Filtration and Molecular Assay of Water Samples for *Cryptosporidium*

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Revision Record

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<td>1</td>
<td>November 2012</td>
<td>Terry Gentry</td>
<td>Initial Release</td>
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Filtration and Molecular Assay of Water Samples for Cryptosporidium

1. Scope and Application
This method is designed to test for the presence of Cryptosporidium in environmental water samples.

2. Method Summary
This methods includes collection of Cryptosporidium oocysts from water samples on Envirochek® HV filters. The concentrated Cryptosporidium oocysts are then eluted from the filters and the eluted volume tested for the presence of Cryptosporidium using a polymerase chain reaction-based assay.

3. Procedures
3.1. Flow rate adjustment
   3.1.1. Connect the sampling system, minus the capsule, to the source water.
   3.1.2. Turn on the pump and adjust the flow rate to 2.0 L/min.
   3.1.3. Allow 2 to 10 L of water to flush the system. Adjust the pump speed as required during this period. Turn off the pump when the flow rate has been adjusted.

3.2. Install the capsule filter in the line, securing the inlet and outlet ends with the appropriate clamps/fittings.

3.3. Record the sample number, sample turbidity, sample type, sample filtration start date and time, and name of analyst filtering the sample on a bench sheet.

3.4. Filtration
   3.4.1. Place the drain end of the sampling system tubing into an empty graduated container with a capacity of 10 to 15 L. This container will be used to determine the sample volume filtered. Alternately, determine the volume by connecting a flow meter downstream of the filter and recording the initial and final meter readings.
   3.4.2. Allow the carboy discharge tube and capsule to fill with sample water. Vent residual air using the bleed valve/vent port, gently shaking or tapping the capsule, if necessary. Turn on the pump to start water flowing through the filter. Verify that the flow rate is 2 L/min.
   3.4.3. After the entire sample has passed through the filter, turn off the pump. Allow the pressure to decrease until flow stops. If excess sample remains in the filter capsule upon receipt in the laboratory, pull the remaining sample volume through the filter before eluting the filter.
   3.4.4. Based on the water level in the graduated container or meter reading, record the volume filtered on the bench sheet to the nearest quarter liter. Discard the contents of the graduated container.

3.5. Disassembly
3.5.1. Disconnect the inlet end of the capsule filter assembly while maintaining
the level of the inlet fitting above the level of the outlet fitting to prevent
backwashing and the loss of oocysts from the filter. If needed, restart the
pump and allow as much water to drain as possible. Turn off the pump.
3.5.2. Loosen the outlet fitting, then cap the inlet and outlet fittings.

3.6. Elution Setup

3.6.1. **NOTE:** The laboratory must complete the elution, concentration, and
purification in one work day. It is critical that these steps be completed in
one work day to minimize the time that any target organisms present in the
sample sit in eluate or concentrated matrix.

3.6.2. Assemble the laboratory shaker with the clamps aligned vertically so that
the filters will be aligned horizontally. Extend the clamp arms to their
maximum distance from the horizontal shaker rods to maximize the
shaking action.

3.6.3. Prepare sufficient quantity of elution buffer to elute all samples. Elution may
require up to 275 mL of buffer per sample.

3.6.4. Designate at least one 250-mL conical centrifuge tube for each sample and
label with the sample number.

3.6.5. Prepare sufficient quantity of 5% sodium hexametaphosphate (NaHMP)
solution to pre-treat all of the designated filters. Pre-treatment may require
up to 150 mL of NaHMP per sample.

3.7. Dispersant Addition

3.7.1. Record the elution date and time on the bench sheet. Using a ring stand or
other means, clamp each capsule in a vertical position with the inlet end
up. **NOTE:** Dispersant Addition cannot be performed on a sampling
capsule through which water can no longer be filtered (i.e. clogged).

3.7.2. Remove the inlet cap, pour NaHMP solution through the inlet opening, and
allow the liquid level to stabilize. Sufficient NaHMP solution must be added
to cover the pleated white membrane with NaHMP solution or NaHMP
solution may be measured to 125 mL. Replace the inlet cap.

3.7.3. Securely clamp the capsule in one of the clamps on the laboratory shaker
with the bleed valve positioned at the top on a vertical axis (in the 12
o’clock position). Turn on the shaker and set the speed to maximum (700 –
900 rpm or per manufacturer’s instructions). Agitate the capsule for
approximately 5 minutes. Time the agitation using a lab timer, rather than
the timer on the shaker to ensure accurate time measurement.

3.7.4. Remove the filter from the shaker, remove the outlet cap, and attach the
capsule filter outlet to tubing, upstream of a pump. Holding the filter upright,
remove the inlet cap, being careful not to pour any liquid from the inlet, turn
on the pump and allow pump to pull all the NaHMP through the filter, turn
off pump. Do not allow the filter pleats to collapse during the pumping
process.
3.7.5. Fill the capsule with reagent water, pinching the outlet hose if necessary, to cover the white pleated membrane and the plastic above the membrane; allow the liquid level to stabilize. Sufficient reagent water must be added to cover the pleated white membrane. Turn on the pump and allow pump to pull all the water through the filter. Turn off the pump.

3.7.6. Replace the inlet cap. Disconnect the outlet tubing from the filter, and replace the outlet cap. Proceed directly to elution within the same working day.

3.8. Elution

3.8.1. Using a ring stand or other means, clamp each capsule in a vertical position with the inlet end up.

3.8.2. Remove the inlet cap, pour elution buffer through the inlet fitting, and allow the liquid level to stabilize. Sufficient elution buffer must be added to cover the pleated white membrane with buffer solution or elution buffer may be measured to ensure the use of one 250-mL centrifuge tube. Replace the inlet cap.

3.8.3. Securely clamp the capsule in one of the clamps on the laboratory shaker with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position). Turn on the shaker and set the speed to maximum (approximately 700 - 900 rpm or per manufacturer's instructions). Agitate the capsule for approximately 5 minutes. Time the agitation using a lab timer, rather than the timer on the shaker to ensure accurate time measurement.

3.8.4. Remove the filter from the shaker, remove the inlet cap, and pour the contents of the capsule into the 250-mL conical centrifuge tube.

3.8.5. Clamp the capsule vertically with the inlet end up and add sufficient volume of elution buffer through the inlet fitting to cover the pleated membrane. Replace the inlet cap.

3.8.6. Return the capsule to the shaker with the bleed valve positioned at the 4 o'clock position. Turn on the shaker and agitate the capsule for approximately 5 minutes.

3.8.7. Leaving the elution buffer in the capsule, re-align the capsule in the shaker with the bleed valve now at the 8 o'clock position. Turn on the shaker and agitate the capsule for a final 5 minutes.

3.8.8. Remove the filter from the shaker, remove the inlet cap and pour the contents into the 250-mL centrifuge tube. Rinse down the inside of the capsule filter walls with reagent water or elution buffer using a squirt bottle with a disposable tip or a serological pipette inserted in the inlet end of the capsule. Invert the capsule filter over the centrifuge tube and ensure that as much of the eluate as possible has been transferred. Replace the inlet cap. Manually swing the filter capsule through an arc of ~180° to retrieve more of the eluate from the filter.

3.8.9. Proceed to step 3.9 for concentration and separation (purification).
3.9. Concentration
3.9.1. The filter eluate is concentrated through centrifugation,
3.9.2. Balance the tubes to within 0.5 g of each other prior to centrifugation and/or use a self-balancing rotor. Centrifuge the 250-mL (or 50-mL) centrifuge tube containing the eluate at a minimum of 1500 × G for 15 minutes. Allow the centrifuge to coast to a stop—do not use the brake. Record the pellet volume (volume of solids) on the bench sheet.
3.9.3. If the packed pellet volume is ≤ 0.5 mL, using a pipette, carefully aspirate the supernatant to 5 mL above the pellet. Aspirate at the air/water interface from the center of the tube using gentle and steady low vacuum pressure (e.g., <5 in. Hg vacuum). Vacuum pressure may be reduced when 30 mL of supernatant remains. Care must be taken to avoid aspirating oocysts and cysts during this step, particularly if the sample is reagent water.
3.9.3.1. Vortex the tube vigorously for 10-15 seconds and/or pipette mix until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Record the resuspended pellet volume and the number of any subsamples. Be sure pellet is completely homogenized immediately before transfer. Visually inspect to ensure complete homogenization and lack of debris aggregates. This is particularly important for samples with high clay content.
3.9.4. If the packed pellet volume is > 0.5 mL, the concentrate must be separated into multiple subsamples: a subsample is equivalent to no greater than 0.5 mL of packed pellet material. No more than 0.5 mL of pellet must be processed at a time. Aspirate the supernatant from the centrifuge tube leaving 5 mL of fluid for every 0.5 mL of pellet or portion of 0.5 mL of pellet. For example, if the packed pellet volume is 1.2 mL, the total volume required is 15 mL. Aspirate at the air/water interface from the center of the tube using gentle and steady low vacuum pressure (e.g., <5 in. Hg vacuum). Vacuum pressure may be reduced when 30 mL of supernatant remains.
3.9.5. Record total in centrifuge tube, which includes the pellet and supernatant. Divide pellet into 40 µl aliquots and store at -80°C until DNA extraction.

3.10. DNA Extraction
3.10.1. Add 20 µl of 1:1 Chelex/molecular grade water (MGW) to 30 µl of each sample.
3.10.2. Lyse the oocysts using 8 cycles, approx. 1 min each, of freezing in liquid nitrogen and thawing at 95°C. Make sure that entire sample freezes and thaws with each cycle.
3.10.3. Quick spin sample, then transfer, including Chelex and foam, to a 0.45 µm cellulose acetate microfuge spin filter. Keep the 0.65 ml sample tube.
3.10.4. Centrifuge at high speed for 30 seconds (spin filter hinge at 12 o’clock position).
3.10.5. Add a 5 µl MGW rinse to the 0.65 ml sample tube, briefly vortex, then quick spin in the microcentrifuge (spin filter hinge at 6 o’clock position). Transfer rinse to the spin filter.

3.10.6. Discard the spin filter insert containing the Chelex resin and foam, then cap the sample tube. The sample volume should be ~40 µl and duplicate 20 µl volumes will be used as template in the PCR.

3.10.7. Proceed immediately to PCR or store at -80°C.

3.11. Polymerase Chain Reaction (PCR) Amplification and Gel Electrophoresis

3.11.1. Prepare PCR master mixes. The volume of master mix can be scaled up or down according to the number of samples that need to be analyzed.

3.11.2. Vortex the master mix after the addition of all ingredients.

3.11.3. Centrifuge for 10 seconds in a microcentrifuge.

3.11.4. Dispense 30 µL of the appropriate mix to the labeled plates or tubes.

3.11.5. Add 20 µL of the appropriate sample to each tube.

3.11.6. Run on a thermal cycler with a setting of 1 cycle at 95°C for 10 minutes followed by 55 cycles of 95°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 30 seconds followed by a final extension at 72°C for 10 minutes.

3.11.7. 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.

3.11.8. 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.

3.11.9. 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).

3.11.10. Compare results with molecular marker to determine the presence or absence of Cryptosporidium in each sample.

4. References:
