Video Article

A Modified EPA Method 1623 that Uses Tangential Flow Hollow-fiber Ultrafiltration and Heat Dissociation Steps to Detect Waterborne *Cryptosporidium* and *Giardia spp.*

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Abstract

Cryptosporidium and *Giardia* species are two of the most prevalent protozoa that cause waterborne diarrheal disease outbreaks worldwide. To better characterize the prevalence of these pathogens, EPA Method 1623 was developed and used to monitor levels of these organisms in US drinking water supplies [12]. The method has three main parts; the first is the sample concentration in which at least 10 L of raw surface water is filtered. The organisms and trapped debris are then eluted from the filter and centrifuged to further concentrate the sample. The second part of the method uses an immunomagnetic separation procedure where the concentrated water sample is applied to immunomagnetic beads that specifically bind to the *Cryptosporidium* oocysts and *Giardia* cysts allowing for specific removal of the parasites from the concentrated debris. These (oo)cysts are then detached from the magnetic beads by an acid dissociation procedure. The final part of the method is the immunofluorescence staining and enumeration where (oo)cysts are applied to a slide, stained, and enumerated by microscopy.

Method 1623 has four listed sample concentration systems to capture *Cryptosporidium* oocysts and *Giardia* cysts in water: Envirochek filters (Pall Corporation, Ann Arbor, MI), Envirochek HV filters (Pall Corporation), Filta-Max filters (IDEXX, Westbrook, MA), or Continuous Flow Centrifugation (Haemonetics, Braintree, MA). However, *Cryptosporidium* and *Giardia* (oo)cyst recoveries have varied greatly depending on the source water matrix and filters used[1,14]. A new tangential flow hollow-fiber ultrafiltration (HFUF) system has recently been shown to be more efficient and more robust at recovering *Cryptosporidium* oocysts and *Giardia* cysts from various water matrices; moreover, it is less expensive than other capsule filter options and can concentrate multiple pathogens simultaneously[1-3,5-8,10,11]. In addition, previous studies by Hill and colleagues demonstrated that the HFUF significantly improved *Cryptosporidium* oocysts recoveries when directly compared with the Envirochek HV filters[4]. Additional modifications to the current methods have also been reported to improve method performance. Replacing the acid dissociation procedure with heat dissociation was shown to be more effective at separating *Cryptosporidium* from the magnetic beads in some matrices[9,13] .

This protocol describes a modified Method 1623 that uses the new HFUF filtration system with the heat dissociation step. The use of HFUF with this modified Method is a less expensive alternative to current EPA Method 1623 filtration options and provides more flexibility by allowing the concentration of multiple organisms.

Video Link

The video component of this article can be found at https://www.jove.com/video/4177/

Protocol

1. Tangential Flow Hollow-fiber Ultrafiltration Procedure

1. Preparation of buffers and solutions:
   Elution Solution (1 L):
   To 1 L of reagent grade water add 0.1 g sodium polyphosphate, 0.1 ml Tween-80 and 0.01 ml Y-30 antifoam.
2. Prepare filter assembly (**Figure 1**) in a biological safety cabinet:
   1. Insert Masterflex I/P 73 lab tubing through a Masterflex I/P Easy-Load pump head connected to a Masterflex I/P precision brushless drive.

2. Secure the tubing to a 0-60 psi, glycerin-filled pressure gauge fitted with a NPT branch T-connector upstream of the pump head with a screw clamp, and to a HDPE T-connector downstream of the pump head.
3. Assemble the retentate bottle by fitting a Nalgene 53-B filling/venting cap with Masterflex L/S 15 lab tubing and a T-connector and securing it to a 1 L Nalgene heavy duty polypropylene bottle.
4. Fit Masterflex L/S 24 tubing with a pinch clamp (pinch clamp 2) and connect it from the retentate bottle to the HDPE T-connector.
5. Connect the Masterflex L/S 24 lab tubing from the pressure gauge to the Asahi Kasei Rexeed 25S high-flux dialyzer with a screw clamp, and from the dialyzer to the retentate bottle. Use custom-made DIN adapters designed to accommodate ¼" ID tubing to connect the tubing to the hollow-fiber ultrafilter.
6. Fit Masterflex L/S 24 lab tubing with a pinch clamp (pinch clamp 1) and connect it to a 10 ml plastic pipette with the tip and cotton plug removed to act as the sample uptake line and then attach the tubing to the HDPE T-connector.
7. Fit one end of Masterflex L/S-36 lab tubing with a ramp clamp and connect the tubing to the effluent port located near the exit end of the filter. Place the other end in the waste container.

3. Add 0.01 % (w/v) sodium polyphosphate to the 10 L water sample and mix for 3 min.
4. Close pinch clamp 2 and remove the vent cap from the retentate bottle. All other clamps should be open.
5. Set the pump direction to move the fluid from the T-connector to the pressure gauge (right to left following **Figure 1**). Adjust the pump speed to 25% of the maximum speed and turn on the pump.
6. When the retentate bottle is 2/3 full, open pinch clamp 2 and quickly replace the vent cap to the retentate bottle. Check all of the lines and fittings to ensure there are no leaks.
7. Slowly increase the pump speed to the desired filtration rate (approximately 1.5 L/min), checking for leaks. Using a graduated cylinder and a timer or flow meter, check the filtrate rate of the water exiting from the effluent line (blue L/S 36 tubing in **Figure 1**). Air bubbles can typically form at the exit end of the filter making it difficult to achieve a stable pressure and filtration rate. This is corrected by pinching the effluent line by hand for a moment. This action will generally coax the air bubble to the filtrate port and to exit via the effluent line. Repeat as needed, keeping in mind that pea size air bubbles are often unavoidable.
8. Monitor the filtration process. Measure and record the pressure and the filtration rate as needed. The pressure should never exceed 20 psi. It is recommended that the filtration rate should not exceed 2.0 L/min. It is important that the volume of water in the retentate bottle be monitored to ensure that it never empties. It is normal for the volume to increase or decrease slightly. If the water volume in the retentate bottle falls below 1/3 full, then remove the vent cap and close pinch clamp 2 on the retentate bottle line. Bring the volume back to about 2/3 full, open the clamp and quickly replace the vent cap, ensuring a tight seal. If the volume in the retentate bottle falls quickly and continuously, then ensure the retentate bottle lid is tight and the vent cap is securely in place, and that the tubing is still in contact with the water sample. If these issues are not occurring, then it is likely that the seal in the retentate bottle lid is bad and should be replaced.
9. When the sample container is empty, immediately close pinch clamp 1, reduce the pump speed to 20% of its maximum, remove the vent cap from the retentate bottle and close the ramp clamp.
10. Adjust the volume of sample in the retentate bottle to approximately 200 ml by tightening or loosening the ramp clamp. After the volume is approximately 200 ml, tighten the ramp clamp for the elution steps (1.11-1.12).
11. Add 500 ml of elution solution to the sample container and rinse the inside of the container. Place the 10 ml pipette connected to the sample uptake line into the container that contains the elution solution. Ensure that the ramp clamp is closed. Open pinch clamp 1 and close pinch clamp 2 momentarily to draw up the elution solution.
12. After the 500 ml of elution solution is drawn up, close pinch clamp 1 and open pinch clamp 2. Allow the elution solution to circulate for 5 minutes with a pump speed of 20% of its maximum.
13. Adjust the volume of sample in the retentate bottle to about 100 ml by tightening or loosening the ramp clamp on the effluent line. Tighten the ramp clamp and allow the sample to circulate for 1 minute. Avoid pulling air into the tubing by ensuring the volume of the sample in the retentate bottle is high enough to cover the L/S 15 tubing entering the retentate bottle.
14. Reverse the direction of the pump which forces the sample into the retentate bottle. Allow the pump to run in reverse for 20 seconds resulting in a total of ~225 ml in the retentate bottle. Turn off the pump.
15. Remove the Masterflex I/P 73 tubing from the pump head and disconnect the pressure gauge. Disconnect the Masterflex L/S 24 tubing exiting the hollow-fiber ultrafilter. Hold the tubing above the retentate bottle to force any remaining sample into the retentate bottle.
16. Disconnect all tubing from the bottle and replace the venting cap with a non-venting cap.
17. Continue to the IMS/IFA procedure with the ~225 ml retentate.

2. Immunomagnetic Separation Procedure

1. Preparation of buffers and solutions:
    1. Allow the buffers included in the Dynabeads: *Cryptosporidium/Giardia* combo kit to reach room temperature.
    2. 1X SL-buffer A: Add 1 ml of 10X SL-buffer A to 9 ml reagent grade water.

2. Transfer the ~225 ml of liquid from the retentate bottle to a labeled 250 ml conical centrifuge tube. Rinse the retentate bottle twice with 10 ml of reagent water, and add the rinses to the conical centrifuge tube. Centrifuge the suspension at 1500 x g for 15 min at 4 °C with no brake.
3. Carefully aspirate the supernatant from the air-water interface to 5 ml above the packed pellet for every 0.5 ml of pellet volume (i.e. aspirate to 15 ml above a pellet volume of 1.3 ml, and aspirate to 5 ml for a pellet of 0.5 ml or less).
4. Thoroughly resuspend the pellet into the supernatant by vortexing and/or pipette mixing. Transfer each 5 ml volume of the liquid to the flat-sided Dynal L10 tube containing 1 ml each of 10X SL-buffer A and 10X SL-buffer B. Rinse the conical centrifuge tube twice with 2.5 ml of reagent water and add the rinse to the L10 tube, bringing the total volume in the L10 tube to 12 ml, including the buffers.
5. Add 100 µl each of well-mixed resuspended anti-*Cryptosporidium* and anti-*Giardia* Dynabeads to the L10 tube. Rotate the L10 tube at 18 rpm for 1 hour at room temperature on a rotator mixer.
6. Place the flat side of the L10 tube against the MPC-6 magnet and gently hand rock the tube end-to-end, 180° for 2 minutes.
7. Keeping the L10 tube in the MPC-6 magnet with the magnet side up, decant the supernatant away from the bead/(oo)cyst complexes bound to the magnet. Remove the L10 tube from the magnet and add 0.5 ml of 1X SL-buffer A to the tube. Transfer the suspension using two additional rinses of 0.5 ml of 1X SL-buffer A into a 1.5 ml microcentrifuge tube held in the MPC-S with the magnet in the vertical position.

8. Gently rock the tube in the MPC-S magnet 180° for 1 minute. With the magnet in place, aspirate the supernatant using a Pasteur pipette directed to the bottom of the microcentrifuge tube.
9. Add 1 ml of 1X PBS to the front side of the microcentrifuge tube, remove the magnet and rock the tube gently just until the beads are resuspended. Replace the magnet in the vertical position and gently rock the tube 180° for 1 minute. Aspirate the PBS rinse, without disturbing the bead pellet, using a Pasteur pipette to remove as much debris as possible.
10. Remove the magnet and add 50 µl of reagent water to the back side of the microcentrifuge tube. Vortex the tube at full speed for 50 seconds, then incubate the tube at 80 °C for 10 minutes followed by a 30 second vortex. Replace the magnet into the MPC-S in the slanted position, binding the beads to the magnet and leaving the (oo)cysts in the liquid. Apply the (oo)cyst suspension to a SingleSpot well slide.
11. Repeat step 2.10, applying the liquid to the same well containing the first dissociation. Place slide on a 37 °C slide warmer for 1 hour to dry the suspension to the slide well.

3. Staining and Examination

1. Preparation of buffers and solutions:
   Working DAPI solution: Add 25 µl of DAPI stock solution (2 mg/ml in methanol) to 25 ml of 1X PBS. Store stock and working solutions between 1 °C and 10 °C in the dark.
2. Apply 50 µl of methanol to the slide well and allow it to dry at room temperature.
3. Add 50 µl of the working DAPI solution to the slide well and incubate for 2 minutes at room temperature.
4. Use a Kimwipe to wick the DAPI from the well. Apply 50 µl of EasyStain. Incubate at 35 °C for 30 minutes.
5. Wick the stain off the well with a Kimwipe, and then slowly add 300 µl of cold EasyStain fixing buffer, allowing it to flow over the well edge. Incubate for 2 minutes at room temperature.
6. Use a Kimwipe to wick the buffer from the well and apply 10 µl of EasyStain Mounting Medium.
7. Carefully apply a cover slip, removing any bubbles that occur. Seal the cover slip with clear nail polish.
8. Scan the entire slide using the FITC filter, at 200X total magnification, for ovoid or spherical apple-green fluorescent objects that resemble an oocyst or cyst. Examine all such objects with the DAPI filter at 1000X total magnification and then with DIC, also at 1000X total magnification. Record the size using a calibrated ocular micrometer and morphological characteristics.
9. Document results.

Note: Additional information about the original procedure can be found in the December 2005 version of EPA Method 1623[12]. The tangential flow hollow-fiber ultrafiltration procedure described is used in place of Section 12.0 of EPA Method 1623. The heat dissociation modifies Section 13.3.3 of EPA Method 1623. The procedure also describes an additional PBS rinse during the IMS process which can be inserted into the December 2005 version of Method 1623 after section 13.3.2.16. The complete list of consumables, reagents and equipment used for EPA Method 1623 including these modifications is listed in the equipment list.

4. Representative Results

Cryptosporidium oocysts and *Giardia* cysts recovered through the processes of filtration and immunomagnetic separation are detected by microscopic analysis. At 200X total magnification, each organism exhibiting a typical staining pattern, size, and shape as shown in **Figure 2** should be further observed using oil immersion at 1000X total magnification. This will allow for measurement and identification of either typical defining features or atypical features that would rule out positive identification. *Cryptosporidium* is an ovoid to spherical object 4 to 6 µm in diameter which exhibits brilliant apple-green FITC fluorescence with brightly highlighted edges (**Figure 3A**). With DAPI UV, an oocyst will exhibit one of the following typical feature categories: light blue internal staining with a green rim and no distinct nuclei (DAPI negative), intense blue internal staining, or up to four distinct, sky-blue nuclei (DAPI positive - **Figure 3B**). Atypical features include deviations in color, structure, or DAPI fluorescence (e.g., too many stained nuclei, red fluorescing internal structures). If the fluorescent object has met criteria for typical FITC and DAPI staining, it is examined using differential interference contrast (DIC). The object is examined for atypical external or internal morphological characteristics such as cell wall ornamentation, or one or two large nuclei filling the cell. If atypical structures are not observed, the object is recorded in the total IFA count and categorized as an empty amorphous structure or with one to four sporozoites present (**Figure 3C**). Similarly, *Giardia*-like objects are examined with regard to FITC and DAPI staining as well as DIC characteristics, like axonemes, median bodies, and nuclei. *Giardia* cysts are round to ovoid brilliant apple-green objects, 8 - 18 µm long by 5 - 15 µm wide with brightly highlighted edges (**Figure 3D**). With DAPI UV, the *Giardia* cyst will exhibit DAPI-negative staining, or DAPI-positive characteristics (**Figure 3E**). The fluorescent object is examined by DIC for typical and atypical features in the same manner as described for *Cryptosporidium*. If atypical features are not observed, the object is recorded in the total IFA count and categorized as empty containing amorphous structure, or with one or more type of internal structures present (**Figure 3F**).

Any organism that is observed to have atypical features should not be counted as an (oo)cyst. Microscopic analysis of environmental samples can be challenging as there are organisms that may auto-fluoresce or cross-react with the FITC-conjugated anti-*Cryptosporidium* and/or anti-*Giardia* antibodies [1]. It is recommended that an analyst be familiar with aquatic microbes and review dozens of slides to gain experience identifying *Cryptosporidium* and *Giardia*. At least three (oo)cysts on the positive staining control slide should be characterized prior to every session at the microscope.

Quality control samples may be spiked with (oo)cysts to determine the percent recovery for each protozoan using the calculation:
(Oo)cyst Percent Recovery = ((QC Sample Count - Count from Unspiked Sample) / Spike) x 100.

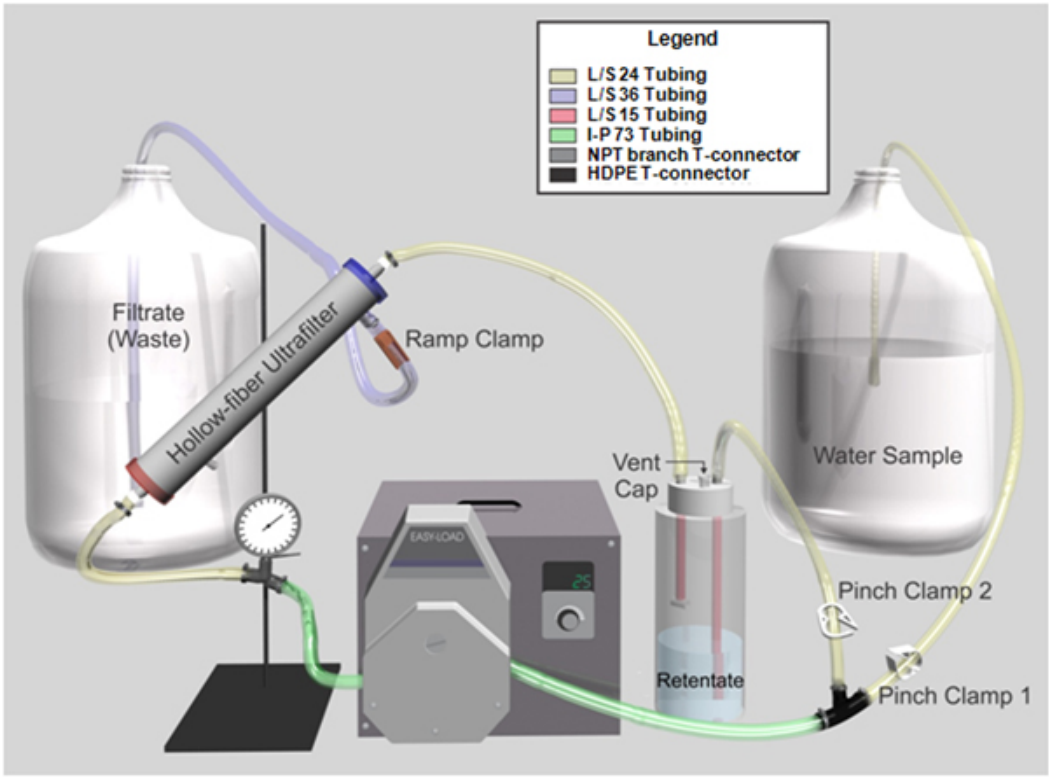

Figure 1. Graphic representation of the tangential flow hollow-fiber ultrafiltration system. The tubing is color coded to aid is assembly of the system.

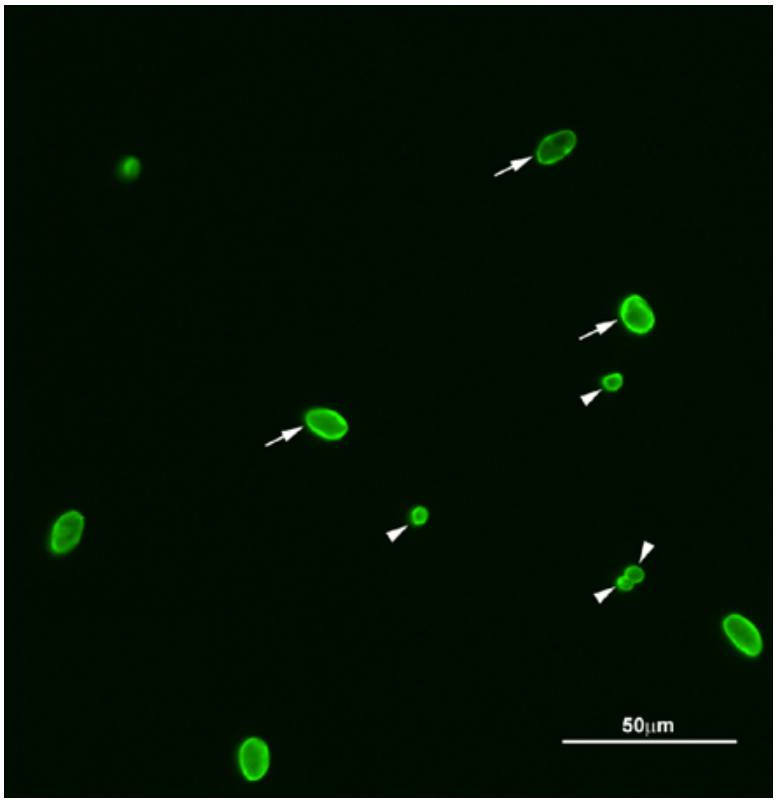

Figure 2. Representative fluorescence image of *Cryptosporidium* and *Giardia* (oo)cysts. *Cryptosporidium* oocysts and *Giardia* cysts were stained with FITC labeled anti-*Cryptosporidium*/*Giardia* antibodies. Arrows, *Giardia* cysts; arrowheads, *Cryptosporidium* oocysts. A total of four *Cryptosporidium* oocysts and six *Giardia* cysts were found in the plane of focus. Samples observed under 200X magnification.

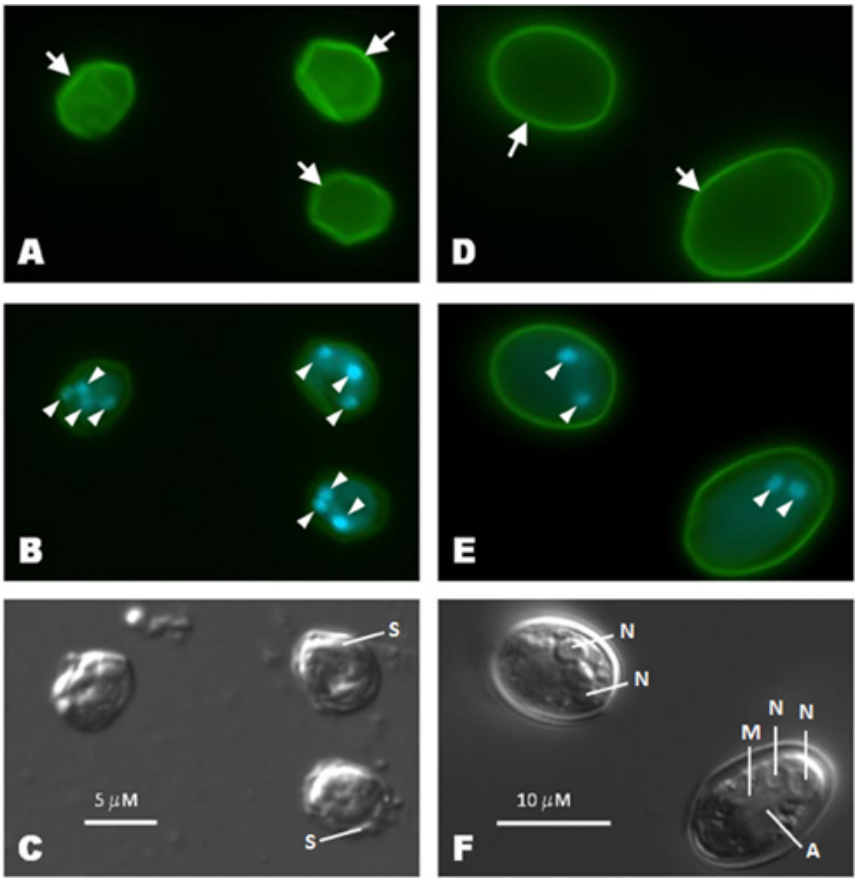

Figure 3. Representative microscopic images of *Cryptosporidium* oocysts and *Giaridia* cysts used for characterization. *Cryptosporidium* oocysts (A - C). Brilliant apple-green FITC fluorescence of spherical objects 4 to 6 µm in diameter with brightly highlighted edges (A) containing up to four distinct, sky-blue DAPI nuclei (B) and one to four sporozoites (S) per oocyst (C). *Giardia* cysts (D - F). Brilliant apple-green FITC fluorescence of round to ovoid objects 8 - 18 µm long by 5 - 15 µm wide with brightly highlighted edges (D) containing up to four sky-blue DAPI nuclei (E) and with one or more discernable internal structure such as nuclei (N), median body (M) and or axonemes(A) (F). White arrows, brilliant apple green fluorescence staining *Cryptosporidium* oocysts and *Giardia* cysts walls; white arrowheads, DAPI positive nuclei. Samples observed under 1000X magnification.

Discussion

Tangential flow hollow-fiber ultrafiltration is an alternative and effective technique for the initial concentration of *Cryptosporidium oocysts* and *Giardia* cysts from water. Hollow-fiber ultrafiltration is less expensive than traditional filters. Since it has the ability to concentrate *Cryptosporidium* oocysts and *Giardia* cysts from a variety of different water matrices it is a useful alternative to the current filtration techniques used for EPA Method 1623. As with most other filtration methods, hollow-fiber ultrafiltration is prone to fouling with extremely turbid samples. High water pressure would result from the filter fouling; therefore it is recommended to monitor the pressure during the filtration run. In addition to *Cryptosporidium* oocysts and *Giardia* cysts, hollow-fiber ultrafiltration has been shown to be capable of concentrating bacteria and viruses[1-3,5,8] . Hollow-fiber ultrafiltration outlined in this method can be used to concentrate multiple organisms in a single sample. It is noteworthy that obtaining a final volume between 200 and 250 ml is the critical final step in the concentration procedure so that extra centrifugation steps, that may result in (oo)cyst loss, are avoided (step 2.2). However, allowing the volume in the bottle to drop too low can have unfavorable effects on the recoveries since there will not be enough liquid volume to force all the oocysts or cysts into the retentate bottle. Therefore it is recommended to maintain a final volume between 200 and 250 ml.

Heat dissociation is an alternative to the acid dissociation step in Method 1623. This alternative step has been shown to improve *Cryptosporidium* oocyst recovery and reduce the method variation when isolated from either river or reagent water[9]. A side-by-side comparison of acid and heat dissociation methods demonstrated that using heat to dissociate the organisms from the immunomagnetic beads produced higher mean recoveries for both *Cryptosporidium* and *Giardia*. In addition, the precision of *Cryptosporidium* and *Giardia* recoveries was better in samples processed with heat dissociation compared with acid dissociation[9].

The incorporation of HFUF as the concentration step allows more flexibility by providing the ability to concentrate multiple organisms. In addition it is a less expensive alternative to current Method 1623 filtration options.

Disclosures

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References

1. DiGiorgio, C.L., Gonzalez, D.A., & Huitt, C.C. *Cryptosporidium* and *Giardia* recoveries in natural waters by using Environmental Protection Agency Method 1623. *Appl. Environ. Microbiol.* **68**, 5952-5 (2002).
2. Hill, V.R., Kahler, A.M., Jothikumar, N., Johnson, T.B., Hahn, D., & Cromeans, T.L. Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples. *Appl. Environ. Microbiol.* **73**, 4218-25 (2007).
3. Hill, V.R., Polaczyk, A.L., Hahn, D., Narayanan, J., Cromeans, T.L., Roberts, J.M., & Amburgey, J.E. Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. *Appl. Environ. Microbiol.* **71**, 6878-84 (2005).
4. Hill, V.R., Polaczyk, A.L., Kahler, A.M., Cromeans, T.L., Hahn, D., & Amburgey, J.E. Comparison of hollow-fiber ultrafiltration to the USEPA VIRADEL technique and USEPA method 1623. *J. Environ. Qual.* **38**, 822-5 (2009).
5. Holowecky, P.M., James, R.R., Lorch, D.P., Straka, S.E., & Lindquist, H.D. Evaluation of ultrafiltration cartridges for a water sampling apparatus. *J. Appl. Microbiol.* **106**, 738-47 (2009).
6. Lindquist, H.D., Harris, S., Lucas, S., Hartzel, M., Riner, D., Rochele, P., & Deleon, R. Using ultrafiltration to concentrate and detect *Bacillus anthracis*, *Bacillus atrophaeus* subspecies *globigii*, and *Cryptosporidium parvum* in 100-liter water samples. *J. Microbiol. Methods.* **70**, 484-92 (2007).
7. Polaczyk, A.L., Roberts, J.M., & Hill, V.R. Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. *J. Microbiol. Methods*. **68**, 260-6 (2007).
8. Rhodes, E.R., Hamilton, D.W., See, M. J., & Wymer, L. Evaluation of hollow-fiber ultrafiltration primary concentration of pathogens and secondary concentration of viruses from water. *J. Virol. Methods.* **176**, 38-45 (2011).
9. Shaw, N.J., Villegas, L.F., Eldred, B.J., Gaynor, D.H., Warden, P.S., & Pepich, B.V. Modification to EPA Method 1623 to address a unique seasonal matrix effect encountered in some U.S. source waters. *J. Microbiol. Methods.* **75**, 445-8 (2008).
10. Simmons, O.D., 3rd, Sobsey, M.D., Heaney, C.D., Schaefer, F.W., 3rd, & Francy, D.S. Concentration and detection of *Cryptosporidium* oocysts in surface water samples by method 1622 using ultrafiltration and capsule filtration. *Appl. Environ. Microbiol.* **67**, 1123-7 (2001).
11. Sobsey, M.D., & Glass, J.S. Influence of water quality on enteric virus concentration by microporous filter methods. *Appl. Environ. Microbiol.* **47**, 956-60 (1984).
12. **USEPA.** Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA., Office of Water, EPA 815-R-05-002 (2005).
13. Ware, M.W., Wymer, L., Lindquist, H.D., & Schaefer, F.W., 3rd. Evaluation of an alternative IMS dissociation procedure for use with Method 1622: detection of *Cryptosporidium* in water. *J. Microbiol. Methods.* **55**, 575-83 (2003).
14. Zuckerman, U. & S. Tzipori. Portable continuous flow centrifugation and method 1623 for monitoring of waterborne protozoa from large volumes of various water matrices. *J. Appl. Microbiol.* **100**, 1220-7 (2006).