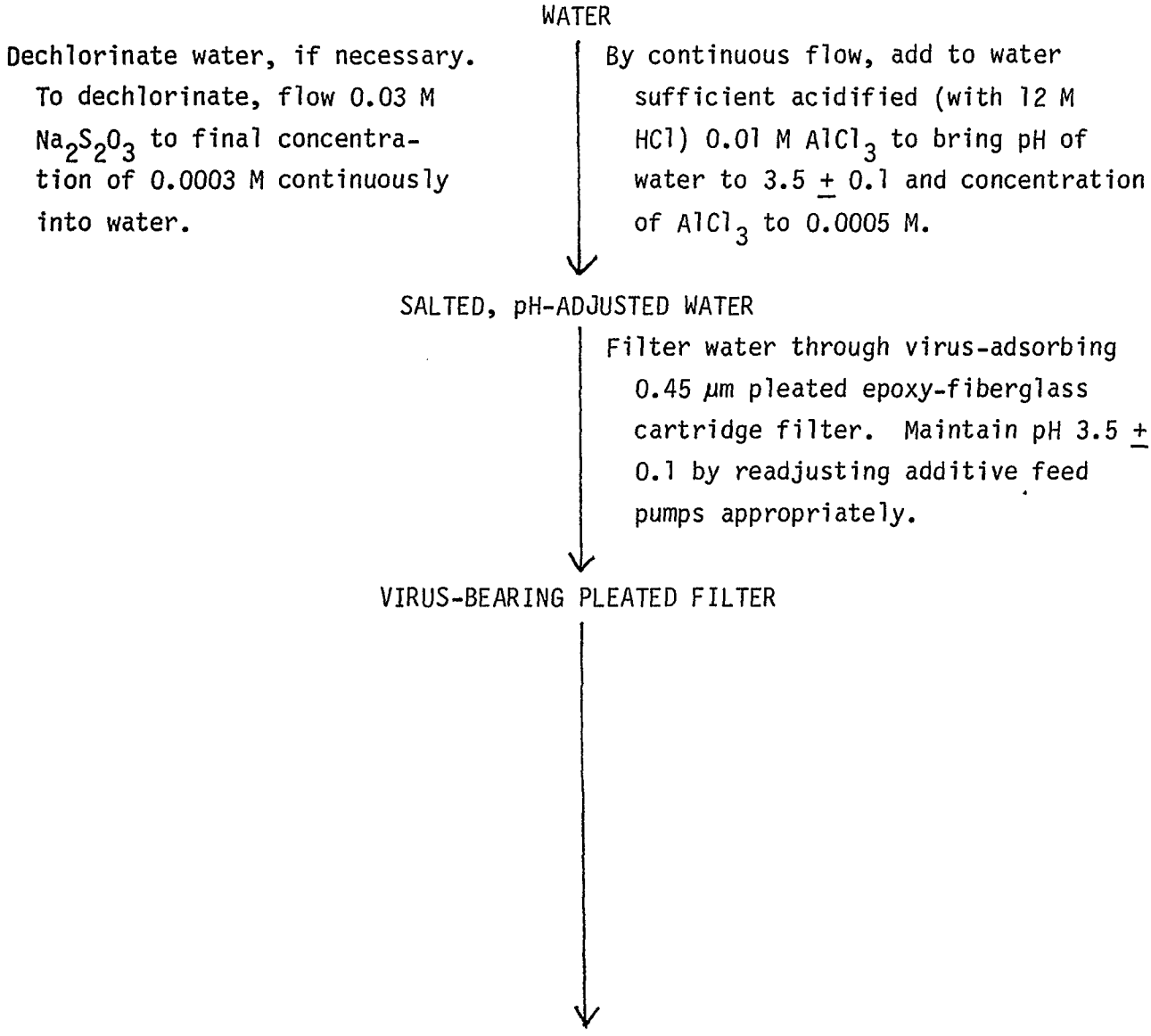

Figure 6-1. Flow Diagram of Method One for Concentrating Viruses from Large Volumes (More than 200 Liters) of Clean Waters.

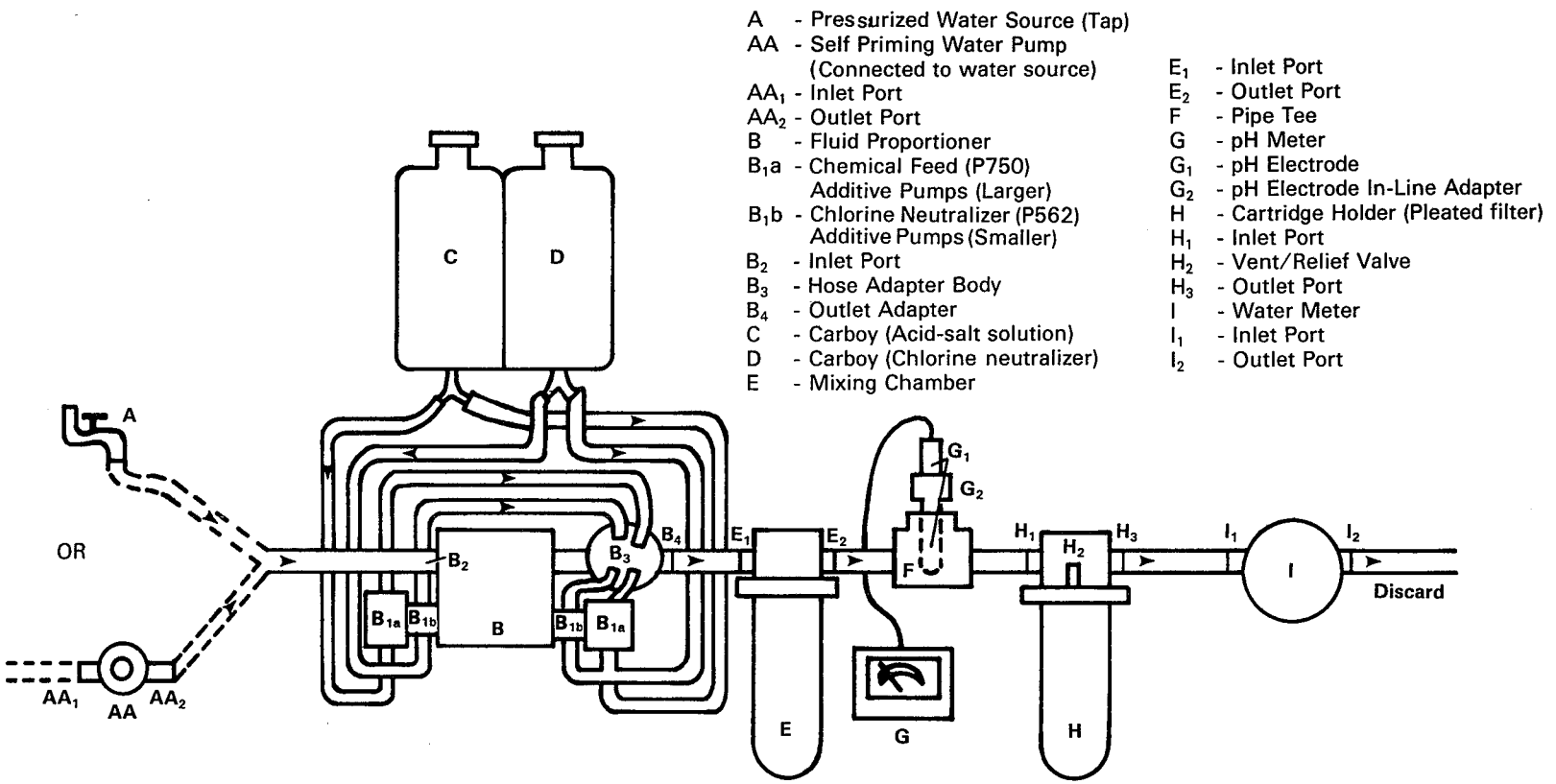

Figure 6-2. Schematic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Cartridge Filter Procedure for Large Volume Filtrations of Clean (Non-turbid) Waters (See Figure 6-3 for Photographic Representation of Apparatus).

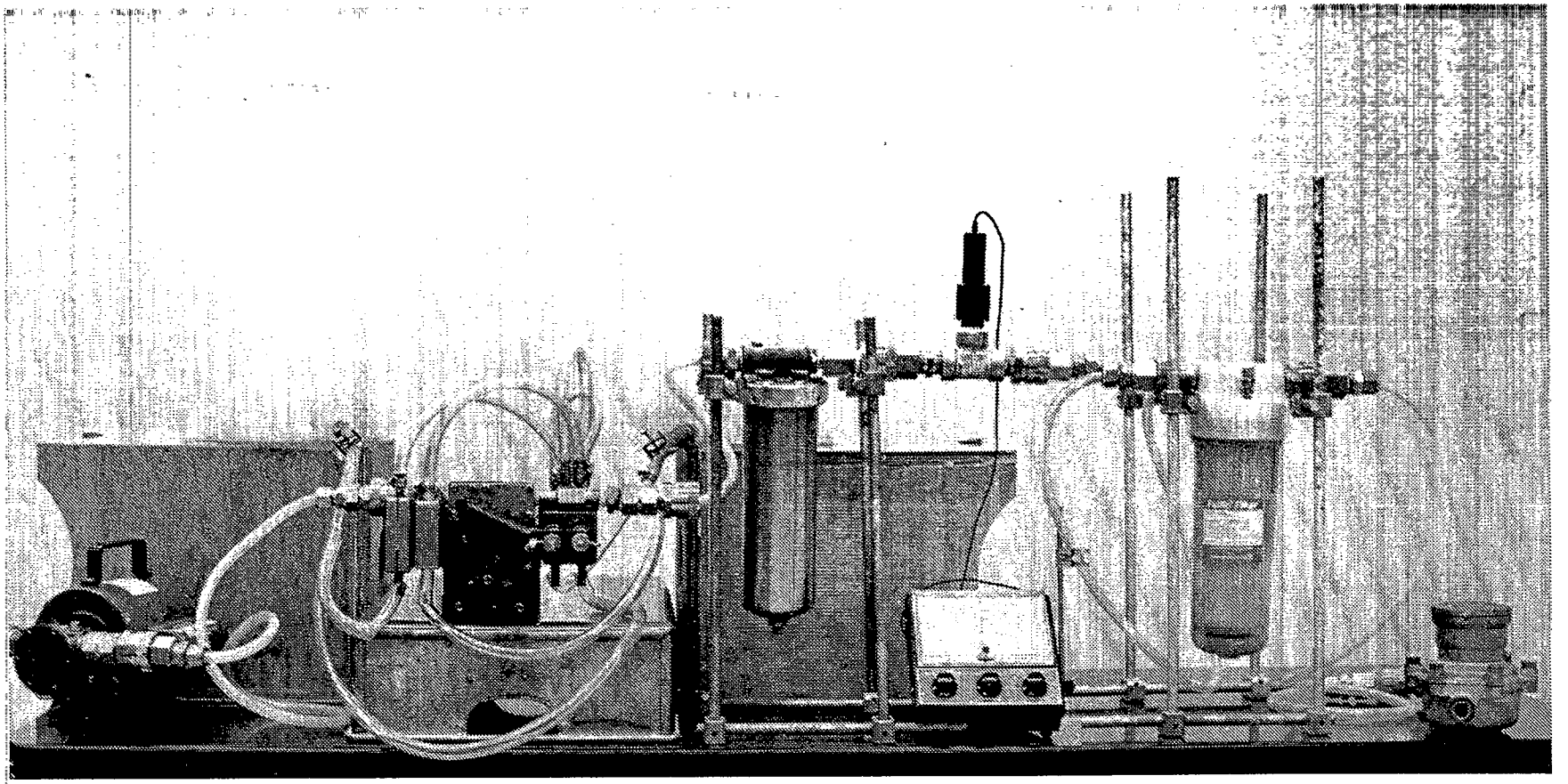

Figure 6-3. Photographic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Cartridge Filter Procedure for Large Volume Filtrations of Clean (Non-turbid) Waters (See Figure 6-2 for Annotated Schematic Representation of Apparatus).

connect outlet port AA_2 of water pump AA to inlet port B_2 of fluid proportioner B.

(a.2) Connect outlet port B_4 of fluid proportioner B to inlet port E_1 of mixing chamber E.

(a.3) Connect outlet port E_2 of mixing chamber E to one arm of pipe tee F.

(a.4) Lock pH electrode G_1 into pH electrode in-line adapter G_2.

The same pH electrode (after sterilization) that is used to adjust pH in Step (c.4) may be used.

(a.5) Connect other arm of pipe tee F to inlet port H_1 of cartridge filter holder H.

(a.6) Connect outlet port H_3 of cartridge filter holder H to inlet port I_1 of water meter I.

(a.7) Connect outlet port I_2 of water meter I to discard.

(b) Preparation of salt supplement

Preparation of sufficient salt supplement for 400 liters of processed water is described below. If more or less water is to be processed, proportionately more or less salt supplement needs to be prepared. When more salt supplement is needed, prepare it in another carboy.

(b.1) Remove cover from 20-liter carboy C.

(b.2) Pour 10 liters of deionized distilled water into carboy C, and add 67 mL of 3 M $AlCl_3$ solution to the deionized distilled water.

(b.3) Replace cover loosely on carboy C.

(c) Preparation of acid for adjustment of pH

(c.1) Pour 380 mL of test water into a 600-mL beaker.

(c.2) Place stir bar into test water.

(c.3) Place beaker on magnetic stirrer, and stir at speed sufficient to develop vortex in test water.

(c.4) Place pH electrode into test water. pH meter must be standardized before it is used.

(c.5) Adjust pH of test water to 3.5 $\pm$ 0.1 with 0.12 M HCl.

(c.6) Record volume of 0.12 M HCl used.

(c.7) Add to carboy C a volume of 12 M HCl equal to 11 times the quantity of 0.12 M HCl needed to reduce the pH in the 380 mL volume of test water to 3.5 $\pm$ 0.1.

(c.8) Bring the volume of acid-salt solution to 20 liters with deionized distilled water, and mix solution well.

(d) Preparation of $Na_2S_2O_3$ solution for dechlorination

Step (d) applies only to chlorinated waters processed directly from a source (e.g., tap water). All chlorinated test waters obtained from sources outside of the processing facility must be dechlorinated immediately when the samples are

obtained (see Page 6-1). Preparation of sufficient $Na_2S_2O_3$ for dechlorinating 400 liters of processed water is described below. If more or less water is to be processed, proportionately more or less $Na_2S_2O_3$ needs to be prepared. When more $Na_2S_2O_3$ is needed, prepare it in another carboy.

(d.1) Remove cover from 20-liter carboy D.

(d.2) Pour 10 liters of deionized distilled water into carboy D.

(d.3) Add 186 mL of 40% $Na_2S_2O_3$ solution to the deionized distilled water in carboy D to give final molarity of 0.03, and mix solution well.

(d.4) Replace cover loosely on carboy D.

(e) Fluid proportioner

(e.1) Connect a long length of tubing to each end of dispensing Y on 20-liter carboy C that contains the acid-salt solution prepared in Step (c.8) above.

Tubing is already in place if additive pumps are sterilized with chlorine (see Section 1.2.1). In this instance, disconnect tubing from bottoms of (larger) additive pumps B_{1a}, and continue with Step (e.2).

(e.2) Remove cover from top of carboy C.

(e.3) Place free end of each tube into mouth of carboy C.

(e.4) Release pinch clamp, and allow acid-salt solution to flow into tubes.

(e.5) Remove tubes from mouth of carboy C, and insert tubes into the inlet (bottom) ports of (larger) additive pumps B_{1a}.
Allow acid-salt solution to flow freely into tubing, but manipulate tubes to prevent overflow.

(e.6) Replace cover loosely on carboy C.

(e.7) Adjust the calibration on the metering rod for each pump B_{1a} to a 3.2 setting.
This calibration equals delivery rate of 1 part of acid-salt solution to each 19 parts of test water. If dechlorination is not necessary, leave the ports of the two remaining (smaller) additive pumps B_{1b} covered (see Section 1.2.1), and go to Step (e.15).

(e.8) Connect a long length of tubing to each end of dispensing Y on 20-liter carboy D that contains the 0.03 M $Na_2S_2O_3$ solution prepared in Steps (d.1-d.4) above.
Tubing is already in place if pumps are sterilized with chlorine (see Section 1.2.1). In this instance, disconnect tubing from bottoms of additive pumps, and continue with Step (e.9).

(e.9) Remove cover from top of carboy D.

(e.10) Place free end of each tube into mouth of carboy D.

(e.11) Release pinch clamp, and allow $Na_2S_2O_3$ solution to flow into tubes.

(e.12) Remove tubes from mouth of carboy D, and insert tubes into the inlet (bottom) ports of (smaller) additive pumps B_{1b}.
Allow $Na_2S_2O_3$ solution to flow freely into tubes, but manipulate tubes to prevent overflow.

(e.13) Replace cover loosely on carboy D.

(e.14) Adjust the calibration on the metering rod for each additive pump B_{1b} to a 1.3 setting. This calibration equals delivery rate of one part of 0.03 M $Na_2S_2O_3$ solution to each 99 parts of test water.

(e.15) Disconnect tube from inlet port E_1 of mixing chamber E, and connect tube to discard.

(e.16) To remove air from tubes, prime all additive pumps by hand-operating metering rods in a reciprocating motion.

(e.17) Reconnect tube from outlet port B_4 of fluid proportioner B to inlet port E_1 of mixing chamber E.

1.2.2 Filtration of Sample

(a) Unscrew base of cartridge filter holder H.

(b) Center 0.45-µm pleated epoxy-fiberglass cartridge filter into base of filter holder H.

(c) Screw base of cartridge filter holder H into its top section, and wrench-tighten to seal.

(d) Make initial reading on water meter I, and record reading.

(e) Open vent/relief valve H_2 on top of cartridge filter holder H.

(f) Open source valve A or start water pump AA to provide maximum flow through system.

(g) Close vent/relief valve H_2 on cartridge filter holder H as soon as water flows through valve.

(h) Wipe up spilled water with laboratory disinfectant.

(i) Read pH meter G to ascertain that proper pH is achieved.

Read meter periodically to be certain that proper pH is maintained. If pH readjustment is necessary, appropriately alter settings on metering rods for (larger) additive pumps B_{1a}.

(j) After required volume of water has been filtered, close source valve A or turn off water pump AA.

(k) Open vent/relief valve H_2 on top of cartridge filter holder H to relieve pressure in system.

(l) Close vent/relief valve H_2.

Wipe up spills with disinfectant, as necessary.

(m) Disconnect tubing from inlet port H_1 of cartridge filter holder H.

Disinfect spills at disconnect.

(n) Connect free end of tubing to discard.

(o) Elevate cartridge filter holder H, and invert to drain.

(p) Make final reading on water meter I, and record reading.

Subtract initial reading from final reading to determine total volume filtered. Subtract volume of acid-salt solution used and volume of $Na_2S_2O_3$ solution, if used, from total volume filtered to determine volume of water sample filtered.

(q) Elute viruses from filter as described in Sections 3 and 4.

2. ADSORPTION -- METHOD TWO

This procedure may be used for waters that require prefiltration.

2.1 Preparation

2.1.1 Apparatus and Materials

Install quick-disconnect connectors on ports of all apparatus except on additive pumps.

(a) Cartridge filter, pleated epoxy-fiberglass -- 10-inch*, 0.45-μm pore size (DUO-FN 10-E-0.45 N-ECIS, Filterite Corp., or equivalent).

(b) Cartridge filter, honeycomb-wound fiberglass yarn -- 10-inch, 1-μm and 5-μm pore sizes (K 27, 1-μm and K 19, 5-μm, Commercial Filter Division, Carborundum Co., or equivalent), as needed.

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

One or more fiberglass-wound filters needs to be used only when it is anticipated that the pleated filter will clog before the filtration procedure is complete. In the absence of experience, honeycomb-wound filters should be used for all waters except tap waters, but may be used for tap waters, if necessary.

(c) Holders for 10-inch cartridge filters (Fulflo, Model No. F15-10, Commercial Filter Division, Carborundum Co., or equivalent).

One holder is needed for pleated filter. An additional holder is needed for each honeycomb-wound cartridge that is to be used.

(d) Plastic-coated drums -- 200-liter capacity, or other containers of size suitable to hold sample, if sample is not pumped directly from source.

(e) Sterilizable self-priming water pump that delivers approximately 25-50 liters per minute.

Pump is not needed if sample water is under pressure, e.g., tap water.

(f) Carboy, autoclavable plastic with nipple on bottom fitted with tubing clamped to a dispensing Y (clamp tubing closed between nipple and Y) -- 20-liter capacity.

If the water at the sampling site is to be drawn directly from a pressurized source and is to be dechlorinated, then two similarly fitted carboys are needed. Otherwise, only one carboy is needed.

Twice the number of carboys is needed, under these conditions, if water volumes greater than 400 liters are to be processed.

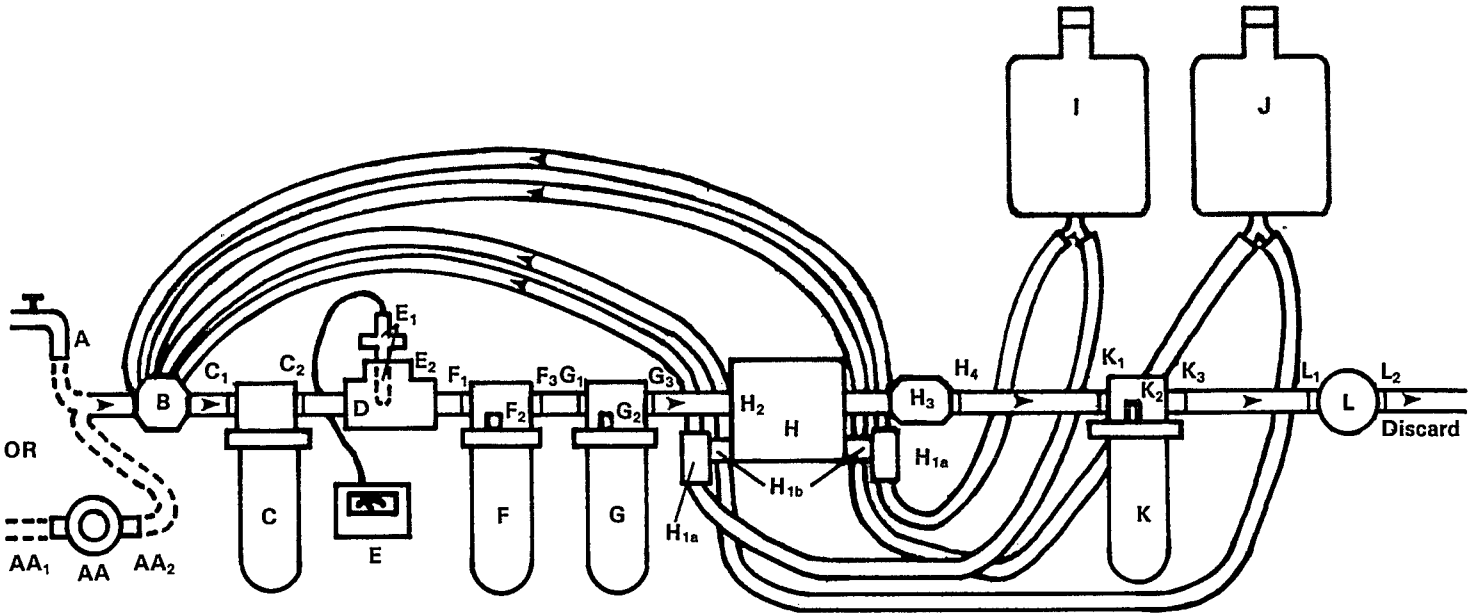
(g) Four stainless steel pipe plugs (Johanson and Son Machine Corp., A40, or equivalent).

(h) Hose adapter for fluid proportioner equipped with four hose fittings (quad system) (Johanson and Son Machine Corp., A34-Q and A33 or equivalent).

(i) Fluid proportioner consisting of fluid-driven motor with four additive pumps (Johanson and Son Machine Corp., Model M 14Q with one P-562 and one P-750 additive pump affixed to each side of the fluid-driven motor, or equivalents).

Assemble fluid proportioner in accordance with the manufacturer's instructions except when otherwise indicated. If four tube fittings with attached tubing are connected to hose adapter H_3, remove the tubing from the fittings, and replace the fittings with four stainless steel pipe plugs (see Figures 6-4 and 6-5 for location of hose adapters). Then, screw the four tube fittings into hose adapter B. Connect a 1.8 meter (6-foot) length of tubing to the top port on each additive pump. Connect the free end of each tube leading from the outlet (top) port of each of the four additive pumps to a tube fitting on hose adapter B.

(j) Mixing chamber (Johanson and Son Machine Corp., C-SS, or equivalent).



A - Pressurized Water Source (Tap)
AA - Self Priming Water Pump (Connected to test water source)
AA_1 - Inlet Port
AA_2 - Outlet Port
B - Hose Adapter Body
C - Mixing Chamber
C_1 - Inlet Port
C_2 - Outlet Port
D - Pipe Tee
E - pH Meter
E_1 - pH Electrode
E_2 - pH Electrode In-Line Adapter
F - Cartridge Holder (Honeycomb filter)
F_1 - Inlet Port
F_2 - Vent/Relief Valve
F_3 - Outlet Port
G - Cartridge Holder (Honeycomb filter)
G_1 - Inlet Port
G_2 - Vent/Relief Valve
G_3 - Outlet Port
H - Fluid Proportioner
H_1a - Chemical Feed (P750) Additive Pumps (Larger)
H_1b - Chlorine Neutralizer (P562) Additive Pumps (Smaller)
H_2 - Inlet Port
H_3 - Hose Adapter Body
H_4 - Outlet Port
I - Carboy (Acid-salt solution)
J - Carboy (Chlorine neutralizer)
K - Cartridge Holder (Pleated filter)
K_1 - Inlet Port
K_2 - Vent/Relief Valve
K_3 - Outlet Port
L - Water Meter
L_1 - Inlet Port
L_2 - Outlet Port

Figure 6-4. Schematic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Cartridge Filter Procedure for Large Volume Filtrations of Turbid Waters (See Figure 6-5 for Photographic Representation of Apparatus).

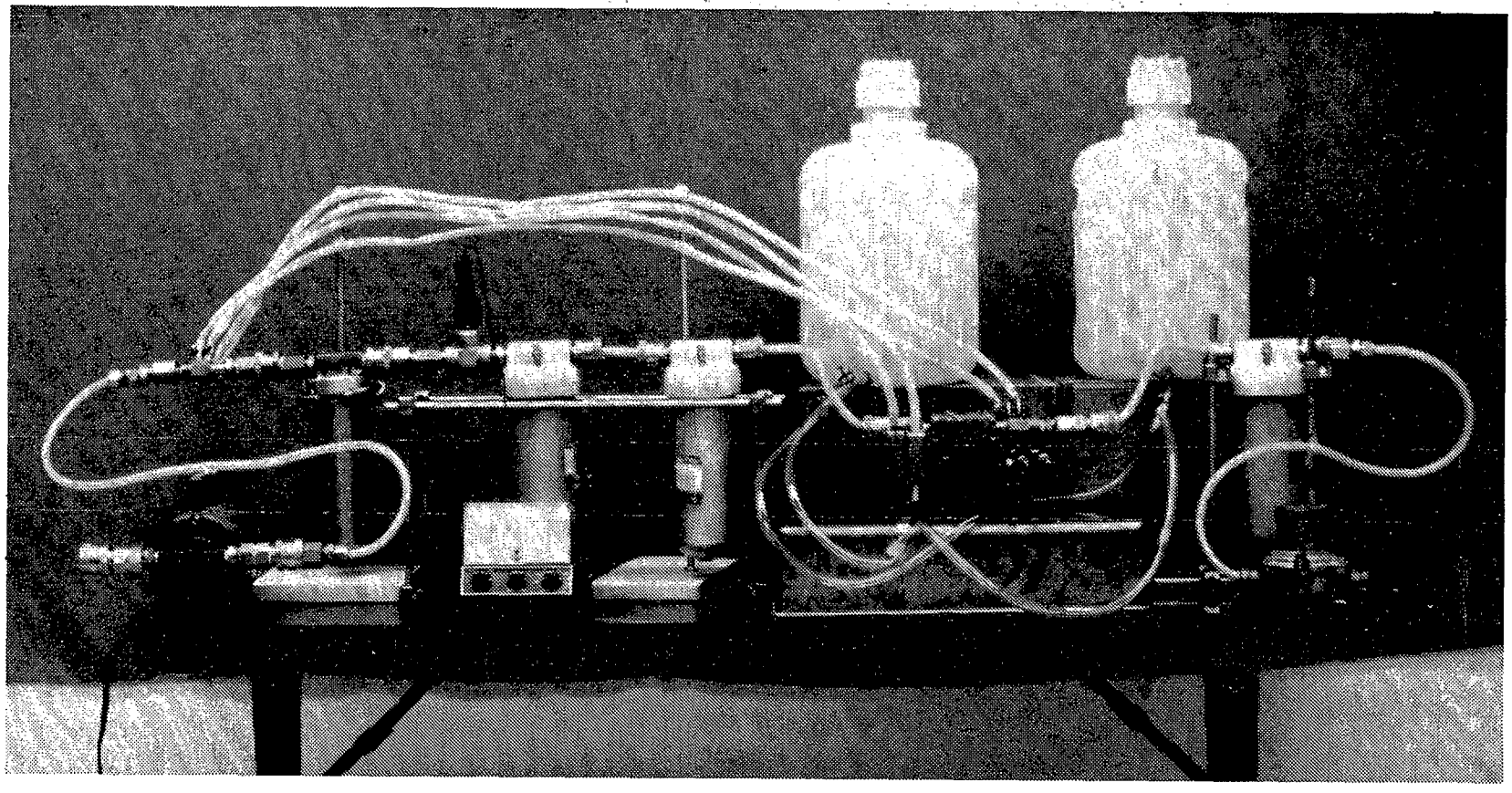


Figure 6-5. Photographic Representation of Apparatus for Recovering Viruses by the Virus Adsorption-Elution (VIRADEL) Cartridge Filter Procedure for Large Volume Filtrations of Turbid Waters (See Figure 6-4 for Annotated Schematic Representation of Apparatus).

(k) pH meter, measuring to an accuracy of at least 0.1 pH unit, equipped with a combination-type electrode (Van London Co., or equivalent, for electrode only).

(l) Tee, stainless steel, with three female NPT ports. Center port equipped with pH electrode in-line adapter (Van London Co., or equivalent, for electrode and adapter only).

(m) Autoclavable inner-braided tubing with metal quick-disconnect connectors for connecting tubing to equipment to be used under pressure.

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

(n) Magnetic stirrer and stir bars.

(o) Water meter (Badger Meter Inc., or equivalent).

(p) Positive pressure source equipped with pressure gauge.

Pressure source, if laboratory air line or pump, must be equipped with oil filter. If source is capable of producing high pressure, deliver to filter holder no more pressure than recommended by manufacturer.

2.1.2 Media and Reagents

(a) Hydrochloric acid (HCl) -- 0.12 and 12 M (concentrated) solutions.

Prepare 100-500 mL of 0.12 M HCl. This solution may be stored for several months at room temperature.

(b) Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) -- 40% (w/v) stock solution (with respect to $Na_2S_2O_3 \cdot 5H_2O$).

Prepare one liter of $Na_2S_2O_3$ solution by dissolving 400 g of $Na_2S_2O_3 \cdot 5H_2O$ in 500 mL of deionized distilled water and bringing final volume of solution to one liter with deionized distilled water. Solution may be stored in dark, rubber-stoppered bottle for up to one month at room temperature.

Solution is to be used for water that cannot be dechlorinated except immediately prior to test procedure (e.g., water tested directly at source). For dechlorinating all other waters, see Page 6-1.

(c) Aluminum chloride ($AlCl_3 \cdot 6H_2O$) -- 3 M stock solution.

Prepare 100 mL of 3M $AlCl_3$ for each 400 liters of water to be processed.

2.2 Procedure (see Figure 6-6 for flow diagram of procedure)

In this procedure, an apparatus is described that can be used for waters so turbid that the volume filtered will clog a 0.45-um pleated epoxy-fiberglass cartridge filter. This apparatus is similar to that described in Method One of this chapter except that honeycomb-wound fiberglass filters are installed in advance of the pleated filter to remove particulate matter in the water, and in-line placement of the equipment is modified to allow adjustment of the pH and salt concentration of the test waters before those waters are prefiltered. Experience usually

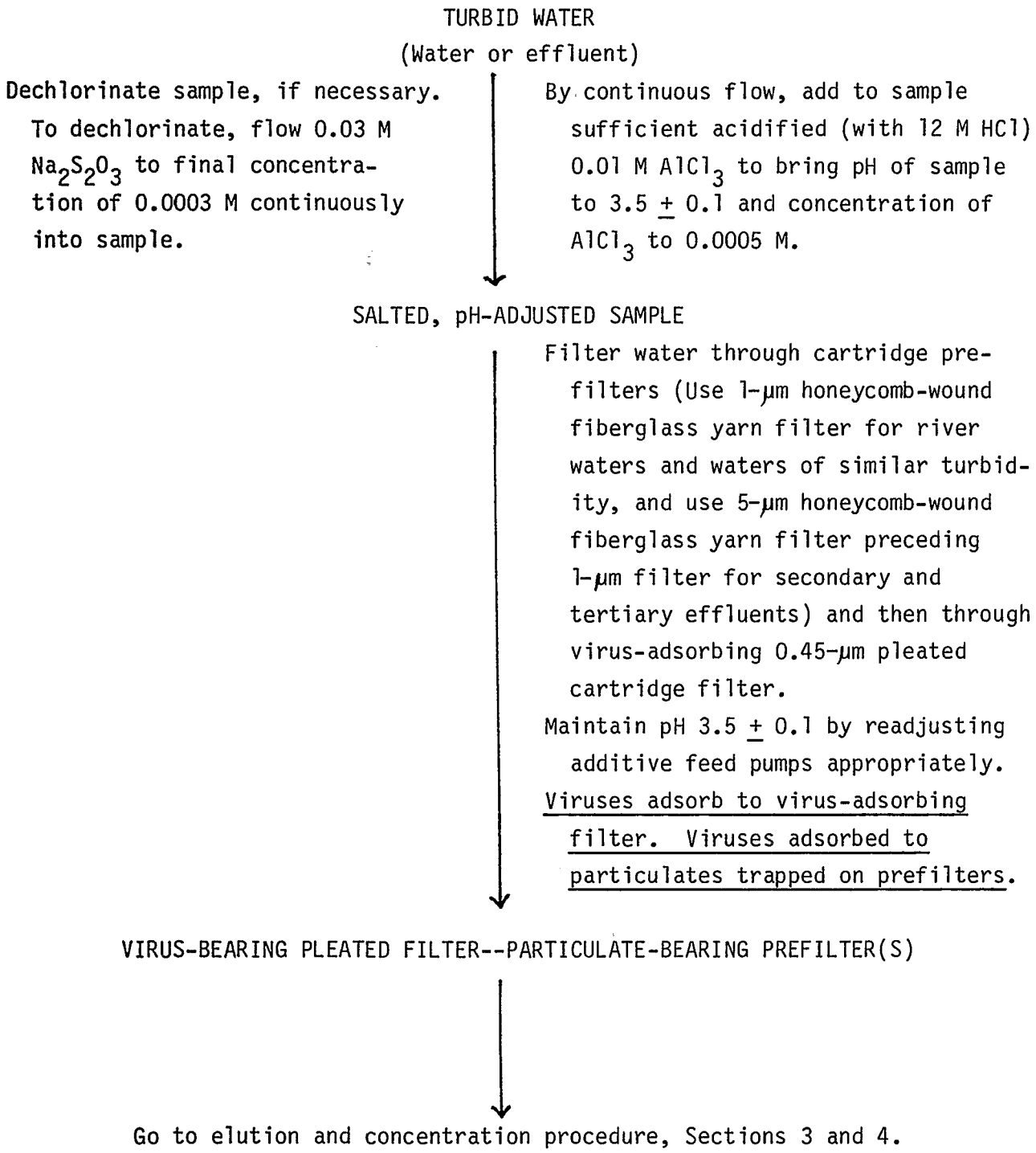


Figure 6-6. Flow Diagram of Method Two for Concentrating Viruses from Large Volumes (More than 200 Liters) of Turbid Waters.

dictates whether prefiltration is needed. In the absence of experience, use procedure in Section 1. ADSORPTION -- METHOD ONE for tap waters and for other waters of similar clarity. Use a 1 μm honeycomb-wound fiberglass yarn cartridge filter preceding the 0.45-μm pleated filter for surface waters and for other waters of similar clarity, and add a 5-μm honeycomb-wound fiberglass yarn cartridge filter preceding the 1-μm filter for secondary and tertiary effluents and for other waters of similar clarity.

2.2.1 Preparation and Implementation

It is usually convenient to sterilize each piece of apparatus and equipment one or more days before it is used (see Chapter 3). It is convenient to sterilize apparatus in small units when sterilization is accomplished by steam or ethylene oxide. It is convenient to assemble and connect all units of apparatus that are to be sterilized by chlorination. The interconnected apparatus can be disassembled after chlorination, the ports covered with aluminum foil and the units stored until used.

(a) Assembly of apparatus (see Figures 6-4 and 6-5)

Use inner-braided tubing fitted with quick-disconnect connectors to make all connections for equipment under pressure.

(a.1) If sample is under pressure, connect water source A to either port of hose adapter B. If sample is not under pressure, connect water source to inlet port AA_1 of self-priming water pump AA. Connect outlet port of water pump AA_2 to either port of hose adapter B.

(a.2) Connect remaining port of hose adapter B to inlet port C_1 of mixing chamber C.

(a.3) Connect outlet port C_2 of mixing chamber C to one arm of pipe tee D.

(a.4) Lock pH electrode E_1 into pH electrode in-line adapter E_2.

Same pH electrode (after sterilization) that is used to adjust pH in Step (c.4) may be used.

(a.5) Connect other arm of pipe tee D to inlet port F_1 of cartridge holder F.

(a.6) Connect outlet port F_3 of cartridge holder F to inlet port G_1 of cartridge holder G.

(a.7) Connect outlet port G_3 of cartridge holder G to inlet port H_2 of fluid proportioner H.

(a.8) Connect outlet port H_4 of fluid proportioner H to inlet port K_1 of cartridge holder K.

(a.9) Connect outlet port K_3 of cartridge holder K to inlet port L_1 of water meter L.

(a.10) Connect outlet port L_2 of water meter L to discard.

(b) Preparation of salt supplement

Preparation of sufficient salt supplement for 400 liters of processed water is described below. If more or less water is to be processed, proportionately more or less salt supplement needs to be prepared. When more salt supplement is needed, prepare it in another carboy.

(b.1) Remove cover from 20-liter carboy I.

(b.2) Pour 10 liters of deionized distilled water into carboy I, and add 67 mL of 3 M $AlCl_3$ solution to the deionized distilled water.

(b.3) Replace cover loosely on carboy I.

(c) Preparation of acid for adjustment of pH

(c.1) Pour 380 mL of test water into a 600-mL beaker.

(c.2) Place stir bar into test water.

(c.3) Place beaker on magnetic stirrer, and stir at speed sufficient to develop vortex in test water.

(c.4) Place pH electrode into test water. pH meter must be standardized before it is used.

(c.5) Adjust pH of test water to 3.5 $\pm$ 0.1 with 0.12 M HCl.

(c.6) Record volume of 0.12 M HCl used.

(c.7) Add to carboy I a volume of 12 M HCl equal to 11 times the quantity of 0.12 M HCl needed to produce the required pH in the 380-mL volume of test water.

(c.8) Bring acid-salt solution to 20-liters with deionized distilled water, and mix solution well.

(d) Preparation of $Na_2S_2O_3$ solution for dechlorination

Step (d) applies only to chlorinated waters processed directly from a source. All chlorinated test waters obtained from sources outside of the processing facility must be dechlorinated immediately when the samples are obtained (see Page 6-1). Preparation of sufficient $Na_2S_2O_3$ for dechlorinating 400 liters of processed water is described below.

If more or less water is to be processed, proportionately more or less $Na_2S_2O_3$ needs to be prepared. When more $Na_2S_2O_3$ is needed, prepare it in another carboy.

(d.1) Remove cover from 20-liter carboy J.

(d.2) Pour 10 liters of deionized distilled water into carboy J.

(d.3) Add 186 mL of 40% $Na_2S_2O_3$ solution to the deionized distilled water in carboy J to give a final molarity of 0.03, and mix solution well.

(d.4) Replace cover loosely on carboy J.

(e) Fluid proportioner

(e.1) Connect a long length of tubing to each end of dispensing Y on 20-liter carboy I that contains the acid-salt solution prepared in Step (c.8) above.

Tubing is already in place if additive pumps are sterilized with chlorine (see Section 2.2.1).

In this instance, disconnect tubing from bottom of additive pumps H_{1a}, and continue with Step (e.2).

(e.2) Remove cover from top of carboy I.

(e.3) Place free end of each tube into mouth of carboy I.

(e.4) Release pinch clamp, and allow acid-salt solution to flow into tubes.

(e.5) Remove tubes from mouth of carboy I, and insert tubes into inlet (bottom) ports of (larger) additive pumps H_{1a}.
Allow acid-salt solution to flow freely into tubing, but manipulate tubes to prevent overflow.

(e.6) Replace cover loosely on carboy I.

(e.7) Adjust the calibration on the metering rod for each pump H_{1a} to a 3.2 setting.
This calibration equals delivery rate of one part of acid-salt solution to each 19 parts of test water. If dechlorination is not necessary, leave the ports of the two remaining additive pumps H_{1b} covered (see Section 2.2.1), and go to Step (e.15).

(e.8) Connect a long length of tubing to each end of dispensing Y on 20-liter carboy J that contains the 0.03 M $Na_2S_2O_3$ solution prepared in Steps (d.1-d.4) above.

Tubing is already in place if pumps are sterilized with chlorine (see Section 2.2.1). In this instance, disconnect tubing from bottoms of additive pumps, and continue with Step (e.9).

(e.9) Remove cover from top of carboy J.

(e.10) Place free end of each tube into mouth of carboy J.

(e.11) Release pinch clamp, and allow $Na_2S_2O_3$ solution to flow into tubes.

(e.12) Remove tubes from mouth of carboy J, and insert tubes into inlet (bottom) ports of (smaller) additive pumps H_{1b}.
Allow $Na_2S_2O_3$ solution to flow freely into tubes, but manipulate tubes to prevent overflow.

(e.13) Replace cover loosely on carboy J.

(e.14) Adjust the calibration on the metering rod for each additive pump H_{1b} to a 1.3 setting. This calibration equals delivery rate of one part of 0.03 M $Na_2S_2O_3$ solution to each 99 parts of test water.

(e.15) Disconnect tube from inlet port C_1 of mixing chamber C, and connect tube to discard.

(e.16) To remove air from tubes, prime additive pumps by hand-operating metering rods in a reciprocating motion.

(e.17) Reconnect tube from outlet port of hose adapter B to inlet port C_1 of mixing chamber C.

2.2.2 Filtration of Sample

(a) Unscrew base of cartridge filter holder F.

(b) Center 5-μm honeycomb-wound fiberglass yarn cartridge filter into base of filter holder F.

(c) Screw base of cartridge filter holder F back into its top section, and wrench-tighten to seal.

(d) Unscrew base of cartridge filter holder G.

(e) Center 1-μm honeycomb-wound fiberglass yarn cartridge filter into base of filter holder G.

(f) Screw base of cartridge filter holder G back into its top section, and wrench-tighten to seal.

(g) Unscrew base of cartridge filter holder K.

(h) Center 0.45-μm pleated epoxy-fiberglass cartridge filter into base of filter holder K.

(i) Screw base of cartridge filter holder K back into its top section, and wrench-tighten to seal.

(j) Make initial reading on water meter L, and record reading.

(k) Open vent/relief valves F_2, G_2, and K_2 on top of cartridge filter holders F, G, and K.

(l) Open source valve A or start water pump AA to provide maximum flow through system.

(m) Close vent/relief valves F_2, G_2, and K_2 on top of cartridge filter holders F, G, and K as soon as water flows through valves.

(n) Wipe up spilled water with laboratory disinfectant.

(o) Read pH meter E to ascertain that proper pH is achieved.

Read meter periodically to be certain that proper pH is maintained. If pH readjustment is necessary, appropriately alter settings on metering rods for (larger) additive pumps H_{1a}.

(p) After required volume of water has been filtered, close source valve A or turn off water pump AA.

(q) Open vent/relief valves F_2, G_2, and K_2 on top of cartridge filter holders F, G, and K to relieve pressure in system.

(r) Close vent/relief valves F_2, G_2, and K_2.
Wipe up spills with disinfectant as necessary.

(s) Disconnect tubing from source A or from water pump outlet AA_2.
Disinfect spills at disconnect.

(t) Connect free end of tubing to discard.

(u) Elevate cartridge filter holders F, G, and K, and invert to drain.

(v) Take final reading on water meter, and record reading.
Subtract initial reading from final reading to determine total volume filtered. Subtract volume of acid-salt solution used and, if used, volume of $Na_2S_2O_3$ solution from total volume filtered to determine volume of water sampled.

(w) Elute viruses from filters as described in Sections 3 and 4.

3. ELUTION AND RECONCENTRATION -- METHOD ONE

This method may be used for eluting viruses not significantly inactivated at pH levels of about 10.5 in 15 minutes at ambient temperatures. To elute viruses that cannot be safely recovered by this procedure, see Section 4.

3.1 Procedure for Eluting Viruses from Cartridge Filters (see Figures 6-7.1 and 6-8.1 for flow diagrams of procedure)

3.1.1 Apparatus and Materials

(a) Positive pressure source equipped with a pressure gauge.

Gauge necessary only if pressure source is capable of producing pressures exceeding tolerances of equipment. Pressure source, if laboratory air line or pump, must be equipped with an oil filter. If source is capable of producing high pressure, deliver to pressure vessel and filter holder no more pressure than recommended by manufacturer.

(b) Dispensing pressure vessel -- 4 liters (Millipore Corp., or equivalent).

(c) Beakers, graduated -- 2 liters.

One beaker is needed for each filter that is eluted.

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

(e) Autoclavable inner-braided tubing fitted with metal quick-disconnect connectors on one end and glass elbow on the other.

Make glass elbow from a 13-cm length (approximate O.D. 6 mm) of glass tubing by making a 40 degree bend about 5 cm from one end. Connect tubing onto

Figure 6-7.1 Virus Elution Procedure

VIRUS-BEARING CARTRIDGE FILTER

↓

Force 1600 mL of basic glycine solution (0.05 M glycine, pH 10.5 ± 0.1) through cartridge filter slowly (Apply pressure no greater than 0.4 kg/cm^2).

Viruses elute from filter.

Check pH of eluate. If pH is less than 9.5, repeat elution procedure with fresh basic glycine solution, and combine eluates.

If precipitate forms in eluate, reconcentrate viruses by $Al(OH)_3$-hydroextraction procedure.

CAUTION: Reconcentration by $Al(OH)_3$-hydroextraction procedure must begin immediately, because pH of eluate must be reduced immediately to prevent inactivation of viruses.

Figure 6-7.2 Virus Reconcentration Procedure

BASIC ELUATE

↓

Mix rapidly into eluate sufficient acid glycine solution (0.05 M glycine, pH 2) to bring pH of eluate to 3.5 ± 0.1.

If eluate becomes turbid during or after acidification, terminate procedure and reconcentrate viruses by $Al(OH)_3$-hydroextraction procedure.

CAUTION: Begin $Al(OH)_3$ procedure immediately, because pH of eluate must be reduced immediately to prevent inactivation of viruses.

ACIDIFIED ELUATE

↓

Filter acidified eluate through 0.45-µm virus-adsorbing disc filter.

Viruses adsorb to filter.

VIRUS-BEARING DISC FILTER

↓

Elute viruses by forcing successively two 5-mL volumes of basic glycine solution (0.05 M glycine, pH 10.5 ± 0.1) through disc filter.

Check pH of eluate. If pH is less than 9.5, repeat elution procedure with fresh basic glycine solution, and combine eluates.

BASIC ELUATE

↓

Adjust pH of combined eluates to 7.0-7.5 with acid glycine solution (0.05 M glycine, pH 2).

NEUTRALIZED ELUATE

↓

Add sufficient fetal calf serum to neutralized eluate to yield final serum concentration of 2%.

STABILIZED NEUTRALIZED ELUATE

↓

Assay for viruses (See Chapter 9).

Figure 6-7. Flow Diagram of High pH Procedure (Basic Glycine, pH 10.5) for Eluting Viruses from Cartridge Filters and for Reconcentrating Viruses from Clear Eluates by the Membrane Filter Procedure.

Figure 6-8.1 Virus Elution Procedure

VIRUS-BEARING CARTRIDGE FILTER

↓ Force 1600 mL of basic glycine solution (0.05 M glycine, pH 10.5 ± 0.1) through cartridge filter slowly (Apply pressure no greater than 0.4 kg/cm^2).
Viruses elute from filter.
Check pH of eluate. If pH is less than 9.5, repeat elution procedure with fresh basic glycine solution, and combine eluates.

Figure 6-8.2 Virus Reconcentration Procedure

BASIC ELUATE

↓ Stir sufficient 0.3 M $AlCl_3$ into eluate to obtain final $AlCl_3$ concentration of 0.003 M.

SALTED ELUATE

↓ Adjust pH of salted eluate to 7.0 ± 0.1 with 1 M Na_2CO_3.
An $Al(OH)_3$ floc forms as Na_2CO_3 is added.
Stir for five minutes more. Allow floc to settle for 30 minutes.

SETTLED VIRUS-BEARING $Al(OH)_3$ FLOC

↓ Aspirate all but several mL of liquid above settled floc, discard aspirated fluid, and centrifuge floc at 1,000 x g for 30 minutes. Save floc, discard supernate.

CENTRIFUGED VIRUS-BEARING $Al(OH)_3$ FLOC

↓ Measure floc volume, and mix three volumes of buffered fetal calf serum (BFCS)-glycine, pH 11.5 ± 0.1 into $Al(OH)_3$ floc.
Viruses elute from floc.

FLOC-BFCS ELUATE MIXTURE

↓ Centrifuge floc-eluate mixture at 1,000 x g for three minutes. Discard floc.

BFCS ELUATE

↓ Adjust pH of BFCS eluate to 7.0 ± 0.1 with acid glycine solution (1 M glycine, pH 2).

NEUTRALIZED BFCS ELUATE

↓ Pour neutralized BFCS eluate into a dialysis bag, and dialyze BFCS against polyethylene glycol at 4° C until 10-20 mL of fluid remains in bag.

DIALYSATE

↓ Suspend bag containing dialysate in chilled phosphate-buffered saline. Maintain at 4°-10° C for one hour.

ISOTONIC DIALYSATE

↓ Add antibiotics.

Assay for viruses (See Chapter 9).

Figure 6-8. Flow Diagram of High pH Procedure (Basic Glycine, pH 10.5) for Eluting Viruses from Cartridge Filters and for Reconcentrating Viruses from Turbid Eluates by the $Al(OH)_3$-Hydroextraction Procedure.

longer end of elbow. One elbow with attached tubing is needed for each filter that is eluted.

(f) Magnetic stirrer and stir bars.

3.1.2 Media and Reagents

(a) Sodium hydroxide (NaOH) -- 10 M.

Prepare 500 mL of 10 M NaOH.

(b) Basic glycine solution -- 0.05 M glycine, adjusted to pH 10.5 $\pm$ 0.1 with 10 M NaOH.

Autoclave glycine solution before adjusting pH.

Prepare 3 liters of basic 0.05 M glycine solution.

(c) Hydrochloric acid (HCl) -- 12 M (concentrated) HCl solution.

(d) Acid glycine solution -- 0.05 M glycine, adjusted to pH 2 with 12 M HCl.

Autoclave glycine solution before adjusting pH.

Prepare 3 liters of acid 0.05 M glycine solution.

3.1.3 Rearrangement of Apparatus

(a) Rearrangement for Method One (see Figures 6-2 and 6-3).

(a.1) Disconnect at pipe tee F, the tubing leading to inlet port H_1 of filter holder H.

(a.2) Connect free end of tubing from inlet port H_1 of filter holder H to outlet port of pressure vessel.

Pressure vessel is not shown in Figures 6-2 and 6-3.

(a.3) Connect inlet port of pressure vessel to positive air pressure source.

(a.4) Disconnect tubing from outlet port H_3 of filter holder H.

(a.5) Hook glass elbow with 40 degree bend onto pouring spout of a 2-liter glass beaker. Raise aluminum foil covering beaker enough to expose only the pouring spout.

(a.6) Connect free end of tubing from glass elbow to outlet port H_3 of filter holder H.

(a.7) Crimp aluminum foil cover over glass elbow.

(a.8) Elute viruses from filter as described in Section 3.1.4 below.

(b) Rearrangement for Method Two (see Figures 6-4 and 6-5).

(b.1) Disconnect at pipe tee D, the tubing leading to the inlet port F_1 of filter holder F.

(b.2) Connect free end of tubing from inlet port F_1 of filter holder F to outlet port of pressure vessel.
Pressure vessel is not shown in Figures 6-4 and 6-5.

(b.3) Connect inlet port of pressure vessel to positive pressure source.

(b.4) Disconnect tubing from outlet port F_3 of filter holder F.

(b.5) Hook glass elbow with 40 degree bend onto pouring spout of a 2-liter glass beaker. Raise aluminum foil covering beaker enough to expose only the pouring spout.

(b.6) Connect free end of tubing from glass elbow to outlet port F_3 of filter holder F.

(b.7) Crimp aluminum foil cover over glass elbow.

(b.8) Elute viruses from filter as described in Section 3.1.4 below.

(b.9) Disconnect tubing from outlet port of pressure vessel.

(b.10) Connect free end of tubing from inlet port G_1 of filter holder G to outlet port of pressure vessel.

(b.11) Disconnect tubing from outlet port G_3 of filter holder G.

(b.12) Hook glass elbow with 40 degree bend onto pouring spout of a 2-liter glass beaker. Raise aluminum foil covering beaker enough to expose only the pouring spout.

(b.13) Connect free end of tubing from glass elbow to outlet port G_3 of filter holder G.

(b.14) Crimp aluminum foil cover over glass elbow.

(b.15) Elute viruses from filter as described in Section 3.1.4 below.

(b.16) Disconnect tubing from outlet port of pressure vessel.

(b.17) Disconnect at outlet port H_4 of fluid proportioner H, the tubing leading to the inlet port K_1 of filter holder K.

(b.18) Connect free end of tubing from inlet port K_1 of filter holder K to outlet port of pressure vessel.

(b.19) Disconnect tubing from outlet port K_3 of filter holder K.

(b.20) Hook glass elbow with 40 degree bend onto pouring spout of a 2-liter graduated glass beaker.

Raise aluminum foil covering beaker enough to expose only the pouring spout.

(b.21) Connect free end of tubing from glass elbow to outlet port K_3 of filter holder K.

(b.22) Crimp aluminum foil cover over glass elbow.

(b.23) Elute viruses from filter as described in Section 3.1.4 below.

3.1.4 Elution Procedure

(a) Remove top of pressure vessel.

(b) Pour into pressure vessel 1600 mL of basic glycine solution (pH 10.5 $\pm$ 0.1).

(c) Replace top of pressure vessel.

(d) Close vent/relief valve on pressure vessel.

(e) Open vent/relief valve on cartridge filter holder.

(f) Apply pressure sufficient to purge trapped air from filter apparatus.

(g) Close vent/relief valve on cartridge filter holder as soon as basic glycine solution begins to flow from valve.

(h) Wipe up spilled liquid with laboratory disinfectant.

(i) Increase pressure to that sufficient to force basic glycine solution through the filter.

Do not exceed a pressure of 0.4 kg/cm^2 so that basic glycine solution passes through cartridge filter slowly thereby maximizing elution contact period.

(j) Turn off pressure at source.

(k) Open vent/relief valve on pressure vessel.

(l) Check pH of eluate.

If pH of eluate is below 9.5, repeat elution procedure with fresh elutant, combine eluates in graduated beaker, and reconcentrate. Instructions for reconcentrating viruses begin in Section 3.2. Reconcentration must begin immediately, because pH of eluate must be reduced immediately to prevent inactivation of viruses.

3.2 Reconcentration -- Method A. Membrane Disc Procedure (see Figure 6-7.2 for flow diagram of procedure)

Where it can be used, the membrane disc procedure is the preferred method for reconcentrating viruses from the eluates resulting from the procedures described in the preceding section (Section 3.1.4). However, in some eluates, a precipitate is present that impedes filtration of the eluate through a membrane filter. Reconcentrate such eluates by the aluminum hydroxide-hydroextraction procedure described in Section 3.3. If, during acidification in the membrane procedure, turbidity occurs in previously clear eluates, discontinue acidification and reconcentrate these eluates by the aluminum hydroxide-hydroextraction procedure. Optionally, for any given sample, all clear eluates may be pooled and all turbid eluates may be pooled for reconcentration.

3.2.1 Apparatus and Materials

(a) High pressure disc filter holders -- 47mm diameter (Millipore Corp., XX4504700, or equivalent).

(b) Virus-adsorbing disc filter, mixed esters of cellulose -- 0.45-μm pore size (Millipore HA, or equivalent).

(c) Dispensing pressure vessel -- 20-liter capacity (Millipore Corp., XX6700L20, or equivalent).

(d) Positive pressure source equipped with pressure gauge.

Gauge necessary only if pressure source is capable of producing pressures exceeding tolerances of equipment. Pressure source, if laboratory air line or pump, must be equipped with oil filter. If source is capable of producing high pressure, deliver to pressure vessel and filter holder no more pressure than recommended by manufacturer.

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

(f) Autoclavable inner-braided tubing with metal quick-disconnect connectors or with thumb-screw-drive-clamps for connecting tubing to equipment.

(g) Magnetic stirrer and stir bar.

(h) Filling bell connected to inner-braided tubing.

3.2.2 Media and Reagents

(a) Hydrochloric acid (HCl) -- 12 M (concentrated) HCl solution.

(b) Acid glycine solution, 0.05 M, adjusted to pH 2 with 12 M HCl.

Autoclave glycine solution before adjusting pH.

(c) Sodium hydroxide (NaOH) -- 10 M.

Prepare 500 mL of 10 M NaOH.

(d) Basic glycine solution, 0.05 M, adjusted to pH 10.5 ± 0.1 with 10 M NaOH.

Prepare 3 liters of basic glycine solution. Autoclave glycine solution before adjusting pH.

(e) Fetal calf serum.

3.2.3 Procedure

(a) Assembly of Apparatus (See Figures 6-9 and 6-10)

(a.1) Remove top of filter holder C.

(a.2) With forceps, lay 0.45-um virus-adsorbing filter onto support screen of holder.

(a.3) Replace and tighten down top of filter holder C.

(a.4) Connect positive pressure source A or AA to inlet port B of pressure vessel B.

(a.5) Connect outlet port B_3 of pressure vessel B to inlet port C_1 of filter holder C.

(a.6) Place filling bell D, with inner-braided tubing attached, over opening of flask E, and connect free end of the tubing to the outlet port C_2 of filter holder C.

(b) Adjustment of pH of eluates from Section 3.1.4, Step (1)

Add with rapid, continuous stirring sufficient acid glycine solution to bring pH of eluate to 3.5 ± 0.1. It is important to mix acid glycine solution into sample rapidly, because slow mixing may result in pH levels sufficiently low in parts of the sample to

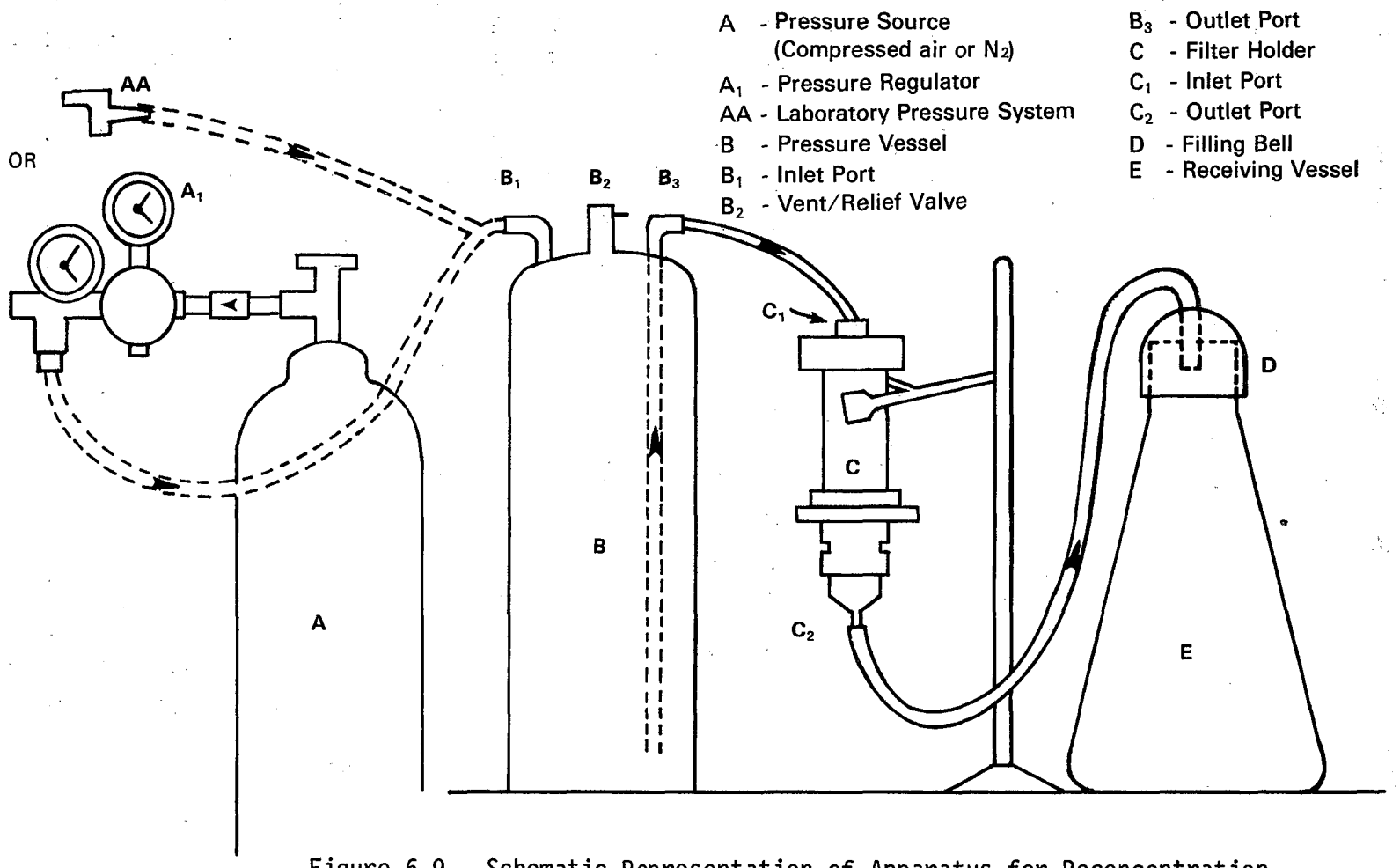


Figure 6-9. Schematic Representation of Apparatus for Reconcentration -- Method A, a Membrane Disc Procedure for Reconcentrating Viruses from Glycine Eluates (See Figure 6-10 for Photographic Representation of Apparatus).

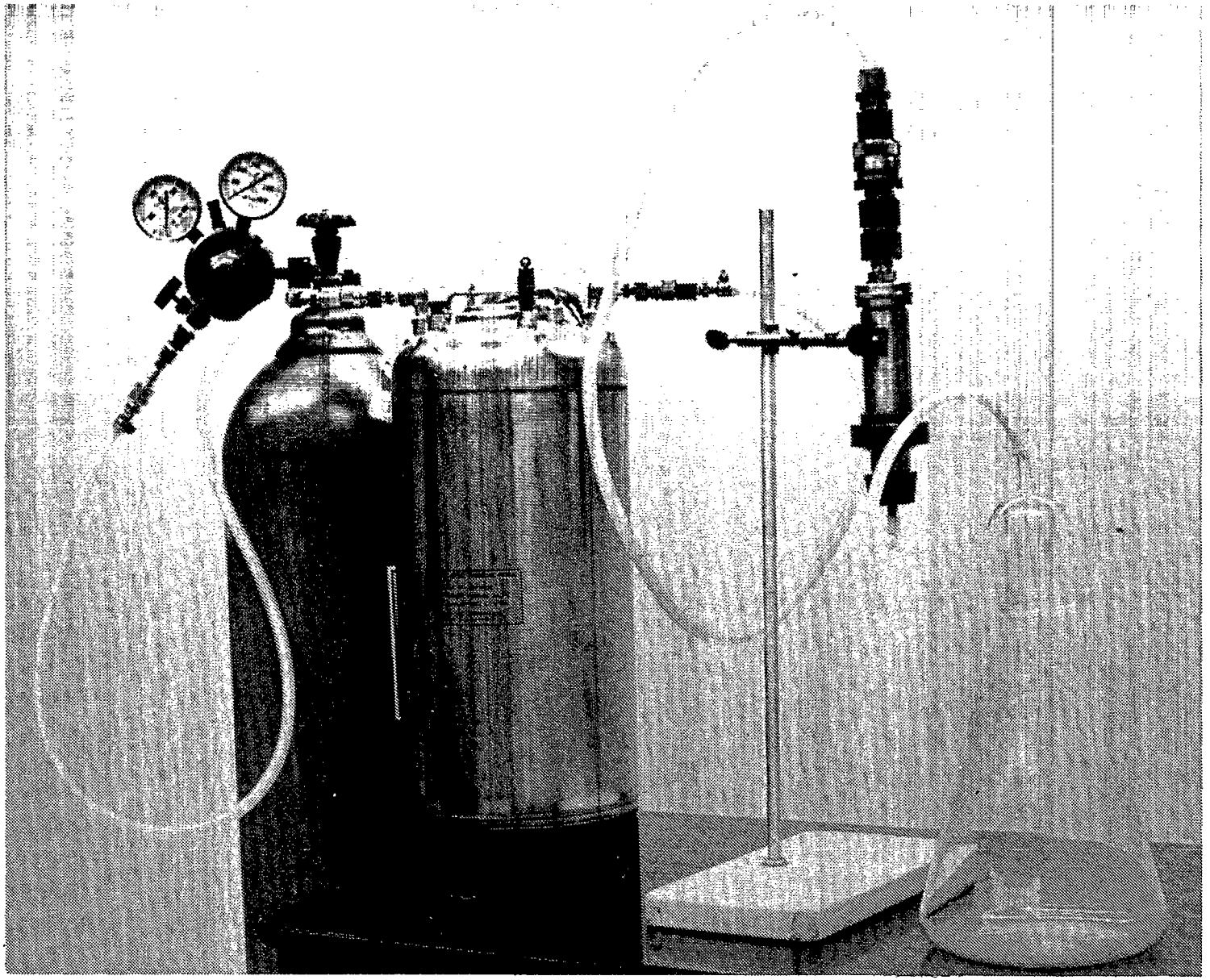


Figure 6-10. Photographic Representation of Apparatus for Reconcentration -- Method A, a Membrane Disc Procedure for Reconcentrating Viruses from Glycine Eluates (See Figure 6-9 for Annotated Schematic Representation of Apparatus).

inactivate viruses. If eluate becomes *turbid during* or after acidification, terminate procedure and reconcentrate viruses by the aluminum hydroxide-hydroextraction procedure described in Section 3.3. Reconcentration with the aluminum hydroxide-hydroextraction procedure must begin immediately, because pH of eluate must be reduced immediately to prevent inactivation of viruses.

(c) Filtration of eluate.

(c.1) Remove top of pressure vessel B.

(c.2) Pour eluate into pressure vessel B.

(c.3) Replace and tighten down top of pressure vessel B.

(c.4) Apply pressure from source A or AA sufficient to force sample through the filter (usually 0.4-1.5 kg/cm^2).

(c.5) Turn off pressure at source A or AA.

(c.6) Open vent/relief valve B_2 of pressure vessel B.

(c.7) When pressure is relieved, close vent/relief valve B_2.

(d) Elution of viruses from filter.

(d.1) Disconnect tubing from outlet port C_2 of filter holder C.

(d.2) Place 100-mL beaker under outlet port C_2 of filter holder C.

(d.3) Disconnect tubing from inlet port C_1 of filter holder C.

(d.4) Pour 5 mL of basic glycine solution into inlet port C_1 of filter holder C.

(d.5) Reconnect tubing to inlet port C_1 of filter holder C.

(d.6) Apply sufficient pressure from source A or AA to force basic glycine solution through filter.

(d.7) Turn off pressure at source A or AA.

(d.8) Open vent/relief valve B_2 on pressure vessel B.

(d.9) When pressure is relieved, close vent/relief valve B_2.

(d.10) Disconnect tubing from inlet port C_1 of filter holder C.

(d.11) Pour another 5 mL of basic glycine solution into inlet port C_1 of filter holder C.

(d.12) Repeat steps (d.5-d.8), collecting total 10 mL of eluates in the same beaker.

Check pH of combined eluates. If pH is below 9.5, repeat steps (d.9-d.12) with fresh basic glycine solution.

(d.13) Adjust pH of combined eluates to 7.0-7.5 with acid glycine solution.

(d.14) Measure total volume of neutralized eluate.

(d.15) Add sufficient fetal calf serum to neutralized eluate to yield a final serum concentration of 2%.

(d.16) Refrigerate neutralized eluate at 4^{0} C immediately, and maintain at that temperature until eluate is assayed for viruses. If assay for viruses cannot be undertaken within eight hours, store eluate immediately at -70^{0} C.

3.3 Reconcentration -- Method B. Aluminum Hydroxide-Hydroextraction Procedure (see Figure 6-8.2 for flow diagram of procedure)

Use the aluminum hydroxide-hydroextraction procedure to reconcentrate viruses from turbid eluates that result from the procedures described in Section 3.1.4 and from eluates that become turbid upon acidification during the membrane disc procedure described in Section 3.2.3, Step (b).

3.3.1 Apparatus and Materials

(a) Magnetic stirrer and stir bars.

(b) pH meter, measuring to an accuracy of at least 0.1 pH unit, equipped with combination-type electrode.

(c) Instrument tray, stainless steel -- overall dimensions 43 cm x 10 cm x 5 cm (Vollrath Co., No. 83170, or equivalent).

(d) Dialyzer tubing, molecular weight cutoff 12,000, 3-cm diameter (Arthur H. Thomas Co., No. 3787-D42, or equivalent).

(e) Clamps, dialyzer tubing (Arthur H. Thomas Co., No. 3787-N30, or equivalent).

(f) Centrifuge tubes, screw-capped, round-bottom -- 50-100 mL.

3.3.2 Media and Reagents

(a) Hydrochloric acid (HCl) -- 12 M (concentrated) HCl solution.

(b) Sodium hydroxide (NaOH) -- 10 M.

Prepare 500 mL of 10 M NaOH.

(c) Aluminum chloride ($AlCl_3$) -- 0.3 M.

Prepare 500 ml of 0.3 M $AlCl_3$.

(d) Sodium carbonate (Na_2CO_3) -- 1 M.

Prepare 100 mL of 1 M Na_2CO_3.

(e) Glycine.

(f) Acid glycine solution -- 1 M glycine adjusted to pH 2 with 12 M HCl.

Prepare 2 liters of acid glycine solution.

(g) Basic fetal calf serum (BFCS) with glycine -- 1 M glycine in fetal calf serum (FCS) adjusted to pH 11.5 $\pm$ 0.1 with 10 M NaOH.

Prepare 100 mL of BFCS with glycine. To prepare BFCS with glycine, autoclave glycine powder in a covered vessel, add FCS, and adjust pH.

(h) Phosphate-buffered saline -- Solution A: Sodium chloride (NaCl), 40 g; potassium chloride (KCl), 1 g; calcium chloride ($CaCl_2$), 0.5 g; magnesium chloride ($MgCl_2 \cdot 6H_2O$), 0.5 g; deionized distilled water to 4 liters. Solution B: Sodium phosphate, dibasic (Na_2HPO_4), 1 g; potassium phosphate, monobasic, (KH_2PO_4), 1 g; deionized distilled water to 1 liter. Prepare solutions A and B separately, then mix them together in a ratio of 1:1.

(i) Polyethylene glycol -- 20,000 MW.

Three kg of polyethylene glycol are required.

(j) Antibiotics

Prepare as indicated for medium in Chapter 9, Section 7.18.

3.3.3 Procedure

(a) Preparation of dialysis bag.

(a.1) Soak a 40-cm length of dialyzer tubing in deionized distilled water for five minutes.

(a.2) Fold one end of the tubing over itself to form a 2-cm overlap.

(a.3) Center dialyzer tubing clamp over overlap, and lock clamp in place to form dialysis bag.

(a.4) Grasp unclamped end of dialysis bag between thumb and forefinger, and rub the facing surfaces against each other.

This procedure separates the facing surfaces and opens the dialysis bag.

(a.5) Fill dialysis bag two-thirds full with deionized distilled water.

(a.6) Fold free end of dialysis bag over itself to form a 2-cm overlap.

(a.7) Center dialyzer tubing clamp over overlap, and lock clamp in place.

(a.8) Squeeze dialysis bag gently to force water against clamped ends.

If water leaks through either clamped end, remove clamp from faulty seal and repeat Steps (a.6-a.8).

(a.9) Squeeze dialysis bag near clamps, exerting sufficient pressure to test the integrity of the tubing.

<u>If water leaks through tubing, discard tubing and repeat Steps (a.1-a.9)</u>.

(a.10) Sterilize dialysis bag by the procedure described in Chapter 3.

(a.11) After sterilization, recheck dialysis bag for leaks by repeating Steps (a.8-a.9).

(a.12) Store dialysis bag in deionized distilled water at 4^{o} C.

(b) Flocculation and hydroextraction.

(b.1) Measure volume of eluate against graduations on beaker (from Section 3.1.4, Step [1]).

(b.2) Place stir bar into eluate.

(b.3) Place beaker on magnetic stirrer, and stir at speed sufficient to develop vortex in eluate.

(b.4) Add sufficient volume of 0.3 M $AlCl_3$ to eluate to obtain a final $AlCl_3$ concentration of 0.003 M.

(b.5) Place pH electrode into eluate.

(b.6) Adjust pH of eluate to 7 $\pm$ 0.1 with 1 M Na_2CO_3.

<u>An $Al(OH)_3$ floc forms as Na_2CO_3 is added</u>.

(b.7) After pH 7 $\pm$ 0.1 is obtained, stir for five minutes.

(b.8) Remove pH electrode.

(b.9) Turn off magnetic stirrer.

(b.10) Allow floc to settle for 30 minutes.

(b.11) Aspirate liquid to 2-3 cm above level of floc.

(b.12) Discard aspirated liquid.

(b.13) Resuspend settled floc in remaining liquid.

Resuspend floc by swirling beaker or by placing beaker on magnetic stirrer and activating stirrer.

(b.14) Pour suspended floc into round bottom centrifuge tube(s).

Screw-capped centrifuge tubes with a capacity of 50-100 mL are usually adequate. To prevent transfer of stir bar into centrifuge tube, hold another stir bar or magnet underneath beaker when decanting contents.

(b.15) Centrifuge tube(s) at 1000 x g for three minutes.

(b.16) Decant or aspirate supernate.

(b.17) Discard supernate.

(b.18) Measure approximate volume of $Al(OH)_3$ residue.

To a centrifuge tube similar to that containing the $Al(OH)_3$ residue, add water to a level equal to the height of the residue, and estimate volume of residue by measuring the volume of water in a graduated cylinder or pipette.

(b.19) Add to each volume of $Al(OH)_3$ residue, three volumes of BFCS-glycine, pH 11.5 $\pm$ 0.1.

(b.20) Shake centrifuge tube(s) for five seconds to mix contents.

(b.21) Centrifuge each $Al(OH)_3$ -- BFCS-glycine suspension for three minutes at 1000 x g.

(b.22) Pour BFCS-glycine supernate(s) into beaker.

(b.23) Place stir bar into beaker.

(b.24) Place beaker on magnetic stirrer, and stir contents of beaker at a speed sufficient to develop vortex.

(b.25) Place pH electrode into BFCS-glycine.

(b.26) Adjust pH of BFCS-glycine to 7 $\pm$ 0.1 with 1 M acid glycine solution.

(b.27) Turn off magnetic stirrer.

(b.28) Remove pH electrode.

(b.29) Discard residue from Step (b.21).

(b.30) Remove dialysis bag from storage, and wipe exterior of bag with towel.

(b.31) Remove clamp from one end of dialysis bag.

(b.32) With sterile scissors, remove end of dialysis bag by cutting across the bag through center of clamp impression.

This procedure removes inside edge of bag that had been exposed to contamination.

(b.33) Discard water from dialysis bag.

(b.34) Pour neutralized BFCS-glycine eluate into dialysis bag.

(b.35) Fold open end of the bag over itself to form a 2-cm overlap.

(b.36) Center dialyzer tubing clamp over overlap, and lock clamp in place.

(b.37) Place a 1-cm layer of polyethylene glycol into instrument tray.

(b.38) Place dialysis bag on polyethylene glycol.

(b.39) Add sufficient polyethylene glycol to cover dialysis bag.

(b.40) Place cover on instrument tray.

(b.41) Maintain instrument tray overnight at 4^{o} C. Hydroextract until approximately 10 to 20 mL of concentrated neutralized BFCS-glycine remain in bag.

(b.42) Rinse polyethylene glycol from outside surface of dialysis bag with deionized distilled water.

(b.43) Pour 900 mL of chilled (4-10^{o} C) phosphate-buffered saline into a 1-liter beaker. Maintain phosphate-buffered saline at 4-10^{o} C; carry out Steps (b.44-b.47) in cold room or in cold by other means available.

(b.44) Place beaker of phosphate-buffered saline on magnetic stirrer.

(b.45) Place stir bar into phosphate-buffered saline, and stir at a speed sufficient to develop vortex.

(b.46) Immerse dialysis bag into phosphate-buffered saline.
Care must be taken not to puncture bag with stir bar.

(b.47) Stir for one hour.

(b.48) Remove dialysis bag from phosphate-buffered saline and wipe exterior of bag with towel.

(b.49) Remove clamp from one end of dialysis bag.

(b.50) With sterile scissors, remove end of dialysis bag by cutting across the bag through center of clamp impression.

This procedure removes inside edge of bag that had been exposed to contamination.

(b.51) Pour concentrate into 100-mL graduated beaker.

(b.52) Hold dialysis bag in fully inverted position over beaker.

(b.53) Place upper end of bag between forefinger and middle finger in a scissors grip.

(b.54) Squeeze bag between fingers in scissors grip.

(b.55) Pull fingers down over length of bag to remove remaining concentrate.

Take care that fingers do not contaminate concentrate.

(b.56) Determine volume of concentrate in beaker.

(b.57) Add antibiotics to concentrate in accordance with instructions given for medium in Chapter 9, Section 7.18.

(b.58) Refrigerate concentrate immediately at 4^{o} C, and maintain at that temperature until concentrate is assayed for viruses.

If assay for viruses cannot be undertaken within eight hours, store concentrate immediately at -70^{o} C.

4. ELUTION AND RECONCENTRATION -- METHOD TWO

This method may be used for eluting viruses from filters that cannot be safely eluted with Method One (this method should be as effective as Method One for eluting viruses sensitive to pH 10.5).

4.1 Procedure for Eluting Viruses from Filters (see Figure 6-11.1 for flow diagram of procedure)

4.1.1 Apparatus and Materials

(a) Positive pressure source equipped with a pressure gauge.

Gauge necessary only if pressure source is capable of producing pressures exceeding tolerances of equipment. Pressure source, if laboratory air line or pump, must be equipped with an oil filter. If source is capable of producing high pressure, deliver to pressure vessel and filter holder no more pressure than recommended by manufacturer.

(b) Dispensing pressure vessel -- 4 liters (Millipore Corp., or equivalent).

(c) Beaker, graduated -- 2 liters.

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

(e) Autoclavable inner-braided tubing fitted with metal quick-disconnect connectors on one end and glass elbow on the other.

Make glass elbow from a 13-cm length (approximate O.D., 6 mm) of glass tubing by making a 40 degree bend about 5 cm from one end. Connect tubing onto longer end of elbow.

(f) Magnetic stirrer and stir bars.

Figure 6-11.1 Virus Elution Procedure

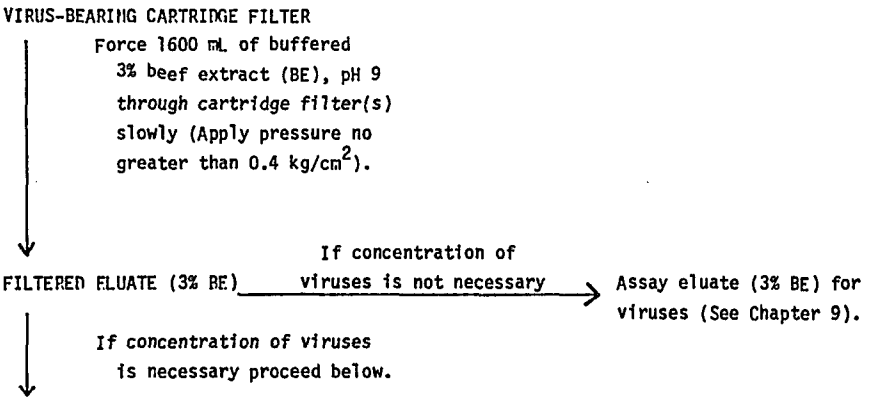


Figure 6-11.2 Virus Concentration Procedure (Katzenelson Organic Flocculation Procedure)

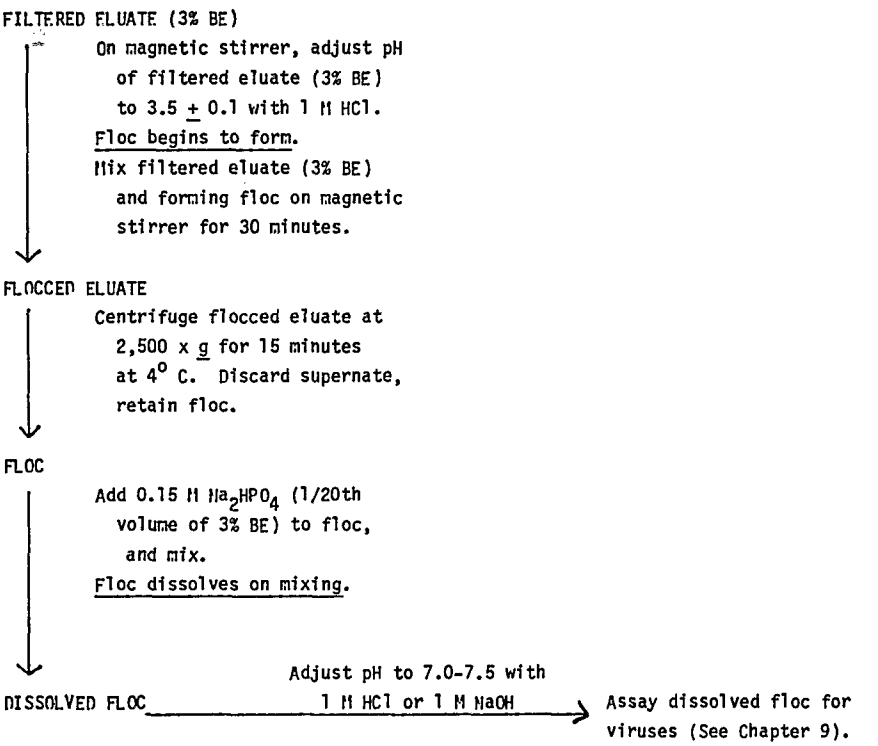


Figure 6-11. Flow Diagram of Beef Extract Method for Eluting Viruses from Cartridge Filters with Buffered 3% Beef Extract and for Concentrating Eluted Viruses by the Katzenelson Organic Flocculation Procedure.

4.1.2 Media and Reagents

(a) Sodium hydroxide (NaOH) -- 1 M.

Prepare 500 mL of 1 M NaOH. This solution may be stored for several months at room temperature.

(b) Glycine.

(c) Beef extract powder (Grand Island Biological Co., or equivalent).

Prepare buffered 3% beef extract by dissolving 60 g of beef extract powder and 7.5 g of glycine (final concentration = 0.05 M) in 2 liters of distilled water. Autoclave beef extract solution, and adjust pH to 9 + 0.1 with 1 M NaOH.

4.1.3 Rearrangement of Apparatus

(a) Rearrangement for Method One (see Figures 6-2 and 6-3).

(a.1) Disconnect at pipe tee F, the tubing leading to inlet port H_1 of filter holder H.

(a.2) Connect free end of tubing from inlet port H_1 of filter holder H to outlet port of pressure vessel.

Pressure vessel is not shown in Figures 6-2 and 6-3.

(a.3) Connect inlet port of pressure vessel to positive air pressure source.

(a.4) Disconnect tubing from outlet port H_3 of filter holder H.

(a.5) Hook glass elbow with 40 degree bend onto pouring spout of a 2-liter glass beaker.

Raise aluminum foil covering beaker enough to expose only the pouring spout.

(a.6) Connect free end of tubing from glass elbow to outlet port H_3 of filter holder H.

(a.7) Crimp aluminum foil cover over glass elbow.

(a.8) Elute viruses from filter as described in Section 4.1.4 below.

(b) Rearrangement for Method Two (see Figures 6-4 and 6-5).

(b.1) Disconnect at pipe tee D, the tubing leading to the inlet port F_1 of filter holder F.

(b.2) Connect free end of tubing from inlet port F_1 of filter holder F to outlet port of pressure vessel.

Pressure vessel is not shown in Figures 6-4 and 6-5.

(b.3) Connect inlet port of pressure vessel to positive pressure source.

(b.4) Disconnect tubing from outlet port G_3 of filter holder G.

(b.5) Disconnect at outlet port H_4 of fluid proportioner H, the tubing leading to the inlet port K_1 of filter holder K.

(b.6) Connect free end of tubing from inlet port K_1 of filter holder K to outlet port G_3 of filter holder G.

(b.7) Disconnect tubing from outlet port K_3 of filter holder K.

(b.8) Hook glass elbow with 40 degree bend onto pouring spout of a 2-liter graduated glass beaker.

Raise aluminum foil covering beaker enough to expose only the pouring spout.

(b.9) Connect free end of tubing from glass elbow to outlet port K_3 of filter holder K.

(b.10) Crimp aluminum foil cover over glass elbow.

(b.11) Elute viruses from filter as described in Section 4.1.4 below.

4.1.4 Elution Procedure

(a) Remove top of pressure vessel.

(b) Pour into pressure vessel 1600 mL of buffered 3% beef extract (pH 9).

(c) Replace top of pressure vessel.

(d) Close vent/relief valve on pressure vessel.

(e) Open vent/relief valve on cartridge filter holder.

If more than one cartridge filter is used, open valves on all holders.

(f) Apply pressure sufficient to purge trapped air from filter apparatus.

(g) Close vent/relief valve on (each) cartridge filter holder as soon as buffered 3% beef extract solution begins to flow from valve.

(h) Wipe up spilled liquid with laboratory disinfectant.

(i) Increase pressure to that sufficient to force buffered 3% beef extract solution through the filter(s).

Do not exceed a pressure of 0.4 kg/cm^2 so that

buffered 3% beef extract solution passes through cartridge filter(s) slowly thereby maximizing elution contact period. When air enters line from pressure vessel, elevate and invert filter holder(s) to permit complete evacuation of buffered 3% beef extract from filters.

(j) Turn off pressure at source.

(k) Open vent/relief valve on pressure vessel.

(l) Proceed to Section 4.2 immediately.

If concentration of viruses cannot be undertaken immediately, eluate may be stored for up to eight hours at 4^{o} C before reconcentration.

If reconcentration cannot be undertaken within eight hours, store eluate immediately at -70^{o} C.

Instructions for reconcentrating viruses begin in Section 4.2.

4.2 Organic Flocculation Concentration Procedure of Katzenelson (see Figure 6-11.2 for flow diagram of procedure)

It is preferable to assay eluted viruses in the beef extract eluate without further concentrating them because some loss of viruses may occur in concentration. However, the numbers of cell cultures needed for assays may be reduced by further concentrating the viruses.

4.2.1 Apparatus and Materials

(a) Magnetic stirrer and stir bars.

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

(c) Refrigerated centrifuge capable of attaining 2500 x g.

Each sample centrifuged at 2500 x g will consist of about 1600 mL.

4.2.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) -- 0.15 M.

(b) Hydrochloric acid (HCl) -- 1 M.

(c) Sodium hydroxide (NaOH) -- 1 M.

4.2.3 Procedure

(a) Place stir bar into graduated beaker containing buffered 3% beef extract eluate from 4.1.4 (1).

(b) Place beaker that contains the beef extract on magnetic stirrer, and stir at a speed sufficient to develop vortex.

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

(c) Insert pH electrode into beef extract eluate.

(d) Add 1 M HCl to flask slowly until pH of beef extract reaches 3.5 $\pm$ 0.1.

A precipitate will form. If pH is accidentally reduced below 3.4, add 1 M NaOH until pH is 3.5 $\pm$ 0.1. Avoid reducing pH below 3.4 because some inactivation of viruses may occur.

(e) Remove pH electrode from beaker, and continue to stir for 30 minutes more.

(f) Remove caps from screw-capped centrifuge bottles.

Glass centrifuge bottles may not be able to withstand g force that will be applied.

(g) Pour contents of beaker into centrifuge bottles. To prevent transfer of stir bar into centrifuge bottle, hold another stir bar or magnet against bottom of beaker when decanting contents.

(h) Replace and tighten down caps on centrifuge bottles.

(i) Centrifuge precipitated beef extract suspension in refrigerated centrifuge (4^{o} C) for 15 minutes at 2500 x g.

(j) Remove caps from screw-capped centrifuge bottles.

(k) Pour supernates into graduate cylinder, and record volumes.

(l) Discard supernates.

(m) Place a stir bar into each centrifuge bottle that contains precipitate.

(n) To each precipitate, add 5 mL of 0.15 M $Na_2HPO_4 \cdot 7H_2O$ for each 100 mL of supernate decanted.

(o) Replace and tighten down caps on centrifuge bottles.

(p) Place each centrifuge bottle on a magnetic stirrer, and stir each precipitate slowly until it has dissolved completely.
Support bottles as necessary to prevent toppling. Avoid foaming which may inactivate or aerosolize viruses. Precipitate may be partially dissipated with spatula before or during stirring procedure.

(q) Remove caps from screw-capped centrifuge bottles.

(r) Remove foil cover from 250-mL beaker.

(s) Combine the dissolved precipitates in beaker.

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

(t) Measure pH of concentrate (dissolved precipitate). If pH is above or below 7.0-7.5, adjust to that range with either 1 M HCl or 1 M NaOH.

(u) Replace foil cover securely on beaker.

(v) Refrigerate concentrate immediately at 4^{o} C, and maintain at that temperature until assay for viruses is undertaken.

If assay for viruses cannot be undertaken within eight hours, store concentrate immediately at -70^{o} C.

(w) Assay for viruses in accordance with instructions given in Chapter 9.

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CHAPTER 7

METHOD FOR RECOVERING VIRUSES FROM SLUDGES (AND OTHER SOLIDS)

The method described below may be used for raw primary and raw activated sludges, and for such sludges after they have been digested mesophilically or thermophilically. Although limited supporting experimental data are available, the method is probably also useful for other sludges, soils, and dredge spoils. See Figure 7-1 for flow diagram of the method. If sludges, soils, or dredge spoils are toxic to cell cultures used for assay of viruses, obtain fresh sample material, and use method described in Chapter 8.

Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (see Chapters 2 and 3).

1. EXTRACTION OF VIRUSES FROM SLUDGES

1.1 Preparation

1.1.1 Apparatus and Materials

(a) Refrigerated centrifuge capable of attaining 10,000 x g and screw-capped centrifuge bottles that can withstand 10,000 x g.

Each sample centrifuged at 10,000 x g will consist of about 100 mL.

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

(c) Magnetic stirrer and stir bars.

(d) Membrane filter apparatus for sterilization -- 47-mm diameter filter holder and 50-mL slip tip syringe (Millipore Corp., Swinnex filter, No. SX0047000, or equivalent for filter holder only).

If final eluate must be concentrated by the organic flocculation procedure of Katzenelson (see Section 2), membrane filter apparatus is not required.

(e) Disc filters, 47 mm diameter -- 3.0-, 0.45-, and 0.25-μm pore size filters (Filterite Corp., Duo-Fine series, or equivalent). Filters must be cut to proper size from sheet filters.

Disassemble Swinnex filter holder. Place filter with 0.25-μm pore size on support screen of filter holder, and stack the remaining filters on top in order of increasing pore size. Reassemble and tighten filter holder. Filters stacked in tandem as described tend to clog more slowly when turbid material is filtered through them. Prepare several filter stacks.

If final eluate must be concentrated by the organic flocculation procedure of Katzenelson (see Section 2), disc filters are not required.

1.1.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$).

(b) Citric acid.

(c) Beef extract powder (Grand Island Biological Co., or equivalent).

Prepare buffered 10% beef extract by dissolving 10 gm beef extract powder, 1.34 g $Na_2HPO_4 \cdot 7H_2O$ and 0.12 g citric acid in 100 mL of deionized distilled water. Dissolve by stirring for about two hours on a magnetic stirrer. Autoclave at 121° C for 15 minutes.

(d) Hydrochloric acid (HCl) -- 5 M.

(e) Aluminum chloride ($AlCl_3 \cdot 6H_2O$) -- 0.05 M.
Autoclave $AlCl_3$ solution at 121° C for 15 minutes.

(f) Sodium hydroxide (NaOH) -- 5 M.

1.2 Procedure (See Figure 7-1.1)

1.2.1 Conditioning of Sludge

In the absence of experience that dictates otherwise, use 100-mL volumes for sludges, 100-mL volumes of a 5% suspension in deionized distilled water for dredge spoils (v/v) or soils (w/v), and 100-g quantities for digested dewatered sludges and for other samples difficult to measure volumetrically.

(a) Measure 100 mL of well-mixed sludge in a graduated 100-mL cylinder.
Sludge must be mixed vigorously immediately before it is poured into cylinder because sludge solids, which contain most of the viruses, begin to settle out immediately after mixing stops.

(b) Place stir bar into a 250-mL beaker.

(c) Pour the 100 mL of measured sludge from the cylinder into the 250-mL beaker.
It may be necessary to pour sludge several times

Figure 7-1.1 Virus Recovery Procedure

SLUDGE (100 mL)

↓ On magnetic stirrer, add $AlCl_3$ (to final concentration 0.0005 M), adjust pH of salted sludge to 3.5 ± 0.1 with 5 M HCl, and mix vigorously for 30 minutes.

SALTED, pH-ADJUSTED SLUDGE

↓ Centrifuge salted, pH-adjusted sludge at 2,500 x g for 15 minutes. Discard supernate, retain sludge solids.

SLUDGE SOLIDS

↓ Add 100 mL of buffered 10% beef extract (BE) (pH 7.0 ± 0.1) to sludge solids. Mix resuspended sludge solids on magnetic stirrer for 30 minutes to elute viruses.

RESUSPENDED SLUDGE SOLIDS

↓ Centrifuge resuspended sludge solids at 10,000 x g for 30 minutes. Discard solids, retain eluate (supernate).

ELUATE

↓ If concentration of viruses is not necessary, filter eluate through membrane stack.
If concentration of viruses is necessary, proceed to Virus Concentration Procedure (Figure 7-1.2)

FILTERED ELUATE (10% BE) → Assay eluate (10% BE) for viruses (See Chapter 9).

Figure 7-1.2 Virus Concentration Procedure

ELUATE

↓ Filter eluate through membrane stack.

FILTERED ELUATE (10% BE)

↓ Add sufficient volume of deionized distilled water to filtered eluate to reduce BE concentration from 10% to 3%.
Begin Katzenelson organic flocculation procedure.

DILUTED FILTERED ELUATE (3% BE)

↓ On magnetic stirrer, adjust pH of filtered eluate (3% BE) to 3.5 ± 0.1 with 1 M HCl.
Floc begins to form.
Mix filtered eluate (3% BE) and forming floc on magnetic stirrer for 30 minutes.

FLOCCED ELUATE

↓ Centrifuge flocced eluate at 2,500 x g for 15 minutes at 4° C. Discard supernate, retain floc.

FLOC FROM ELUATE

↓ Add 0.15 M Na_2HPO_4 (1/20th volume of diluted beef extract) to floc, and mix.
Floc dissolves on mixing.

DISSOLVED FLOC ← Adjust pH to 7.0-7.5 with 1 M HCl or 1 M NaOH → Assay dissolved floc for viruses (See Chapter 9).

Figure 7-1. Flow Diagram of Method for Recovering and Concentrating Viruses in Sludges

from beaker to cylinder and back in order to remove all sludge solids to beaker.

CAUTION: Take care to avoid formation of aerosols.

(d) Place beaker on magnetic stirrer, and stir at speed sufficient to develop vortex.

(e) Add 1 ml of 0.05 M $AlCl_3$ to mixing sludge.
Final concentration of $AlCl_3$ in sludge is approximately 0.0005 M.

(f) Place combination-type pH electrode into mixing sludge.
pH meter must be standardized at pH 4.

(g) Adjust pH of sludge to 3.5 $\pm$ 0.1 with 5 M HCl.
If pH falls below 3.4, readjust it with 5 M NaOH.
When sludge adheres to electrodes, clean electrodes by moving them up and down gently in mixing sludge.

(h) Continue mixing for 30 minutes more.
The pH of the sludge should be checked at frequent intervals. If the pH drifts up, readjust it to 3.5 $\pm$ 0.1 with 5 M HCl. If the pH drifts down, readjust it with 5 M NaOH.

(i) Turn off stirrer, and remove pH electrode from sludge.

(j) Remove cap from a screw-capped centrifuge bottle.
Glass centrifuge bottles may not withstand 10,000 x g force that will be applied.

(k) Pour conditioned sludge into centrifuge bottle.
To prevent transfer of stir bar into centrifuge bottle when decanting sludge, hold another stir bar

or magnet against bottom of beaker. Sludge that adheres to stir bar in the beaker may be removed by manipulation with a pipette. It may be necessary to pour sludge several times from centrifuge bottle to beaker and back in order to remove all sludge solids to bottle.

CAUTION: Take care to avoid formation of aerosols.

(l) Replace and tighten down cap on centrifuge bottle.

(m) Centrifuge conditioned sludge at 2,500 x g for 15 minutes at 4° C.

(n) Remove cap from centrifuge bottle.

(o) Decant supernate into beaker.

(p) Replace cap on centrifuge bottle.

(q) Discard supernate.

1.2.2 Elution of Viruses from Sludge Solids

(a) Remove cap from centrifuge bottle that contains sedimented, conditioned sludge [from Section 1.2.1, Step (p)].

(b) Place stir bar into centrifuge bottle.

(c) Add 100 mL of buffered 10% beef extract to the sedimented, conditioned sludge.

The volume of buffered 10% beef extract used to elute viruses from the conditioned sludge is equal to the original volume of the sludge sample [Section 1.2.1, Step (a)].

(d) Replace and tighten down cap on centrifuge bottle.

(e) Place centrifuge bottle on magnetic stirrer, and

stir at speed sufficient to develop vortex. To minimize foaming (which may inactivate viruses), do not mix faster than necessary to develop vortex. Care must be taken to prevent bottle from toppling. Stabilize bottle as necessary.

(f) Continue mixing for 30 minutes.

(g) Turn off stirrer.

(h) Remove cap from centrifuge bottle.

(i) With long forceps or magnet retriever, remove stir bar from centrifuge bottle.

(j) Replace and tighten down cap on centrifuge bottle.

(k) Centrifuge conditioned sludge-eluate mixture at 10,000 x g for 30 minutes at 4^{o} C.

(l) Remove cap from centrifuge bottle.

(m) Decant eluate into beaker, discard sludge sediment. The number of cell cultures necessary for the viral assay may be reduced by concentrating the viruses in the beef extract by the organic flocculation procedure of Katzenelson. Some loss of viruses may occur with this procedure. If viruses in eluates are to be concentrated, proceed immediately to Section 2. If concentration is not required, proceed to Step (n).

(n) Place a filter holder that contains a filter stack on a 250-mL Erlenmeyer receiving flask.

(o) Load 50-mL syringe with eluate.

(p) Place tip of syringe into filter holder.

(q) Force eluate through filter stack into 250-mL receiving flask.

Take care not to break off tip of syringe and to minimize pressure on receiving flask because such pressure may splinter or topple the flask. If filter stack begins to clog badly, empty loaded syringe into beaker containing unfiltered eluate, fill syringe with air, and inject air into filter stack to force residual eluate from filters. Continue filtration procedure with another filter holder and filter stack. Discard contaminated filter holders and filter stacks. Steps (n) thru (q) may be repeated as often as necessary to filter entire volume of eluate. Disassemble each filter holder and examine bottom filters to be certain they have not ruptured. If a bottom filter has ruptured, repeat Steps (n) through (q) with new filter holders and filter stacks.

(r) Refrigerate eluate immediately at 4^{o} C, and maintain it at that temperature until it is assayed for viruses.

If assay for viruses cannot be undertaken within eight hours, store eluate immediately at -70^{o} C.

2. CONCENTRATION OF VIRUSES FROM SLUDGE ELUATES

2.1 Organic Flocculation Concentration Procedure of Katzenelson*

It is preferable to assay eluted viruses in the beef extract eluate without concentrating them because some loss of viruses may occur in concentration. However, the numbers of cell cultures needed for assays may be reduced by concentrating the viruses in the eluate.

2.1.1 Apparatus and Materials

(a) Magnetic stirrer and stir bars.

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

(c) Refrigerated centrifuge capable of attaining 2,500 x g and screw-capped centrifuge bottles. Each sample centrifuged at 2,500 x g will consist of about 330 mL.

(d) Membrane filter apparatus for sterilization -- 47-mm diameter filter holder and 50-mL slip tip syringe (Millipore Corp., Swinnex filter, No. SX0004700, or equivalent, for filter holder only).

(e) Disc filters, 47 mm diameter -- 3.0-, 0.45-, and 0.25-µm pore size filters (Filterite Corp., Duo-Fine series, or equivalent). Filterite must be cut to proper size from sheet filters.

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*Katzenelson, E., B. Fattal, and T. Hostovesky. 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl. Environ. Microbiol. 32:638-639.

Disassemble Swinnex filter holder. Place filter with 0.25 μm pore size on support screen of filter holder, and stack the remaining filters on top in order of increasing pore size. Reassemble and tighten filter holder. Filters stacked in tandem as described tend to clog more slowly when turbid material is filtered through them. Prepare several filter stacks.

2.1.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) -- 0.15 M.

(b) Hydrochloric acid (HCl) -- 1 M.

(c) Sodium hydroxide (NaOH) -- 1 M.

2.1.3 Procedure (See Figure 7-1.2)

(a) Place a filter holder that contains a filter stack on a 250-mL Erlenmeyer receiving flask.

(b) Load 50-mL syringe with eluate from Section 1.2.2, Step (m).

(c) Place tip of syringe into filter holder, and force eluate through filter stack.

Take care not to break off tip of syringe and to minimize pressure on receiving flask, because such pressure may splinter or topple the flask. If filter stack begins to clog badly, empty loaded syringe into beaker containing unfiltered eluate, fill syringe with air, and inject air into filter stack to force residual eluate from filters. Continue filtration procedure with another filter holder and filter stack. Discard contaminated filter holders and

filter stacks. Steps (a) thru (c) may be repeated as often as necessary to filter entire volume of eluate. Disassemble each filter holder and examine bottom filters to be certain they have not ruptured. If a bottom filter has ruptured, repeat Steps (a) through (c) with new filter holders and filter stacks.

(d) Remove filter holder from top of Erlenmeyer flask, pour eluate into graduated cylinder, and record volume.

(e) Pour eluate into 600-mL beaker.

(f) For every 3 mL of beef extract eluate, add 7 mL of deionized distilled water to the 600-mL beaker. The concentration of beef extract is now 3%. This dilution is necessary because 10% beef extract often does not process well by the organic flocculation concentration procedure.

(g) Record the total volume of the diluted filtered beef extract.

(h) Place stir bar in beaker that contains diluted filtered beef extract.

(i) Place beaker that contains the diluted filtered beef extract on magnetic stirrer, and stir at a speed sufficient to develop vortex.
To minimize foaming (which may inactivate viruses), do not mix faster than necessary to develop vortex.

(j) Insert combination-type pH electrode into diluted, filtered beef extract.
pH meter must be standardized at pH 4.

(k) Add 1 M HCl to flask slowly until pH of beef extract reaches 3.5 ± 0.1.

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

(l) Continue to stir for 30 minutes more.

(m) Turn off stirrer.

(n) Remove caps from screw-capped centrifuge bottles.

Use one or more bottles, as needed. Glass centrifuge bottles may not withstand 2,500 x g force that will be applied.

(o) Remove electrode from beaker, and distribute contents of beaker evenly among centrifuge bottles.

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

(p) Replace and tighten down caps on centrifuge bottles.

(q) Centrifuge precipitated beef extract suspensions at 2,500 x g for 15 minutes at 4^{o} C.

(r) Remove caps from centrifuge bottles.

(s) Pour off, and discard supernates.

(t) Place a small stir bar into each centrifuge bottle that contains precipitate.

(u) Replace covers loosely on centrifuge bottles.

(v) Raise caps from tops of centrifuge bottles and divide a volume of 0.15 M $Na_2HPO_4 \cdot 7H_2O$ equal to 1/20 of the volume recorded in Step (g) equally among

the precipitates in the centrifuge bottles.

The volume of 0.15 M $Na_2HPO_4 \cdot 7H_2O$ in which the precipitate will be dissolved is equal to 5 mL for each 100 mL of diluted beef extract.

(w) Replace and tighten down caps on centrifuge bottles.

(x) Place each bottle on a magnetic stirrer, and stir each precipitate slowly until precipitate has dissolved completely.

Support bottles as necessary to prevent toppling. Avoid foaming which may inactivate or aerosolize viruses. Precipitate may be partially dissipated with spatula before or during stirring procedure.

(y) Remove caps from centrifuge bottles.

(z) Combine the dissolved precipitates in a small beaker.

To prevent transfer of stir bars into beaker, hold another stir bar or magnet against the bottom of each centrifuge bottle when decanting dissolved precipitates.

(aa) Measure pH of dissolved precipitates.

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

(bb) Refrigerate dissolved precipitates immediately at 4° C, and maintain at that temperature until assay for viruses is undertaken.

If assay for viruses cannot be undertaken within eight hours, store dissolved precipitates immediately at -70° C.

(cc) Assay for viruses in accordance with instructions given in Chapter 9.

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CHAPTER 8

METHOD FOR RECOVERING VIRUSES FROM TOXIC SLUDGES AND SOLIDS

The method described below may be used for raw primary sludges and for other sludges toxic to cells used for assaying viruses. Although limited experimental support is available, the method is probably also useful for toxic soils and toxic dredge spoils. See Figure 8-1 for flow diagram of the method.

Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (see Chapters 2 and 3).

1. EXTRACTION OF VIRUSES FROM SLUDGES

   This procedure, which requires Freon, must be done in a hood that is vented to the outdoors.

   1.1 Preparation

      1.1.1 Apparatus and Materials

         (a) pH meter, measuring to an accuracy of 0.1 pH unit, equipped with a combination-type electrode.

         (b) Magnetic stirrer and stir bars.

         (c) Funnel, Buchner, porcelain, Coors, plate diameter 126 mm (American Scientific Co., F7300-8, or equivalent).

Equip funnel with rubber stopper and insert into 2-liter filtering flask. CAUTION: Use only flask that can withstand vacuum applied. Connect rubber tube from sidearm of filtering flask through disinfectant trap to laboratory vacuum line.

(d) Disc filter, AP25, 127 mm diameter (Millipore Corp., AP series, or equivalent).
Place AP25 filter onto plate of Buchner funnel.

(e) Membrane filter apparatus for removing bacteria and fungi -- 47-mm diameter filter holder and 50-mL slip tip syringe (Millipore Corp., Swinnex filter, No. SX0047000, or equivalent for filter holder only).
If final eluate must be concentrated by the organic flocculation procedure of Katzenelson (see Section 2), membrane filter apparatus is not required.

(f) Disc filters, 47-mm diameter -- 3.0-, 0.45-, and 0.25-μm pore size filters (Filterite Corp., Duo-Fine series, or equivalent). Filters must be cut to proper size from sheet filters.
Disassemble Swinnex filter holder. Place filter with 0.25-μm pore size on support screen of filter holder, and stack the remaining filters on top in order of increasing pore size. Reassemble and tighten filter holder. Filters stacked in tandem as described tend to clog more slowly when turbid material is filtered through them. Prepare several filter stacks.

If final eluate must be concentrated by the organic flocculation procedure of Katzenelson (see Section 2), disc filters are not required.

1.1.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$).

(b) Citric acid.

(c) Beef extract powder (Grand Island Biological Co., or equivalent).

Prepare buffered 10% beef extract by dissolving 10 g beef extract powder, 1.34 g $Na_2HPO_4 \cdot 7H_2O$ and 0.12 g citric acid in 100 mL of deionized distilled water. Dissolve by stirring for about two hours on a magnetic stirrer.

(d) Hydrochloric acid (HCl) -- 5 M and 1 M.

(e) Aluminum chloride ($AlCl_3 \cdot 6H_2O$) -- 0.05 M.

(f) Aluminum chloride ($AlCl_3 \cdot 6H_2O$) -- 0.0005 M, pH 3.5 $\pm$ 0.1.

Prepare 500 mL of 0.0005 M $AlCl_3$, and autoclave it at 121° C for 15 minutes. Adjust to pH 3.5 $\pm$ 0.1 with 1 M HCl.

(g) Sodium hydroxide (NaOH) -- 5 M.

(h) Freon (DuPont Freon TF, or equivalent).

Freon does not require sterilization.

1.2 Procedure (See Figure 8-1.1)

1.2.1 Conditioning of Sludge

In the absence of experience that dictates otherwise, use 100-mL volumes for sludges, 100-mL volumes of a 5% suspension in deionized distilled water for dredge spoils

Figure 8-1.1 Virus Recovery Procedure

SLUDGE (100 mL)

↓ On magnetic stirrer, add $AlCl_3$ (to final concentration 0.0005 M), adjust pH of salted sludge to 3.5 ± 0.1 with 5 M HCl, and mix vigorously for 30 minutes.

SALTED, pH-ADJUSTED SLUDGE

↓ Add 75 mL of Freon to mixing sludge, and continue vigorous mixing for 5 minutes.

FREON-TREATED, SALTED, pH-ADJUSTED SLUDGE

↓ Pour sludge-Freon mixture into Buchner funnel that contains an AP25 filter, and apply vacuum. Safely dispose of chemically-microbiologically contaminated flask and contents.

SLUDGE SOLIDS

↓ When solids on filter appear dry, wash solids five times with 100-mL amounts of 0.0005 M $AlCl_3$, pH 3.5 ± 0.1.

WASHED SLUDGE SOLIDS

↓ Pour 100-mL of buffered 10% beef extract (BE) (pH 7.0 ± 0.1) onto washed sludge solids, wait 10 minutes, and apply suction.

ELUATE

↓ If concentration of viruses is not necessary, filter eluate through membrane stack.
If concentration of viruses is necessary, proceed to Virus Concentration Procedure (Figure 8-1.2)

FILTERED ELUATE (10% BE) → Assay eluate (10% BE) for viruses (See Chapter 9).

Figure 8-1.2 Virus Concentration Procedure

ELUATE

↓ Filter eluate through membrane stack.

FILTERED ELUATE (10% BE)

↓ Add sufficient volume of deionized distilled water to filtered eluate to reduce BE concentration from 10% to 3%.
Begin Katzenelson organic flocculation procedure.

DILUTED FILTERED ELUATE (3% BE)

↓ On magnetic stirrer, adjust pH of filtered eluate (3% BE) to 3.5 ± 0.1 with 1 M HCl.
Floc begins to form.
Mix filtered eluate (3% BE) and forming floc on magnetic stirrer for 30 minutes.

FLOCCED ELUATE

↓ Centrifuge flocced eluate at 2,500 x g for 15 minutes at 4° C. Discard supernate, retain floc.

FLOC FROM ELUATE

↓ Add 0.15 M Na_2HPO_4 (1/20th volume of diluted beef extract) to floc, and mix.
Floc dissolves on mixing.

DISSOLVED FLOC — Adjust pH to 7.0-7.5 with 1 M HCl or 1 M NaOH → Assay dissolved floc for viruses (See Chapter 9).

Figure 8-1. Flow Diagram of Method for Recovering and Concentrating Viruses in Toxic Sludges

(v/v) or soils (w/v), and 100-g quantities for digested dewatered sludges and for other samples difficult to measure volumetrically.

(a) Measure 100 mL of well-mixed sludge in a graduated cylinder.

Sludge must be mixed vigorously immediately before it is poured into cylinder because sludge solids, which contain most of the viruses, begin to settle out immediately after mixing stops.

(b) Place stir bar into a 600-mL beaker.

(c) Pour the 100 mL of measured sludge from the 100-mL cylinder into the 600-mL beaker.

It may be necessary to pour sludge several times from beaker to cylinder and back in order to remove all sludge solids to beaker.

CAUTION: Take care to avoid formation of aerosols.

(d) Place beaker on magnetic stirrer, and stir at speed sufficient to develop vortex.

(e) Add 1 mL of 0.05 M $AlCl_3$ to mixing sludge.

Final concentration of $AlCl_3$ in sludge is approximately 0.0005 M.

(f) Place combination-type pH electrode into mixing sludge.

pH meter must be standardized at pH 4.

(g) Adjust pH of sludge to 3.5 $\pm$ 0.1 with 5 M HCl.

If pH falls below 3.4, readjust it with 5 M NaOH.

When sludge adheres to electrodes, clean electrodes by moving them up and down gently in mixing sludge.

(h) Continue mixing for 30 minutes more.

The pH of the sludge should be checked at frequent intervals. If the pH drifts up, readjust it to 3.5 ± 0.1 with 5 M HCl. If the pH drifts down, readjust it with 5 M NaOH.

(i) Remove pH electrode from sludge.

(j) Add 75 mL of Freon to mixing sludge.

All procedures involving Freon must be done in hood vented to outdoors.

(k) Readjust magnetic stirrer to speed sufficient for thorough mixing, and mix for five minutes.

Because Freon is heavier than water and settles to bottom, care must be taken that aqueous and Freon layers intermix thoroughly.

(l) Turn off stirrer.

(m) Turn on vacuum connected to sidearm flask that holds Buchner funnel containing AP25 filter.

(n) Pour sludge-Freon mixture into Buchner funnel.

To prevent transfer of stir bar into Buchner funnel, hold another stir bar or magnet against bottom of beaker when decanting contents.

(o) As soon as sludge solids on AP25 filter begin to appear dry, pour 100 mL of 0.0005 M $AlCl_3$ (pH 3.5 ± 0.1) onto solids.

To avoid possible inactivation of viruses, do not allow filtered solids to dry. Cover all solids on filter in order to ensure thorough wash and maximal removal of toxic substances.

(p) When all wash liquid has passed through solids on filter, repeat wash procedure in Step (o) four more times.

(q) Turn off vacuum.

(r) Replace filtering flask.

Eluted viruses will be collected in second filtering flask. Safely dispose of chemically-microbiologically contaminated flask and contents.

1.2.2 Elution of Viruses from Sludge Solids

(a) Pour 100 mL of buffered 10% beef extract onto solids on AP25 filter.

Cover all solids on filter in order to ensure proper elution. Allow beef extract to remain on solids for ten minutes before going to Step (b).

(b) Turn on vacuum.

(c) When all beef extract has passed through solids on filter, turn off vacuum.

(d) Disconnect tube from sidearm of filtering flask.

(e) Remove Buchner funnel from filtering flask, and discard funnel and contents.

(f) Decant eluate from filtering flask into 250-mL beaker.

The number of cell cultures necessary for the viral assay may be reduced by concentrating the viruses in the beef extract by the organic flocculation procedure of Katzenelson. Some loss of viruses may occur with this procedure. If viruses in eluate

are to be concentrated, proceed immediately to Section 2. If concentration is not required, proceed to Step (g).

(g) Place a filter holder that contains a filter stack on a 250-mL receiving flask.

(h) Load 50-mL syringe with eluate.

(i) Place tip of syringe into filter holder.

(j) Force eluate through filter stack into 250-mL receiving flask.

Take care not to break off tip of syringe and to minimize pressure on receiving flask because such pressure may splinter or topple the flask. If filter stack begins to clog badly, empty loaded syringe into beaker containing unfiltered eluate, fill syringe with air, and inject air into filter stack to force residual eluate from filters. Continue filtration procedure with another filter holder and filter stack. Discard contaminated filter holders and filter stacks. Steps (g) through (j) may be repeated as often as necessary to filter entire volume of eluate. Disassemble filter holder and examine bottom filter to be certain it has not ruptured. If bottom filter has ruptured, repeat Steps (g) through (j) with another filter holder and filter stack.

(k) Refrigerate eluate immediately at 4^{o} C, and maintain it at that temperature until it is assayed for viruses.

If assay for viruses cannot be undertaken within eight hours, store eluate immediately at -70° C.

2. CONCENTRATION OF VIRUSES FROM SLUDGE ELUATES

2.1 Organic Flocculation Concentration Procedure of Katzenelson*

It is preferable to assay eluted viruses in the beef extract eluate without concentrating them because some loss of viruses may occur in concentration. However, the numbers of cell cultures needed for assays may be reduced by concentrating the viruses in the eluate.

2.1.1 Apparatus and Materials

(a) Magnetic stirrer and stir bars.

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

(c) Refrigerated centrifuge capable of attaining 2,500 x g and screw-cap centrifuge bottles.
Each sample centrifuged at 2,500 x g will consist of about 330 mL.

(d) Membrane filter apparatus for sterilization -- 47-mm diameter filter holder and 50-mL slip tip syringe (Millipore Corp., Swinnex filter, No. SX0004700, or equivalent for filter holder only).

(e) Disc filters, 47-mm diameter -- 3.0-, 0.45-, and 0.25-μm pore size filters (Filterite Corp., Duo-Fine

*Katzenelson, E., B. Fattal, and T. Hostovesky. 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl. Environ. Microbiol. 32:638-639.

series, or equivalent). Filters must be cut to proper size from sheet filters.

Disassemble Swinnex filter holder. Place filter with 0.25-μm pore size on support screen of filter holder, and stack the remaining filters on top in order of increasing pore size. Reassemble and tighten filter holder. Filters stacked in tandem as described tend to clog more slowly when turbid material is filtered through them. Prepare several filter stacks.

2.1.2 Media and Reagents

(a) Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) -- 0.15 M.

(b) Hydrochloric acid (HCl) -- 1 M.

(c) Sodium hydroxide (NaOH) -- 1 M.

2.1.3 Procedure (See Figure 8-1.2)

(a) Place filter holder on 250-mL Erlenmeyer receiving flask.

(b) Load 50-mL syringe with eluate from Section 1.2.2, Step (f).

(c) Place tip of syringe into filter holder, and force eluate through filter stack.

Take care not to break off tip of syringe and to minimize pressure on receiving flask because such pressure may splinter or topple the flask. If filter stack begins to clog badly, empty loaded syringe into beaker containing unfiltered eluate, fill syringe with air, and inject air into filter

stack to force residual eluate from filters. Continue filtration procedure with another filter holder and filter stack. Discard contaminated filter holders and filter stacks. Steps (a) through (c) may be repeated as often as necessary to filter entire volume of eluate. Disassemble filter holder and examine bottom filter to be certain it has not ruptured. If bottom filter has ruptured, repeat Steps (a) through (c) with another filter stack.

(d) Remove filter holder from top of Erlenmeyer flask, pour eluate into graduated cylinder, and record volume.

(e) Pour eluate into 600-mL beaker.

(f) For every 3 mL of beef extract eluate, add 7 mL of deionized distilled water to the 600-mL beaker. The concentration of beef extract is now 3%. This dilution is necessary because 10% beef extract does not always process well by the organic flocculation concentration procedure.

(g) Record the total volume of the diluted, filtered beef extract.

(h) Place stir bar in beaker that contains diluted, filtered beef extract.

(i) Place beaker that contains the diluted filtered beef extract on magnetic stirrer, and stir at a speed sufficient to develop vortex.

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

(j) Insert combination-type pH electrode into diluted, filtered beef extract.

pH meter must be standardized at pH 4.

(k) Add 1 M HCl to flask slowly until pH of beef extract reaches 3.5 ± 0.1.

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

(l) Continue to stir for 30 minutes more.

(m) Turn off stirrer.

(n) Remove caps from screw-capped centrifuge bottles.

Use one or more bottles, as needed. Glass centrifuge bottles may not be able to withstand 2,500 x g force that will be applied.

(o) Remove electrode from beaker, and distribute contents of beaker evenly among centrifuge bottles.

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

(p) Replace and tighten down caps on centrifuge bottles.

(q) Centrifuge precipitated beef extract suspensions at 2,500 x g for 15 minutes at 4^{o} C.

(r) Remove caps from centrifuge bottles.

(s) Pour off and discard supernates.

(t) Place a small stir bar into each of the centrifuge bottles that contains precipitate.

(u) Replace covers loosely on centrifuge bottles.

(v) Raise caps from tops of centrifuge bottles, and divide a volume of 0.15 M Na_2HPO_4 equal to 1/20 of the volume recorded in Step (g) equally among the precipitates in the centrifuge bottles.

The volume of 0.15 M Na_2HPO_4 in which the precipitate will be dissolved is equal to 5 mL for each 100 mL of diluted beef extract.

(w) Replace and tighten down caps on centrifuge bottles.

(x) Place each bottle on a magnetic stirrer, and stir each precipitate slowly until precipitate has dissolved completely.

Support bottles as necessary to prevent toppling. Avoid foaming which may inactivate or aerosolize viruses. Precipitate may be partially dissipated with spatula before or during stirring procedure.

(y) Remove caps from centrifuge bottles.

(z) Combine the dissolved precipitates in a small beaker.

To prevent transfer of stir bars into beaker, hold another stir bar or magnet against the bottom of each centrifuge bottle when decanting dissolved precipitates.

(aa) Measure pH of dissolved precipitates.

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

(bb) Refrigerate dissolved precipitates immediately at 4^{o} C, and maintain at that temperature until assay for viruses is undertaken.

If assay for viruses cannot be undertaken within eight hours, store dissolved precipitates immediately at -70^{o} C.

(cc) Assay for viruses in accordance with instructions given in Chapter 9.

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CHAPTER 9

PREPARATION AND USE OF CELL CULTURES

1. INTRODUCTION

This chapter outlines procedures and media for culturing Buffalo green monkey (BGM) kidney cells* and for assaying viruses recovered in these cells. BGM cells are a continuous line derived from African Green monkey kidney cells. The characteristics of this line were described by A. L. Barron, C. Olshevsky, and M. M. Cohen in 1970.** Use of BGM cells for recovering viruses from environmental samples was described by D. R. Dahling, G. Berg, and D. Berman in 1974.***

This chapter is intended as guidance for the competent virologist who is preparing to recover, assay, and identify viruses in environmental samples. Cells other than BGM may be used when deemed preferable by a competent virologist. Although BGM cells are very sensitive to many enteroviruses, these cells are not sensitive

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*BGM cells are available to qualified laboratories and may be obtained from Dr. R. S. Safferman, Chief of Virology, EMSL, U. S. EPA, Cincinnati, Ohio 45268.

**Barron, A. L., C. Olshevsky, and M. M. Cohen. 1970. Characteristics of the BGM Line of Cells from African Green Monkey Kidney. Archiv. for Die Gesamte Virusforschung. 32:389-392.

***Dahling, D. R., G. Berg, and D. Berman. 1974. BGM, A Continuous Cell Line More Sensitive Than Primary Rhesus and African Green Kidney Cells for the Recovery of Viruses from Water, Health Laboratory Sciences. 11:275-282.

to other enteroviruses or to certain other viruses that may occur in environmental samples. Thus, to maximize the numbers of viruses recovered from environmental samples, several cell lines may need to be used.

In this chapter, only the plaque technique for assaying viruses is described. Liquid culture procedures may also be used. Plaque procedures allow greater counting accuracy. Liquid cultures often yield greater sensitivity.

2. PREPARATION

2.1 Apparatus and Materials

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

Storage vessels must be equipped with air-tight closures.

2.1.2 Magnetic stirrer and stir bars.

2.1.3 Autoclavable inner-braided tubing with metal quick-disconnect connectors for tubing to be connected to equipment under pressure.

2.1.4 Positive pressure air or nitrogen source equipped with pressure gauge.

Pressure source, if laboratory air line or pump, must be equipped with oil filter. Deliver to filter holder no more pressure than recommended by manufacturer.

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

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

Use only pressure type filter holders.

2.1.7 Virus-adsorbing disc filters -- 0.22-μm pore size (Millipore Corp., GS series, or equivalent).

2.1.8 Fiberglass prefilters (Millipore Corp., AP15 and AP20, or equivalents).

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

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

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

2.1.10 Screw caps, black with rubber liners (24-414 for 6 oz.* prescription (Rx) bottles, Brockway Glass Co., Inc., or equivalent).

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

Caps for larger culture bottles usually supplied with bottles.

2.1.11 Roller apparatus (7730-Series, Bellco Biological Glassware, or equivalent).

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

2.1.13 Incubator capable of maintaining the temperatures of cell cultures at $36.5^{\circ} \pm 1^{\circ}$ C.

2.1.14 Waterbath, equipped with circulating device to assure even heating at $36.5^{\circ} \pm 1^{\circ}$ C, $56^{\circ} \pm 1^{\circ}$ C, and $60^{\circ} \pm 1^{\circ}$ C.

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

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

2.2 Media and Reagents

To avoid exposure of cells to toxic chemical contaminants, chemicals applied to cell cultures must be reagent grade or equivalent in purity. Compounds such as neutral red, trypan blue, and phenol red that are not usually sold at the reagent grade level must be obtained in the purest form available.

2.2.1 Fetal calf serum, filter-sterilized, heat-inactivated at 56° C for 30 minutes, certified free of viruses and mycoplasma (Grand Island Biological Co., or equivalent). Test toxicity of sample of serum on cells before purchasing serum lot.

2.2.2 Agar (Bacto-Agar, Difco Laboratories, or equivalent).

2.2.3 Milk, sterile, homogenized, whole, fluid (Real-Fresh Brand, or equivalent).

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

2.2.5 Sodium (Tetra) Ethylenediamine Tetraacetate Powder (Versene), technical grade, (Fisher Scientific Company, or equivalent).

2.2.6 Thioglycollate medium (Bacto Dehydrated Fluid Thioglycollate Medium, Difco Laboratories, or equivalent).

2.2.7 Water, sterile, distilled, deionized.
See Chapter 4.

2.2.8 Fungizone, Mycostatin, and Neomycin (E. R. Squibb and Sons, or equivalent), Gentamicin (Schering-Plough Corp., or equivalent), Penicillin G and Dihydrostreptomycin sulfate (Eli Lilly and Co., or equivalent), Tetracycline (Pfizer, Inc., or equivalent).
Use antibiotics of tissue culture or injection grade only.

2.2.9 Vitamins, amino acids, salts, acids, dyes, research grade or best grade available (Sigma Chemical Co., or equivalent).

3. PROCEDURE FOR PREPARATION OF BGM CELL CULTURES

3.1 General Procedures

The BGM cell line grows readily on the inside surfaces of plastic flat-sided vessels, glass bottles, and glass test tubes.

BGM cell cultures can be purchased from several companies for plaque assay or plaque confirmation procedures. Although it is labor intensive for a laboratory to maintain the BGM cell line and to prepare cultures, it is much less expensive to prepare cultures than to purchase them. To reduce the risk of contamination, cell cultures should be prepared in controlled facilities used for no other purpose.

3.1.1 Pass and maintain BGM stock cultures in 16 to 32 oz. (or equivalent in growth area), flat-sided, glass bottles or in plastic cell culture flasks.
If available, roller bottles and roller apparatus units are preferable to flat-sided bottles or flasks for growing cells because roller cultures require less medium than flat-sided bottles per unit of cell monolayer surface. For growing cells in roller bottles, adjust roller apparatus rotation speed to one-half revolution per minute.

3.1.2 Prepare cell cultures for plaque assays in vessels with growth areas of 45 sq. cm (6 oz.) or larger.
Vessels with relatively large surface areas are used to accommodate the large sample inoculums in environmental virology studies. Vessels with smaller surface areas may be used, if necessary. Only flat-sided cell culture vessels can be used for plaque assays.

3.1.3 Prepare cultures for plaque confirmation in 16 x 150 mm glass or plastic cell culture tubes.

3.1.4 Except during handling operations, maintain BGM cells at $36.5^{o} \pm 1^{o}$ C in air-tight cell culture vessels.

(a) Maintain in constant motion roller bottles that contain cells.

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

(c) Maintain cell culture tubes in either stationary racks or in rotating drums slanted upwards at an angle of approximately 15^{o} (almost horizontal) so that the fluid inside extends upward one-third to two-thirds the length of the tube.

Cell culture vessels must be stored in a way such that their liquid contents do not touch the inner surface of the vessels' caps.

3.1.5 To reduce shock to cells, warm growth media, maintenance media, washing solutions used for removing toxic materials from inoculated cell cultures, and all other solutions to $36.5^{o} \pm 1^{o}$ C before placing them on cell monolayers.

3.1.6 Test all media and solutions to be used in cell culture operations to assure their microbiological sterility (see Chapter 4).

3.1.7 Introduce only trypsin-EDTA solution and sample inoculums directly onto cell monolayer surface.

Inoculums are introduced gently onto monolayer surfaces directly to minimize loss of viruses through adherance of inoculums onto other surfaces inside the cell culture vessels.

3.1.8 Introduce all materials other than trypsin-EDTA solution or inoculum onto a part of the inside container wall that is not covered by cell monolayer surface.

This precaution is taken to dissipate force used to introduce fluids.

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

3.1.10 Prepare cultures for plaque assay and plaque confirmation three to seven days before cultures are to be inoculated with virus-containing material.

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

3.2 Procedure for Passage of BGM Cells

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

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

3.2.2 Add to the cell cultures a volume of warm ($36.5^{o} \pm 1^{o}$ C) trypsin-EDTA solution equal to 40% of the volume of medium replaced (see Table 9-1).

Pour the trypsin-EDTA solution directly onto the cells.

3.2.3 Allow trypsin-EDTA solution to remain in contact with the cells at $36.5^{o} \pm 1^{o}$ C until cell monolayer can be shaken loose from inner surface of cell culture vessel (about 10 minutes).

If necessary, a sterile rubber policeman (or scraper) may be used to physically remove the cell sheet from the

TABLE 9-1

Guide for Determining Volume of Cell Suspension, Cell Culture Medium, Virus Sample Inoculum, and Overlay Medium to be Used with Various Sized Cell Culture Vessels

Vessel Type and Size (Flat-Sided Glass or Plastic Bottle)	Volume of Fluid in mL		
	Cell Suspension, Growth, or Maintenance Medium	Virus Sample Inoculum	Agar Overlay Medium
1 oz.*	4	0.1	5
2 oz. or 25 cm^2	8	0.2	10
4 oz.	12	0.3-0.4	15
6 oz.	15	0.5-1.0	20
8 oz. or 75 cm^2	20	0.5-1.5	25
16 oz. or 150 cm^2	40	1.0-3.0	50
32 oz.	50	not commonly used	not commonly used
Roller Apparatus (Glass or Plastic Bottle)			
64 oz.	100	cannot be used	cannot be used
Cell Culture Tube (Glass or Plastic)			
16 x 150 mm	2	0.1-1.0	cannot be used

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

bottle. However, this procedure should be used only as a last resort because of the risk of cell culture contamination inherent in such manipulations.

3.2.4 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.

3.2.5 Centrifuge cell suspension at 1,000 x g for 10 minutes to pellet cells.

3.2.6 Pour off and discard the supernate.

3.2.7 Suspend the pelleted cells in growth medium. The quantity of medium used for resuspending pelleted cells varies from 10 mL to more than 1 liter, depending upon the volume of the individual laboratory's need for cell cultures. Resuspend passaged cells in large volumes of medium to allow thorough mixing of cell pellets (to reduce sampling error) and to minimize the significance of the loss of the 1 mL of cell suspension required for the cell counting procedure. Do not dilute cells to a concentration of less than 6×10^5 per mL, because viable cell counts (see Section 3.3) cannot be done with lesser concentrations of cells.

3.2.8 Perform a viable cell count on this concentrated suspension (see Section 3.3).

3.2.9 Dilute the cell suspension in growth medium to a concentration of approximately 2.5×10^5 viable cells per mL of medium.

3.2.10 Dispense the cell suspension into cell culture vessels.

The quantities of suspension that must be used for culture vessels of different sizes are shown in Table 9-1.

3.3 Procedure for Performing Viable Cell Counts

3.3.1 Add 0.5 mL of cell suspension (or diluted cell suspension) to 1.0 mL of 0.5% trypan blue solution.

Dilution compensated for in Section 3.3.6, Step (b).

3.3.2 Disperse cells by repeated pipetting.

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

3.3.3 With a capillary pipette, carefully fill hemocytometer chambers on both sides of a slip-covered hemocytometer slide.

Do not under or over fill the chambers.

3.3.4 Rest slide on flat surface for about 10 minutes to allow trypan blue to penetrate cell membranes of nonviable cells.

3.3.5 Under 100X total magnification, count and total the cells in the four large corner sections of both hemocytometer chambers.

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 only viable cells, count only cells that are clear in color. Do not count cells that are blue.

3.3.6 Determine the concentration of viable cells in the cell suspension (or diluted cell suspension) with the following equations:

(a)	total number of viable cells in the 8 sections	÷ 8	=	average number of viable cells per section
(b)	average number of viable cells per section	x 3 x 10,000	=	average number of viable cells per mL of cell suspension (or per mL of diluted cell suspension)

To obtain an accurate cell count, the optimal total number of cells per hemocytometer section should be between 20 and 50. This range is equivalent to between 6.0×10^5 and 1.5×10^6 cells per mL of cell suspension. If the cell suspension contains a larger concentration of cells, that portion of the cell suspension to be used for the counting procedure may first require dilution with growth medium. If such a dilution is made, be certain to factor this dilution into the cell count.

3.4 Procedure for Changing Medium on Cultured Cells

Three to four days after seeding with an appropriate number of cells, monolayers normally become 95 to 100% confluent, and growth medium becomes acidic. Growth medium on confluent stock cultures should then be replaced with maintenance medium.

If stock culture cell monolayers have not reached 95 to 100% confluency by this time and the medium on these cultures has not become acidic, then the medium should not be changed until the monolayers do reach 95 to 100% confluency. If three to four days after passage, monolayers are not yet 95 to 100% confluent and the medium in which they are immersed has become acidic, then the medium must be replaced with fresh growth medium instead of with maintenance medium.

It should not be necessary to replace growth medium on cultures for plaque assay or plaque confirmation until four hours or less before cultures are to be inoculated with viruses. If these cultures are not needed for plaque assay or plaque confirmation at the time they become confluent and the growth medium acidic, replace the growth medium with maintenance medium.

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

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

4. PLAQUE PROCEDURE FOR RECOVERING OR TITRATING VIRUSES

To titrate viruses, inoculate multiple dilutions in appropriate numbers of replicate cell cultures.

4.1 Inoculating Sample onto Cell Cultures

4.1.1 From one to four hours before cultures are to be inoculated, replace medium in culture vessels with an equal volume of maintenance medium.

4.1.2 Maintain cultures at $36.5^{\circ} \pm 1^{\circ}$ C until they are to be inoculated.

4.1.3 Before culture vessels are inoculated, identify them with an indelible marker.

4.1.4 Pour medium from cell culture vessels, and discard medium.

4.1.5 Carefully inoculate into each culture vessel the volume of sample which is correct for vessels of that size.

See Table 9-1 for inoculum sizes appropriate for commonly used cell culture vessels.

If inoculum may be toxic, see Section 4.3.

4.1.6 Immediately rock inoculated culture vessel gently to achieve uniform distribution of sample on surface of cell monolayer.

Uniform distribution of sample inoculum results in uniform distribution of plaques and thereby facilitates accurate plaque counting.

4.1.7 Incubate inoculated cell cultures for two hours at room temperature (22-25^{0} C) to permit viruses to adsorb onto and to infect cells.

4.1.8 Apply warm (46^{0} C) agar overlay medium to each culture vessel.

See Table 9-1 for the amount of overlay medium that should be added.

Pour medium onto side of cell culture vessel opposite the cell monolayer, allow medium to cool momentarily, and then place the culture vessel, monolayer side down, on a stationary table or bench at room temperature (22-25^{0} C) so that the agar will distribute evenly as it solidifies.

4.1.9 Cover cell culture vessels with a sheet of aluminum foil, a tightly woven cloth, or some other suitable cover to prevent photoinactivation of virions.

Agar begins to harden almost immediately and fully solidifies in 30 to 60 minutes.

4.1.10 Invert culture vessels, and incubate in the dark at $36.5^{o} \pm 1^{o}$ C.

4.2 Counting Viral Plaques

4.2.1 Begin counting and marking plaques in cultures two days after overlaying, and repeat procedure every two days for a total of 10 days (for enteroviruses) after overlaying.

4.2.2 Record plaque counts at each reading.

Virus titers are calculated from total count.

4.2.3 Calculate virus titers (plaque-forming units [PFU]) for each inoculated virus-containing sample.

Total number of plaques per culture vessel (or average number of plaques per culture vessel if several vessels have been inoculated with the same sample) to obtain the virus titer of sample in terms of PFU per inoculum volume. To obtain PFU per mL, multiply the number of PFU by the reciprocal of the inoculum volume (and by the dilution, if a dilution was made).

4.3 Reduction of Sample-Associated Toxicity

This procedure may result in the loss of virions and is to be used only if there is a likelihood that inoculum will be toxic to cell cultures.

4.3.1 Inoculate cell cultures with samples that contain viruses.

4.3.2 Incubate inoculated cultures for two hours at room temperature (22-25^{0} C) to allow virions to adsorb onto and to infect cells.

4.3.3 Pour inoculum from cell culture vessels, discard inoculum, and add to each culture 1 mL of washing solution [0.85% (w/v) NaCl containing 2% (v/v) fetal calf serum] for each 5-cm^{2} of cell surface area.

4.3.4 Gently rock the washing solution twice across the cell monolayer, and pour off and discard the washing solution.

4.3.5 Overlay washed cultures with agar overlay medium.
See Section 4.1.8.
See Table 9-1 for quantity of overlay required.

5. PROCEDURE FOR VERIFYING STERILITY OF LIQUIDS

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

5.1 Procedure for Verifying Sterility of Small Volumes of Liquids

5.1.1 Inoculate 5-20 mL, as appropriate, of the material to be

tested for sterility into sterile thioglycollate broth (20-100 mL, as appropriate).

5.1.2 Shake the mixture, and incubate it at $36.5^{\circ} \pm 1^{\circ}$ C.

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

Vessels that contain thioglycollate medium must be tightly sealed before and after medium is inoculated. In some instances, growth medium that contains $NaHCO_3$ but no antibiotics may be used as detection medium.

5.2 Procedure for Verifying Sterility of Large Volumes of Liquids

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

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

5.2.3 Incubate Petri dish at $36.5^{\circ} \pm 1^{\circ}$ C, and examine filter surface daily for seven days to determine whether growth of contaminating organisms has occurred.

6. PREPARATION OF CELL CULTURE MEDIA

This section is a guide for preparation of media for growing BGM cells and for performing agar overlay plaque assays for viruses that multiply in BGM cells.

6.1 Technique

6.1.1 Equipment Care

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

6.1.2 Disinfection of Work Area

Thoroughly disinfect surfaces on which media preparation equipment is to be placed.

6.1.3 Aseptic Technique

Use aseptic technique when preparing and handling media or media components.

6.1.4 Dispensing Filter-Sterilized Media

To avoid post-filtration contamination, dispense filter-sterilized media into storage vessels in a microbiological laminar flow hood.

Dispense sterile media into storage containers through clear glass filling bells.

6.2 Sterility Testing

6.2.1 Coding Media

Assign a lot number to each batch of media or media component prepared.

6.2.2 Sterility Test

Test each lot of medium and medium components to confirm sterility before the lot is used for cell culture or plaque assay (See Section 5).

6.2.3 Storage of Media and Media Components

Store media and media components in clear air-tight containers.

6.2.4 Sterilization of $NaHCO_3$-containing Solutions

Sterilize media and other solutions that contain $NaHCO_3$ by positive pressure filtration.

Negative pressure filtration of such solutions increases their pHs and reduces their buffering capacities.

6.3 Media Formulations

6.3.1 Sources of Cell Culture Media

Commercially-prepared liquid cell culture media and media components are available from several sources. Cell culture media can also be purchased in powder form that requires only dissolution in deionized distilled water and sterilization. Media from commercial sources are usually quality controlled carefully and quite adequate. However, media can also be prepared in the laboratory from chemicals. Such preparations are labor intensive and may be expensive but allow quality control of the process at the level of the preparing laboratory.

6.3.2 Constraints, Modifications, and Conditions in Media Formulations

(a) Do not attempt to prepare Leibovitz's L-15 medium in a form more concentrated than that normally used for growing cells (1X concentration).

(b) Prepare Eagle's minimum essential medium (MEM) in concentrations indicated below (up to 10X greater than that normally used for growing cells). Certain components for Eagle's MEM may be prepared in concentrations up to 100X greater than those normally used for growing cells (See below).

7. PREPARATION OF MEDIA AND STAINS FOR CELL CULTURES

7.1 Growth Medium

7.1.1 Formula (Preparation of 1 liter)

Eagle's MEM with Hanks' BSS	450.0 mL
Leibovitz's L-15 medium	450.0 mL
$NaHCO_3$, 7.5% solution	7.0 mL
Fetal calf serum	100.0 mL
Penicillin-streptomycin, stock solution	1.0 mL
Tetracycline, stock solution	0.5 mL
Fungizone, stock solution	0.2 mL

7.1.2 Procedure

(a) Filter through a 0.22-μm membrane any ingredient in the formula that is not sterile.

(b) Place 450 mL of Eagle's MEM with Hanks' BSS into a clear glass vessel.

(c) Maintain continuous stirring.

(d) Add Leibovitz's L-15 medium and 7.5% $NaHCO_3$ to the Eagle's MEM with Hanks' BSS.

(e) Store medium at 4^{o} C until needed.

(f) Add fetal calf serum and antibiotics to medium immediately before medium is used.

7.2 Maintenance Medium

7.2.1 Formula (Preparation of 1 liter)

If medium is to be used for washing cells before cell cultures are to be inoculated with viruses, replace the 50 mL of fetal calf serum with 50 mL of deionized distilled water.

Deionized distilled water	850.0 mL
Earle's BSS 10X stock	100.0 mL
Phenol red, 0.5%	1.0 mL
$NaHCO_3$, 7.5%	7.0 mL
Fetal calf serum	50.0 mL
Penicillin-streptomycin, stock solution	1.0 mL
Tetracycline, stock solution	0.5 mL
Fungizone, stock solution	0.2 mL

7.2.2 Procedure

(a) Filter through a 0.22-μm membrane any ingredient in the formula that is not sterile.

(b) Place 850 mL of deionized distilled water into a clear glass vessel.

(c) Maintain continuous stirring.

(d) Add Earle's BSS 10X stock, phenol red, and 7.5% $NaHCO_3$ to the deionized distilled water.

(e) Store medium at 4° C until needed.

(f) Add fetal calf serum and antibiotics to medium immediately before medium is used.

7.3 Agar Overlay Medium

7.3.1 Formula (Preparation of 1 liter)

Mixture A

2X Eagle's MEM without phenol red for use in overlay medium	415.0 mL
Fetal calf serum	20.0 mL
$NaHCO_3$, 7.5%	30.0 mL
$MgCl_2$, 1%	10.0 mL
Neutral red, 0.1%	15.0 mL
Penicillin-streptomycin, stock solution	1.0 mL
Tetracycline, stock solution	0.5 mL
Fungizone, stock solution	0.2 mL

Mixture B

Agar	15.0 g
Deionized distilled water	500.0 mL

7.3.2 Procedure

Do not prepare Mixtures A and B in advance of the day on which they are to be used.

(a) Mixture A

(a.1) Filter through a 0.22-μm membrane any ingredient in the formula that is not sterile.

(a.2) Place 415 mL of 2X Eagle's MEM without phenol red into a clear glass vessel.

(a.3) Maintain constant stirring.

(a.4) Add the fetal calf serum, $NaHCO_3$, $MgCl_2$, neutral red, and antibiotics to the 2X Eagle's MEM (Mixture A).

(a.5) Warm Mixture A to $36.5^{\circ} \pm 1^{\circ}$ C in a water bath.

If Mixture A is prepared more than one hour before it is to be used, store it at 4° C, and warm it to $36.5^{\circ} \pm 1^{\circ}$ C about 30 minutes before it is needed.

(b) Mixture B

(b.1) Place 500 mL of deionized distilled water into a glass vessel that can withstand autoclaving.

(b.2) Add the agar to the deionized distilled water.

(b.3) Autoclave agar and water at 121° C for 15 minutes.

(b.4) Cool dissolved agar to $56^{\circ} \pm 1^{\circ}$ C in a water bath.

(c) Immediately before overlay medium is to be placed on cell cultures, combine Mixtures A and B and add 10 mL of sterile whole milk.

(d) Mix quickly by swirling.

If a large number of cell cultures is to be overlayed, maintain medium in a $36.5^{\circ} \pm 1^{\circ}$ C water bath during overlay procedure.

(e) Overlay cells immediately.

See Table 9-1 for quantity of overlay required.

7.4 Eagle's Minimum Essential Medium (MEM) with Hanks' Balanced Salt Solution

7.4.1 Formula (Preparation of 1 liter)

This medium may also be prepared as a ten-fold (10X) concentrate, and components of the formula may be prepared in even higher concentrations (See below). Formulations for preparing this medium in 1X and 2X concentrations (the latter for use in preparing overlay medium for plaque assays) from more concentrated sub-components are presented in later sections.

Deionized distilled water	750.0 mL
Inorganic salts	
$CaCl_2$	140.0 mg
KCl	400.0 mg
KH_2PO_4	.60.0 mg
$MgCl_2 \cdot 6H_2O$	100.0 mg
$MgSO_4 \cdot 7H_2O$	100.0 mg
NaCl	8000.0 mg
Na_2HPO_4	60.0 mg
Amino Acids	
L-Arginine HCl	84.0 mg
L-Cystine	48.0 mg
L-Glutamine	300.0 mg
Glycine	30.0 mg
L-Histidine HCl-H_2O	42.0 mg
L-Isoleucine	105.0 mg
L-Leucine	105.0 mg
L-Lysine HCl	146.2 mg

L-Methionine	30.0 mg
L-Phenylalanine	66.0 mg
L-Serine	42.0 mg
L-Threonine	95.0 mg
L-Tryptophan	16.0 mg
L-Tyrosine	73.0 mg
L-Valine	93.6 mg

Vitamins

Choline chloride	4.0 mg
Folic acid	4.0 mg
i-Inositol	7.0 mg
Nicotinamide	4.0 mg
Pantothenic acid	4.0 mg
Pyridoxal HCl	4.0 mg
Riboflavin	0.4 mg
Thiamine HCl	4.0 mg

Other components

Glucose	1000.0 mg
Phenol red	5.0 mg

7.4.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add the ingredients listed to the deionized distilled water.

Allow each ingredient to go into solution before adding the next ingredient.

(d) Adjust the volume of the solution to 1 liter with deionized distilled water.

(e) Filter-sterilize medium through a 0.22-μm membrane.

(f) Store medium at 4^{o} C.

7.5 Eagle's Minimum Essential Medium for Use in Preparing Growth Medium

7.5.1 Formula (Preparation of 1 liter)

Solution A

Deionized distilled water	750 mL
Hanks' BSS 10X stock	100 mL
Phenol red, 0.5%	1 mL
Vitamins 100X stock	10 mL
Amino acids 100X stock	10 mL

Solution B

Deionized distilled water	25 mL
L-Tyrosine	73 mg

Solution C

Deionized distilled water	25 mL
L-Cysteine	48 mg
NaOH, 1N	As needed

7.5.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add to the deionized distilled water the ingredients for Solution A.

Allow each ingredient to go into solution before adding the next ingredient.

(d) Dissolve 73 mg of L-tyrosine in 25 mL of deionized distilled water (Solution B).

Boil over bunsen burner or hot plate until the L-tyrosine goes into solution.

(e) Cool Solution B, and add Solution B to Solution A.

(f) Dissolve 48 mg of L-cysteine into 25 mL of deionized distilled water, and add 1N NaOH until L-cysteine is neutralized (Solution C).

(g) Add Solution C to Solutions A and B.

(h) Adjust volume of medium (Solution A, B, C) to 1 liter with deionized distilled water.

(i) Filter-sterilize medium through a 0.22-μm membrane.

(j) Store medium at 4^{o} C.

7.6 2X Eagle's Minimum Essential Medium Without Phenol Red for Use in Overlay Medium

7.6.1 Formula (Preparation of 1 liter)

Solution A

Deionized distilled water	625 mL
Hanks' BSS 10X stock	200 mL
Vitamins 100X stock	20 mL
Amino acids 100X stock	20 mL

Solution B

Deionized distilled water	50 mL
L-Tyrosine	146 mg

Solution C

Deionized distilled water	50 mL
L-Cysteine	96 mg
NaOH, 1N	As needed

7.6.2 Procedure

(a) Place 625 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add to the deionized distilled water the ingredients for Solution A.
Allow each ingredient to go into solution before adding the next ingredient.

(d) Dissolve 146 mg of L-tyrosine into 50 mL of deionized distilled water (Solution B).

Boil over bunsen burner or hot plate until the L-tyrosine goes into solution.

(e) Cool Solution B, and add Solution B to Solution A.

(f) Dissolve 96 mg of L-cysteine into 50 mL of deionized distilled water, and add 1N NaOH until L-cysteine is neutralized (Solution C).

(g) Add Solution C to Solutions A and B.

(h) Adjust volume of medium (Solutions A, B, C) to 1 liter with deionized distilled water.

(i) Filter-sterilize medium through a 0.22-μm membrane.

(j) Store medium at 4^{o} C.

7.7 Hanks' Balanced Salt Solution (Hanks' BSS) 10X Stock

7.7.1 Formula (Preparation of 1 liter)

Deionized distilled water	750 mL
NaCl	80000 mg
KCl	4000 mg
$MgSO_4 \cdot 7H_2O$	1000 mg
Na_2HPO_4	600 mg
KH_2PO_4	600 mg
$MgCl_2 \cdot 6H_2O$	1000 mg
$CaCl_2$	1400 mg
Glucose	10000 mg

7.7.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add ingredients to the deionized distilled water in the order listed.

Allow each ingredient to go into solution before adding the next one.

(d) Adjust volume of solution to 1 liter with deionized distilled water.

(e) Filter-sterilize medium through a 0.22-μm membrane.

(f) Store medium at 4° C.

7.8 100X Amino Acids Stock for Eagle's Minimum Essential Medium (Without Cysteine and Tyrosine)

7.8.1 Formula (Preparation of 1 liter)

Deionized distilled water	750 mL
L-Arginine HCl	8400 mg
L-Histidine HCl·H_2O	4200 mg
L-Isoleucine	10500 mg
L-Leucine	10500 mg
L-Lysine HCl	14620 mg
L-Methionine	3000 mg
L-Phenylalanine	6600 mg
L-Threonine	9500 mg
L-Tryptophan	1600 mg
L-Valine	9360 mg
Glycine	3000 mg
L-Serine	4200 mg
L-Glutamine	30000 mg

7.8.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel, and bring water to $60^{o} \pm 1^{o}$ C in a water bath.

(b) Add an amino acid (other than L-Glutamine) to the water.

(c) Remove flask from waterbath, and stir over magnetic stirrer until amino acid dissolves completely.

(d) Return vessel to water bath, warm solution in vessel to $60^{\circ} \pm 1^{\circ}$ C, weigh out another amino acid, and repeat procedure until all amino acids (except L-glutamine) have been dissolved.

(e) Cool solution of amino acids to 4° C.

(f) Add L-glutamine, and stir solution until the L-glutamine has dissolved.

(g) Adjust volume of solution (amino acid stock) to 1 liter with deionized distilled water.

(h) Filter-sterilize solution through a 0.22-μm membrane.

If amino acid stock is to be used for preparing a medium that subsequently will be filter-sterilized, filter-sterilization of amino acids stock is unnecessary.

(i) Store stock solution at 4° C.

If amino acid stock solution is to be stored without filter sterilization, store at -20° C.

7.9 100X Vitamins Stock for Eagle's Minimum Essential Medium

7.9.1 Formula (Preparation of 1 liter)

Solution A

Deionized distilled water	750 mL
Choline chloride	400 mg
Nicotinamide	400 mg
Pantothenic acid	400 mg
Pyridoxal HCl	400 mg
Thiamine HCL	400 mg
Riboflavin	40 mg
i-Inositol	700 mg

Solution B

Deionized distilled water	125 mL
Folic acid	400 mg
NaOH, 1N	As needed

7.9.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add to the deionized distilled water the ingredients listed under Solution A. Allow each ingredient to go into solution before adding the next one.

(d) Dissolve the folic acid in 125 mL of deionized distilled water by continuous stirring.

(e) Add 1N NaOH slowly to the folic acid solution until that solution becomes clear (Solution B).

(f) Add Solution B to Solution A.

(g) Adjust volume of combined solution (vitamin stock) to 1 liter with deionized distilled water.

If vitamin stock solution appears turbid, add 1N NaOH until solution becomes clear.

(h) Filter-sterilize stock solution through a 0.22-μm membrane.

If vitamin stock is to be used for preparing a medium that subsequently will be filter-sterilized, filter-sterilization of vitamin stock is unnecessary.

(i) Store vitamin stock solution at 4^{o} C.

If vitamin stock solution is to be stored without filter-sterilization, store at -20^{o} C.

7.10 Leibovitz's L-15 Medium

7.10.1 Formula (Preparation of 1 liter)

This medium can be prepared only in a 1X concentration; it cannot be prepared in more concentrated form.

Deionized distilled water	750 mL
INORGANIC SALTS	
$CaCl_2$	140.0 mg
KCl	400.0 mg
KH_2PO_4	60.0 mg
$MgCl_2 \cdot 6H_2O$	200.0 mg
$MgSO_4 \cdot 7H_2O$	200.0 mg
NaCl	8000.0 mg
Na_2HPO_4	190.0 mg
AMINO ACIDS	
L-Alanine	225.0 mg
L-Arginine	500.0 mg
L-Asparagine	250.0 mg
L-Cysteine	120.0 mg
L-Glutamine	300.0 mg
Glycine	200.0 mg
L-Histidine	250.0 mg
L-Isoleucine	125.0 mg
L-Leucine	125.0 mg
L-Lysine	75.0 mg
L-Methionine	75.0 mg
L-Phenylalanine	125.0 mg
L-Serine	200.0 mg
L-Threonine	300.0 mg
L-Tryptophan	20.0 mg
L-Tyrosine	300.0 mg
L-Valine	100.0 mg

VITAMINS

DL-Ca Pantothenate	1.0 mg
Choline chloride	1.0 mg
Folic acid	1.0 mg
i-Inositol	2.0 mg
Nicotinamide	1.0 mg
Pyridoxine HCl	1.0 mg
Riboflavin-5'-phosphate, sodium	0.1 mg
Thiamine monophosphate	1.0 mg

OTHER COMPONENTS

D (+) Galactose	900.0 mg
Phenol red	10.0 mg
Sodium pyruvate	550.0 mg

7.10.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add the ingredients listed to the deionized distilled water.

Allow each ingredient to go into solution before adding the next one. Add the phenol red last so that complete dissolution of each component can be ascertained.

(d) Adjust volume of medium to 1 liter with deionized distilled water.

(e) Filter-sterilize medium through a 0.22-μm membrane.

(f) Store medium at 4^{o} C.

7.11 Earle's Balanced Salt Solution (Earle's BSS) 10X Stock

7.11.1 Formula (Preparation of 1 liter)

Deionized distilled water	625 mL
NaCl	68000 mg
KCl	4000 mg
$MgSO_4 \cdot 7H_2O$	2000 mg
$NaH_2PO_4 \cdot H_2O$	1250 mg
$CaCl_2$	2000 mg
Glucose	10000 mg

7.11.2 Procedure

(a) Place 625 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add ingredients, in the order listed, to the deionized distilled water.

Allow each ingredient to go into solution before adding the next one.

(d) Adjust volume of solution to 1 liter with deionized distilled water.

(e) Filter-sterilize stock solution through a 0.22-μm membrane.

(f) Store stock solution at 4^{o} C.

7.12 Sodium Bicarbonate, 7.5%

7.12.1 Formula (Preparation of 1 liter)

Deionized distilled water . . . . . 750 mL

$NaHCO_3$ . . . . . 75 g

7.12.2 Procedure

(a) Place 750 mL of COLD deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add $NaHCO_3$ to the deionized distilled water, and stir until the $NaHCO_3$ is completely dissolved.

(d) Adjust volume to 1 liter with deionized distilled water.

(e) Filter-sterilize solution through a 0.22-μm membrane.

Use positive pressure filtration only.

(f) Dispense solution into glass vessels immediately after filtration.

Use only vessels with air-tight rubber stoppers, or with air-tight screw caps.

(g) Store solution at 4^{o} C.

7.13 Magnesium Chloride, 1%

7.13.1 Formula (Preparation of 1 liter)

Deionized distilled water . . . . . 1 liter

$MgCl_2 \cdot 6H_2O$ . . . . . 10 g

7.13.2 Procedure

(a) Place 1 liter of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add the $MgCl_2 \cdot 6H_2O$ to the deionized distilled water.

Stir until the $MgCl_2$ is completely dissolved.

(d) Autoclave solution at 121° C for 30 minutes.

(e) Store solution at 4° C.

7.14 Trypsin-EDTA ($Na_2EDTA \cdot 2H_2O$)* Solution

7.14.1 Formula (Preparation of 1 liter)

(a) Solution A

Deionized distilled water	220 mL
Trypsin, 1:250	3000 mg

Trypsin 1:300 may be substituted for trypsin 1:250. If trypsin 1:300 is used, use 2.5 g of trypsin instead of 3.0 g.

(b) Solution B

Deionized distilled water	778 mL
NaCl	8000 mg
KCl	200 mg
KH_2PO_4	200 mg
$Na_2HPO_4 \cdot 7H_2O$	1150 mg
Glucose	5000 mg
EDTA* (Versene)	1250 mg

*Disodium EDTA dihydrate ($Na_2C_{10}H_{14}O_8N_2 \cdot 2H_2O$)

(c) Additional components

HCl, 1N . . . . . As needed

NaOH, 1N . . . . . As needed

7.14.2 Procedure

(a) Solution A

(a.1) Place 220 mL of deionized distilled water into a clear glass vessel.

(a.2) Maintain constant stirring.

(a.3) Add the trypsin to the deionized distilled water.

(a.4) Stir trypsin in water on a magnetic stirrer until all of the trypsin is dissolved. Expect to stir for at least two hours.

(b) Solution B

(b.1) Place 778 mL of deionized distilled water into a clear glass vessel.

(b.2) Maintain constant stirring.

(b.3) Add NaCl, KCl, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, glucose, and EDTA to the deionized distilled water.

(b.4) Stir until all ingredients are dissolved. Expect to stir for at least two hours.

(c) Add Solution A to Solution B.

(d) Stir for two hours.

(e) Adjust pH of trypsin solution (Solution A and Solution B combined) to 7.5-7.7 with HCl or NaOH.

(f) Filter-sterilize trypsin solution through a 0.22-μm membrane.

(g) Store trypsin solution at 4^{o} C or at -20^{o} C.

7.15 Neutral Red, 0.1%

7.15.1 Formula (Preparation of 1 liter)

Deionized distilled water . . . . . 1 liter

Neutral red . . . . . 1 g

7.15.2 Procedure

(a) Place 1 liter of deionized distilled water into a clear glass vessel.

(b) Maintain constant mixing.

(c) Add neutral red to the deionized distilled water. Stir until the neutral red is completely dissolved.

(d) Filter-sterilize neutral red solution through a 0.22 μm membrane.

(e) Store neutral red solution in the dark at ambient temperatures.

7.16 Phenol Red, 0.5%

7.16.1 Formula (Preparation of 1 liter)

Deionized distilled water . . . . . 1 liter

Phenol red . . . . . 5 g

NaOH, 1N . . . . . As needed

7.16.2 Procedure

(a) Place 750 mL of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add phenol red to the deionized distilled water.

(d) Place pH electrodes into the mixture, and adjust pH to 7 with 1N NaOH.

Stir until phenol red has dissolved completely.

(e) Adjust volume of phenol red solution to 1 liter with deionized distilled water.

(f) Autoclave phenol red solution at 121° C for 15 minutes.

(g) Store phenol red solution at 4° C.

7.17 Trypan Blue Solution (0.5%) for Cell Counting Procedure

7.17.1 Formula (Preparation of 1 liter)

Deionized distilled water . . . . 1 liter

Trypan blue . . . . . 5.0 g

NaCl . . . . . 8.5 g

7.17.2 Procedure

(a) Place 1 liter of deionized distilled water into a clear glass vessel.

(b) Maintain constant stirring.

(c) Add the trypan blue and NaCl to the deionized distilled water.

Stir until trypan blue and NaCl have dissolved completely.

(d) Autoclave trypan blue stain at 121° C for 15 minutes.

(e) Store trypan blue stain at ambient room temperature.

7.18 Stock Solutions of Antibiotics for Cell Culture and Overlay Media

7.18.1 Formula (Stock Solutions)

Amphotericin B (Fungizone)	5 mg/mL
Gentamicin sulfate	50 mg/mL
Nystatin (Mycostatin)	50,000 units/mL
Neomycin sulfate	10 mg/mL
Penicillin-streptomycin	
Penicillin G	100,000 units/mL
Dihydrostreptomycin sulfate (80% active)	125 mg/mL
Tetracycline hydrochloride	25 mg/mL

7.18.2 Procedure

(a) Prepare stock antibiotic solutions and suspensions according to manufacturer's instructions.
<u>If stock antibiotic solutions are not purchased in a sterile form, they must be filter-sterilized through a 0.22-μm membrane before they are used.</u>

(b) Store antibiotic solutions at 4° C except amphotericin B.
<u>Store amphotericin B at -20° C.</u>

7.18.3 Use Levels for Stock Solutions of Antibiotics/100 mL of Medium

(a)	Amphotericin B	0.02 mL
(b)	Gentamicin sulfate	0.10 mL
(c)	Nystatin*	0.20 mL

*Nystatin may be used in place of or in addition to amphotericin B to control fungi.

(d) Penicillin G-dihydrostreptomycin sulfate . . 0.15 mL

(e) Tetracycline hydrochloride . . . . . 0.05 mL

(f) Neomycin sulfate** . . . . . 0.10 mL

**Neomycin may be used in agar overlays when microorganisms resistant to the antibiotics normally used in the overlays are encountered.

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CHAPTER 10

VIRUS PLAQUE CONFIRMATION PROCEDURE

The technique described in this chapter may be used for confirming viral plaques in cell cultures adhering to glass or plastic surfaces or in cells suspended in overlay agar.

Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (see Chapters 2 and 3).

1. RECOVERY OF VIRUS FROM PLAQUE

1.1 Apparatus and Materials

1.1.1 Disposable Pasteur pipettes -- 22.9 cm (long tip).

Flame pipette gently about 2 cm from end of tip until tip bends to approximate angle of 45^{o}.

1.1.2 Rubber bulb -- 1 mL capacity.

1.1.3 Cell culture in roller tube.

Use culture appropriate for virus likely to be recovered.

1.1.4 Test tube rack for roller tube cultures.

1.1.5 Storage medium, Earle's balanced salt solution containing 2% heat-inactivated fetal calf serum (see Chapter 9).

Storage medium is necessary only if plaque sample material is to be stored before confirmation procedure is completed. Whenever possible, plaque sample material should be inoculated onto a cell culture immediately, because storage of such sample material even at -70^{o} C may result in some reduction in confirmation counts.

1.1.6 Five-mL screw-capped (with rubber insert) vial.

Place 2 mL of storage medium in 5-mL screw-capped vial.

1.2 Procedure

1.2.1 Procedure for obtaining viruses from plaque.

Decision to test plaque material for viruses immediately or to store material at -70° C for later testing must be made before proceeding further.

(a) Place rubber bulb onto top of Pasteur pipette.

(b) Remove screw cap or stopper from plaque bottle (If plaque is in petri dish, raise cover from dish sufficiently to allow entry into dish).

(c) Squeeze rubber bulb on top of Pasteur pipette to expel air.

(d) Penetrate agar directly over edge of plaque with tip of Pasteur pipette.

(e) Gently force tip of pipette through agar to surface of vessel, and scrape cells from edge of plaque.
If cells are present as a monolayer on the surface of the vessel, surface must be repeatedly scratched and gentle suction applied to insure that virus-cell-agar plug enters pipette. If cells are suspended in the agar, scraping of vessel surface with pipette is unnecessary.

(f) Aspirate plug from plaque into pipette.

(g) Remove pipette from plaque bottle (or petri dish).

(h) Replace and tighten down screw cap or stopper on plaque bottle (If plaque is in petri dish, replace cover on dish).

If sample is to be tested in cell culture immediately, proceed to Section 1.2.2, Step (a). If sample must be stored, proceed to Section 1.2.2, Step (b).

1.2.2 Procedure for inoculating viruses obtained from plaques onto cell cultures.

(a) Procedure for samples tested immediately.

(a.1) Remove cap from cell culture tube.

(a.2) Place tip of Pasteur pipette containing virus-cell-agar plug into medium in cell culture tube.

Tilt cell culture tube as necessary to facilitate procedure.

(a.3) Force agar from Pasteur pipette into cell culture medium by gently squeezing rubber bulb.

Squeeze bulb repeatedly to wash contents of pipette into cell culture medium.

(a.4) Withdraw pipette from cell culture tube, replace and tighten down screw-cap on tube, and discard pipette.

(a.5) Place cell culture tube in rack for roller tube cultures.

(a.6) Incubate cell culture at 36.5^{o} C $\pm$ 1^{o} C, and examine cells daily for cytopathic effects (CPE).

Incubate under conditions and for a period of time appropriate for the virus likely to be recovered. See Chapter 9 for cell culture and cell culture examination techniques.
If confirmation is to be completed by identifying viruses (enteroviruses) recovered, proceed to Chapter 11.

(b) Procedure for samples stored at -70° C before testing.

(b.1) Thaw vial containing storage medium in a 36° C water bath, and remove cap from vial.

(b.2) Place tip of Pasteur pipette containing virus cell-agar plug into storage medium.

(b.3) Force agar from Pasteur pipette into storage medium by gently squeezing rubber bulb. Squeeze bulb repeatedly to wash contents of pipette into storage medium.

(b.4) Withdraw pipette from vial, replace and tighten down screw-cap onto vial, and discard pipette.

(b.5) Store vial at -70° C.
When confirmation is to be completed, thaw sample quickly in warm water, and proceed to Step (b.6).

(b.6) Remove cap from cell culture tube.

(b.7) Remove cap from storage vial containing thawed sample.

(b.8) With a 2-mL pipette, inoculate complete contents of vial containing sample into cell culture tube.

Take care to wash total contents of pipette into cell culture medium.

(b.9) Withdraw pipette from cell culture tube, replace and tighten down screw-cap on tube, discard pipette and sample vial.

(b.10) Place cell culture tube in rack for roller tube cultures.

(b.11) Incubate cell culture at 36.5^{o} C $\pm$ 1^{o} C, and examine cells daily for cytopathic effects (CPE).

Incubate under conditions and for a period of time appropriate for the virus likely to be recovered. See Chapter 9 for cell culture and cell culture examination techniques.

If confirmation is to be completed by identifying viruses (enteroviruses) recovered, proceed to Chapter 11.

CHAPTER 11

IDENTIFICATION OF ENTEROVIRUSES

1. PROCEDURE FOR TYPING VIRUSES

A neutralization test for enteroviruses is described in this chapter. The test procedure utilizes Lim Benyesh-Melnick (LB-M) antiserum pools A-H for the identification of 37 enteroviruses, a single antiserum preparation for the identification of coxsackievirus B3 and LB-M pools J-P for the identification of 19 type A coxsackieviruses not identified by pools A-H. The antiserum pools, with instructions for rehydration and storage and with virus identification tables, were available until recently from the National Institutes of Health (NIH). These antiserum pools have now been depleted. New pools are being produced by the World Health Organization (WHO) and should be available by the time this manual is published. The method described herein, with some modification to accomodate differences in pool design, should be appropriate for the new pools.

The microtiter method described herein is a modification of the method described in the literature accompanying the NIH pools. The two methods work equally well, but the microtiter method requires much less antiserum.

1.1 Apparatus and Materials

1.1.1 Microtiter plates, 96 well, flat bottom.

1.1.2 Sealing tapes for microtiter plates if plates are to be incubated in a non-CO_2 incubator (recommended method), or

plastic lids for microtiter plates if plates are to be incubated in a CO_2 incubator.

1.1.3 Micro-pipettors or pipettes capable of dispensing volumes of 0.025 and 0.05 mL.

1.1.4 Cornwall syringe, or equivalent, capable of delivering 0.2 mL quantities.

1.1.5 Cotton-tipped applicators.

1.1.6 Magnetic stirrer and stir bars.

1.1.7 Narrow-tip felt marking pen.

1.2 Media and Reagents

1.2.1 Earle's Balanced Salt Solution (EBSS) (for dilution).

Prepare 40 mL for each virus to be identified.

1.2.2 Antiserum pools A-H and coxsackievirus B3 antiserum diluted and prepared as described in NIH instruction sheets.

Store at -20° C until used.

1.2.3 Growth medium containing 5% gamma globulin-free or normal fetal calf serum.

Prepare 30 mL of medium for each microtiter plate to be used.

Prepare antiserum pools J-P only when needed to type viruses not identified by pools A-H or coxsackievirus B3 antiserum.

1.3 Procedure

1.3.1 Preparation of Microtiter Plates

Arrange each plate as indicated in Figures 11-1 and 11-2.

(a) With a narrow-tip felt marking pen, draw lines between every two columns along the length of the plate.

(b) On one end of each plate, mark identification code of samples tested.

Four viruses can be identified simultaneously on one

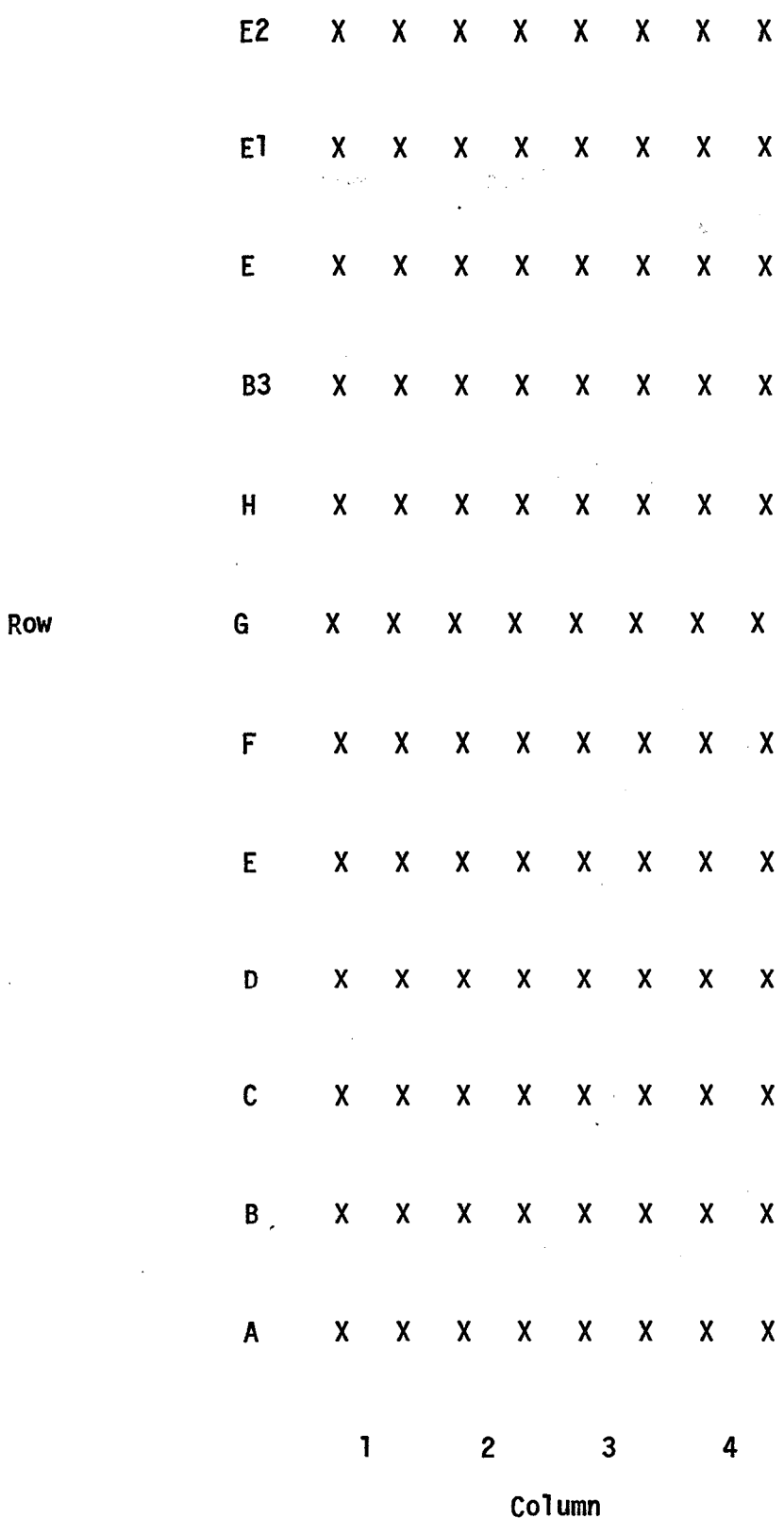

Figure 11-1. Schematic Representation of Microtiter Plate Preparation (See Figure 11-2 for Photographic Representation of Microtiter Plate Preparation).

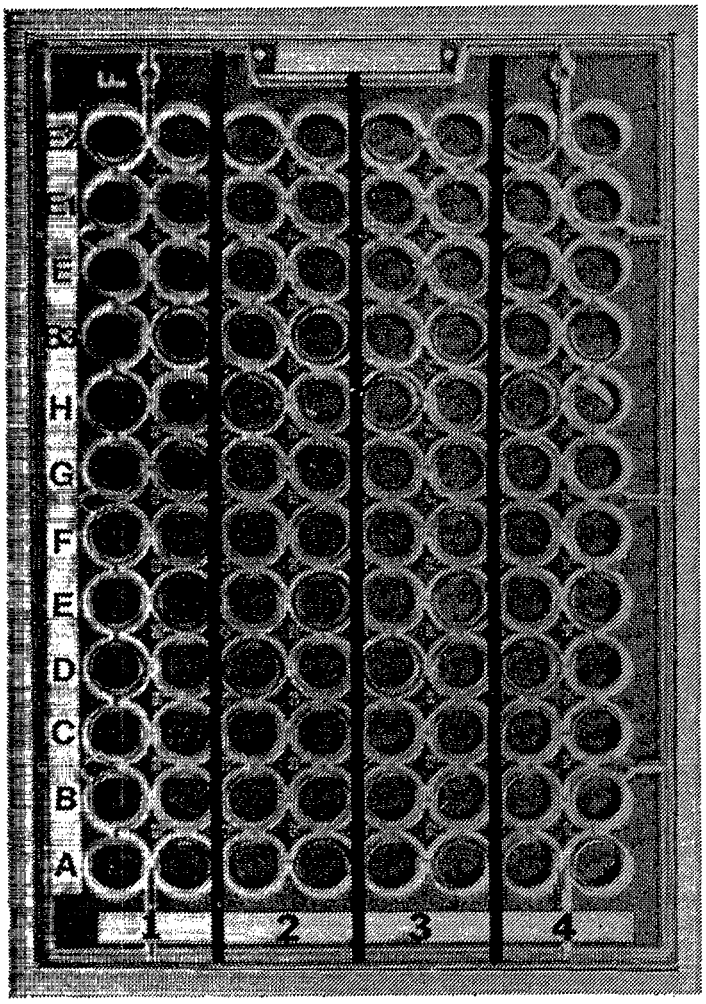

Figure 11-2. Photographic Representation of Microtiter Plate Preparation (See Figure 11-1 for Schematic Representation of Microtiter Plate Preparation).

plate. Thus, number the columns 1, 2, 3, and 4 to designate duplicate wells for each virus.

(c) Mark identity of each antiserum on left side of plate next to each row of wells.

(See Figure 11-1). Designate the first eight rows as A-H to indicate LB-M pools A-H, designate row 9 as B3 to indicate coxsackievirus B3 antiserum, designate row 10 as E to indicate virus control dilution made in Earle's balanced salt solution (EBSS) (see Chapter 9), and designate rows 11 and 12 as E1 and E2, respectively, to indicate serial 10-fold dilutions of virus control in row 10.

1.3.2 Preparation of Virus for Identification

(a) Remove virus isolate from storage in -70° C freezer, thaw, and mix well. Designate the virus isolate as No. 1.

(b) Dilute thawed virus to 10^{-5} in EBSS.

Prepare 2 mL of 10^{-5} dilution of virus. The 10^{-5} dilution is the working dilution of virus that will be mixed with the antiserum pools in the microtiter plate wells.

(c) From the 10^{-5} dilution, prepare a 1:2 dilution in EBSS.

This dilution will be transferred to row E of the microtiter plate later.

(d) From the 1:2 dilution of virus prepare two serial 10-fold dilutions (1:20 and 1:200).

These dilutions will be transferred to rows E1 and E2 of microtiter plate later.

(e) Repeat Steps (a)-(d) with each virus isolate to be

identified, designate these isolates 2-4, and proceed to Section 1.3.3.

1.3.3 Addition of Antiserum Pools to Microtiter Plate

(a) Thaw the antiserum pools, and mix each antiserum pool well.

(b) With a micro-dilutor tip or pipette, dispense 0.025 mL of antiserum from pool A into each well in row A.
It is important to place tip of dilutor or pipette into the bottom of the well and to expel all of the antiserum in the pipette into the well.

(c) Repeat Section 1.3.3, Steps (a) and (b) with antiserum pools B-H and with the antiserum for coxsackievirus B3, placing antiserums into designated wells, and proceed to Section 1.3.4.

1.3.4 Addition of Virus to Microtiter Plates

(a) Add 0.025 mL of the 10^{-5} dilution of virus No. 1 [from Section 1.3.2, Step (b)] to each well in rows A-B3 of column 1.
Take care to introduce the virus at the top of the wells. Do not allow tip of dilutor or pipette to touch an antiserum and thereby possibly cross-contaminate other antiserums.

(b) Into the two wells marked E in column 1, add 0.05 mL of the 1:2 dilution of virus No. 1 from Section 1.3.2, Step (c).

(c) Into the two wells marked E1 in column 1, add 0.05 mL of the 1:20 dilution of virus No. 1 from Section 1.3.2, Step (d).

(d) Into the two wells marked E2 in column 1, add 0.05 mL of the 1:200 dilution of virus No. 1 from Section 1.3.2, Step (d).

(e) Repeat steps (a)-(d) with viruses No. 2-4, adding the appropriate dilutions of the viruses to the appropriate wells (See Figure 11-1).

(f) Gently tap the sides of the microtiter plate with index finger to mix the contents of the wells.

(g) Cover microtiter plates with lids or with a loose sterile cover, and incubate plates at 36.5^{o} C $\pm$ 1^{o} C for two hours.

1.3.5 Preparation of Cell Suspension and Completion of Microtiter Test

Many host cell types, primary and continuous, are available for propagating viruses. Usually, the host cell type in which a virus is recovered from the environment is suitable for identifying that virus by the microtiter neutralization test. See Chapter 9 for methods for preparation of BGM cell cultures. See Lennette, E. H. and Schmidt, N. J., Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, American Public Health Association, Washington, D. C., 1979, for methods for preparation of primary and other continuous cell types, for suckling mouse procedures necessary for identifying most Group A coxsackieviruses, and for methods for identifying viruses other than enteroviruses.

(a) Trypsinize sufficient cells to yield a final cell count appropriate for the cells used in the test.

For BGM cells a count of 30,000-50,000 cells per 0.2 mL of cell culture medium is appropriate. The number of

cells required for this test differs with different cell types.

(b) Mix cells in medium for at least 15 minutes.
A longer period of mixing will generally not injure cells.

(c) After virus-antiserum mixtures have incubated for two hours (Section 1.3.4, Step G), with a Cornwall syringe, dispense 0.2 mL of cell suspension into each well.
Do not allow tip of syringe to touch contents of a well and thereby possibly cross-contaminate the contents of other wells. With cotton-tipped applicators, wipe up spilled cells on the top of plates between and around wells.

(d) Remove backing from sealing tape, seal each plate, and incubate plates at 36.5^{o} C $\pm$ 1^{o} C.
If plates are to be incubated in a CO_2 incubator, do not seal plates.

(e) After three days of incubation, examine cells in wells daily for five more days for cytopathic effects (CPE).
Use an inverted microscope to examine cells.

(f) When CPE develops, use identification tables provided with antiserum pools to identify viruses.
If all wells evidence CPE and identification cannot be made with virus identification tables, titrate virus and repeat entire test with a virus dilution calculated to add 200 infective doses to each well in Row E. Follow this same procedure if all virus control wells in Rows 1 and 2 are negative and the pattern of results does not allow identification with identification tables.

If virus control wells show that an appropriate quantity of virus infective doses has been used in test and cells in at least one well containing antiserum show no CPE and identification cannot be made with identification tables, repeat tests with antiserum pools A-H and B3.

If, under this circumstance, CPE appears in all wells containing virus and antiserum, repeat test but with antiserum pools J-P instead of A-H and B3.

2. BIBLIOGRAPHY

Laboratory Manual in Virology, Edition Two. Ontario Ministry of Health, Toronto, Ontario, Canada, 1974. 375 pp.

Lennette, E. H. and Schmidt, N. J. 1979. Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, American Public Health Association, Washington, D.C. 1138 pp.

APPENDIX

VENDORS*

American Scientific Products
2410 McGaw Road
Obetz, Ohio 43207
614-491-0050

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Flow Products Division
4545 West Brown Deer Road
Milwaukee, Wisconsin 53223
414-355-0400

Becton, Dickinson and Company
Rutherford, New Jersey 07070
201-460-2232

Bellco Biological Glassware
Vineland, New Jersey 08360
609-691-1075

Bristol Laboratories
Division of Bristol-Myers Company
P. O. Box 657
Syracuse, New York 13201
315-432-2000

Brockway Glass Company, Inc.
Parkersburg, West Virginia 26101
304-295-9311

Carborundum Company
Commercial Filters Division
Lebanon, Indiana 46052
317-482-3900

The Clorox Company
P. O. Box 24305
Oakland, California 94623
415-271-7000

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*List of vendors only indicates one possible source for products used in this Manual. In most instances, many other vendors can supply the same materials listed or acceptable alternatives.

Corning Glass Works
P. O. Box 5000
Corning, New York 14831
607-974-9000

Costar Division
Data Packaging Corporation
205 Broadway
Cambridge, Massachusetts 02139
617-492-1110

Department of Health and Human Services
United States Public Health Service
National Institutes of Health
Building WW, Room 7A03
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202-496-2131

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Detroit, Michigan 48232
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Cincinnati, Ohio 45202
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201-773-6160

Kansas City Biological, Inc.
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Lenexa, Kansas 66215
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Lederle Laboratories Division
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914-735-5000

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209-732-8005

Schering-Plough Corporation
Galloping Hill Road
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201-931-2000

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800-325-3010

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Sheboygan, Wisconsin 53081
414-457-4851

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