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CHAPTER 7

METHOD FOR RECOVERING VIRUSES FROM SLUDGES, SOILS, SEDIMENTS AND OTHER SOLIDS

1. INTRODUCTION

This chapter describes a procedure that may be used to monitor for the presence of solids-associated viruses. Its design requires that the virus be eluted directly from solids. Samples which may have a portion of the virus content suspended unattached in a liquid matrix, such as in sludges and sediments, must first be treated to bind the free viruses to the solids. Thus, initial steps in this procedure will differ based on whether or not the solids are suspended in liquid. Because of the naturally high association of viruses with solids, the virus content of the liquid matrix is likely to represent only a small fraction of the viruses present in a sample.

Processed sludges, soils, and sediment samples are assayed in cell culture for plaque-forming viruses. If the inocula is observed to be or suspected of being toxic to the cell culture, or is so darkly colored as to result in inaccurate plaque counts, use the method described in Chapter 8 (April 1986 revision) to reduce interference.

The procedures in this chapter require dispensing the entire sample volume into a centrifuge bottle. If bottles of sufficient capacity are unavailable, the sample should be divided and then recombined after centrifugation.

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

2. CONDITIONING OF SUSPENDED SOLIDS

The procedure in this Section is used only for processing of suspended solids. Conditioning of such samples (e.g. sludges) is required as a means of adsorbing to the solids those viruses that are present in the liquid matrix. Non-suspended solids, such as soil, are not to be conditioned. Begin processing these samples by following the procedures given in Section 3.

2.1 Preparation

2.1.1 Apparatus and Materials

(a) 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 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.

2.1.2 Media and Reagents

(a) Hydrochloric acid (HCl)--5 M.

(b) Aluminum chloride (AlCl3 - 6H2O)--0.05 M. Autoclave AlCl3 solution at 121 degrees C for 15 minutes.

(c) Sodium hydroxide (NaOH)--5 M.

2.2 Procedure. Flow diagram of procedure to condition suspended solids is given in Figure 1. In the absence of experience that dictates otherwise, use 100-mL volumes per sample.

2.2.1 Measure 100 mL of well-mixed sample in a graduated 100-mL cylinder. Sample must be mixed vigorously immediately before it is poured into graduated cylinder because solids, which contain most of the viruses, begin to settle out immediately after mixing stops.

2.2.2 Place stir bar into a 250-mL beaker.

2.2.3 Pour the 100 mL of measured sample from the graduated cylinder into the 250-mL beaker. It may be necessary to pour

sample several times back and forth from beaker to graduated cylinder to obtain all solids in the beaker. CAUTION: Avoid formation of aerosols. Slowly pour sample down the inner wall of vessel to avoid splatter.

2.2.4 Place beaker on magnetic stirrer, cover loosely with aluminum foil, and stir at speed sufficient to develop vortex.

2.2.5 Add 1 ml of 0.05 M AlCl3 to mixing sample. Final concentration of AlCl3 in sample is approximately 0.0005 M.

2.2.6 Place combination-type pH electrode into mixing sample. pH meter must be standardized at pH 4.

2.2.7 Adjust pH of sample to 3.5 plus or minus 0.1 with 5 M HCl. If pH falls below 3.4, readjust it with 5 M NaOH. When solids adhere to electrodes, clean electrodes by moving them up and down gently in mixing sample.

2.2.8 Continue mixing for 30 minutes. The pH of the sample should be checked at frequent intervals. If the pH drifts up, readjust it to 3.5 plus or minus 0.1 with 5 M HCl. If the pH drifts down, readjust it with 5 M NaOH.

2.2.9 Pour conditioned sample into centrifuge bottle. To prevent transfer of stir bar into centrifuge bottle when decanting sample, hold another stir bar or magnet against bottom of beaker. Solids that adhere to stir bar in the beaker may be removed by manipulation with a pipette. It may be necessary to pour sample several times back and forth from centrifuge bottle to beaker to obtain all solids in the bottle. CAUTION: Take care to avoid formation of aerosols. Slowly pour sample down the inner wall of vessel to avoid splatter.

2.2.10 Centrifuge conditioned sample at 2,500 x g for 15 minutes at 4 degrees C.

2.2.11 Decant supernatant into beaker and discard.

2.2.12 Replace cap on centrifuge bottle.

2.2.13 Elute viruses from solids by following the procedure described in Section 4.

3. PREPARATION OF NON-SUSPENDED SOLIDS

In the absence of experience that dictates otherwise, use 100 g of sample (e.g. soil).

3.1 Weigh two 100-g portions for each test sample. Test sample

is transferred to a large bottle and is thoroughly mixed by manually shaking and tumbling before it is weighed.

3.2 Place one of the 100-g portions in a 250-mL beaker and cover loosely with aluminum foil.

3.3 Elute viruses from the above 100-g sample by following the procedure described in Section 4.

3.4 Place other 100-g portion in a tared weighing pan.

3.5 Place pan and its contents in an oven maintained at 103-105 degrees C.

3.6 Heat sample to dryness.

3.7 Cool sample to room temperature in a desiccator.

3.8 Weigh sample.

3.9 Record dry weight of sample.

3.10 Repeat Sections 3.5 through 3.9 until loss in weight is no more than 4 percent of the previous weight. Re-dry the sample for 1 hour before repeating the cooling and weighing cycle. These data will be used in Chapter 10 (December 1987 revision) to calculate viral content of non-suspended solids in plaque forming units per gram of dry weight.

4. ELUTION OF VIRUSES FROM SOLIDS

4.1 Preparation

4.1.1 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. A refrigerated centrifuge capable of attaining 2,500 x g and screw-cap centrifuge bottles that can withstand 2,500 x g may be used for samples such as soils, which settle readily at lower centrifugation speeds. If the eluates resulting from such centrifugation are not easily forced through the membrane filters used for sterilization (see Step (e) in Section 4.1.1), then centrifuge at 10,000 x g.

(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).

(e) Disc filters, 47-mm diameter--3.0-, 0.45-, and 0.25-micrometer pore size filters (Filterite Corp., Duo-Fine series, or equivalent). Filters may be cut to the proper diameter from sheet filters. Disassemble Swinnex filter holder. Place filter with 0.25-micrometer 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.

4.1.2 Media and Reagents

(a) Sodium phosphate, dibasic ($Na_2HPO_4 - 7H_2O$).

(b) Citric acid.

(c) Beef extract powder (BBL Microbiology Systems, or equivalent). Prepare buffered 10 percent beef extract by dissolving 10 g beef extract powder, 1.34 g $Na_2HPO_4 - 7H_2O$ and 0.12 g citric acid in 100 mL of deionized distilled water. Dissolve by stirring on a magnetic stirrer. Autoclave beef extract solution for 15 minutes at 121 degrees C.

(d) Hydrochloric acid (HCl)--5 M.

(e) Aluminum chloride ($AlCl_3 - 6H_2O$)--0.05 M. Autoclave $AlCl_3$ solution at 121 degrees C for 15 minutes.

(f) Sodium hydroxide (NaOH)--5 M.

4.1.3 Procedure. Flow diagram of the virus elution procedure is given in Figure 2.

(a) Place stir bar into vessel containing the solids (from either Section 2.2.13 or Section 3.3.) Vessel may be a centrifuge bottle (Section 2.2.13) or a 250-mL beaker (Section 3.3).

(b) Add 100 mL of buffered 10 percent beef extract to the vessel containing the solids.

(c) Place vessel 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.

(d) Continue mixing for 30 minutes.

(e) If solids were processed in beaker, pour contents into centrifuge bottle. To prevent transfer of stir bar into centrifuge bottle when decanting sample, hold another stir bar or magnet against bottom of beaker. Solids that adhere to stir bar in the beaker may be removed by manipulation with a pipette. It may be necessary to pour sample several times back and forth from centrifuge bottle to beaker to obtain all solids in the bottle. Use appropriate centrifuge bottles for the centrifugal force that will be applied. CAUTION: Take care to avoid formation of aerosols. Slowly pour sample down the inner wall of vessel to avoid splatter.

(f) If solids were processed in centrifuge bottle, remove stir bar from bottle with long forceps or a magnet retriever. Determine if centrifuge bottle is appropriate for the centrifugal force that will be applied.

(g) Centrifuge solids-eluate mixture at either 10,000 x g or 2,500 x g for 30 minutes at 4 degrees C. Use a centrifugal force of 2,500 x g for mixtures such as soil-eluate mixtures, which settle readily at lower centrifugation speeds. If the eluates resulting from such centrifugation are not easily forced through the membrane filters used for sterilization, then centrifuge at 10,000 x g. Sludge-eluate mixtures generally require centrifugation at 10,000 x g for 30 minutes.

(h) Decant supernatant fluid (eluate) into beaker and discard solids.

(i) Place a filter holder that contains a filter stack on a 250-mL Erlenmeyer receiving flask.

(j) Load 50-mL syringe with eluate from Section 4.1.3, Step (h).

(k) Place tip of syringe into filter holder.

(l) 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 crack 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

(i) thru (l) 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 (i) through (l) with new filter holders and filter stacks.

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 (reference cited in Section 5.1). Some loss of viruses may occur with this procedure. If viruses in eluates are to be concentrated, proceed immediately to Section 5. If concentration is not required, proceed to Step (m).

(m) Refrigerate eluate immediately at 4 degrees 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 degrees C.

5. CONCENTRATION OF VIRUSES FROM ELUATES

5.1 Organic Flocculation Concentration Procedure (Katzenelson et al., 1976) 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 number of cell cultures needed for assays may be reduced by concentrating the viruses in the eluate. Floc formation capacity of the powdered beef extract reagent must be pretested. Some powdered beef extracts may not produce sufficient floc resulting in significantly reduced virus recoveries. Where it has been predetermined that insufficient floc is formed, the reagent is fortified with floc from paste beef extract. Procedure for preparing the additional floc is described in Section 5.1.3.

5.1.1 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.

5.1.2 Media and Reagents

(a) Sodium phosphate, dibasic ($Na_2HPO_4 - 7H_2O$)--0.15 M.

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

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

(d) Paste beef extract (Difco Laboratory, Bacto beef extract or equivalent)--3 percent. Paste beef extract is not needed if the 10 percent powdered beef extract reagent used for the elution process in Section 4.1.2, Step (c) produces sufficient floc when processed by the organic flocculation concentration procedure of Katzenelson. Prepare the 3 percent paste beef extract stock solution by dissolving 30 g of paste beef extract in 1,000 mL deionized distilled water. Autoclave the stock solution at 121 degrees C for 15 minutes and use at room temperature. From this stock solution, one 330-mL aliquot is removed for each sample requiring supplementation with paste beef floc. Although the paste beef extract stock solution may be stored at 4 degrees C for an extended time period, it is advisable to prepare solutions on a weekly basis, thereby lessening the possibility of microbial contamination.

5.1.3 Preparation of Floc from Paste Beef Extract Reagent
Prepare required paste beef floc before proceeding to Section 5.1.4 for those samples in which the powdered beef extract reagents had been determined to produce insufficient floc when processed by the organic flocculation concentration procedure. Flow diagram of procedure to prepare reagent floc is given in Figure 3.

(a) Place stir bar in 600-mL beaker.

(b) Pour 330 mL of a 3 percent paste beef extract stock solution into beaker and cover loosely with aluminum foil.

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

(d) Insert combination-type pH electrode into paste beef extract stock solution. pH meter must be standardized at pH 4.

(e) Add 1 M HCl to flask slowly until pH of beef extract reaches 3.5 plus or minus 0.1. A precipitate will form.

(f) Remove electrode from beaker, and pour contents of beaker into a 1,000-mL centrifuge bottle. To prevent transfer of stir bar into a centrifuge bottle, hold another stir bar or magnet against bottom of beaker when decanting contents.

(g) Centrifuge precipitated beef extract suspensions at 2,500 x g for 15 minutes at 4 degrees C.

(h) Pour off and discard supernatant.

(i) Retain floc in centrifuge bottle at 4 degrees C for subsequent mixing with the non-flocculating buffered beef extract.

5.1.4 Procedure. Flow diagram of the virus concentration procedure is given in Figure 4.

(a) Pour filtered eluate from Section 4.1.3, Step (l) into graduated cylinder, and record volume.

(b) Pour filtered eluate into 600-mL beaker and cover loosely with aluminum foil.

(c) 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 percent. This dilution is necessary because 10 percent beef extract often does not process well by the organic flocculation concentration procedure.

(d) Record the total volume of the diluted, filtered beef extract. Proceed to Step (h) only if the powdered beef extract reagent used for the virus elution process is known to form sufficient floc to undertake eluate processing by the organic flocculation concentration procedure without addition of paste floc. Where the addition of paste floc is required, add the diluted beef extract to the floc as described in Steps (e) through (g) before proceeding to Step (h).

(e) Pour extract from beaker into centrifuge bottle containing floc from Section 5.1.3, Step (i).

(f) Disperse floc manually using a pipette until it is dissolved in the extract.

(g) Pour contents into a 600-mL beaker.

(h) Place stir bar in beaker that contains diluted, filtered beef extract from either Step (d) or Step (g).

(i) Place beaker on magnetic stirrer, cover loosely with aluminum foil, and stir at a speed sufficient to develop vortex. To minimize foaming (which may inactivate viruses), do not mix faster than necessary to develop vortex.

(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 plus or minus 0.1. A precipitate will form. If pH is accidentally reduced below 3.4, add 1 M NaOH until pH is 3.5 plus or minus 0.1. Avoid reducing pH below 3.4 because some inactivation of viruses may occur.

(l) Continue to stir for 30 minutes.

(m) Remove electrode from beaker, and pour contents of beaker into a 1,000-mL centrifuge bottle. To prevent transfer of stir bar into a centrifuge bottle, hold another stir bar or magnet against bottom of beaker when decanting contents.

(n) Centrifuge precipitated beef extract suspensions at 2,500 x g for 15 minutes at 4 degrees C.

(o) Pour off and discard supernatant.

(p) Place a stir bar into centrifuge bottle that contains precipitate.

(q) Add to centrifuge bottle a volume of 0.15 M Na2HPO4 - 7H2O equal to 1/20 of the volume recorded in Step (d). The volume of 0.15 M Na2HPO4 - 7H20 in which the precipitate will be dissolved is equal to 5 mL for each 100 mL of diluted beef extract.

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

(t) Refrigerate dissolved precipitates immediately at 4 degrees 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 degrees C.

u) Assay for viruses in accordance with instructions given in Chapter 10 (December 1987 revision).

6. BIBLIOGRAPHY

Berg, G., D. Berman, and R. S. Safferman. 1982. A Method for

Concentrating Viruses Recovered from Sewage Sludges. Can. J. Microbiol. 28:553-556.

Berg, G., and D. R. Dahling. 1980. Method for Recovering Viruses from River Water Solids. Appl. Environ. Microbiol. 39:850-853.

Berman, D., G. Berg, and R. S. Safferman. 1981. A Method for Recovering Viruses from Sludges. J. Virol. Methods. 3:283-291.

Bitton, G. 1987. Recovering Viruses from Soils and Aquatic Sediments, p. 68-75. In G. Berg (ed), Methods for Recovering Viruses from the Environment. CRC Press, Inc., Boca Raton, FL.

Brashear, D. A., and R. L. Ward. 1982. Comparison of Methods for Recovering Indigenous Viruses from Raw Wastewater Sludge. Appl. Environ. Microbiol. 43:1413-1418.

Farrah, S. R. 1982. Isolation of Viruses Associated with Sludge Particles, p. 161-170. In C.P. Gerba and S. M. Goyal (eds), Methods in Environmental Virology. Marcel Dekker, Inc., New York, NY.

Gerba, C. P. 1982. Detection of Viruses in Soil and Aquatic Sediments, p. 151-160. In C.P. Gerba and S. M. Goyal (eds), Methods in Environmental Virology. Marcel Dekker, Inc., New York, NY.

Goddard, M. R., J. Bates, and M. Butler. 1981. Recovery of Indigenous Enteroviruses from Raw and Digested Sewage Sludges. Appl. Environ. Microbiol. 42:1023-1028.

Hurst, C. J. 1987. Recovering Viruses from Sewage Sludges and from Solids in Water, p. 25-51. In G. Berg (ed), Methods for Recovering Viruses from the Environment. CRC Press, Inc., Boca Raton, FL.

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.

Nielsen, A., and B. Lydholm. 1980. Methods for the Isolation of Virus from Raw and Digested Wastewater Sludge. Water Res. 14:175-178.

Pancorbo, O. C., P. R. Scheuerman, S. R. Farrah, and G. Bitton. 1981. Effect of Sludge Type on Poliovirus Association with and Recovery from Sludge Solids. Can. J. Microbiol. 27:279-287.

Rao, V. C. and J. L. Melnick. 1987. Human Viruses in Sediments, Sludges, and Soils. CRC Press, Inc., Boca Raton, FL. 241 pp.

Safferman, R. S., M. E. Rohr and T. Goyke. 1988. Assessment of recovery efficiency of beef extract reagents for concentrating viruses from municipal wastewater sludge solids by the organic flocculation procedure. Appl. Environ. Microbiol. 54:309-316.

Subrahmanyan, T. P. 1977. Persistence of Enteroviruses in Sewage Sludge. Bull. World Health Org. 55:431-434.

Wellings, F. M., A. L. Lewis, and C. W. Mountain. 1976. Demonstration of Solids-Associated Virus in Wastewater and Sludge. Appl. Environ. Microbiol. 31:354-358.

FIGURES

Figure 1. Flow Diagram of Method for Conditioning Suspended Solids

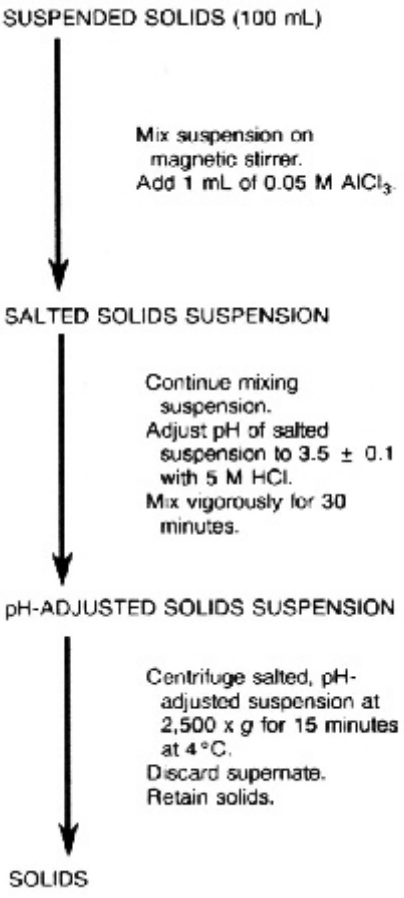


Figure 2. Flow Diagram of Method for Elution of Virus from Solids

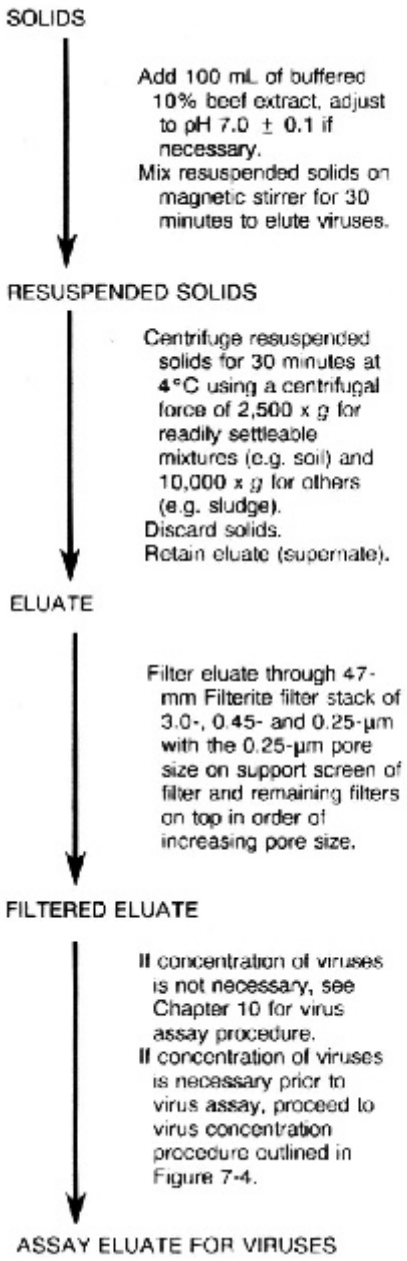


Figure 3. Flow Diagram of Method for Preparation of Floc from Paste Beef Extract Reagent.

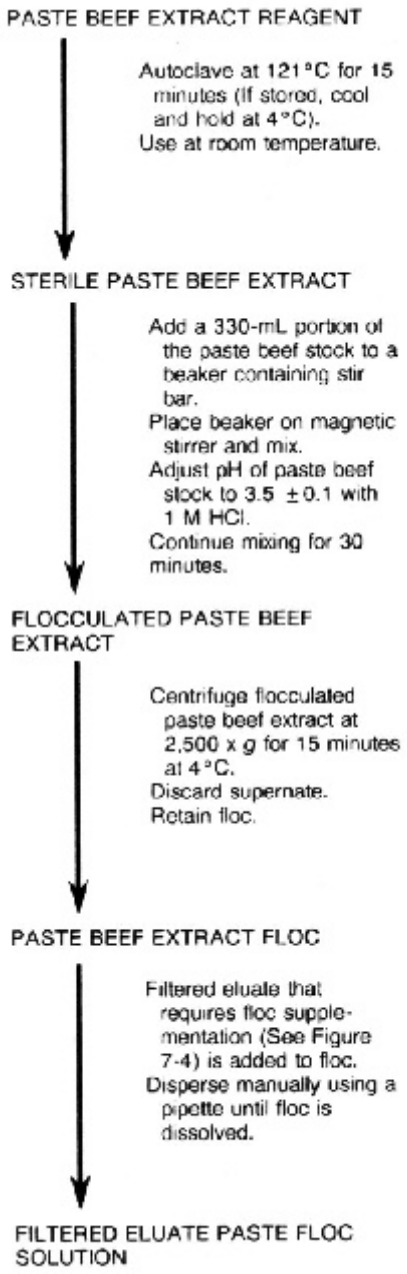
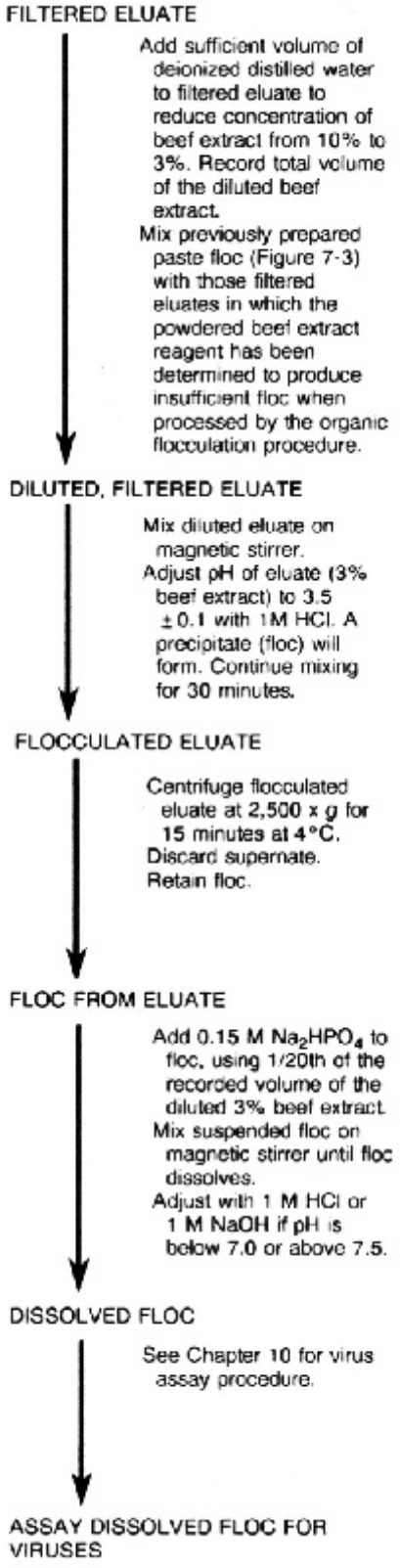


Figure 4. Flow Diagram of Method for Concentration of Viruses from Beef Extract Eluate.



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