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Standard Practice for Recovery of Viruses from Wastewater Sludges¹

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1. Scope

1.1 This practice is used for the recovery of viruses from wastewater sludges and favors the enteroviruses.

1.2 Both procedures are applicable to raw, digested, and dewatered sludges.

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1.3 This practice was tested on standardized sludges as described in 10.1 and 17.1. It is the user's responsibility to ensure the validity of this practice for untested matrices.

1.4 *This standard may involve hazardous materials, operations, and equipment. This standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.5 *Only adequately trained personnel should be allowed to perform these procedures and should use safety precautions recommended by the U.S. Public Health Service, Center for Disease Control,² for work with potentially hazardous biological organisms.*

2. Referenced Documents

- 2.1 *ASTM Standards:*
D 1129 Definitions of Terms Relating to Water³
D 1193 Specification for Reagent Water³

3. Terminology

3.1 *Definitions*—For definitions of terms used in this practice, refer to Definitions D 1129.

4. Significance and Use

4.1 Although many laboratories are presently isolating viruses from sludge, a valid comparison of data generated has not been possible because of the lack of a standard test method(s).

5. Purity of Reagents

5.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the

Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.⁴ Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

5.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type II.

PROCEDURE A—ADSORPTION

6. Summary of Procedure

6.1 The adsorption procedure relies upon adsorption of viruses from the liquid phase to the sludge solids, which are concentrated by centrifugation. The supernatant is discarded. Viruses are desorbed from the solids by physicochemical means and further concentrated by organic flocculation. Decontamination is accomplished by filtration.

7. Apparatus

7.1 *Centrifuge(s)*, refrigerated, capable of attaining 10 000 $\times g$, screw-capped 100-mL centrifuge bottles that can withstand 10 000 $\times g$, and 250-mL screw-capped centrifuge bottles capable of withstanding 2 500 $\times g$.

7.2 *pH Meter*, measuring to an accuracy of at least 0.1 pH unit, equipped with a combination-type electrode. Calibrate with standard buffers.

7.3 *Filter Apparatus*, for membrane sterilization,⁵ with 47-mm diameter filter holder and 50-mL slip-tip syringe (see 8.7 for type of filter material).

8. Reagents and Materials

8.1 *Aluminum Chloride Solution* (12.07 g/L)—Dissolve 12.07 g of aluminum chloride ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$) in 500 mL of water and dilute to 1000 mL. Autoclave AlCl_3 solution at 121°C for 15 min.

8.2 *Buffered Beef Extract Solution*—Dissolve 10 g of beef extract powder,⁶ 1.34 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.12 g of citric acid in 100 mL of water in a screw-cap flask by stirring for about 2 h on a magnetic stirrer. Autoclave at 121°C for 15 min.

8.3 *Disodium Hydrogen Phosphate Solution* (4 g/100

¹ This practice is under the jurisdiction of ASTM Committee D-19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology. Current edition approved Oct. 27, 1989. Published March 1990.

² Richardson, J. H., and Barkley, W. E., *Biological Safety in Microbiological and Biomedical Laboratories*, 2nd. edition, U.S. Dept. of Health and Human Services, Public Health Service, Center for Disease Control, and National Institutes of Health and Human Services, 1988.

³ *Annual Book of ASTM Standards*, Vol 11.01.

⁴ "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

⁵ The Swinnex filter (No. SX0047000, available from Millipore Corp., 80 Ashby Rd., Bedford, MA 01730, or equivalent, has been found suitable for this purpose.

⁶ Extract available from Grand Island Biological Corp., 3175 Staley Rd, Grand Island, NY 14072, or equivalent, has been found suitable for this purpose.

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mL)—Dissolve 4 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) in 100 mL of water and autoclave at 121°C for 15 min.

8.4 *Hydrochloric Acid (1+1)*—Add 1 volume of concentrated HCl (sp gr 1.19) to 1 volume of water.

8.5 *Hydrochloric Acid (1+9)*—Add 1 volume of concentrated HCl (sp gr 1.19) to 9 volumes of water.

8.6 *Sodium Hydroxide Solution (4 g/100 mL)*—Dissolve 4.0 g of dry sodium hydroxide (NaOH) in water and dilute to 100 mL.

8.7 *Filters, Disc, Membrane, 47-mm—3.0-, 0.45-, and 0.25- μm pore size* which must be cut to proper size from sheet filters.⁷ Disassemble 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.

9. Procedure

9.1 *Conditioning of Sludge*—In the absence of experience that dictates otherwise, use 100-mL volumes for liquid sludges and 100-g quantities for digested, dewatered sludges.

9.1.1 Measure 100 mL of well-mixed sludge in a graduated 100-mL cylinder. Mix sludge 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.

9.1.2 Place stir bar into a 250-mL beaker.

9.1.3 Pour the 100-mL of measured sludge from the cylinder into the 250-mL beaker. If necessary, pour sludge several times from beaker to cylinder and back to remove all sludge solids to beaker. Take care to avoid formation of aerosols.

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

9.1.5 Add 1 mL of AlCl_3 solution to sludge. Final concentration of AlCl_3 in sludge is approximately 0.0005 M.

9.1.6 Place combination-type pH electrode into sludge and adjust pH of sludge to 3.5 ± 0.1 with HCl (1+1). If pH falls below 3.5, readjust with NaOH solution (4 g/100 mL). If sludge adheres to electrodes, clean electrodes by moving them up and down gently in mixing sludge. pH meter must be standardized at pH 4.

9.1.7 Continue mixing for 30 min. Check pH of the sludge at frequent intervals. If the pH drifts up, readjust to 3.5 ± 0.1 with HCl (1+9). If the pH drifts down, readjust with NaOH solution (4 g/100 mL).

9.1.8 Turn stirrer off and remove pH electrode from sludge.

9.1.9 Remove cap from a screw-capped centrifuge bottle and 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. Remove sludge that adheres to stir bar in the beaker by manipulation with a stirring rod. If necessary,

pour sludge several times from centrifuge bottle to beaker and back to remove all sludge solids to bottle. Take care to avoid formation of aerosols.

9.1.10 Replace and tighten cap on centrifuge bottle.

9.1.11 Centrifuge conditioned sludge at $2\,500 \times g$ for 15 min at 4°C . Discard supernatant.

9.2 *Elution of Viruses from Sludge Solids:*

9.2.1 Add stir bar to the centrifuge bottle that contains sedimented, conditioned sludge.

9.2.2 Add 100 mL of buffered beef extract solution to the sedimented, conditioned sludge. The volume of buffered beef extract solution used to elute viruses from the conditioned sludge is equal to the original volume of the sample volume (see 9.1).

9.2.3 Replace and tighten cap on centrifuge bottle.

9.2.4 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.

9.2.5 Continue mixing for 30 min.

9.2.6 Turn stirrer off and remove stir bar from centrifuge bottle.

9.2.7 Replace and tighten cap on centrifuge bottle and centrifuge conditioned sludge-eluate mixture at $10\,000 \times g$ for 30 min at 4°C .

9.2.8 Remove cap from centrifuge bottle. Decant supernatant fluid (eluate) into beaker and discard sediment.

9.2.9 Place a filter holder that contains a filter stack as described in 8.7 on a 250-mL Erlenmeyer receiving flask.

9.2.10 Load 50-mL syringe with eluate.

9.2.11 Place tip of syringe into filter holder.

9.2.12 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. Repeat 9.2.9 through 9.2.12 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 9.2.10 through 9.2.12 with new filter holders and filter stacks.

9.2.13 Refrigerate eluate immediately at 4°C , and maintain at that temperature until it is assayed for viruses (see 9.3). 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. Some loss of virus may occur with this procedure. If viruses in eluates are to be concentrated, proceed immediately to 9.4. If further concentration is not required and if assay for viruses cannot be undertaken within 8 h, distribute eluate into sterile sample bottles, cap tightly, and store immediately at -70°C .

9.3 *Viral Assay:*

9.3.1 At time of viral assay, rapidly thaw the frozen concentrate at 37°C and proceed with usual viral assay. At least 10 % of the isolates should be confirmed by second passage.

⁷ Duo-Fine series sheet filters, available from Filterlite Corp., 2033 Green Spring Dr., Timonium, MD 21093, or equivalent, have been found suitable for this purpose.

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9.4 Procedure for Concentrating Viruses from Sludge Eluates (Organic Flocculation Concentration)—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. Significant further loss of viruses may occur with the currently available beef extract which may not produce sufficient floc to adsorb all of the suspended virions.

9.4.1 Pour eluate from 9.2.13 into a graduated cylinder and record the volume.

9.4.2 Pour eluate into 600-mL beaker.

9.4.3 For every 3 mL of beef extract eluate, add 7 mL of sterile 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.

9.4.4 Pour the diluted, filtered beef extract into a graduated cylinder and record the total volume.

9.4.5 Decant diluted filtered beef extract into 600-mL beaker and add a stir bar.

9.4.6 Place beaker 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.

9.4.7 Insert combination-type pH electrode into diluted, filtered beef extract and add HCl (1+9) slowly until pH of beef extract reaches 3.5 ± 0.1 . A flocculate or precipitate will form. If pH drops below 3.4, add NaOH solution (4 g/100 mL) until pH is 3.5 ± 0.1 . Avoid reducing pH below 3.4 because some inactivation of viruses may occur. Continue to stir for 30 min.

9.4.8 Turn stirrer off, 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.

9.4.9 Replace and tighten caps on centrifuge bottles and centrifuge the flocculated beef extract suspension at $2\ 500 \times g$ for 15 min at 4°C. Pour off and discard supernatants.

9.4.10 Place a small stir bar into each centrifuge bottle that contains flocculate and replace covers loosely.

9.4.11 Measure a volume of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ solution equal to $\frac{1}{20}$ of the volume recorded in 9.4.4. Divide this volume equally among the flocculates in the centrifuge bottles.

9.4.12 Replace and tighten-down caps on centrifuge bottles, and place each on a magnetic stirrer. Stir flocculates slowly until dissolved completely. Support bottles as necessary to prevent toppling. Avoid foaming which may inactivate or aerosolize viruses. Flocculates may be partially dissipated with spatula before or during stirring procedure.

9.4.13 Remove caps from centrifuge bottles and combine the dissolved flocculates in a small beaker. To prevent transfer of stir bars into beaker, hold another stir bar or magnet against the bottom of centrifuge bottle when decanting dissolved flocculates.

9.4.14 Measure pH of dissolve flocculate. If pH is above or below 7.0 to 7.5, adjust to within this range with either HCl (1+9) or NaOH solution (4 g/100 mL).

9.4.15 Refrigerate final concentrate immediately at 4°C,

and maintain at that temperature until assay for viruses is undertaken. If assay for viruses cannot be undertaken within 8 h, transfer dissolved precipitates to sterile sample bottles, cap tightly, and store immediately at -70°C .

9.4.16 At the time of viral assay, rapidly thaw the frozen concentrate at 37°C and proceed with usual viral assay. At least 10 % of the isolates should be confirmed by second passage.

10. Precision and Bias

10.1 Eight independent laboratories participated in the evaluation of this recovery procedure for viruses in sludges. Five standardized sludges were utilized in the study: (1) Anaerobic, high rate, digested (mesophilic), (2) Anaerobic, standard rate, digested (mesophilic), (3) Anaerobic, digested, dewatered, (4) Aerobic, digested, and (5) Primary, undigested.

10.1.1 Sludge aliquots of each type were prepared by one laboratory and were shipped on-ice to participating laboratories. Triplicate analyses were performed on each sludge within 72 h after receipt by each laboratory utilizing its own equipment, media and reagents, and cell culture assay procedures. Two sets of triplicate analyses were done on one day and a third was done on the next day.

10.2 Bias—No bias statement is possible from the study data because each sludge was a natural material containing only indigenous viruses. However, the following geometric means give some idea of the count ranges studied:

Sludge Type	Geometric Mean Count (PFU ⁴ /L) With Test Method A
Anaerobic, high rate, digested (mesophilic)	89.1
Anaerobic, standard rate, digested (mesophilic)	550
Anaerobic, digested, dewatered	302
Aerobic, digested	17.4
Primary, undigested	1445

⁴ Plaque Forming Units.

10.3 Precision:

10.3.1.1 Intralaboratory Precision:

10.3.1.1 Single-operator precision was estimated by the standard deviation among the logarithms to the base 10 of the replicate analyses within each laboratory for each sludge type. There were no statistical differences among these estimates across laboratories or sludges, each of which had a different mean recovery; the following pooled estimate was made:

$$S_o \text{ as a } \log_{10} = 0.26$$

10.3.2 The total standard deviation was also estimated from the logarithms to the base 10 of the study data for each sludge. Since there were no significant differences among the sludges, the following pooled estimate was made:

$$S_T \text{ as a } \log_{10} = 0.41$$

10.3.3 More specific information and data regarding this round robin evaluation of the viruses in sludge recovery procedures may be found in other publications.⁸

⁸ See Goyal, S. M., et al., "Round Robin Investigation of Methods for Recovering Human Enteric Viruses from Sludge," *Journal of Applied and Environmental Microbiology*, Vol 48:3, 1984, pp. 531-538.