Method 1623 IMS Procedure - Supplemental Information

July 10, 2007

The immunomagnetic separation (IMS) procedure of EPA Method 1623[1] is designed to separate *Cryptosporidium* oocysts and *Giardia* cysts from the debris in the sample and eliminate the transfer of the debris to the microscope slide. When problems with the IMS process are identified, review the IMS techniques and standardized protocols carefully. It is important to standardize each IMS processing step to maximize recovery and precision. This supplemental document does not replace Method 1623, but provides processing tips, special techniques, and quality control recommendations that laboratories may implement to improve recovery and precision in problematic samples. Before processing field samples with any of the modifications described in Section II of this document, laboratories should demonstrate acceptable performance of the modified method (see Section III).

PLEASE NOTE:

- *All Method 1623 section references are from the 2005 version.*
- *Inclusion of individual vendor or product/equipment does not indicate an endorsement by EPA, other vendors and equivalent products/equipment may be used.*
- *The MPC®-M is no longer available for purchase and the MPC®-S has a stronger magnet. Accordingly, only the MPC®-S is referred to in this document. Product information for beads and magnets can be obtained from the manufacturer*[2, 3].

I. Processing Tips

Pellet Transfer to Flat-sided Tube – Method 1623 Section 13.3.2.1

- Completely homogenize resuspended pellet immediately prior to transfer. Vortex vigorously for 10-15 seconds and/or pipette mix, then visually inspect to ensure complete homogenization and lack of debris aggregates. This is particularly important for samples with high clay content.
- The volume of the transferred sample plus 2 reagent water rinses should total 10 mL. The addition of 2 mL of buffers brings the total in the flat-sided tube to 12 mL.
- Measure the volume being transferred using a serological or graduated transfer pipette to determine the maximum volume that can be used for the centrifuge tube rinses.
- Do not rinse the pellet prior to transfer, as oocysts and cysts may be lost.
- Be sure IMS reagents are at room temperature before use.
- Do not refrigerate samples between the completion of centrifugation and the start of IMS.

Decanting Supernatant from Flat-sided Tube – Method 1623 Sections 13.3.2.10 - 13.3.2.11

- Do not let the flat-sided tubes sit motionless before decanting; continuous movement of the tubes prevents binding of low-mass, magnetic, or magnetizable material.
- Use a clean, lint-free tissue to blot the end of each flat-sided tube after decanting to remove more matrix debris.

- Allow the flat-sided tube to sit undisturbed in the MPC for 30-60 seconds after decanting; then aspirate residual liquid and debris from the bottom of the tube.
- If using MPC®-6, rock each side through a 90° angle 3 times before decanting the remaining tubes.
- With the MPC®-6, the supernatant may be decanted from 1 to 3 flat-sided tubes at one time; maximum 3 tubes at once.

Transfer to Microcentrifuge Tube – Method 1623 Section 13.3.2.12 - 13.3.2.13

- Add 0.5 mL of 1X SL Buffer A directly to the flat side of the tube to resuspend the bead pellet, avoid any debris present on the round side of the tube.
- Pipette mix gently to completely resuspend the bead pellet prior to initial transfer and each rinse transfer.
- During each pipette mix step, release the liquid down the flat side of the tube to further rinse the tube.
- Allow the flat-sided tube to sit undisturbed for 60 seconds after the last rinse to collect residual liquid for transfer to the microcentrifuge tube.
- Microcentrifuge tubes should be in the MPC®-S with the magnet in the vertical position during transfer to produce an immediate bead pellet which aids in the separation of the debris.
    - The MPC®-S magnet has two notch positions for the placement of the magnet (**Figure 1**).
    - The vertical position is used for producing the bead-organism pellet prior to dissociation (**Figure 2 top**).

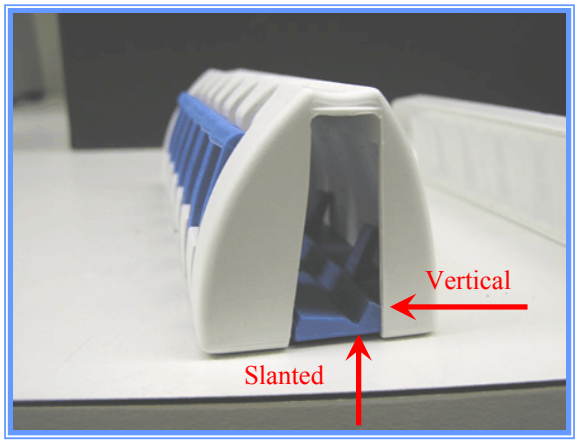


Figure 1

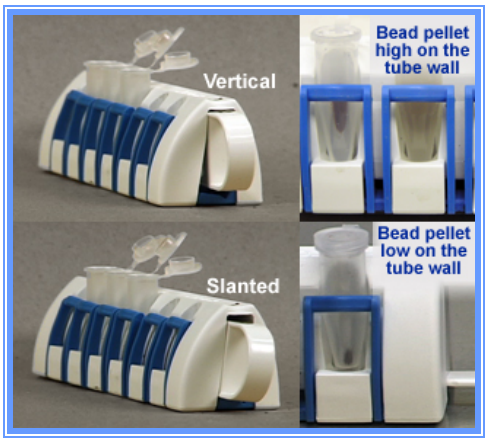


Figure 2

Aspiration and Dissociation in Microcentrifuge Tube – Method 1623 Sections 13.3.2.16 and 13.3.3.6

- Perform the aspiration described in Section 13.3.2.16 with the magnet in the vertical position, aspirate the waste liquid to just below the bead pellet and discard; without disturbing the bead pellet gently pipette mix the remaining liquid and debris to resuspend the debris and improve the removal.
- The slanted magnet position (**Figure 1 and Figure 2 bottom**) should be used in Section 13.3.3.6 when collecting the bead pellet during acid dissociation.

II. Special Techniques: Potential Method Modifications

NOTE: These protocols are to be used only by experienced analysts with successful OPRs and requisite quality control for using method modifications (Section III)!

IMS may not perform consistently when applied to certain source waters and special techniques may be necessary when matrix spike recoveries are low, pellet sizes are large, and/or extraneous debris carries over to successive steps. Modifications to Method 1623 may be used to increase recovery (Section 9.1.2). Some special techniques are not routinely used, and not firmly established, but may be beneficial for some source waters. It is often helpful to discuss technique modifications with other experienced analysts before trying them. Always practice and test modified techniques prior to use on field samples. Performing the appropriate QC validates the use of the modification (see III. Quality Control Recommendations). The proposed protocols included in this document address:

- Removing magnetic minerals
- Adjusting pH
- Additional rinse of flat-sided tube
- Pellet wash in microcentrifuge tube
- Heat dissociation
- Modified sample application to microscope slide

Implementation of any individual special technique should be performed independently from other modifications; do not attempt to incorporate multiple changes at once. Application of any special technique should be based on laboratory experience analyzing samples from a particular source.

1) Removing Magnetic Materials[4] – This modification to Method 1623 Section 13.3 must meet QC requirements (See Section III below).

Some source water samples can contain high concentrations of iron and other magnetic material that may interfere with IMS. Some laboratories have determined the removal of extraneous magnetic material from the sample, prior to the addition of beads, can improve recoveries. The sample is processed through filtration, elution, and concentration then extraneous magnetic material is removed. The remaining sample is carried through the IMS process.

Process sample(s) according to Method 1623 through Section 13.2.4.2; then proceed at 13.3.1 with the following substitutions and additions:

13.3.1 Preparation and addition of reagents

13.3.1.1 Same as method

13.3.1.2 Same as method

13.3.1.3 Same as method with following steps added:

13.3.1.3.1 Use a graduated, 10-mL pipette that has been pre-rinsed with elution buffer to transfer the water sample concentrate from Section 13.2 to another flat-sided tube(s) not containing IMS buffers. If all of the concentrate is used, rinse the centrifuge tube twice with reagent water and add the rinsate to the flat-sided tube containing the concentrate (or to the tube containing the first subsample, if multiple subsamples will be processed). Each of the two rinses should be half the volume needed to bring the total volume in the flat-sided sample tube to 8 mL. (For example, if 5 mL of sample was transferred after resuspension of the pellet, the centrifuge tube would be rinsed twice with 1.5 mL of reagent water to bring the total volume in the flat-sided tube to 8 mL.) Visually inspect the centrifuge tube after completing the transfer

to ensure that no concentrate remains. If multiple subsamples will be processed, bring the volume in the remaining flat-sided tubes to 8 mL with reagent water. Label the flat-sided tube(s) with the sample number (and subsample letters).

13.3.1.3.2 Place the flat-sided tube in the MPC®-1 or MPC®-6; check the tube is tight against the magnet.

13.3.1.3.3 Rock the magnet and tube gently and smoothly through a 90° angle for 2 minutes with approximately one 90° rock per second. Ensure the tilting action is continued throughout the 2 minute period.

13.3.1.3.4 If the sample is allowed to stand motionless for more than 10 seconds, remove the tube from the magnet, shake to resuspend all materials, replace the sample tube in the magnet, and repeat the 2 minute rocking.

13.3.1.3.5 Return tube to upright position and immediately remove the cap.

13.3.1.3.6 Keeping the flat side of tube on top, pour off supernatant into another flat-sided tube containing Buffers A and B. Without removing the tube from the MPC, rinse the round side of tube twice with 1 mL of reagent water. This tube now contains 12 mL of the sample and buffers ready to continue through the IMS process. Label this flat-sided tube(s) with the sample number (and subsample letters).

13.3.2 Oocyst and cyst capture

13.3.2.1 Omit this step

13.3.2.2 Same as method, to completion of the method

NOTE: The flat-sided tube remaining in the magnet should contain extraneous iron and other magnetic material removed by the magnet. This extraneous material may be discarded as waste and the tube either discarded or cleaned for reuse per laboratory standard operating procedure.

2) Adjusting pH[4] – This modification to Method 1623 Section 13.3.2.1 must meet QC requirements (See Section III below).

Some source water samples may produce a pellet with non-neutral pH characteristics. Low recoveries could result if acidity or alkalinity of the pellet is not adequately buffered during the IMS process. Some laboratories have determined the addition of HCl or NaOH to neutralize the sample, after buffers have been added, can improve recoveries.

Process sample(s) according to Method 1623 through Section 13.3.2.1, transferring all sample(s) to flat-sided tube(s) with buffers; then proceed with the following additions:

13.3.2.1 Same as method

13.3.2.1.1 Gently mix the buffers with the transferred sample by inverting the flat-sided tube 3 times.

13.3.2.1.2 Read and record the pH of the suspension.

13.3.2.1.3 Adjust the pH of the suspension with 1N HCl or 1N NaOH as needed to establish pH = 7.

13.3.2.2 Same as method, to completion of the method.

NOTE: The pH of the sample could also be checked and adjusted before buffers are added (Section 13.2.2), and after the 1-hour rotation (Section 13.3.2.7) to ensure the pH is stable. Some laboratories have used IQ Scientific Instruments-handheld pH meter with Micro Probe (PH17-SS) to check the pH in the flat-sided tube. Alternative pH measurement techniques may be used.

3) Additional Rinse of Flat-sided Tube – This modification to Method 1623 Section 13.3.2.11 must meet QC requirements (See Section III below).

When source water pellets contain visible excess debris after aspiration of the supernatant (Section 13.3.2.11), debris carryover may interfere with recoveries and an additional rinse of the flat-sided tube may be performed. If this additional rinse of the flat-sided tube is performed, then the additional modification of a pellet wash in the microcentrifuge tube (described later) should not be performed, to prevent over-rinsing and loss of organisms.

Process sample(s) according to Method 1623 through Section 13.3.2.11, remove supernatant from flat-sided tube; then proceed with the following additions:

13.3.2.11 Same as method with following steps added:
- 13.3.2.11.1 Orient the tube horizontally with bead pellet and magnet on top.
- 13.3.2.11.2 Gently add 10 mL of PBS or PBST (0.05% Tween 20) to the rounded side of the tube opposite the beads; do not disturb the bead pellet.
- 13.3.2.11.3 Gently tip the rinse solution over the side of the tube opposite of the bead pellet, three times.
- 13.3.2.11.4 Decant while continuing to keep magnet and flat-side of tube up.

13.3.2.12 Same as method, to completion of the method.

4) Pellet Wash in Microcentrifuge Tube – This modification to Method 1623 Section 13.3.2.16 must meet QC requirements (See Section III below).

Do not perform the pellet wash in the microcentrifuge tube if an additional rinse at the flat-sided tube was performed to prevent over-rinsing and loss of organisms.

Recoveries for source water matrices that produce debris carryover are usually improved by additional rinse of the flat-sided tube (see above). However, some source water matrices are better improved by a pellet wash in the microcentrifuge tube. This wash may be used with difficult matrices that exhibit visible debris trapped in the bead pellet at the back or bottom of the microcentrifuge tube after aspiration of the supernatant.

Remember: Any debris remaining in the microcentrifuge tube will end up on the slide!

Process sample(s) according to Method 1623 through Section 13.3.2.16, remove supernatant from microcentrifuge tube; then proceed with the following additions:

13.3.2.16 Same as method with following steps added:
- 13.3.2.16.1 Remove the magnetic strip from the MPC®-S after aspiration of supernatant.
- 13.3.2.16.2 Gently add 1 mL of PBS or PBST (0.05% Tween 20) to the bead suspension.
- 13.3.2.16.3 Gently invert the tube 5-10 times to completely resuspend the bead pellet.
- 13.3.2.16.4 Replace the magnetic strip in the MPC®-S and repeat Sections 13.3.2.15-13.3.2.16
- 13.3.2.16.5 After aspirating the wash, allow the microcentrifuge tube to sit undisturbed for 30-60 seconds before aspirating any remaining liquid.

13.3.3 Dissociation of beads/oocyst/cyst complex; same as method, to completion of the method.

5) Heat Dissociation[5] – This modification to Method 1623 Section 13.3.3 must meet QC requirements (See Section III below).

The addition of acid to some source water matrices for dissociation may drive chemical reactions that interfere with the method. Heat can be used instead of acid to inhibit reformation of the bead oo/cyst complexes and potentially improve recoveries.

Process sample(s) according to Method 1623 through Section 13.3.3.1; then revise13.3.3.2 through 13.3.3.10 as follows:

13.3.3.2	Add 50 µL of reagent water [instead of HCl], then vortex at the highest setting for approximately 50 seconds.
13.3.3.3	Place tube(s) in heat block stabilized at 80°C for 10 minutes.
13.3.3.4	Remove tube(s) from heat block, and vortex at the highest setting for approximately 30 seconds.
13.3.3.5	Same as method
13.3.3.6	Same as method
13.3.3.7	Same as method
13.3.3.8	DELETE this step in the method.
13.3.3.9	Same as method except omit "with the NaOH".
13.3.3.10	Replace "acid" with "heat"; (second dissociation may not be necessary)
13.3.3.11	Same as method, to completion of the method.

NOTE: Some laboratories have used a Multi Block Heater, Model 2050 or Grant UBD1 heat block. The Grant UBD1 heat block has options of various block sizes to accommodate different plasticware including a 1.5 mL microtube interchangeable block BB-E1 that can hold 24 tubes at once.

6) Increasing Surface Area for Sample Application to Slide – This modification to Method 1623 Section 13.3.3.7 must meet QC requirements (See Section III below).

Matrix debris may result in loss of organisms during the staining process and visual obstruction of oo/cysts on the slide. Increasing the surface area for sample application to slides can reduce interference from debris.

- Use slides with larger diameter wells to spread the debris and organisms over a larger surface area. Commercially available microscope slides have well diameters ranging from 9 mm to 15 mm.
- Split the dissociation volumes from each sample evenly between two slide wells.

To split each dissociation volume, process sample(s) according to Method 1623 through Section 13.3.3.6 (sample is in the microcentrifuge tube in the MPC®-S); then proceed at 13.3.3.7 with the following substitutions and additions:

13.3.3.7	Prepare two separate wells for each sample.
13.3.3.8	OMIT this step
13.3.3.9	Same as method, except apply half of the dissociation volume, 25 µL, to one well and the second half to a second well (See **Figure 3**; apply 25 µL to Well A and 25 µL to Well B)
13.3.3.10	Same as method, except apply splits of the dissociation volume to the two slide wells as before (modified 13.3.3.9 above, See **Figure 3**).
13.3.3.11	Add 5 µL of 1.0 N NaOH to each of the two wells after applying the samples
13.3.3.12	Same as method, to completion of the method.

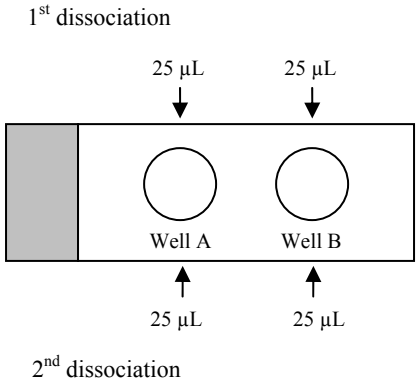


Figure 3

III. Quality Control Recommendations

Method 1623 is a performance-based method and modifications are allowed if the laboratory can meet applicable QC criteria (Section 9.1.2). Before modifications with the special techniques described above are incorporated for processing field samples, initial and on-going QC samples (described below) should be analyzed to demonstrate acceptable performance of the modified method. Laboratories should repeatedly perform the modified techniques and demonstrate acceptable organism recovery using these modified procedures with reagent and matrix samples prior to use on field samples.

Initial Demonstration of Acceptable Performance:

- Required, Section 9.4: Initial Precision and Recovery (IPR) samples consisting of 4 reagent water organism-spiked samples and one method blank (Section 9.6).
- Strongly Recommended, Section 9.5: Matrix spike, matrix spike duplicate, and unspiked field sample analyses.

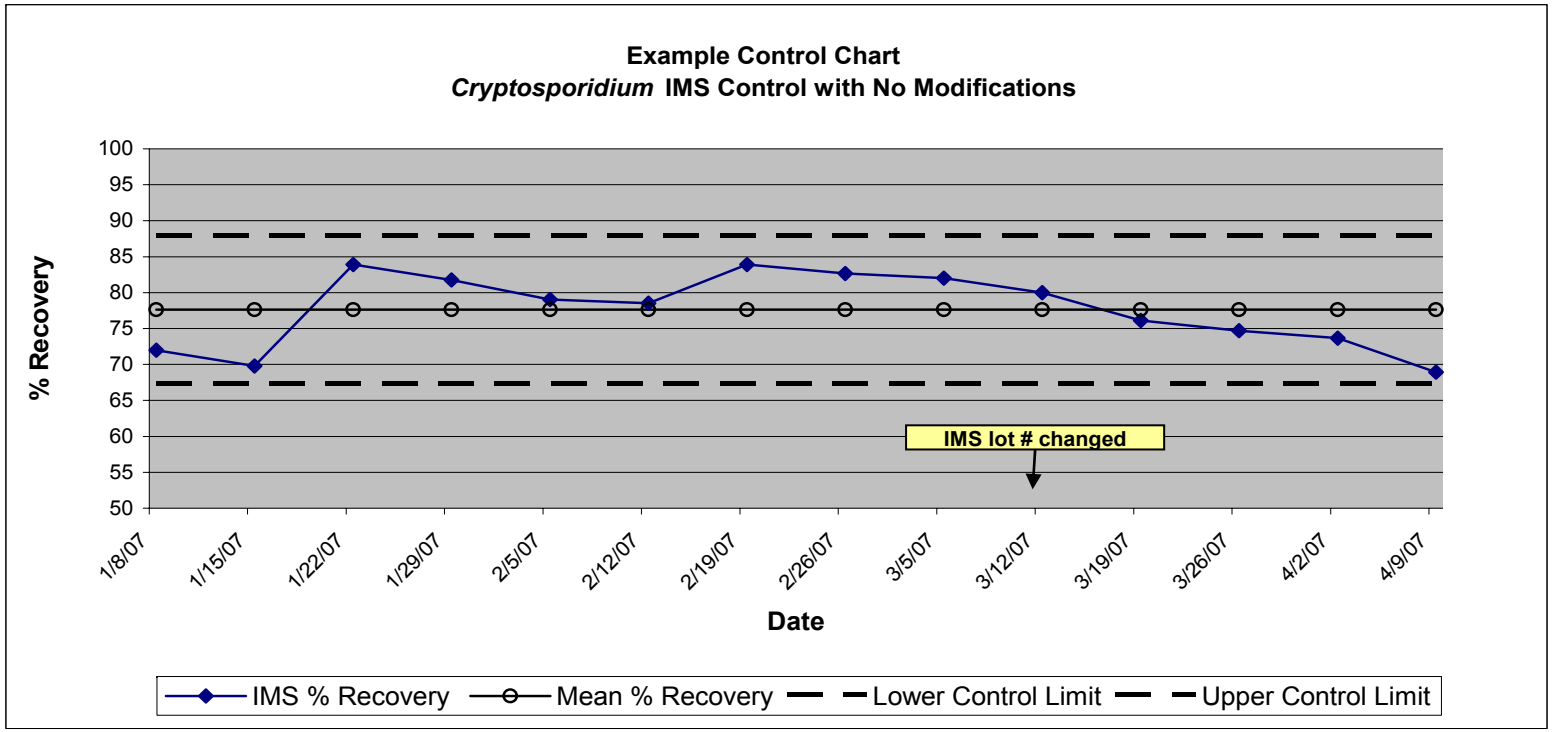
On-going Demonstration of Acceptable Performance, Section 9.7:

- Process matrix spike sample using the same modification as the associated field sample
- Process On-going Precision and Recovery (OPR) samples using the special techniques at the same percentage as the modification is used on field samples

  Example: If 25% of the field samples are processed with an additional rinse, every 4th OPR would be analyzed using the same type of additional rinse.

Processing IMS controls helps to monitor performance of modifications, new IMS kits, spike organisms and staining kits, as well as troubleshoot low recoveries in OPR and proficiency test samples**.** An IMS control is a 10-mL reagent water sample that is spiked with a known concentration of organisms and processed through IMS and staining to determine recoveries. Percent recovery should be at least 70% and typically is >80%. If the sample matrix is suspected of interfering with recovery, an IMS control may be analyzed using a 10-mL concentrate from the suspect source to help pinpoint the interference. The IMS control should be run weekly or with every 20 samples. An IMS control may also be run before processing field samples with a new lot of reagents (IMS kit, spike organisms, or stain). An IMS control may be used as a quality control check when processed with each batch of samples, if desired.

Control charts may be used to determine if problems are related to processing, reagent lots, equipment, and/or analysts. Control charts should include reagent lot numbers, analyst names, and method modifications. See **Figure 4** for an example of an IMS Control Chart. **Further guidance is available in Sections 3.3.5, 3.3.12.3 and 3.3.13 of the Laboratory Guidance Manual[6].**



Date	Total Count	Estimated # Spiked	IMS % Recovery	Mean % Recovery	Standard Deviation	Lower Control Limit	Upper Control Limit	Analyst	Spiking Suspension Lot #	IMS Kit Lot #	Stain Lot #	HCl Lot #	NaOH Lot #
1/8/07	108	150	72.0	77.64	5.14	67.35	87.93	TK	061220	48473	250050.111	H4056	NH0789
1/15/07	104	149	69.8	77.64	5.14	67.35	87.93	TK	070108	48473	250050.111	H4056	NH0789
1/22/07	125	149	83.9	77.64	5.14	67.35	87.93	TK	070108	48473	250050.111	H4056	NH0789
1/29/07	121	148	81.8	77.64	5.14	67.35	87.93	JS	070122	48473	250050.111	H4056	NH0789
2/5/07	117	148	79.1	77.64	5.14	67.35	87.93	JS	070122	48473	250050.111	H4056	NH0789
2/12/07	117	149	78.5	77.64	5.14	67.35	87.93	JS	070205	48473	250050.111	H4056	NH0789
2/19/07	125	149	83.9	77.64	5.14	67.35	87.93	JS	070205	48473	250050.111	H4056	NH0789
2/26/07	124	150	82.7	77.64	5.14	67.35	87.93	TK	070219	48473	250050.111	H4056	NH0789
3/5/07	123	150	82.0	77.64	5.14	67.35	87.93	TK	070219	48473	250050.111	H4056	NH0789
3/12/07	120	150	80.0	77.64	5.14	67.35	87.93	TK	070219	48474	250050.111	H4056	NH0789
3/19/07	119	150	76.1	77.64	5.14	67.35	87.93	JS	070219	48474	250050.111	H4056	NH0789
3/26/07	112	148	74.7	77.64	5.14	67.35	87.93	JS	070319	48474	250050.112	H4056	NH0789
4/2/07	109	148	73.6	77.64	5.14	67.35	87.93	JS	070319	48474	250050.112	H4056	NH0789
4/9/07	102	148	68.9	77.64	5.14	67.35	87.93	TK	070319	48474	250050.112	H4056	NH0789

Figure 4

IV. References

1. *Method 1623*: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA, Office of Water, Office of Ground Water and Drinking Water Technical Support Center, U.S. Environmental Protection Agency, Cincinnati, OH (2005) EPA 815-R-05-002 http://www.epa.gov/microbes/1623de05.pdf

2. Invitrogen Water and Environmental Testing Product Selection Guide; http://www.invitrogen.com/content.cfm?pageid=11110.

3. Aureon Biosystems or Immtech, Inc. (US distributor); http://www.aureonbio.com/ProductList.htm, http://www.immtech.net/html/imskr.html

4. Kuhn, R.C., C. M. Rock, and K. H. Oshima. Effects of pH and Magnetic Material on Immunomagnetic Separation of *Cryptosporidium* Oocysts from Concentrated Water Samples. Appl Environ Microbiol. 2002 April; 68(4): 2066–2070. doi: 10.1128/AEM.68.4.2066-2070.2002. Copyright © 2002, American Society for Microbiology. http://www.pubmedcentral.nih.gov/picrender.fcgi?tool=pmcentrez&artid=123889&blobtype=pdf

5. Ware, M. W., L. Wymer, H. D. A. Lindquist, and F. W. Schaefer III, 2003. Evaluation of an Alternative IMS Dissociation Procedure for Use with Method 1622: Detection of *Cryptosporidium* in Water. Journal of Microbiological Methods 55**:**575-583.

6. *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*, Office of Water, Office of Ground Water and Drinking Water Technical Support Center, U.S. Environmental Protection Agency, Cincinnati, OH (2006) EPA 815-R06-006 http://www.epa.gov/safewater/disinfection/lt2/pdfs/guide_lt2_microbialguidancemanual.pdf