**EPA Method 1615. Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. II. Total Culturable Virus Assay**

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**Abstract**

A standardized method is required when national studies on virus occurrence in environmental and drinking waters utilize multiple analytical laboratories. The U.S Environmental Protection Agency’s (USEPA) Method 1615 was developed with the goal of providing such a standard for measuring Enterovirus and Norovirus in these waters. Virus is concentrated from water using an electropositive filter, eluted from the filter surface with beef extract, and then concentrated further using organic flocculation. Herein we present the protocol from Method 1615 for filter elution, secondary concentration, and measurement of total culturable viruses. A portion of the concentrated eluate from each sample is inoculated onto ten replicate flasks of Buffalo Green Monkey kidney cells. The number of flasks demonstrating cytopathic effects is used to quantify the most probable number (MPN) of infectious units per liter. The method uses a number of quality controls to increase data quality and to reduce interlaboratory and intralaboratory variation. Laboratories must meet defined performance standards. Method 1615 was evaluated by examining virus recovery from reagent-grade and ground waters seeded with Sabin poliovirus type 3. Mean poliovirus recoveries with the total culturable assay were 111% in reagent grade water and 58% in groundwaters.

**Video Link**

The video component of this article can be found at http://www.jove.com/video/52437/

**Introduction**

Enteric viruses are a diverse group of viruses that infect the human intestinal system and which are transmitted via the fecal-oral route. These viruses enter surface and ground waters through sewage treatment plant and septic tank effluents, improperly designed or broken septic tanks, broken sewer lines, combined sewer overflows, and other point and non-point sources. Human infections and disease from waterborne viruses occur via consumption of contaminated or inadequately disinfected water or through recreational water contact. Disease symptoms may involve mild to severe gastroenteritis; conjunctivitis; fever; upper respiratory distress; hand, foot and mouth disease; myocarditis; aseptic meningitis; encephalitis; paralysis; sepsis, and death.

USEPA Method 1615 provides a procedure to measure infectious enteric virus particles in environmental and drinking waters. These waters can contain a mixture of infectious and non-infectious virions, but only the infectious particles pose a potential health hazard. Infectious virus particles lose infectivity over time in environmental and drinking waters from loss of protein capsid integrity, damage to nucleic acids due to UV radiation from sunlight, and damage due to any disinfectants that may be present. The total culturable virus procedure provided in the method is based upon the production of cytopathic effects (CPE) in the Buffalo Green Monkey kidney (BGM) cell line. This cell line was chosen because of its widespread use in the environmental virology field, even though the range of infectious virus types detected are restricted primarily to certain enteroviruses. The purpose of this paper is to describe Method 1615’s procedures for elution of five-inch electropositive cartridge filters, secondary concentration, and measurement of total culturable viruses. An evaluation of the overall method is described in Cashdollar et al.

**Protocol**

NOTE: Please see supplemental materials section S1 for a list of definitions. The QA procedures associated with USEPA Method 1615 are described in the supplemental materials section S2.

1. **Filter Elution Procedure**

   1. First Elution
   
      1. Place 500 ml of buffered 1.5% beef extract, pH 9.0, warmed to room temperature in a graduated cylinder. Open the filter cartridge housing and add a sufficient amount of beef extract to cover the electropositive filter completely. Replace the filter housing lid and pour the remaining beef extract into a sterile beaker.
2. Organic Flocculation Concentration Procedure

1. Sterilize a combination-type pH electrode with 0.525% sodium hypochlorite for at least 5 min. Rinse the electrode with sterile dH₂O and then dechlorinate with 0.05 M sodium thiosulfate. Calibrate the pH meter using pH 4 and 7 standards.

2. Place the beaker containing the eluate on a stir plate. Turn on the plate and increase the stirring speed until a vortex is formed.

3. Adjust the pH of the eluate to 3.5 ± 0.1 slowly by drop wise addition of 1.2 M HCl to the eluate. Add the HCl drop-wise because rapid addition will inactivate virus. During this time the eluate will become cloudy as a precipitate begins to form.

4. Reduce the mixing speed to a slow stir and then continue to monitor and maintain the pH of the eluate at 3.5 ± 0.1 at room temperature for 30 min.

5. Pour the precipitated beef extract suspension into one or more centrifuge bottles and centrifuge for 15 min at 2,500 x g at 4 °C. Remove the supernatant.

6. Re-sterilize a combination type pH electrode with 0.525% sodium hypochlorite and dechlorinate with 0.05 M sodium thiosulfate as described in Step 2.1. Calibrate the pH meter using pH 7 and 10 standards. Slowly adjust the pH of the completely dissolved precipitate to 9.0 with 1 M NaOH and then stir for 10 min.

7. Remove the stir bar and centrifuge the dissolved precipitate for 10 min at 4,000–10,000 x g and 4 °C.

8. Carefully pour the supernatant into a glass beaker without disturbing the pellet. Add a stir bar to the beaker and discard the pellet.

9. Place the beaker onto a magnetic stirrer, and stir the solution. Add 1.2 M HCl drop wise to adjust the pH to 7.0–7.5.

10. Filter sterilize the supernatant by passage through a sterilizing filter containing a prefilter that has been pretreated with 15 ml of 1.5% beef extract, 0.05 M glycine, pH 7.0–7.5.

11. Assay Sample Volume (S) calculations:

   \[ S = \frac{D}{TSV} \times FCSV \]  

   where D (Volume of Original Water Sample Assayed) is 500 L for groundwater, TSV (Total Sample Volume) is the volume of field sample passed through the 5-inch electropositive cartridge filter received by the laboratory, and FCSV (Final Concentrated Sample Volume) is the volume following filtration in Step 2.10. An example is shown in supplemental materials Section S4.1.

12. Divide the FCSV into three subsamples.

   1. Prepare subsamples 1 and 2 with a volume equal to 1.04 times the Assay Sample Volume. Freeze these subsamples at or below -70 °C if they cannot be analyzed using the total culturable virus assay (Subsample 1, Step 4) or processed for the molecular assays (not shown) within 24 hr; otherwise, hold at 4 °C.

   2. Freeze the remaining volume (subsample 3) at or below -70 °C.

13. Calculate the Inoculum Volume by dividing the S by 10.

3. Total Culturable Virus Quantal Assay

   NOTE: For all steps always add solutions carefully to avoid disturbing the cell monolayer.

1. Decant or aspirate media from test vessels containing a monolayer of BGM cells at 3–6 days post splitting and then add a volume of balanced salt solution equal to the media removed.

2. Decant or aspirate the balanced salt solution from the cell culture test vessels being used and then inoculate the cell culture test vessels
1. Inoculate 10 test vessels for each test sample with a volume of subsample 1 equal to the **Inoculum Volume** along with the total culturable virus quantal assay controls (supplemental materials section S2.4).

2. For the LFB and the Lab Fortified Sample Matrix (LFSM; i.e., seeded water matrix sample), prepare 5-, 25-, and 125-fold dilutions using subsample 3 and 0.15 M sodium phosphate, pH 7.0–7.5 as a diluent. An example of a procedure for making the dilutions is given in supplemental materials section S3. Inoculate 10 washed cell culture test vessels for each dilution series using an **Inoculum Volume** on each test vessel in addition to the vessels inoculated with undiluted Subsample 1 in Step 3.2.1.

3. For any test sample from Step 3.2.1 (other than those inoculated in Step 3.2.2) that has CPE in all 10 replicates after 14 days of incubation (see Step 3.3), prepare 5-, 25-, and 125-, and 625-fold dilutions of subsample 3. Inoculate 10 washed cell culture test vessels for each dilution series using an **Inoculum Volume** on each test vessel along with a new set of the total culturable virus quantal assay controls (supplemental materials section S2.4).

4. Distillate the inoculum over the surface of the cell monolayers by tilting the vessels back and forth. Incubate the test vessels at room temperature for 80–120 min on a mechanical rocking platform at 1–5 oscillations/min or with rocking of the vessels every 15–20 min to allow any virus present to adsorb to cells.

5. Add prewarmed maintenance medium, and then incubate the test vessels at 36.5 ± 1 °C.

6. Identify individual test vessels as virus positive when they show CPE in both the 1st and positive in the 2nd passage and positive on the 2nd as described in Step 3.3. Perform a 3rd passage as described for the 2nd passage using only the negative assay controls and cell cultures that were negative during the 1st passage and positive on the 2nd passage.

7. Use USEPA's Most Probable Number Calculator with the default program settings set as shown in Table S2 to calculate the virus titer of all test samples.

   1. Input the number of virus positive replicate test vessels from Step 3.6 for each test sample into the calculator to determine the MPN/ml value (\(M_{\text{ml}}\)) and the upper (\(CL_{\text{u}}\)) and lower (\(CL_{\text{l}}\)) 95% confidence limits/ml values.
   2. Obtain the MPN/L value (\(M_L\)) of the corresponding test sample using Equation 2.

   \[M_L = \frac{M_{\text{ml}} S}{D}\]

   \(M_{\text{ml}}\) is the MPN/ml value in Step 3.7, \(S\) is the **Assay Sample Volume**, and \(D\) is the **Volume of Original Water Sample Assayed**.

   3. Calculate the upper confidence limit/L by substituting the \(CL_{\text{u}}\) value for the \(M_{\text{ml}}\) value. Calculate the lower confidence limit/L by substituting the \(CL_{\text{l}}\) value for the \(M_{\text{ml}}\) value. An example calculation is shown in supplemental materials Section S4.2.

   4. Report \(M_{\text{u}}\) values of 0 as ≤ 1/D. For example, ≤ 0.002 MPN/L (≤ 1/500 L) for groundwater samples.

   5. Calculate the MPN and 95% confidence limit values for each Lab Fortified Blank and Lab Reagent Blank by first multiplying the values/ml obtained in the calculator by \(S\) and then dividing the result by 0.3.

**Representative Results**

Virus was concentrated from the source groundwater of three drinking water treatment plants and a private well using electrophoretic filters. Two sample sets, consisting of a field sample and LFSM control, were collected from the treatment plants on separate occasions, and one sample set was collected from the private well. Overall virus recovery was determined using LFSM samples from two of the plants and the private well (two samples from one plant and one sample from another were excluded from the calculation because the MPN value of the seed used with each sample could not be determined accurately due to abnormal CPE results among replicate flasks). Mean poliovirus recovery from groundwater samples averaged 58% with a coefficient of variation of 79% (Figure 2). No culturable virus was detected in any of the duplicate unseeded ground water field samples.

Method performance also was measured using LFB samples modified by using two different seed levels. A “low” titer of 300 MPN of poliovirus was used to evaluate performance at levels less than the “standard” 500 MPN LFB level used in USEPA Method 1615. A “high” titer of 1,000 MPN of poliovirus was used to test performance at the level of the LFSM control. These controls performed similarly with a mean recovery of 111% and a coefficient of variation of 100% (Figure 2). All LRB samples were negative and all LFB samples performed within the acceptance range (supplemental materials Table S1).
Figure 1. Filter Elution. A schematic for using a pressure container for cartridge filter elution is shown. Positive air pressure is used to push beef extract solution in the pressure container through the cartridge housing containing the electropositive cartridge filter. A peristaltic pump can be substituted for the pressure container, with the inlet of the pump being placed in the container holding the beef extract solution.

Figure 2. Mean Poliovirus Recovery (%) from Ground and Reagent-Grade Water. The mean percent recovery is shown for poliovirus from ground (n=4) and reagent grade water (n=12) samples. The twelve reagent grade water samples included six seeded with 300 MPN and six seeded with 1,000 MPN of poliovirus. Error bars represent standard error.

Discussion

USEPA Method 1615 was developed for use during the Unregulated Contaminant Monitoring Regulation's third monitoring cycle (UCMR3) and designed primarily for measuring virus occurrence in groundwater. It shares a number of common steps with the Information Collection Rule (ICR) method, but has two minor differences. Both use quantal assays to measure virus that produce CPE on BGM cell monolayers with quantitation being based upon Most Probable Number (MPN) calculations. Method 1615 allows the use of a newer electropositive filter for concentrating virus from various water matrices and reduces the number of cell culture test vessel replicates per dilution from 20 to 10. Both minor changes reduce overall method costs. The reduction in the number of replicates reduces labor, but results in a slightly lower detection limit. Although groundwaters are expected to have lower concentrations of virus than surface waters, the amount of sample assayed is five times that of surface water, compensating in part for the differences. The use of fewer replicates will be adequate for most surface waters, but some will require sample dilution.

Method 1615 has several critical steps and limitations. Beef extracts vary from lot to lot. Each lot should be tested for effectiveness of the virus elution and the capacity for virus concentration through the secondary concentration steps as described in supplemental materials section S2.3. The method uses precise formulas for calculating the amount of sample to inoculate onto BGM cell cultures and to determine virus titers. Inaccurate results will be generated if these formulas are not rigorously followed. Proper aseptic technique must be used for maintaining cell cultures. Uninfected BGM cell culture controls that show distress during the 14-day incubation period likely indicate problems with cell culture maintenance. Great care also must be taken during pipetting steps involved with inoculation of and medium addition to cell culture flasks to avoid cross-contamination. The quality controls described in supplemental materials Section S2 must be rigorously followed. Section S2 also provides troubleshooting advice for quality issues.

The primary mechanism of virus adsorption to electropositive filters is a charge interaction related to the strength of the positive charge on the filter and the strength of the virus’ negative charge related to its isoelectric point and the pH of the water being tested. Elution from filters also is
affected by the strength of these interactions. Because they vary among virus types and even among strains within the same type, elution from the filters is not uniform. This means that any result may underestimate the actual level of virus present in environmental waters. The use of the single BGM cell line also underestimates virus occurrence. The range of enteric virus that can produce CPE in this cell line primarily is limited to polioviruses and Enterovirus B species serotypes as well as some reoviruses. Other infectious virus types will not be detected.

Poliovirus recoveries from ground and reagent grade waters met the USEPA Method 1615 performance acceptance criteria for both Performance Evaluation (PE; i.e., seeded reagent grade water samples with titers unknown to an analyst that are used to evaluate the performance of the analyst prior to the start of a study) and LFB samples (supplemental materials Table S1). The 58% recovery from groundwater using the culture procedure is similar to that reported by others using tap water. The mean recovery from the LFB samples of 111% with a coefficient of variation (CV) of 100% also met the method performance acceptance criteria even though they are higher than that observed for PE samples during the ICR. ICR mean interlaboratory recovery was 56% with a coefficient of variation (CV) of 92% while mean intralaboratory recoveries varied from 36% to 85% (CVs 58% to 131%; unpublished data from the ICR PE database). Higher recoveries were observed in this study for low seed LFB samples than for higher seed samples (122 versus 42%). At the time that the ICR was being planned, it was expected that PE samples receiving low seed values would have lower recovery than those receiving high seeds. Similar to that observed here for the LFB samples, poliovirus recovery for ICR PE samples were 71% (CV 100%), 54% (CV 69%), and 44% (CV 71%) for seed values ≤300 MPN, 300–1,500 MPN, and >1,500 MPN, respectively.

There are many methods for measuring infectious virus in water samples. This method is significant in respect to other methods in the degree of standardization. The standardization not only includes quality and performance controls, but also uses defined volumes and formulas to ensure that all analytical laboratories perform the method identically. Without standardization, it is difficult to compare results across laboratories, and therefore standardization is essential when conducting large scale studies in multiple analytical laboratories. With the built-in standardization this method could be expanded in the future to include additional virus types and cell lines. Research is underway to provide data for inclusion of adenovirus into the method.

Disclosures
The authors have nothing to disclose.

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