Designation: D4994 – 89 (Reapproved 2014)

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.

	Sections
Procedure A—Adsorption	6 to 10
Procedure B—Sonication	11 to 15

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 and health 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.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

3. Terminology

3.1 *Definitions*—For definitions of terms used in this practice, 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 2 500 × *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, 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.

[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.

Current edition approved Jan. 1, 2014. Published March 2014. Originally approved in 1989. Last previous edition approved in 2009 as D4494 – 89 (2009). DOI: 10.1520/D4994-89R14.

[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.

[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.

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.

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.

[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.

9.2.9 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.

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[A] /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

[A] Plaque Forming Units.

10.3 *Precision:*

10.3.1 *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.[9]

PROCEDURE B—SONICATION

11. Summary of Procedure

11.1 The sonication procedure relies upon elution of sludge-associated viruses through sonication of the sludge in the presence of beef extract at pH 9 to preclude readsorption of viruses to sludge solids. Following centrifugation, the solids are discarded and the viruses in the supernatant fluid is concentrated by organic flocculation. Decontamination and detoxification are accomplished by physicochemical means.

12. Apparatus

12.1 *Blender,* with high- and low-speed capability, and blender jar, 1000-mL capacity with cover. All glassware must be sterilized before use. Cover or apply lids and caps loosely to all glassware before sterilization.

12.2 *Sonicator,* with probe capable of 100 W output power. Disinfect probe by immersion in HCl (1 + 9) for 5 min, and rinse thoroughly with water.

12.3 *Magnetic Stirrer* and 2-in. polytetrafluoroethylene-coated stir bars.

12.4 *pH Meter,* measuring to an accuracy of at least 0.1 pH units. Calibrate with standard buffers, pH 4.0 to pH 10 range; disinfect probe similarly as for sonicator.

12.5 *Centrifuge,* refrigerated, capable of attaining 10 000 × *g*, screw-capped 250-mL centrifuge tubes rated to 10 000 × *g*, and screw-capped 50-mL centrifuge tubes rated to 10 000 × *g* (chloroform compatible).[10]

13. Reagents and Materials

13.1 *Antibiotics* (stock 100 X: 10 000 of IU penicillin, 10 000 µg of streptomycin, 500 µg of tetracycline, and 500 µg of amphotericin-B per millilitre)—Prepare aseptically according to instructions on bottle and stored at – 20°C.

13.2 *Antifoam-B Solution* [5,11]—Use as supplied.

13.3 *Beef Extract, paste or powder* [5,7]—Used as supplied.

13.4 *Calcium Chloride Solution* (1 g/L)—Dissolve 0.1 g of calcium chloride ($CaCl_2$) in 100 mL of water and autoclave at 121°C for 15 min.

13.5 *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.

13.6 *Dithizone/Chloroform Reagent (Concentrated Stock)*—Dissolve 100 mg of ACS reagent-grade diphenylthiocarbazone[5,12] in 1000 mL of chloroform (ACS-approved for suitability in dithizone test); store at 4°C in amber bottle (shelf life is approximately 30 days).

13.6.1 *Dithizone/Chloroform Reagent (Working Stock)*—Dilute concentrated stock (13.6) 1 + 10 in chloroform. Prepare fresh daily.

13.7 *Hydrochloric Acid* (1 + 4)—Add 1 volume of concentrated HCl (sp gr 1.19) to 4 volumes of water.

13.8 *Hydrochloric Acid* (1 + 49)—Add 1 volume of concentrated HCl (sp gr 1.19) to 49 volumes of water.

13.9 *Sodium Hydroxide Solution* (8 g/100 mL)—Dissolve 8 g of dry sodium hydroxide (NaOH) in water and dilute to 100 mL.

13.10 *Sodium Hydroxide Solution* (0.8 g/100 mL)—Dissolve 0.8 g of dry sodium hydroxide (NaOH) in water and dilute to 100 mL.

14. Procedure

14.1 *Suspension of Sludge Solids:*

14.1.1 Measure a quantity of chilled (4°C) sludge sufficient to yield 20 g of dry sludge solids and pour into a blender jar. Adjust to 400 mL final volume with chilled (4°C) sterile distilled water. For sludges containing less than 5 % dry solids, measure 400 mL into blender jar.

14.1.2 Add 9.6 g of beef extract powder or 12 g of beef extract paste as supplied.

[9] 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, No. 3, 1984, pp. 531–538.

[10] Glass, fluorocarbon, or equivalent tubes.

[11] The sole source of supply of the apparatus, Cat. No. CS-283-4M, known to the committee at this time is Fisher Scientific, 711 Forbes Ave., Pittsburgh, PA 15219.

[12] The sole source of supply of the apparatus, Eastman No. 3092, known to the committee at this time is Eastman-Kodak, Inc., 343 State St., Rochester, NY 14650.

14.1.3 Add 0.5 mL of antifoam-B to prevent foaming and blend for 2 min at low speed, then 1 min at maximum speed.

14.1.4 Transfer sludge suspension to sterile beaker containing stir bar.

14.1.5 Repeat 14.1.1 to 14.1.3 with a second aliquot of sludge and combine with first aliquot in beaker, resulting in a total sample volume of 800 mL.

14.1.6 Stir combined sludge and adjust to pH 9 by dropwise addition of NaOH solution (8 g/100 mL). Stir and monitor pH for an additional 10 min. Maintain at pH 9 by dropwise addition of NaOH solution (8 g/100 mL) or HCl (1 + 4), as necessary.

14.2 *Sonication:*

14.2.1 Dispense sludge suspension equally into four sterile 250-mL centrifuge bottles in an ice bath.

14.2.2 Insert sonicator probes about 1 cm below liquid surface of suspension in centrifuge bottles, and sonicate each aliquot at 100 W power for 2 min.

14.2.3 Centrifuge the sonicated aliquots at 10 000 × *g* for 30 min at 4°C.

14.2.4 Pour the supernatants into a sterile beaker with a stir bar. Discard the sediments.

14.3 *Concentration (Organic Flocculation Procedure):*

14.3.1 Place beaker on magnetic stirrer and adjust supernatant in beaker to pH 3.5 by dropwise addition of HCl (1 + 4).

14.3.2 Monitor pH for 30 min, readjusting pH as required to maintain pH 3.5, by dropwise addition of HCl (1 + 4) or NaOH solution (8 g/100 mL). A floc will form.

14.3.3 Dispense flocculate suspension equally into four sterile 250-mL centrifuge bottles and centrifuge at 10 000 × *g* for 30 min at 4°C.

14.3.4 Discard supernatants, taking care not to disturb flocculates.

14.3.5 Redissolve each pelleted flocculate in 5 mL of $Na_2HPO_4{\cdot}7H_2O$ solution by repeated pipetting; pool redissolved flocculates (final concentrate), and adjust to pH 6.0 to pH 8.0 by dropwise addition of NaOH solution (0.8 g/100 mL) or HCl (1 + 49).

14.4 *Detoxification/Decontamination:*

14.4.1 Divide concentrates equally in two chloroform-compatible centrifuge tubes.

14.4.2 Add 10 mL of working stock dithizone/chloroform solution to each tube. Mix rapidly on vortex mixer for 1 min and centrifuge at 10 000 × *g* for 30 min at 4°C.

14.4.3 With pipet, remove upper phase (not the opaque interface) from each tube, pool in one sample bottle, and discard interface and lower phases.

14.4.4 Add 0.05 mL of $CaCl_2$ solution; aerate gently (approximately 1 bubble/s) through a sterile cotton-plugged Pasteur pipet for 10 min.

14.4.5 Add 0.1 mL each of antibiotic stock solutions.

14.4.6 Pour the concentrate into a graduated cylinder and record final volume.

14.4.7 Distribute concentrate into sufficient number of bottles to preclude breakage on freezing. Cap bottles tightly.

14.4.8 Store at −70°C until assayed for virus.

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

15. Precision and Bias

15.1 Eight independent laboratories participated in the evaluation of this recovery procedure for virus 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.

15.1.1 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.

15.2 *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 [A] /L) With Test Method B
Anaerobic, high rate, digested (mesophilic)	41.7
Anaerobic, standard rate, digested (mesophilic)	288
Anaerobic, digested, dewatered	295
Aerobic, digested	4.9
Primary, undigested	646

[A] Plaque Forming Units.

15.3 *Precision:*

15.3.1 *Intralaboratory Precision:*

15.3.1.1 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. 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:

$$S_o \text{ as a } \log_{10} = 0.24$$

15.3.2 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:

$$S_T \text{ as a } \log_{10} = 0.48$$

15.3.3 More specific information and data regarding this round-robin evaluation of the viruses in sludge recovery procedures may be found in other publications.[9]