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Detection of *Cryptosporidium* Recovered from Large-Volume Water Samples Using Dead-End Ultrafiltration

Amy M. Kahler¹, Vincent R. Hill²

¹Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Diseases Control and Prevention, Atlanta, GA, USA.

²Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Diseases Control and Prevention, Atlanta, GA, USA.

Abstract

The procedure described here provides instructions for detection of *Cryptosporidium* recovered from large-volume water samples. Water samples are collected by dead-end ultrafiltration in the field and ultrafilters are processed in a laboratory. Microbes recovered from the filters are further concentrated and subjected to *Cryptosporidium* isolation or nucleic acid extraction methods for the detection of *Cryptosporidium* oocysts or *Cryptosporidium* DNA.

Keywords

Cryptosporidium; Dead-end ultrafiltration; IMS; Microscopy; Real-time PCR; Water

1 Introduction

The procedure described here provides instructions for detection of *Cryptosporidium* recovered from large-volume water samples. Water samples are collected using a hollow-fiber, dead-end ultrafiltration method (DEUF). The hollow-fiber ultrafilters used in this procedure have pore sizes on the order of 10 nm (~30 kiloDaltons (kDa)) and thus can be used to recover protozoan parasites, bacteria, viruses, and large toxins from water samples [1–3]. A specific ultrafilter type (REXEED 25S) has been identified in this protocol because of its large filter fiber surface area (2.5 m²) and reported performance effectiveness by multiple research laboratories. However, alternative hollow fiber ultrafilters may be used if they have similar specifications and performance characteristics.

The DEUF method is designed to be performed in the field with minimal equipment and setup. In the DEUF procedure, water flows into the ultrafilter, through the hollow-fiber membranes, and out of the ultrafilter pores while microbes are captured within the hollow fibers. The ultrafilters are capable of filtering 10–50 L of turbid surface water or hundreds of

liters of finished drinking water. The volume of water filtered will depend on water quality characteristics and the suspected concentrations of target microorganisms. After ultrafiltration, the ultrafilter is processed in a laboratory. The ultrafilter is backflushed with 500 mL of a solution containing 0.5% Tween 80, 0.01% sodium polyphosphate, and 0.001% Antifoam Y-30 emulsion to recover the microbes from the ultrafilter. If the concentration of the target microbes is sufficiently high, the resulting backflushed solution can be analyzed directly. If the concentration of the target microbes is low or unknown, the backflushed solution can be further concentrated to achieve a volume that is amenable to downstream detection methods.

For *Cryptosporidium* detection, the sample concentrate may be subjected to immunomagnetic separation (IMS) and immunofluorescence assay (FA) microscopy for observation of *Cryptosporidium* oocysts [4] and/or nucleic acid extraction and real-time PCR for detection of *Cryptosporidium* DNA [5, 6]. The choice of detection methods should be determined by the goals of the study and/or the unique characteristics of the water type being analyzed. The performance recovery efficiency of each methodological step (DEUF, secondary concentration, IMS, nucleic acid extraction) may vary depending on water quality and composition [1, 2]. Therefore, it is recommended that the complete method be evaluated and validated before processing real-world samples to ensure that effective detection can be achieved.

2 Materials (See Note 1)

2.1 Dead-End Ultrafiltration

1. Insulated cooler.
2. Reusable freezer packs.
3. REXEED 25S dialysis filter (Asahi Kasei Medical America Inc., Glenview, IL).
4. Masterflex L/S 36 tubing (platinum-cured silicone or C-Flex ULTRA) (Cole-Parmer, Vernon Hills, IL).
5. Female DIN to 3/8 barb fitting adapters, polypropylene (Molded Products, Inc., Harlan, IA).
6. SNP-8 acetal copolymer tubing clamps.
7. Blood port kidney storage cap (Molded Products).
8. Flow totalizing meter, 1/2" schedule 80 PVC NPT turbine, 3.8–38 L/min (Omega Engineering, Inc. Swedesboro, NJ) fitted with two straight barbed to male NPT threaded adapters, 3/4" NPTM $\times$ 3/8" tubing ID.
9. 20 L collapsible cubitainers, optional.
10. Long nose pliers.
11. Scissors.
12. 500 mL bottles (Nalgene).

13. Sodium thiosulfate.
14. Geotech Geopump peristaltic pump (Geotech Environmental Equipment, Inc., Denver, CO) with EZ-Load II pump head placed on the Series 2 drive (for non-pressurized water sources).
15. Alcohol wipe or equivalent.
16. 60 mL syringe.
17. 1/2" ID hose × swivel FGHT nylon swivel female insert.
18. SNP-12 acetal copolymer tubing clamps.
19. Masterflex I/P 89 platinum-cured silicone tubing (Cole-Parmer).
20. Reducing connector 5/8" to 3/8".
21. SNP-28 acetal copolymer tubing clamps.
22. SNP-24 acetal copolymer tubing clamps.
23. SNP-19 acetal copolymer tubing clamps.

2.2 Backflushing

1. Masterflex L/S digital pump system with Easy-Load II pump head, 600 rpm, 115/230 V (or use the same pump as for field collection) (Cole-Parmer).
2. Lab jack with 10" × 10" top.
3. Ultra flex support with base plate, 18" arm length.
4. Masterflex L/S 36 tubing (Platinum-cured silicone or C-Flex ULTRA) (Cole-Parmer).
5. Blunt-edge forceps.
6. Faceshield.
7. Long nose pliers.
8. SNP-12 or SNP-14 acetal copolymer tubing clamps.
9. 500 mL bottle.
10. 1 L bottle.
11. 10 mL plastic serological pipette.
12. Deionized or distilled (DI) water.
13. Tween 80.
14. Sodium hexametaphosphate.
15. Antifoam Y-30 emulsion.

2.3 Secondary Concentration

1. Centrifuge capable of accepting 200–500 mL conical centrifuge tubes.
2. 200 mL or 500 mL conical centrifuge bottles.
3. 50 mL plastic serological pipette.
4. 0.01 M PBS, pH 7.2–7.4 (1×) or equivalent buffer solution for resuspension.

2.4 Immuno-magnetic Separation (IMS) and Fluorescent Antibody (FA) Microscopy

1. Glass Leighton tubes or Dynabeads L-10 tubes (Applied Biosystems Inc., Beverly, MA).
2. Deionized or distilled (DI) water.
3. Anti-*Cryptosporidium* magnetic beads (Dynabeads anti-*Cryptosporidium* or Dynabeads GC-Combo (Applied Biosystems).
4. Rotary mixer for immunomagnetic beads (Dynabeads rotary mixer, Applied Biosystems).
5. Magnet for magnetic bead capture in Leighton tubes or 10–30 mm tubes (MPC-6 magnetic particle concentrator, Applied Biosystems).
6. 1 mL or 2 mL plastic serological pipettes.
7. 1.5 mL nuclease-free (NF) microcentrifuge tubes (Invitrogen Corp., Carlsbad, CA).
8. Magnet for magnetic bead capture in microcentrifuge tubes (MPC-S magnetic particle concentrator (Applied Biosystems).
9. 0.01 M PBS, pH 7.2–7.4 (1×).
10. 0.1 N HCl.
11. 1 N NaOH.
12. Two-well microscope slides with adhesive coating (SuperStick slides, Waterborne Inc., New Orleans, LA).
13. Heat block or slide warmer.
14. EasyStain *Cryptosporidium* oocyst labeling reagent (bioMérieux Inc., Durham, NC).
15. Coverslips, 22 × 60 mm.
16. Clear nail polish.
17. Fluorescent microscope with FITC filter.

2.5 Nucleic Acid Extraction and Real-Time PCR

1. Molecular grade ethanol, 200 proof.
2. Nuclease-free (NF) water (or molecular grade water).

3. 0.2 mm zirconium oxide beads, Y₂O₃-stabilized, 95% (Union Process, Inc., Akron, OH).
4. 0.5 mm zirconium oxide beads, Y₂O₃-stabilized, 95% (Union Process, Inc.).
5. 0.1 N HCl.
6. Oven.
7. 0.5 mL nuclease-free mL tubes.
8. Double-ended micro-tapered stainless steel spatula.
9. Twist ties.
10. FastPrep-24 bead beater (MP Biomedicals, LLC, Santa Ana, CA).
11. FastPrep compatible 2 mL empty bead beating tubes (MP Biomedicals).
12. FastPrep compatible caps for 2 mL beating tubes (MP Biomedicals).
13. UNEX lysis buffer (Microbiologics, Inc., St. Cloud, MN).
14. Proteinase K, 600 mAU/mL.
15. Silica HiBind RNA minicolumn RNACOL (Omega Bio-tek, Inc., Norcross, GA).
16. OneStep PCR inhibitor removal (Zymo Research, Irvine, CA).
17. 2 mL collection tube.
18. 1.5 mL nuclease-free microcentrifuge tubes.
19. Tris-EDTA (TE) buffer, pH 8.0, molecular biology grade.
20. TaqMan Environmental Master Mix 2.0 (Life Technologies Corp., Carlsbad, CA).
21. Oligonucleotides
Forward primer: ATG ACG GGT AAC GGG GAA T
Reverse primer: CCA ATT ACA AAA CCA AAA AGT CC
Probe: 6FAM-CGC GCC TGC TGC CTT CCT TAG ATG-BHQ1
22. T4 gene 32 protein (gp32).
23. Bovine serum albumin (BSA, molecular biology grade).
24. TaqMan Exogenous Internal Positive Control Kit (Life Technologies Corp.).
25. Aerosol barrier pipette tips.
26. Applied Biosystems 7500 real-time PCR thermocycler (Life Technologies Corp.).
27. MicroAmp optical 96-well reaction plate or MicroAmp optical 8-tube strip (Life Technologies Corp.).

28. Optical adhesive film (MicroAmp 96-well format) or MicroAmp optical 8-cap strip (Life Technologies Corp.).
29. Laminar flow cabinet or similar PCR-compatible workstation.
30. Plate or strip spinner.
31. RNase AWAY surface decontaminant (Thermo Fisher Scientific, Inc., Norcross, GA).

3 Methods

3.1 Reagent Preparation

1. Sodium thiosulfate solution (1% w/v): add 5 g sodium thiosulfate to a 500 mL Nalgene bottle, add 500 mL DI water or ultrafilter effluent as described in Subheading 3.2, **step 15**.
2. Backflush solution (0.5% Tween 80, 0.01% sodium polyphosphate (NaPP), and 0.001% Antifoam Y-30 emulsion): add 10 mL DI water to a screw-cap tube, add 1 g NaPP and 100 μ L Antifoam (*see* Note 2) to the DI water (10% NaPP, 1% Antifoam), shake vigorously to dissolve the NaPP (*see* Note 3). This stock solution can be kept at room temperature or at 4 °C. Add 500 mL DI water to a 500 mL bottle. Add 2.5 mL Tween 80 and 500 μ L of the NaPP/Antifoam stock solution to the DI water. Swirl to dissolve the Tween 80. If desired, the backflush solution can be prepared a day in advance and stored at 4 °C. Warm to room temperature before use.
3. BSA/gp32 solution (10 mg/mL BSA, 500 μ g/mL gp32): add 10 mL TE buffer to 100 mg of BSA and dissolve by swirling. Aliquot 1 mL into 1.5 mL microcentrifuge tubes and store at 4 °C. Thaw gp32 and add 950 μ L BSA solution and invert to mix, store at 4 °C.

3.2 Ultrafiltration

1. Prepare the flow totalizer: screw in a tubing adapter to each end of the totalizer, take a length of L/S 36 tubing and cut in half, push tubing onto both sides of the meter, and no tubing clamps are needed.
2. Label ultrafilter with an indelible marker.
3. Fill out pertinent information on chain of custody form or sample collection bench sheet: pertinent information may include: sample location and/or sample ID, measured water quality parameters, water type, site description, etc.
4. Remove one end port cap and screw in a DIN adapter (this is the influent port).

5. Push L/S 36 tubing onto the DIN adapter and secure with a SNP-8 clamp. The length of influent tubing needed will be determined by the distance to the water source.
6. Close off the side port closest to the influent port by pushing the cap until it clicks.
7. Screw in a blood port cap to the end port opposite the influent port.
8. Remove the cap from the effluent port (furthest from the influent port) and save. Push the flow totalizer tubing onto the effluent port, making sure that the arrow on the flow totalizer points away from the ultrafilter. No tubing clamp is needed.
9. Record the initial flow totalizer meter reading or reset to zero.
10. Place the effluent tubing in an empty 20 L cubitainer or equivalent container, downstream of the water being collected, or near a floor drain. The effluent water will be free of microbial contamination so it can be released into the environment as conditions allow (*see* Note 4).
11. Non-pressurized water sources (Fig. 1): Place the influent tubing into the water source (or water that has been collected in a 20 L cubitainer). Ensure that the end of the tubing will stay below the surface of the water (*see* Note 5). Feed the influent tubing through the pump head and close the pump head using the lever. Plug in the “battery” power cord into the pump and the other end into the battery (*see* Note 6). The Geopump comes with alligator clips so that any external battery that is 12–18 V DC @ 70 watts or 90–260 V AC @ 47–65 Hz can be used. Place the battery in a location where it will not get wet. Position the toggle switch on the pump for the appropriate direction of flow. Ensure the speed dial is

set to zero and turn on the pump. Gradually increase the speed to the maximum setting (*see* Note 7).

12. Pressurized water sources (Fig. 2): wipe the faucet with an alcohol wipe or other sanitizer. Turn on the faucet and purge the water for 2–3 min. Turn off the faucet before setting up the ultrafilter. If sampling from a standard garden faucet (hose bib), screw on a FGHT adapter. Push influent tubing onto the adapter and secure with a SNP-12 clamp. If sampling from a non-standard faucet, push 4–6 inches of I/P 89 tubing over the faucet head and secure with a SNP-28 clamp. Use a reducing connector to connect the I/P 89 and L/S 36 influent tubing, and secure both sides with SNP-19 and SNP-8 clamps, respectively. Turn on the faucet and gradually increase the flow until the desired flow rate is achieved (3–5 L/min) (*see* Note 8).
13. If the flow totalizer is not reset to zero, calculate the desired flow totalizer end reading by adding the volume of water collected to the starting reading (*see* Note 9).
14. During filtration, visually inspect the flow rate from the effluent tubing. Dramatic changes in flow rate will indicate filter clogging, which can be due to water quality or entrapment of an object in the influent tubing.
15. Prepare the sodium thiosulfate solution if a chlorine residual is present (or suspected) in the water source. If water was not already added to the sodium thiosulfate bottle during preparation, fill the bottle to the 500 mL mark using the ultrafilter effluent. Shake to dissolve and set aside.
16. Once the desired volume of water has been filtered, turn off the pump or faucet.
17. For water sources with a chlorine residual, follow the chlorine quenching steps below. For non-chlorinated water sources, proceed to **step 20**.
18. Non-pressurized water sources: place the influent tubing into the sodium thiosulfate solution. Turn on the pump to draw the sodium thiosulfate solution into the ultrafilter. Continue until the sodium thiosulfate solution has been drawn through most of the influent tubing, but do not allow air to be pumped into the ultrafilter. Turn off the pump and release lever on the pump head.
19. Pressurized water sources: remove the influent tubing from the faucet and cut the tubing so that only 2–3 inches remain attached to the ultrafilter. Remove the plunger from the 60 mL syringe. Insert the tip of the syringe into the influent tubing and secure with an SNP-8 clamp. Pour 60 mL of the sodium thiosulfate solution into the syringe and insert the plunger to push the solution through the ultrafilter. Remove the SNP-8 clamp and take the syringe out of the tubing. Remove the plunger from the syringe. Pulling out the plunger before removing the syringe from the tubing will create negative pressure and draw liquid out of the ultrafilter. Repeat sodium thiosulfate injection (as described above) one more time.

20. Unscrew the DIN adapter with influent tubing. Screw a blood port cap into the influent port.
21. Place influent tubing and clamps into a waste bag for used supplies. Return to the lab for disposal or decontamination.
22. Remove the effluent tubing and re-cap the effluent port using the cap supplied with the ultrafilter. The effluent tubing and flow totalizer can be reused for subsequent samples.
23. Place the sealed ultrafilter in a cooler for shipment or transport to the laboratory. Use ice packs or bags of ice to keep the cooler chilled. The ideal temperature range is 2–8 °C.

3.3 Backflushing and Secondary Concentration

1. Prepare the backflush solution.
2. Assemble the system as shown in Fig. 3 (*see Note 10*): set the pump on the lab jack, raise the lab jack so that the pump head will be at the same height as the side port of the ultrafilter where the backflush will enter. Clamp ultrafilter vertically into support base so that end ports are at the top and bottom and side ports are pointed in the direction of the pump. Ensure the ultrafilter is high enough that a 1 L bottle can be placed under the end port. Ensure a blood port storage cap is screwed into the top port. Remove the cap from the highest side port. Ensure the remaining side port cap is secure.
3. Cut a 10–12" length of L/S 36 tubing.
4. Push the tubing onto the open side port and secure with an SNP-12 or SNP-14 clamp.
5. Feed the tubing through the pump head.
6. Remove the cotton plug from a 10 mL serological pipette using a pair of forceps. Break off the conical tip of the plastic pipette (*see Note 11*).
7. Push the influent tubing onto the top of the pipette and place the pipette into the backflush solution.
8. Close the pump head and ensure the direction flow is correct. Set the pump speed to 650 mL/min (*see Note 12*).
9. Remove the blood port storage cap from the lower end port and place a sterile 1 L bottle under the port.
10. Turn on the pump and pump the entire backflush solution through the ultrafilter.
11. Turn off the pump when no backflush solution remains in the bottle and the flow out of the ultrafilter slows to a trickle. Do not continue pumping once the flow of liquid from the ultrafilter has stopped. Doing so will cause pressure to build up and increase the risk of the side port cap to burst off.
12. Open the pump head slowly to release the pressure in the influent tubing.

13. Remove the tubing clamp and save for subsequent ultrafilters.
14. Release the ultrafilter from the support base and cap the bottle containing the sample concentrate.
15. Unscrew the blood port caps and save for decontamination.
16. Measure and record the backflush concentrate volume, if necessary.
17. Centrifuge the backflush concentrate at $1500\text{--}2000 \times g$ for 15 min according to USEPA Method 1623.1 (*see* Note 13).
18. Carefully remove the supernatant using a 50 mL pipette, leaving 1–5 mL of supernatant above the pellet.
19. Measure and record the packed pellet volume and total concentrate volume.
20. Proceed to Subheadings 3.4–3.5 for *Cryptosporidium* detection by immunofluorescence assay microscopy or Subheadings 3.6–3.7 for *Cryptosporidium* detection by real-time PCR. If both detection methods are utilized, split the centrifuge concentrate for processing by each method (*see* Note 14).

3.4 Immuno-magnetic Separation(IMS, Per USEPA Method 1623.1)

1. If the packed pellet volume is 0.5 mL, the entire sample concentrate, or up to 10 mL, can be processed by IMS. If the packed pellet volume is >0.5 mL, process no more than the equivalent of 0.5 mL. If desired, the sample concentrate can be split into subsamples in order to process the entire volume. If the packed pellet volume is 0.5 mL, but the total sample concentrate volume is >10 mL, the sample concentrate can be split into subsamples in order to process the entire volume, if desired.
2. Prepare a 1× dilution of SL-A buffer from the provided 10× buffer stock using DI water. For each sample processed, 1.5 mL of 1× SL-A buffer will be required.
3. Add the sample concentrate to an L-10 tube. Bring the volume up to 10 mL with DI water if processing <10 mL sample concentrate.
4. Add 1 mL each of the 10× SL-A and SL-B buffers to the L-10 tube.
5. Vortex the Dynabeads for 10 s and add 100 µL to the L-10 tube.
6. Place the L-10 tube on the rotary mixer and rotate at 18 rpm for 1 h at room temperature.
7. Place the L-10 tube in the MPC-6, ensuring the flat side of the tube is flush against the magnet.
8. Starting in an upright position, gently rock the tube by hand through 180° for 2 min, with approximately 1 tilt per second.

9. Remove the cap from the L-10 tube and pour the supernatant into a collection beaker. Allow more supernatant to settle and remove with a 1 mL or 2 mL serological pipette.
10. Remove the L-10 tube from the MPC-6 and resuspend the sample in 0.5 mL of 1× SL-A buffer by repeatedly releasing the liquid down the flat side of the tube. Do not vortex.
11. Transfer the solution to a 1.5 mL microcentrifuge tube. Repeat rinsing procedure two more times.
12. Place the microcentrifuge tube in the MPC-S with the magnet in place.
13. Starting in an upright position, gently rock the tube by hand through 180° for 1 min, with approximately 1 tilt per second.
14. Aspirate the supernatant from the tube using a 1000 µL micropipettor.
15. Add 1 mL of 0.01 M PBS to the tube, taking care not to disturb the beads at the back of the tube.
16. Remove the magnet from the MPC-S and gently resuspend the beads.
17. Replace the magnetic strip in the MPC-S.
18. Starting in an upright position, gently rock the tube by hand through 180° for 1 min, with approximately 1 tilt per second.
19. Aspirate the supernatant from the tube using a 1000 µL micropipettor.
20. Let the tube stand undisturbed for 1 min, then aspirate any remaining liquid from the tube.
21. Remove the magnet from the MPC-S and add 50 µL of 0.1 N HCl to the tube. Vortex on high for 50 s.
22. Allow the tube to stand undisturbed in an upright position for 10 min at room temperature.
23. Vortex on high for 30 s.
24. Tap the sample into the base of the tube and place in the MPC-S.
25. Replace the magnetic strip in the MPC-S and allow the tube to stand undisturbed for at least 10 s.
26. Add 5 µL NaOH to two sample wells on a two-well adhesive microscopy slide, or 10 µL to one well if the volume from the two dissociation steps will be combined to the same well.
27. While the tube is still in the MPC-S, transfer the sample volume from the tube to a microscopy slide well.
28. Repeat the dissociation process (Subheading 3.4, **steps 21–27**); add the sample volume to a second sample well or combine with the volume from the first dissociation.

29. Proceed directly to fluorescent antibody microscopy or store the slides up to 24 h in a closed container at 4 °C.

3.5 Fluorescent Antibody (FA) Microscopy

1. Place the Fixing Buffer from the EasyStain kit into a beaker of ice water, hold at 4 °C until use.
2. Dry the sample suspension on the two-well adhesive microscope slides on a slide warmer or heat block (60 °C, usually 10 min). Alternatively, the slides can be dried inside a 37 °C incubator.
3. Add 50 µL EasyStain reagent to the well.
4. Incubate at room temperature for 30 min or in a box containing a moist tissue at 37 °C for 15 min (*see* Note 15).
5. Place the slide onto a paper towel and tilt the slide to absorb the stain from the well.
6. Add 300 µL ice-cold Fixing Buffer to the well, ensuring the entire well is covered. Let stand for 2 min.
7. Place the slide onto a Kimwipe or paper towel and tilt the slide to absorb the Fixing Buffer from the well.
8. Add one drop of Mounting Medium to the well and apply the coverslip (*see* Note 16).
9. Apply clear nail polish to the edges of the coverslip to seal the coverslip to the slide.
10. Examine the slide under 200×–400× magnification using a fluorescent microscope with a FITC filter. *Cryptosporidium* oocysts will appear as ~5 µm circles with a bright green fluorescent oocyst wall.

3.6 Nucleic Acid Extraction

1. Prepare 70% ethanol by combining 35 mL of 200-proof ethanol and 15 mL of NF water in a 50 mL conical tube. Store the solution at room temperature.
2. Prepare the extraction beads: submerge the 0.2 mm and 0.5 mm beads separately in 0.1 N HCl in a 250 mL glass beaker and mix for 30 s. Let it sit for 10 min, then decant the HCl. Rinse the beads by adding enough deionized or DI water to submerge the beads. Mix for 30 s and decant the DI water. Repeat the DI rinsing procedure four more times. Transfer the beads to separate clean 400 mL glass beakers. Bake the beads in a laboratory oven at 200 °C for 30 min (or until completely dry) and allow to cool to room temperature. Transfer the beads to a container with a cap, such as a 50 mL conical tube, and store at room temperature.
3. Cut the cap off a 0.5 mL microcentrifuge tube and secure with a twist tie onto the end of a double-ended spatula.

4. Scoop one capful each of the 0.2 mm and 0.5 mm acid-wash beads into a bead beating tube.
5. Add 750 μ L water sample concentrate to the prepared beat beating tube (*see* Note 17). Add 750 μ L UNEX lysis buffer and 75 μ L Proteinase K, vortex to mix.
6. Allow the sample to sit at room temperature for at least 15 min for Proteinase K activity.
7. Place the bead beating tube securely in the bead beater. Balance with a similarly weighted bead beating tube if processing one sample.
8. Beat the sample at 6.0 m/s for 60 s.
9. Centrifuge the bead beating tube at $10,000 \times g$ for 30 s.
10. Prepare the silica column by placing into a provided 2 mL collection tube.
11. Transfer 700 μ L of supernatant from the bead beating tube into a prepared silica column.
12. Centrifuge the silica column at $10,000 \times g$ for 1 min.
13. Pour the filtrate from the collection tube into an autoclave waste container, place the silica column back into the collection tube.
14. Repeat **steps 11–13** to process the remaining volume, being careful to avoid the beads and any pellet material while removing the supernatant.
15. Add 500 μ L of 100% ethanol to the silica column.
16. Centrifuge the silica column at $10,000 \times g$ for 1 min.
17. Pour the filtrate from the collection tube into an autoclave waste container, place the silica column back into the collection tube.
18. Add 500 μ L of 70% ethanol to the silica column.
19. Centrifuge the silica column at $10,000 \times g$ for 1 min.
20. Pour the filtrate from the collection tube into an autoclave waste container, place the silica column back into the collection tube.
21. Centrifuge the silica column at $10,000 \times g$ for 1 min. This is a dry spin to remove any residual ethanol.
22. Discard the collection tube and place the silica column into a labeled 1.5 mL microcentrifuge tube.
23. Add 80 μ L TE buffer to the silica column.
24. Centrifuge the silica column at $9000 \times g$ for 1 min.
25. Insert a OneStep PCR inhibitor removal column into a provided collection tube.
26. Add 600 μ L of the Prep-Solution and centrifuge at $8000 \times g$ for 3 min.
27. Transfer the column to a labeled 1.5 mL microcentrifuge tube.

28. Transfer the eluted TE buffer from the silica column to the OneStep column.
29. Centrifuge the OneStep column at $8000 \times g$ for 1 min (*see Note 18*).
30. Discard the OneStep column and store the nucleic acid eluate at -20 or -80 °C until PCR analysis.

3.7 Real-Time PCR

1. Thaw frozen reagents in a laminar flow or PCR-compatible cabinet.
2. Prepare the master mixture solution by adding the PCR reagents to a 1.5 mL microcentrifuge tube (Table 1). Mix the solution inverting the tube several times or briefly vortexing.
3. Add 45 μ L of the master mixture into each reaction tube in a 96-well plate or 8-tube strip.
4. Add 5 μ L template DNA to the appropriate reaction tubes. Triplicate reaction tubes are recommended for environmental samples (*see Note 19*).
5. Add 5 μ L NF water as a no-template control to the appropriate reaction tubes. Additional no-template controls using the internal control blank can also be analyzed.
6. Add 2–5 μ L positive control, depending on the concentration and desired Ct value. If adding less than 5 μ L, add additional NF water to bring the total reaction volume to 50 μ L.
7. Briefly spin tube strips or 96-well plates to move liquid to the bottom of the tubes.
8. Run samples with the following cycling conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 55 °C for 1 min. Fluorescence acquisition is done during the 55 °C step (*see Note 20*).
9. Analyze amplification curves visually to obtain detect/non-detect status of each sample. Calculate Ct values by manually or automatically setting the threshold level for the run. When comparing Ct values between real-time PCR runs, ensure the threshold level is the same for each run.

3.8 Decontamination and Disposal

1. Discard the ultrafilter and side port caps in an autoclave waste container.
2. Remove tubing clamps, faucet adapters, reducing connectors, and DIN adapters from influent sample collection tubing. Discard the tubing in an autoclave waste container.
3. Inspect and clean the effluent tubing and flow totalizer. The setup can remain assembled for future use. Discard tubing that has become cracked or worn. Store in a cool dry place.

4. Place reusable plastic DEUF components into a solution of 10% bleach for 1 h. Rinse thoroughly with DI water and air dry.
5. Discard centrifuge supernatant in a beaker containing 10% (v/v) sodium hypochlorite and dispose of according to the laboratory's procedures.
6. UV sterilize laminar flow or PCR-compatible cabinet and wipe down pipettes and equipment with RNase AWAY.

4 Notes

1. Catalog numbers are included for reference; substitutions can be made, provided the substitute item has the same specifications and equivalent performance as the suggested item.
2. In order to more easily pipette the Antifoam solution, cut off the tip of a 1000 μ L pipette tip to create a larger opening.
3. NaPP requires considerable shaking to dissolve and a wrist shaker is ideal for this purpose. Alternatively, the stock solution can be prepared in advance to allow time to dissolve or the volume can be scaled up to 100 mL and the solution can be stirred in a beaker on a magnetic stir plate.
4. For sampling non-flowing bodies of water, the ultrafilter effluent should be directed away from the location where the influent tubing is drawing water.
5. It is important that the tubing stays submerged during filtration. A bit of air in the line will not cause a problem, but drawing in too much air will cause pumping to fail. When this occurs, the pump will not draw any water, even when the tubing is re-submerged. Turn off the pump and open the pump head to release the pressure. Pumping can resume once the tubing is re-submerged in the water. For natural water sources (e.g., lakes, rivers), it can be helpful to loosely attach the tubing to something in the water using a zip-tie. If that is not possible, a rock or other object can be gently placed over the tubing to secure it, provided the tip of the tubing can be positioned such that it will not draw in sediment.
6. Charge pump batteries 24 h before use. Non-United States outlets require a step-down converter with an outlet adapter.
7. If a Geotech pump (or equivalent) is not used, ensure the filtrate rate does not exceed 5 L/min.
8. Certain power sources may not provide enough power to achieve a flow rate of 3.8 L/min. When this is the case, the pump should be run at full speed to achieve the highest flow rate possible. The flow totalizer will provide a less accurate (but still useful) estimate of the volume passed through the ultrafilter as the flow rate decreases. It may also be useful to purge any air from the totalizer at the beginning of the sample collection by briefly orienting the totalizer so the outlet is pointed "up" and visually noting the disappearance or absence of bubbles exiting the totalizer.

9. If you do not have a flow totalizer, you can manually calculate the flow rate using a graduated cylinder or 1 L bottle and a stopwatch.
10. There is no directionality to the ultrafilter. The end ports are color coded for hemodialysis, but there is no difference in functionality for water sampling.
11. The 10 mL pipette gives more control during the backflushing, but it is not necessary. If opting out using of the pipette, make the tubing long enough to place the end directly into the backflush bottle.
12. If the pump used for backflushing does not have a display for the speed setting, the desired pumping rate of 650 mL/min can be determined by pumping water through a length of tubing and measuring the flow rate. This setting can be marked on the pump for future reference. If the Geotech pump is used for backflushing, it requires going above the 650 mL/min setting to achieve pumping. Once pumping is initiated, the dial should immediately be turned back to the 650 mL/min setting.
13. A centrifugation speed up to $4000 \times g$ can be used if there are other analytes in the sample that require higher g forces to pellet.
14. For potable or groundwater samples (i.e., small concentrate volume), the pellet can be split 50:50 to nucleic acid extraction and IMS. For surface water samples (i.e., larger concentrate volume), up to 750 μ L can be used for nucleic acid extraction and a portion of the remaining volume can be used for IMS.
15. An empty micropipette tip box makes a suitable storage container for slides. For staining, remove the pipette tip tray and place a folded Kimwipe or paper towel in the bottom of the box. Mist or squirt DI water to moisten the towel. Replace the pipette tip tray to hold the slide. After the slide is stained, remove the moist towel. If the pipette tip box lid is not opaque, wrap the lid with aluminum foil. Store the stained slide in the dark box until microscopy is performed.
16. It is important to minimize the formation of bubbles when placing the coverslip, as they will interfere with examination under the microscope. It is helpful to connect the coverslip with the mounting medium at an angle. Place the long edge coverslip along one end of the slide and lower down at an angle until it is resting on top. Alternatively, lower the slide down onto the coverslip at an angle.
17. For low-quality water samples, or when sample volume is limited, less than 750 μ L can be extracted. Add an equal volume of UNEX lysis buffer and 10% Proteinase K (10% of sample volume, not total volume after UNEX buffer is added).
18. Some water concentrates may produce a nucleic acid extract that is not completely recovered from the OneStep column after 1 min. Subsequent spins (1 min at a time) can be performed to recover a larger volume.
19. Because PCR inhibitors are sometimes present, it may be useful to also test template samples diluted 1:10.

20. Cycling times will vary depending on master mix and real-time thermocycler used. Follow the manufacturer's instructions for the appropriate cycling times.

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Disclosures: Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Fig. 1.
DEUF assembly for non-pressurized water sources

Fig. 2.
DEUF assembly for pressurized water sources

Fig. 3.
DEUF assembly for backflushing and recovery of captured sample concentrate

Table 1*Cryptosporidium* real-time PCR master mixture recipe

Reagent	Initial concentration	Volume per 50 μ L reaction (μ L)	Final concentration
Environmental Master Mix 2.0	2 $\times$	25	1 $\times$
Forward primer	10 μ M	1.25	250 nM
Reverse primer	10 μ M	1.25	250 nM
Probe	4 μ M	1.25	100 nM
BSA/gp32	10 mg/mL BSA; 500 μ g/mL gp32	2.5	0.5 mg/mL BSA; 25 μ g/mL gp32
Internal control mix	10 $\times$	5	1 $\times$
Internal control DNA	50 $\times$	1	1 $\times$
NF water	NA	7.75	NA