

**Soil and Aquatic Microbiology Laboratory
Texas AgriLife Research / Texas A&M University**

Standard Operating Procedure SAML-12-104

Filtration and Molecular Assay of Water Samples for Enterovirus

Author: _____
Laboratory Director Terry Gentry

Approved: _____
Laboratory Director Terry Gentry

Revision Record

Revision	Date	Responsible Person	Description of Change
1	November 2012	Terry Gentry	Initial Release

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 1 of 10

Filtration and Molecular Assay of Water Samples for Enterovirus

1. Scope and Application

This method is designed to test for the presence of enteroviruses in environmental water samples.

2. Method Summary

This methods includes collection of viruses from water samples on 1 MDS (positively charged filters). The concentrated viruses are then eluted from the filters and the eluted volume tested for the presence of enteroviruses using a reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay.

3. Procedures

3.1. Sample Collection

3.1.1. Filter sampling apparatus sterilization

- 3.1.1.1. Before each use, wash, and then sterilize the intake and cartridge housing modules, tubing, and pumps.
- 3.1.1.2. Cover the apparatus ends and port(s) with sterile aluminum foil.
- 3.1.1.3. Place tubing and smaller parts into a sterile bag or wrap them in such a way that they may be removed without contaminating them.

3.1.2. Preparation for sample collection

Note: Individuals collecting water samples for virus analysis must wear surgical gloves and avoid conditions that can contaminate a sample with virus. Gloves should be changed after touching human skin or handling components that may be contaminated (e.g., water taps, other environmental surfaces). Care must be taken to ensure that cartridge filters are properly seated in the housings. Housings with properly seated filters must not leak. Filters should be checked for proper seating upon opening the housing at the analytical laboratory by examining the gaskets for depressions that do not extend beyond the edge of the filter.

- 3.1.2.1. Purge the well before connecting the filter apparatus. Continue purging for 2-3 minutes or until any debris that has settled in the line has cleared.
- 3.1.2.2. Remove the foil from the backflow regulator. Loosen the swivel female insert slightly to allow it to turn freely and connect the backflow regulator to the tap. Retighten the swivel female insert. Disconnect the cartridge housing module at the quick connect, if connected, and cover the open end with sterile foil.
- 3.1.2.3. Remove the foil from the ends of the discharge module and connect it to the regulator module. Place the end of the regulator module or the tubing connected to the outlet of the regulator module into a 1 L plastic bottle.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 2 of 10

- 3.1.2.4. Slowly increase the flow until the flow meter/totalizer reads 10 L/min. If this flow rate cannot be achieved, adjust the valve to achieve the maximum flow rate. Slower flow rates will result in longer sampling times.
- 3.1.2.5. Flush the apparatus assembly with at least 10 L of the water to be sampled. While the system is being flushed, measure pH, temperature, and other field parameters.
- 3.1.2.6. Record the pH, temperature, and other field parameters onto a sample data sheet.
- 3.1.2.7. Turn off the pump.

3.1.3. Virus collection

- 3.1.3.1. Remove the foil from the cartridge housing module (containing a 1MDS filter) and connect it to the end of the regulator module, or if used, the injector module. Connect the discharge module to the outlet of the cartridge housing module.
- 3.1.3.2. If the water sample has turbidity greater than 75 NTU, remove the foil from each end of the prefilter module and connect the prefilter module between the regulator module or, if used, the injector module and the cartridge housing module.
- 3.1.3.3. Record the unique sample number, location, date, time of day and initial totalizer reading onto a sample data sheet.
- 3.1.3.4. If an injector module is being used, turn on the metering pump.
- 3.1.3.5. With the filter housing placed in an upright position, slowly increase the water flow to desired rate (Note: If the cartridge housing has a vent button, press it while opening the tap to expel air from the housing. When the air is totally expelled from the housing, release the button, and open the sample tap completely. If the housing does not have a vent button, allow the housing to fill with water before allowing maximum flow).
- 3.1.3.6. Using the totalizer readings, pass the 20 to 100 L of water through the apparatus.
- 3.1.3.7. Turn off the flow of water at the end of the sampling period and record the date, time of day, and totalizer reading onto a sample data sheet.
- 3.1.3.8. Loosen the swivel female insert on the regulator module and disconnect the backflow regulator from the tap. Disconnect the cartridge housing module and the prefilter housing module, if used, from the other modules.
- 3.1.3.9. Turn the filter housing(s) upside down and allow excess water to flow out. Turn the housing(s) upright and cover the quick connects on each end of the modules with sterile aluminum foil.

3.2. Transport of Samples

- 3.2.1. Pack the cartridge housing module(s) into an insulated shipping box.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 3 of 10

- 3.2.1.1. Add 6-8 small ice packs (prefrozen at -20°C) or double bagged ice cubes around the cartridge housings to keep the sample cool in transit (the number of ice packs or bags may have to be adjusted based upon experience to ensure that the samples remain cold, but not frozen).
- 3.2.2. Drain and add the regulator and injector modules used.
- 3.2.3. Drain and then cover the ends of the discharge module with foil. The discharge module may remain at the sampling site, if samples will be taken on a routine basis. If not, pack the module into the shipping box.
- 3.2.4. Filters must be refrigerated immediately upon arrival. Ideally, viruses should be eluted from filters within 24 h of the start of the sample collection, but all filters must be eluted within 72 h of the start of the sample collection.

3.3. Filter Elution Procedure

3.3.1. Elution Equipment Setup

- 3.3.1.1. Attach sections of braided tubing to the inlet and outlet ports of the cartridge housing containing the cartridge filter. Note: If a prefilter or more than one electropositive filter was used to collect a sample, each filter must be eluted and analyzed separately using the procedures below.
- 3.3.1.2. Place the sterile end of the tubing connected to the outlet of the cartridge housing into a sterile 2 L glass or polypropylene beaker.
- 3.3.1.3. Connect the free end of the tubing from the inlet port of the cartridge housing to the outlet port of a sterile pressure vessel and connect the inlet port of the pressure vessel to a positive air pressure source.

3.3.2. Elution

3.3.2.1. First elution

- 3.3.2.1.1. Elute 1MDS filters with 500 mL or 1,000 mL of buffered 1.5% beef extract, pH 9.0 (prewarmed to room temperature), respectively, by opening the cartridge housing and adding a sufficient amount of beef extract to cover the filter. Close the housing and pour the remaining beef extract into the pressure container. An acceptable alternative to the use of a pressure vessel is to use a peristaltic pump and sterile tubing to push the remaining beef extract through the filter.
- 3.3.2.1.2. Replace the top of the pressure vessel and close its vent/relief valve.
- 3.3.2.1.3. Open the vent/relief valve (if present) on the cartridge housing and slowly apply sufficient pressure to fill the housing with beef extract. Close the vent/relief valve (if present) as soon as the buffered beef extract solution begins to flow from it. Carefully observe housings without vents to ensure that all trapped air has been purged.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 4 of 10

- 3.3.2.1.4. Turn off the pressure and allow the solution to contact the filter for 1 minute. Wipe up spilled liquid with disinfectant-soaked sponge.
 - 3.3.2.1.5. Increase the pressure to force the buffered beef extract solution through the filter(s). The solution should pass through the filter slowly to maximize the elution contact period. When air enters the line from the pressure vessel, elevate and invert the filter housing to permit complete evacuation of the solution from the filters. Note: Slow passage of the solution also minimizes foaming, which may inactivate some viruses; the addition of a few drops of sterile antifoam to minimize foaming is optional.
 - 3.3.2.1.6. Turn off the pressure at the source and open the vent/relief valve on the pressure vessel.
- 3.3.2.2. Second elution
- 3.3.2.2.1. Place the buffered beef extract from the 2 L beaker back into the cartridge housing and pressure vessel and repeat sections 3.3.2.1.1 to 3.3.2.1.6.
 - 3.3.2.2.2. Turn off the pressure at the source and open the vent/relief valve on the pressure vessel. Record the total volume. Thoroughly mix the eluate and proceed to step 3.4 immediately.

3.4. Organic Flocculation Concentration Procedure

3.4.1. Organic Flocculation

- 3.4.1.1. Place a sterile stir bar into the beaker containing the buffered beef extract eluate from the cartridge filter. Place the beaker onto a magnetic stirrer, and stir at a speed sufficient to develop a vortex. Minimize foaming (which may inactivate viruses) throughout the procedure by not stirring or mixing faster than necessary to develop a vortex.
- 3.4.1.2. Sterilize the electrode of a combination-type pH electrode. Calibrate the pH meter at pH 4 and 7.
- 3.4.1.3. Insert the sterile pH electrode into the beef extract eluate. Add 1.2 M HCl to the eluate dropwise while moving the tip of the pipette in a circular motion away from the vortex to facilitate mixing. Continue adding 1.2 M HCl until the pH reaches 3.5 ± 0.1 .
- 3.4.1.4. While continuing to monitor the pH, slowly stir the eluate for 30 minutes at room temperature. A precipitate will form during the 30 minutes stirring period. If pH falls below 3.4, add 1 M NaOH to bring it back to 3.5 ± 0.1 . Exposure to a pH below 3.4 may result in virus inactivation.
- 3.4.1.5. Remove the electrode from the beaker, and pour the contents of the beaker into a centrifuge bottle. To prevent the transfer of the stir

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 5 of 10

bar into a centrifuge bottle, hold another stir bar or magnet against the bottom of the beaker while decanting the contents. The beef extract suspension will usually have to be divided into several centrifuge bottles.

- 3.4.1.6. Cap the bottle and centrifuge the precipitated beef extract suspension at 2,500 x g for 15 minutes at 4°C.
- 3.4.1.7. Carefully pour off or aspirate the supernatant so as to not disturb the pellet, including any loose floc on top of the pellet, and then discard the supernatant.

3.4.2. Reconcentrated Eluate

- 3.4.2.1. Place a stir bar into the centrifuge bottle that contains the precipitate.
- 3.4.2.2. Add 30 mL of 0.15 M sodium phosphate, pH 9.0.
- 3.4.2.3. Place the bottle onto a magnetic stirrer, and stir slowly until the precipitate has dissolved completely. Continue stirring for at least 10 minutes before proceeding. When the centrifugation is performed in more than one bottle, dissolve the precipitates in a total of 30 mL and combine into one bottle before proceeding to the next step. Significant virus loss can occur if the precipitates are not dissolved completely. Precipitates that prove to be difficult to dissolve can be treated using any of the following techniques:
 - 3.4.2.3.1. Break up the precipitate with a sterile spatula before or during the stirring procedure.
 - 3.4.2.3.2. Use a pipette repeatedly to draw the solution up and down during the stirring.
 - 3.4.2.3.3. Shake the precipitate at 160 rpm for 20 minutes on an orbital shaker in place of stirring.
 - 3.4.2.3.4. If the above procedures take longer than 20 minutes to dissolve the precipitate or if experience with the water matrix shows that precipitates are always difficult to manage, either slowly adjust the pH to 7.0 - 7.5 with 1.2 M HCl or resuspend the precipitate initially in 0.15 M sodium phosphate, pH 7.0 - 7.5. Use one of the above techniques to dissolve the precipitate and then slowly re-adjust the pH to 9.0 with 1 M NaOH. Mix for 10 minutes at room temperature before proceeding.
 - 3.4.2.3.5. Check the pH and readjust to 9.0 with 1 M NaOH, as necessary. Remove the stir bar.
 - 3.4.2.3.6. Centrifuge the dissolved precipitate at 4,000 x g for 10 minutes at 4°C. The centrifugation speed may be increased to 10,000 x g for 10 minutes at 4°C to facilitate the filtration step below.
 - 3.4.2.3.7. Remove and collect the supernatant and discard the pellet. Adjust the pH of the supernatant to 7.0-7.5 slowly with 1.2 M HCl.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 6 of 10

- 3.4.2.3.8. Pretreat a sterilizing filter or, for samples that are difficult to filter, a sterilizing filter stack with 10-15 mL of 1.5% beef extract. Load the supernatant into a 50 mL syringe and force it through the filter. If the sterilizing filter or filter stack begins to clog badly, empty the loaded syringe into the bottle containing the unfiltered supernatant, fill the syringe with air, and inject air into filter to force any residual sample from it. Continue the filtration procedure with another filter.
- 3.4.2.3.9. Divide the final eluate into three equal volumes and store the samples at -80°C for subsequent PCR-based analyses.

3.5. Enterovirus Molecular Assay

3.5.1. Preliminary procedures

- 3.5.1.1. Prepare 100 µM stock solutions of each oligonucleotide primer (probes should come from the manufacturer at 100 µM solutions).
 - 3.5.1.1.1. Centrifuge the vials containing primers in a microcentrifuge for 30 seconds.
 - 3.5.1.1.2. Dissolve the primers in a volume of molecular or PCR grade water (e.g., Roche Cat. No. 03 315 932 001) that equals the number of nanomoles of primer shipped (as identified on the specification sheet from the manufacturer) times 10 (in µl; e.g., if a primer contains 51.0 nmol; resuspend in 510 µl).
 - 3.5.1.1.3. Aliquot the stock solutions into small quantities to avoid multiple freeze thaw cycles and store at -20°C.
- 3.5.1.2. Prepare 10 µM primer and probe working solutions by diluting the stock solutions 1:10 in molecular or PCR grade water. Aliquot stocks and store at -20°C.
- 3.5.1.3. Note: Preparation of primers and probes must be performed in a clean room or other location to minimize the possibility of false positive reactions.

3.5.2. Nucleic Acid Isolation

3.5.2.1. Preliminary procedures

- 3.5.2.1.1. Add 310 µL of Buffer AVE to a vial of carrier RNA to give a final concentration of 1 µg/µL.
- 3.5.2.1.2. Aliquot and store at -20°C. Prepare a sufficient number of aliquots so that each aliquot does not have to be frozen and thawed more than three times.
- 3.5.2.1.3. Add carrier RNA to Buffer AVL (note: do not use the Buffer AL that comes with the Qiagen kit) to give a concentration of 0.027 µg/µL by adding, per sample, 5.6 µL of carrier RNA to 200 µL of Buffer AVL (e.g., 5.6 µL carrier RNA x number of samples + 200 µL Buffer AVL x number of samples).

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 7 of 10

- 3.5.2.2. For each sample to be processed, label a 1.5 mL microcentrifuge tube and add 200 μ L of the appropriate sample vortex briefly to mix. Freeze any remaining sample at -80°C .
- 3.5.2.3. Add 200 μ L of Buffer AVL with carrier RNA. Vortex for 15 seconds.
- 3.5.2.4. Incubate at 56°C for 10 minutes.
- 3.5.2.5. Centrifuge at $>5,000$ rpm for 5 seconds in a microcentrifuge.
- 3.5.2.6. Add 200 μ L of ethanol. Vortex for 15 seconds and then centrifuge for about 5 seconds.
- 3.5.2.7. Add the mixture to a QIAamp Mini Spin column, but avoid wetting the rim of the tube. Close the cap.
- 3.5.2.8. Centrifuge at $6,000 \times g$ for 1 minute. Check to determine if the sample has completely passed through the column. If it has not, centrifuge again for 1 minute at $10,000$ - $20,000 \times g$ or for longer times until the sample has completely passed through the column.
- 3.5.2.9. Place the Mini Spin column into a clean 2 mL collection tube and discard the collection tube containing the filtrate.
- 3.5.2.10. Add 500 μ L of Buffer AW1 without touching the tube rim.
- 3.5.2.11. Centrifuge at $6,000 \times g$ for 1 minute. Again, transfer the column to a clean collection tube and discard the tube containing the filtrate.
- 3.5.2.12. Add 500 μ L of Buffer AW2 without touching the tube rim.
- 3.5.2.13. Centrifuge at $20,000 \times g$ for 3 minutes. Again, transfer the column to a clean collection tube and discard the tube containing the filtrate.
- 3.5.2.14. Centrifuge at $20,000 \times g$ for 1 minute.
- 3.5.2.15. Add 40 units of RNasin to a clean microcentrifuge tube. Transfer the column from the collection tube to the 1.5 mL microcentrifuge tube and discard the collection tube. Alternatively, RNasin can be added to an amount of Buffer AE sufficient for the number of samples to be eluted at a concentration of 400 units/mL in place of adding it to the microcentrifuge tubes.
- 3.5.2.16. Add 50 μ L of Buffer AE to the column. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at $6,000 \times g$.
- 3.5.2.17. Repeat step 3.5.2.16. Remove and discard the column.
- 3.5.2.18. Proceed immediately to step 3.5.3 or prepare aliquots and store the RNA at -80°C until it can be assayed.

3.5.3. Reverse Transcription (RT)

- 3.5.3.1. Preliminary procedures (to be performed in a clean room)
 - 3.5.3.1.1. Label PCR plates or tubes with appropriate sample numbers.
 - 3.5.3.1.2. Prepare RT master mix 1 and 2. The amounts shown for the volume per master mix can be scaled up or down according to the number of samples that need to be analyzed.
 - 3.5.3.1.3. Vortex the master mixes after the addition of all ingredients.
 - 3.5.3.1.4. Centrifuge at $>5,000$ rpm for 10 seconds in a microcentrifuge.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 8 of 10

- 3.5.3.2. Aliquot 16 μL of RT master mix 1 to the labeled PCR tubes or plate wells.
 - 3.5.3.2.1. Run every environmental water and QC sample in triplicate by adding 6.7 μL of the appropriate sample to each of the tubes or plate wells labeled for that sample.
 - 3.5.3.2.2. Add 6.7 μL of molecular grade water to one or more tubes or plate wells as no template controls (NTC). Include at least one NTC for the replicates associated with every fourth water sample run on a plate; NTC controls must be distributed throughout the plate. Note: If any NTC control is positive, the cause of the false positive value should be investigated. After fixing the cause of the problem, all samples on the plate must be rerun.
 - 3.5.3.2.3. Close the tubes or seal the plates and heat at 99°C for 4 minutes, followed by quenching on ice or a hold temperature of 4°C.
 - 3.5.3.3. Add 17.3 μL of RT mix 2 to each tube or well.
 - 3.5.3.4. Centrifuge at >5,000 rpm for 10 seconds in a microcentrifuge at 4°C.
 - 3.5.3.5. Run at 25°C for 15 minutes, 42°C for 60 minutes, 99°C for 5 minutes, and then hold at 4°C for up to 2 hours until PCR is performed.
 - 3.5.3.6. Centrifuge at >5,000 rpm for 10 seconds in a microcentrifuge.
 - 3.5.3.7. Proceed immediately to step 3.5.4 or store reverse transcribed samples at -80°C until they can be processed.
- 3.5.4. Polymerase Chain Reaction (PCR) Amplification and Gel Electrophoresis
- 3.5.4.1. Preliminary procedures
 - 3.5.4.1.1. Label PCR plates or tubes with appropriate sample numbers.
 - 3.5.4.1.2. Prepare PCR master mixes. The amounts shown for the volume per master mix can be scaled up or down according to the number of samples that need to be analyzed.
 - 3.5.4.1.3. Vortex the master mix after the addition of all ingredients.
 - 3.5.4.1.4. Centrifuge at >5,000 rpm for 10 seconds in a microcentrifuge.
 - 3.5.4.1.5. Dispense 14 μL of the appropriate mix to the labeled plates or tubes.
 - 3.5.4.2. Add 6 μL of the appropriate sample to each tube.
 - 3.5.4.3. Run on a thermal cycler with a setting of 1 cycle at 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.
 - 3.5.4.4. Prepare a 2% agarose gel in 1XTBE buffer. Add the appropriate amount of ethidium bromide to the slightly cooled molten agarose, then pour the agarose in the casting tray. Let the agarose solidify, then transfer the tray containing the solidified gel to the electrophoresis tank containing 1XTBE buffer.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 9 of 10

- 3.5.4.5. Mix 10 µl of each PCR product with 2 µl of each gel electrophoresis sample loading buffer. Load samples into the agarose gel wells, always loading molecular weight marker into the first and last wells used.
- 3.5.4.6. Run the gel for 60 minutes at room temperature and at a constant 5 V per cm of gel length (e.g. a 20 cm gel would be run at a constant 100 V).
- 3.5.4.7. Compare results with Armored RNA standard to determine the presence or absence of enteroviruses in each sample.

References:

Di Giovanni, G.D., R.M. Hoffman, and G.D. Sturbaum. 2010. *Cryptosporidium* genotyping method for regulatory microscopic slides. Web Report #4099. Water Research Foundation, Denver, CO.

Fout, G.S., N.E. Brinkman, J.L. Cashdollar, S.M. Griffin, B.R. McMinn, E.R. Rhodes, E.A. Varughese, M.R. Karin, A.G. Grimm, S.K. Spencer, and M.A. Borchardt. 2010. Method 1615. Measurement of eneterovirus and norovirus occurrence in water by culture and RT-qPCR. EPA/600/R-10/181. USEPA, Cincinnati, OH.

Soil & Aquatic Microbiology Laboratory	Filtration and Assay of Water Samples for Enterovirus	
Texas AgriLife Research	SOP: SAML-12-104	Rev. No. 1
Texas A&M University	Date: November 2012	Page 10 of 10