Designation: D 4994 – 89 (Reapproved 2002)

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

This standard is issued under the fixed designation D 4994; 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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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 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.*

1.5 *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 Terminology Relating to Water[3]

D 1193 Specification for Reagent Water[3]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this practice, refer to Terminology 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. 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] 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.[5] 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 D 1193, Type II.

[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] *Annual Book of ASTM Standards*, Vol 11.01.

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

[5] *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.

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, [6] 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. [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.

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 2 500 × *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.

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

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

[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.