Designation: D4994 – 19

Standard Practice for Recovery of Viruses from Wastewater Sludges[1]

This standard is issued under the fixed designation D4994; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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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Procedure A—Adsorption	7 to 10
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1.3 This practice was tested on standardized sludges as described in 10.1. It is the user's responsibility to ensure the validity of this practice for untested matrices.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.6 *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.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]
D1129 Terminology Relating to Water
D1193 Specification for Reagent Water

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminology D1129.

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. Apparatus

5.1 *Centrifuge(s),* refrigerated, capable of attaining 10 000 × *g*, screw-capped 100-mL centrifuge bottles that can withstand 10 000 × *g*, and 250-mL screw-capped centrifuge bottles capable of withstanding 2500 × *g*.

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

5.3 *Filter Apparatus,* for membrane sterilization,[4,5] with 47-mm diameter filter holder and 50-mL slip-tip syringe (see 7.7 for type of filter material).

6. Purity of Reagents

6.1 *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,

[1] This practice is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[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] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[4] The sole source of supply of the apparatus, Swinnex filter (No. SX0047000), known to the committee at this time is Millipore Corp., 80 Ashby Rd., Bedford, MA 01730.

[5] If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

*A Summary of Changes section appears at the end of this standard

where such specifications are available.[6] 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.

6.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type II.

PROCEDURE A—ADSORPTION

7. Reagents and Materials

7.1 *Aluminum Chloride Solution* (12.07 g/L)—Dissolve 12.07 g of aluminum chloride ($AlCl_3 \cdot 6H_2O$) in 500 mL of water and dilute to 1000 mL. Autoclave $AlCl_3$ solution at 121°C for 15 min.

7.2 *Buffered Beef Extract Solution*—Dissolve 10 g of beef extract powder,[5,7] 1.34 g of $Na_2HPO_4 \cdot 7H_2O$, 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.

7.3 *Disodium Hydrogen Phosphate Solution* (4 g/100 mL)—Dissolve 4 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) in 100 mL of water and autoclave at 121°C for 15 min.

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

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

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

7.7 *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.[5,8] 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.

8. Summary of Procedure

8.1 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.

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 2500 × *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.

[6] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

[7] The sole source of supply of the apparatus, extract, known to the committee at this time is Grand Island Biological Corp., 3175 Staley Rd, Grand Island, NY 14072.

[8] The sole source of supply of the apparatus, Duo-Fine series sheet filters, known to the committee at this time is Filterlite Corp., 2033 Green Spring Dr., Timonium, MD 21093.

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 × *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 7.7 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.

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 2500 × *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 $Na_2HPO_4 \cdot 7H_2O$ solution equal to 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.