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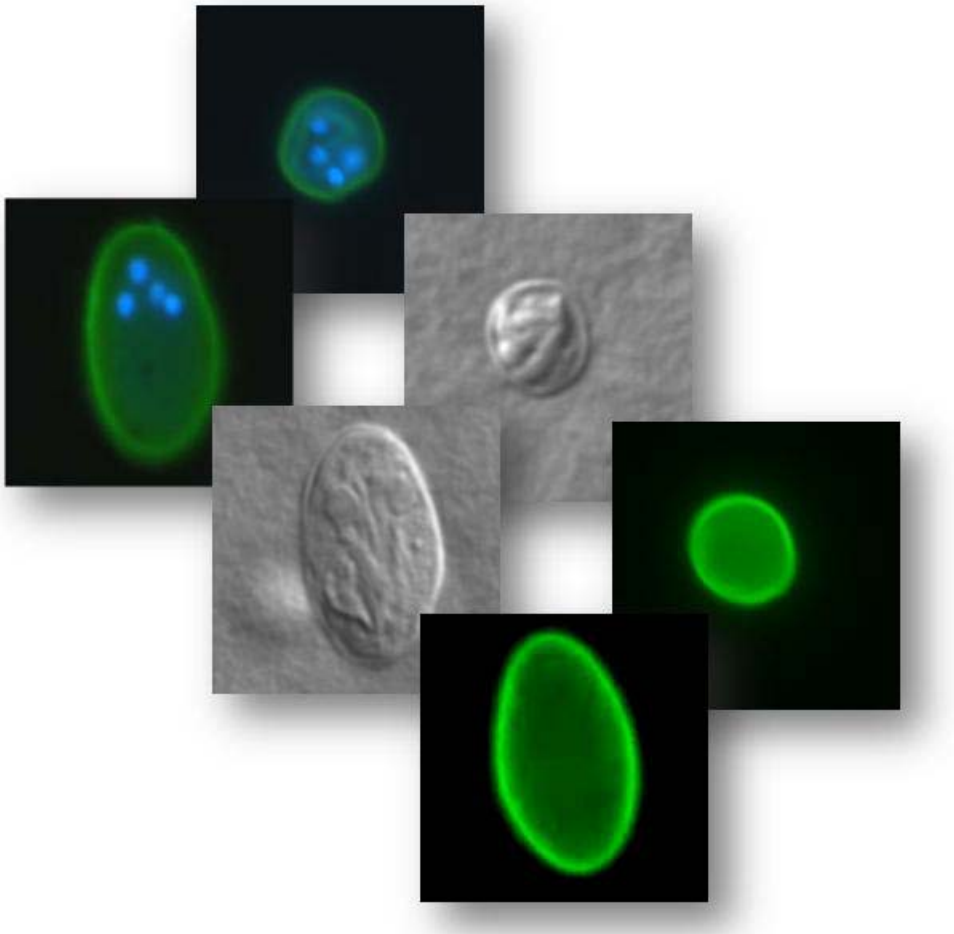
Environmental Protection
Agency

Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA



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Disclaimer

This method has been reviewed by U.S. EPA Office of Water and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Introduction

To support future regulation of protozoa in drinking water, the Safe Drinking Water Act Amendments of 1996 require U.S. Environmental Protection Agency (EPA) to evaluate the risk to public health posed by drinking water contaminants, including waterborne parasites, such as *Cryptosporidium spp.* and *Giardia spp.* To implement these requirements, EPA must assess *Cryptosporidium* and *Giardia* occurrence in raw surface waters used as source waters for drinking water treatment plants. EPA Method 1623 was developed to support this assessment.

Note: Throughout the rest of this document, the genera Cryptosporidium spp. *and* Giardia spp. *are referred to by their generic names (*Cryptosporidium *and* Giardia*) without reference to species, and without providing a scientific authority. This has been done for the sake of clarity and brevity given the intended audience. The authors acknowledge that this is an atypical example of scientific nomenclature.*

Method Development and Validation

EPA initiated an effort in 1996 to identify new and innovative technologies for protozoan monitoring and analysis. After evaluating potential alternatives to the then-current method through literature searches, discussions with research and commercial laboratories, and meetings with experts in the field, the Engineering and Analysis Division within the Office of Science and Technology within EPA's Office of Water developed draft Method 1622 for *Cryptosporidium* detection in December 1996. This *Cryptosporidium*-only method was validated through an interlaboratory study in August 1998, and was revised as a final, valid method for detecting *Cryptosporidium* in water in January 1999.

Although development of an acceptable immunomagnetic separation (IMS) system for *Giardia* lagged behind development of an acceptable system for *Cryptosporidium*, an acceptable system was identified in October 1998, and EPA validated a method for simultaneous detection of *Cryptosporidium* and *Giardia* in February 1999 and developed quality control (QC) acceptance criteria for the method based on this validation study. To avoid confusion with Method 1622, which already had been validated and was in use both domestically and internationally as a stand-alone *Cryptosporidium*-only detection method, EPA designated the new combined procedure EPA Method 1623.

The interlaboratory validated versions of Method 1622 (January 1999; EPA-821-R-99-001) and Method 1623 (April 1999; EPA-821-R-99-006) were used to analyze approximately 3,000 field and QC samples during the Information Collection Rule Supplemental Surveys (ICRSS) between March 1999 and February 2000. Method 1622 was used to analyze samples from March 1999 to mid-July 1999; Method 1623 was used from mid-July 1999 to February 2000.

Changes in the April 2001 Versions of the Methods

Both methods were revised in April 2001, after completion of the ICRSS and multiple meetings with researchers and experienced laboratory staff to discuss potential method updates. Changes incorporated in the April 2001 revisions of the methods (EPA-821-R-01-025 and EPA-821-R-01-026) included the following:

- Nationwide approval of modified versions of the methods using the following components:
    - (a) Whatman Nuclepore CrypTest™ filter
    - (b) IDEXX Filta-Max® filter
    - (c) Waterborne Aqua-Glo™ G/C Direct FL antibody stain
    - (d) Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains
- Clarified sample acceptance criteria
- Modified capsule filter elution procedure

- Modified concentrate aspiration procedure
- Modified IMS acid dissociation procedure
- Updated QC acceptance criteria for initial precision and recovery (IPR) and ongoing precision and recovery (OPR) tests
- Addition of a troubleshooting section for QC failures
- Modified holding times
- Inclusion of flow cytometry–sorted spiking suspensions

Changes in the June 2003 Versions of the Methods

Both methods were revised again in June 2003 to support proposal of EPA's Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule). Changes incorporated include:

- Nationwide approval of a modified version of the methods using the Pall Gelman Envirochek® HV filter
- Removal of Whatman Nuclepore CrypTest™ filter from the methods as a result of discontinuation of the product by the manufacturer
- Nationwide approval of the use of BTF EasySeed™ irradiated oocysts and cysts for use in routine QC samples
- Minor clarifications and corrections
- Rejection criteria for sample condition upon receipt
- Guidance on measuring sample temperatures
- Clarification of QC sample requirements and use of QC sample results
- Guidance on minimizing carry-over debris onto microscope slides after IMS

Changes in the December 2005 Versions of the Methods

Both methods were revised again in 2005 to support promulgation of EPA's LT2 Rule. Changes incorporated include:

- Nationwide approval of the use of portable continuous-flow centrifugation as a modified version of the method. The product met all method acceptance criteria for *Cryptosporidium* using 50-L source water samples (but not *Giardia*, however, individual laboratories are permitted to demonstrate acceptable performance for *Giardia* in their laboratory).
- Addition of BTF EasyStain™ monoclonal antibody stain as an acceptable reagent for staining in Methods 1622/1623. The product was validated through an interlaboratory validation study using the Pall Envirochek® HV filter.
- Clarification of the analyst verification procedure
- Clarification of sample condition criteria upon receipt

Changes in Method 1623.1

Method 1623 was revised in January 2012 to become Method 1623.1. Changes incorporated include:

- Nationwide approval of the use of Waterborne AccuSpike™-IR irradiated oocysts and cysts for use in routine QC samples
- Nationwide approval of the use of Invitrogen's Dynal® MX7 Mixer during IMS procedures
- Clarification of procedural components approved for use; summarized in Table 1
- Deletion of single-laboratory (Tier 1) validated method modifications
- Addition of minimum QC requirements summary (Table 2)
- Clarification of biosafety recommendations (Sections 5.3 and 5.4)
- Updated shipping requirement for samples containing known infectious materials to reflect changes in U.S. Department of Transportation Hazardous Materials Regulations (HMR; 49 CFR Parts 171-180) (Section 5.5)
- Updated product and reagent lists with current catalog numbers (Sections 6 and 7)
- Clarification of fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI) filter specifications for all microscope vendors (Section 6.7)
- Updated suggestions for measuring sample temperature, to include temperature recorders (Section 8.1.4)
- Clarification of Initial Demonstration of Capability (IDC) (Section 9.2)
- Clarification of control chart procedures (Section 9.12)
- Requirement for flow cytometer-enumerated *Cryptosporidium* and *Giardia* spiking suspensions (Section 11.0)
- Dispersant addition using sodium hexametaphosphate as required for capsule filter elution procedure (Sections 7.6.1.1 and 12.2.7)
- Requirement for bead pellet wash step during IMS procedure (Section 13.3.2.17)
- Clarified text for adjustment of pellet volume (Section 13.2)
- Clarified requirements for *Cryptosporidium* and *Giardia* characterizations (Sections 15.2.2 and 15.2.3)
- Clarified oocyst/cyst reporting requirements (Section 15.2.5)
- Inclusion of updated acceptance criteria for IPR, OPR, and MS/MSD samples (Section 17.0 and Tables 3 and 4)
- Addition of Figure 4 to clarify *Cryptosporidium* and *Giardia* reporting requirements (Section 15.2.2.1, 15.2.3.1, Figure 4)
- Organizational changes to improve ease of use (Sections 9, 10 and Appendices)

To enhance program-wide data quality and consistency, and guard against the use of sample processing shortcuts that could compromise data quality, the updated method provides laboratories with the flexibility to select from options for various procedural components that do not require an alternate test

procedure study (ATP) (Table 1). However, each option must be performed according to the procedures described in the method, which were used during the multi-laboratory validation study or historically documented SOP. Any additional ATPs should follow a process for conducting side-by-side method comparisons and for conducting quality control acceptance criteria-based method studies (Reference 20.1).

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Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

1.0 Scope and Application

1.1 This method is for the detection of *Cryptosporidium* (CAS Registry number 137259-50-8) and *Giardia* (CAS Registry number 137259-49-5) in water by concentration, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. *Cryptosporidium* and *Giardia* are further characterized using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy.

1.2 This method is designed to meet the survey and monitoring requirements of U.S. Environmental Protection Agency (EPA). It was originally based on laboratory testing of recommendations by a panel of experts convened by EPA. The 2012 revisions reflect the improved protocols for recovery and detection of protozoa evaluated by EPA Office of Ground Water and Drinking Water's Technical Support Center.

1.3 This method identifies the genera, *Cryptosporidium* or *Giardia*, but not the species. The method cannot determine the host species of origin, nor can it determine the viability or infectivity of detected oocysts and cysts.

1.4 This method is for use only by persons experienced in the determination of *Cryptosporidium* and *Giardia* by filtration, IMS, and FA. Experienced persons are defined in Section 22.2 as analysts or principal analysts. Laboratories unfamiliar with analyses of environmental samples by the techniques in this method must obtain training and experience using water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using bright-field and DIC microscopy prior to implementing the method. Training may be obtained through a variety of sources including other laboratories, experienced analysts, regulatory agencies, on-line modules, etc. Experience may be gained by performing the method in a variety of matrices under the supervision of an experienced analyst. Document training and experience according to the individual laboratory's standard operating procedure (SOP).

1.5 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures (Reference 20.1).

1.6 The laboratory is permitted to select from options for various procedural components in the method including sample collection, spiking, filtration/elution, concentration/aspiration, IMS, and staining. The options available for each component are detailed in the method and summarized in **Table 1**. To change between options, the laboratory must perform the procedures outlined in the Initial Demonstration of Capability (IDC, **Table 2**, Sections 9.5, 9.6, and 9.7) which includes acceptable performance in at least one matrix of interest and verify that all quality control (QC) acceptance criteria are met (Sections 9.1 and 21.0, **Tables 3 and 4**). Although different options can be used by different laboratories, each option must be performed according to the procedures specified in this method or the manufacturers' instructions. These procedures were approved by EPA based on the multi-laboratory validation studies using multiple matrices or historical demonstration of accuracy and precision at multiple laboratories. To guard against the use of sample processing shortcuts or cost-cutting that could compromise data quality, no changes to these validated procedures are permitted without demonstrating acceptability through a multi-laboratory validation study using multiple matrices, as per Reference 20.1.

This description of method flexibility is an abbreviated summation. Specific detail is provided throughout the method, which supersedes the above general guidance.

2.0 Summary of Method

2.1 A water sample is filtered and the oocysts, cysts, and extraneous materials are retained on the filter. Although EPA has only validated the method using laboratory filtration of bulk water samples shipped from the field, field-filtration also may be used.

2.2 Elution and separation

2.2.1 Materials on the filter are eluted and the eluate is centrifuged to pellet the oocysts and cysts, and the supernatant fluid is aspirated.

2.2.2 The oocysts and cysts are made paramagnetic (magnetic only when exposed to a magnetic field) by attachment of paramagnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. The paramagnetic oocysts and cysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The paramagnetic bead complex is then detached from the oocysts and cysts.

2.3 Enumeration

2.3.1 The oocysts and cysts are stained on well slides with fluorescently labeled monoclonal antibodies and DAPI. The stained sample is examined using fluorescence and DIC microscopy.

2.3.2 Qualitative analysis is performed by scanning each slide well for objects that meet the size, shape, and fluorescence characteristics of *Cryptosporidium* oocysts or *Giardia* cysts.

2.3.3 Quantitative analysis is performed by counting the total number of objects on the slide that meet the FA, DAPI, and DIC criteria for oocysts or cysts.

2.4 Quality is assured through reproducible calibration and testing of the filtration, IMS, staining, and microscopy systems. Detailed information on these tests is provided in Sections 9.0 and 10.0, and Appendix B.

3.0 Definitions

3.1 *Cryptosporidium* is a genus of protozoan parasites potentially found in water and other media. *Cryptosporidium* oocysts are defined in this method as objects exhibiting brilliant apple green fluorescence under UV light [after staining with fluorescein isothiocyanate (FITC)-conjugated antibodies (FA-positive)], typical size (4 to 6 µm) and shape (round to oval), and no atypical characteristics by FA, DAPI fluorescence, or DIC microscopy. Examination and characterization using fluorescence (FITC and DAPI stain) and DIC microscopy are required for exclusion of atypical organisms (e.g., those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).

3.2 *Giardia* is a genus of protozoan parasites potentially found in water and other media. *Giardia* cysts are defined in this method as objects exhibiting brilliant apple green fluorescence under UV light [after staining with FITC-conjugated antibodies (FA-positive)], typical size (8 to 18 µm long by 5 to 15 µm wide) and shape (oval to round), and no atypical characteristics by FA, DAPI fluorescence, or DIC microscopy. Examination and characterization by fluorescence (FITC and DAPI stain) and DIC microscopy are required for exclusion of atypical organisms (e.g., those

possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).

3.3 Definitions for other terms used in this method are given in the glossary (Section 22.0).

4.0 Contamination, Interferences, and Organism Degradation

4.1 Turbidity caused by inorganic and organic debris can interfere with the concentration, separation, and examination of the sample for *Cryptosporidium* oocysts and *Giardia* cysts. In addition to naturally-occurring debris (e.g., clays and algae), chemicals (e.g., iron, alum coagulants) and polymers added to source waters during the treatment process may result in additional interference.

4.2 Organisms and debris that autofluoresce or demonstrate non-specific immunofluorescence, such as algal and yeast cells, when examined by epifluorescent microscopy, may interfere with the detection of oocysts and cysts and contribute to false positives by FA (Reference 20.2).

4.3 Solvents, reagents, labware, and other sample-processing hardware may yield artifacts that may cause misinterpretation of microscopic examinations for oocysts and cysts. All materials used must be demonstrated to be free from interferences under the conditions of analysis by running a method blank (negative control sample) initially and a minimum of every week or after changes in source of reagent water. Specific selection of reagents and purification of solvents and other materials may be required.

4.4 Freezing samples, filters, eluates, concentrates, or slides may interfere with the detection and/or identification of oocysts and cysts.

4.5 Clean all equipment according to manufacturers' instructions, or according to SOP used in the laboratory. Use disposable supplies wherever possible.

5.0 Safety

5.1 The biohazard associated with, and the risk of infection from, oocysts and cysts is high in this method because live organisms are handled. This method does not purport to address all of the safety problems associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. In particular, laboratory staff must know and observe the safety procedures required in a microbiology laboratory that handles pathogenic organisms while preparing, using, and disposing of sample concentrates, reagents and materials, and while operating sterilization equipment (Reference 20.3).

5.2 The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, it is prudent to treat each chemical compound as a potential health hazard. Reduce exposure to these compounds to the lowest possible level. The laboratory is responsible for maintaining current knowledge of Occupational Safety and Health Administration regulations regarding the safe handling of the chemicals specified in this method. Make a reference file of material safety data sheets available to all personnel involved in these analyses. Additional information on laboratory safety can be found in References 20.3 through 20.7.

5.3 Samples may contain high concentrations of biohazards and toxic compounds, and must be handled with gloves. Reference materials and standards containing oocysts and cysts must also be handled with gloves and laboratory staff must never place gloves in or near the face after exposure to solutions known or suspected to contain oocysts and cysts. Laboratory personnel

must change gloves after handling filters and other contaminant-prone equipment and reagents. Gloves must be removed or changed before touching any other laboratory surfaces or equipment.

5.4 Avoid any potential contamination from concentrated samples of control suspensions that may results in accidental ingestion. Do not mouth-pipette. Take appropriate precautions to ensure that no aerosol is generated by any step. Performing the Method in a biological safety cabinet for as many steps as logistically possible is recommended to prevent exposure (Reference 20.3).

5.5 The U.S. Department of Transportation (DOT) Hazardous Material Regulation (49 CRF Parts 171-178) classifies biological hazards as two categories. *Cryptosporidium* and *Giardia* are Category B: An infectious substance not in a form generally capable of causing permanent disability, life-threatening, or fatal disease in otherwise healthy humans or animals when exposure occurs. Any sample known or suspected to contain *Cryptosporidium* or *Giardia* should be shipped as Biological substance, Category B, UN3373 (Reference 20.8) or by regulations controlling the shipment that are in effect at the time of shipment. Environmental samples that are not considered to pose a significant health risk are not subject to the requirements of the Hazardous Material Regulation.

6.0 Equipment and Supplies

NOTE: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials when stated as "or equivalent," but demonstration of equivalent performance that meets the requirements of this method (Section 9.2 and Table 1) is the responsibility of the laboratory.

6.1 Sample collection equipment for shipment of bulk water samples for laboratory filtration. Collapsible low-density polyethylene (LDPE) cubitainer for collection of 10-L bulk sample(s)—Cole Parmer cat. no. U-06100-30 or equivalent. Fill completely to ensure collection of a full 10-L sample. Discard after one use. Alternatively, use clean, 10-L carboy with bottom delivery port (½"), Cole-Parmer cat. no. 06080-42, or equivalent; calibrate to 10.0 L and mark level with waterproof marker.

6.2 Equipment for sample filtration. Three options have been demonstrated to be acceptable for use with Method 1623.1: Envirochek® HV Sampling Capsule, Filta-Max® Foam Filter, and Portable Continuous Flow Centrifuge (PCFC).

6.2.1 Cubitainer spigot to facilitate laboratory filtration of sample—Cole Parmer cat. no. U-06061-01, or equivalent.

6.2.2 Tubing—Glass, polytetrafluoroethylene (PTFE), high-density polyethylene (HDPE), or other tubing to which oocysts and cysts will not easily adhere, Tygon formula R-3603, or equivalent. If rigid tubing (glass, PTFE, HDPE) is used and the sampling system uses a peristaltic pump, a minimum length of compressible tubing may be used in the pump. Between samples, the tubing must be autoclaved, thoroughly rinsed with detergent solution, followed by repeated rinsing with reagent water to minimize sample contamination. Alternately, decontaminate using hypochlorite solution, sodium thiosulfate, and multiple reagent water rinses. Dispose of tubing after one use whenever possible or when wear is evident.

6.2.3 Flow control valve—0.5 gpm (0.03 L/s) Bertram Controls, Plast-O-Matic cat. no. FC050B½-PVor equivalent

6.2.4 Pump—peristaltic, centrifugal, impeller, or diaphragm pump; MasterFlex I/P® EasyLoad® peristaltic pump (Cole-Parmer cat. no. EW-77963-10) with EW-77601-10 pumphead, EW-77410-10 drive unit, and EW-06429-73 Tygon LFL tubing; Dayton, model number 3YU61 (available through Grainger), Jabsco Flexible Impeller Pump (Cole-Parmer cat. no.EW-75202-00); Simer, model number M40; or equivalent. It is recommended that the pump be placed on the effluent side of the filter, to reduce the risk of contamination and the amount of tubing replaced or cleaned.

6.2.5 Flow meter—Omega flow meter, Stamford, CT, model FTB4105; or equivalent. Alternatively, use a graduated carboy(s) (See Section 6.16).

6.2.6 Stir bar—Fisher cat. no. 14-513-66, or equivalent

6.2.7 Stir plate—VWR cat. no. 47751-150, or equivalent

6.2.8 Envirochek® HV Sampling Capsule, Pall Corporation, Ann Arbor, MI, part no. 12099 (individual filter) or part no.12098 (box of 25 filters)

6.2.8.1 Laboratory shaker for agitation of sampling capsules —Pall Corporation part no. 4821or equivalent

6.2.8.2 Side arms for laboratory shaker

6.2.9 Filta-Max® foam filter, IDEXX, Westbrook, ME. Filter module cat. no. FMC 10603

NOTE: Check at least one filter per batch to ensure that the filters have not been affected by improper storage or other factors that could result in brittleness or other problems. At a minimum confirm that the test filter expands properly in water before using the batch or shipping filters to the field.

6.2.9.1 Filta-Max® starter kit, IDEXX, Westbrook, ME, cat. no. FMC 11002

6.2.9.1.1 Manual wash station with clamp set, cat. no. FMC 10101 or FMC 10106 or Automatic wash station cat. no. FMC 10103

6.2.9.1.2 Quick connect tubing set (includes elution tube, quick connect kit, concentrator tube, concentrator base (with line tap), steel tube and all o-rings), cat. no. FMC 10307

6.2.9.1.3 Vacuum set (includes plastic hand pump, waste bottle, tubing and magnetic stirrer bar), cat. no. FMC 10401

6.2.9.1.4 MKII filter housing with appropriate fittings, cat. no. FMC 10504

6.2.9.1.5 Housing tools cat. no. FMC 10506

6.2.9.2 Filter membranes, 100 pk, IDEXX, Westbrook, ME, cat. no. FMC 10800

6.2.9.3 Forceps—blunt end

6.2.9.4 Rubber stoppers, large and small, IDEXX, Westbrook, ME, cat. no. FMC 10508 and FMC 10302

6.2.10 PCFC requirements (for use with procedures described in Section 12.4). The technique is based on technology from Haemonetics Corporation, Braintree, MA.

6.2.10.1 Portable continuous-flow centrifuge, Scientific Methods, Inc., Granger, IN, cat. no. CFC-201

6.2.10.2 Disposable Bowl, 25 pk, Scientific Methods, Inc., Granger, IN, cat. no. CFC-210

6.2.10.3 Tubing, 25 pk, Scientific Methods, Inc., Granger, IN, cat. no. CFC-220

6.3 IMS apparatus

6.3.1 Sample mixer—Available through IDEXX or Invitrogen, Dynal® Sample Mixer, cat. no. 94701, or equivalent

6.3.2 Magnetic particle concentrator for 10-mL test tubes—Available through IDEXX or Invitrogen, Dynal® MPC®-1, cat. no. 12001D or MPC®-6, cat. no. 12002D, or equivalent

6.3.3 Magnetic particle concentrator for microcentrifuge tubes—Available through IDEXX, Dynal® MPC®-S, cat. no. A13346 or equivalent

6.3.4 Flat-sided sample tubes—16 × 125 mm Leighton-type tubes with 60 × 10 mm flat-sided magnetic capture area, available through IDEXX or Invitrogen, Dynal L10, cat. no. 74003, or equivalent

6.3.5 Microcentrifuge tubes—conical, siliconized (coated with silicone to reduce adhesion to walls) or low-retention, 1.5-mL, Fisher cat. no. 13-6987-91 or equivalent

6.4 Powder-free latex gloves—Fisher cat. no. 11-394-5B, or equivalent

6.5 Graduated cylinders, autoclavable—10-, 100-, and 1000-mL

6.6 Centrifuges

6.6.1 Centrifuge capable of accepting 50- to 250-mL conical centrifuge tubes and achieving at least 1500 × G—Allegra® X-15R benchtop centrifuge with swinging bucket SX4750A ARIES™ Smart Balance Rotor with bucket adaptors, Beckman Coulter, Brea, CA, or equivalent

6.6.2 Centrifuge tubes—Conical, graduated, 50- and 250-mL, Fisher cat. no. 06-443-20 and 05-538-53, or equivalent

6.7 Microscope

6.7.1 Epifluorescence/DIC with stage and ocular micrometers and 20X (N.A.=0.4) to 100X (N.A.=1.3) objectives.

6.7.2 Excitation/band-pass filters for FA—Filter set to examine FITC (peak excitation 490- to 495-nm, peak emission 516- to 525-nm), optimized with microscope, DAPI filter set, and slide preparations; general specifications: 450- to 495-nm excitation filter, 505-nm dichroic mirror, and 515- to 520-nm emission, barrier, or suppression filter; or equivalent

6.7.3 Excitation/band-pass filters for DAPI—Filter set to examine DAPI (peak excitation 345- to 359-nm, peak emission 455- to 470-nm), optimized with microscope, FITC filter set, and slide preparations; general specifications: 325- to 380-nm excitation

filter, 390- to 415-nm dichroic mirror, 420- to 485-nm emission, barrier, or suppression filter; or equivalent

6.8 Ancillary equipment for microscopy

6.8.1 Well slides—Dynal® Spot-On well slides, Invitrogen, cat. no. 740-04; treated, 12-mm diameter well slides, Meridian Diagnostics Inc., Cincinnati, OH, cat. no. R2206; or equivalent.

6.8.2 Glass coverslips—22 × 50 mm or appropriate size

6.8.3 Nonfluorescing immersion oil—low fluorescence, Type LDF, HF, or FF Cargille cat. no. 16241, 16245, 16212, or equivalent

6.8.4 Micropipette, adjustable (See Appendix A):

6.8.4.1 0- to 10-µL with 0- to 10-µL tips

6.8.4.2 10- to 100-µL, with 10- to 200-µL tips

6.8.4.3 100- to 1000-µL with 100- to 1000-µL tips

6.8.5 Humid chamber—A tightly sealed plastic container containing damp paper towels on top of which the slides are placed

6.9 Pipettes—Glass or plastic

6.9.1 1-, 5-, 10-, and 25-mL

6.9.2 Pasteur, disposable, internal diameter of orifice 0.80 to 1.5 mm

6.10 Balances

6.10.1 Analytical—Capable of weighing 0.1 mg

6.10.2 Top loading—Capable of weighing 10 mg

6.11 pH meter

6.12 Incubator—Fisher Scientific Isotemp™, or equivalent

6.13 Vortex mixer—Scientific Industries Vortex-Genie, or equivalent

6.14 Vacuum source—Capable of maintaining 25 in. Hg, equipped with shutoff valve and vacuum gauge

6.15 Miscellaneous labware and supplies

6.15.1 Appropriate tube racks

6.15.2 Flasks—Suction, Erlenmeyer, and volumetric, various sizes

6.15.3 Beakers—Glass or plastic, 5-, 10-, 50-, 100-, 500-, 1000-, and 2000-mL

6.15.4 Lint-free tissues

6.16 10- to 15-L graduated container—Fisher cat. no. 02-961-50B, or equivalent; calibrate to 9.0, 9.5, 10.0, 10.5, and 11.0 L and mark levels with waterproof marker

6.17 Filters for filter-sterilizing reagents—Sterile Acrodisc, 0.45 µm, Pall Corporation, cat. no. 4184, or equivalent

7.0 Reagents and Standards

7.1 Reagents for adjusting pH

7.1.1 Sodium hydroxide (NaOH)—ACS reagent grade, 6.0 N

7.1.2 Hydrochloric acid (HCl) —ACS reagent grade, 6.0 N

7.2 Solvents—Acetone, glycerol, ethanol, and methanol, ACS reagent grade

7.3 Reagent water—Water in which oocysts and cysts and interfering materials and substances, including magnetic minerals, are not detected by this method. See Reference 20.9 (Part 9020) for reagent water requirements.

7.4 Anti-microbial supplies—bleach, 3% hydrogen peroxide, ethanol wipes, and commercial multi-surface cleaner. Use cleaner appropriate for surface and biohazard (Reference 20.3).

7.5 Phosphate buffered saline (PBS), pH 7.4—Sigma Chemical Co. cat. no. P-3813, or equivalent. Alternately, prepare 1 X PBS by adding the following to 800 mL of reagent water: 8 g NaCl; 0.2 g KCl; 1.15 g Na_2HPO_4, anhydrous; and 0.2 g KH_2PO_4. Adjust pH to 7.4 with HCl or NaOH. Dilute to a final volume of 1000 mL using regent water. Store in a plastic or glass container at room temperature or 4°C. Discard if microbial growth is apparent or after a specific time determined by the laboratory.

7.6 Reagents for eluting filters

NOTE: Store prepared eluting solution for no more than 1 week or until noticeably turbid, whichever comes sooner.

7.6.1 Reagents for eluting Envirochek® HV sampling capsules (Section 6.2.8)

7.6.1.1 5% w/v NaHMP solution—Dissolve 50 g of sodium hexametaphosphate ($(NaPO_3)_n$ – Sigma 71600 or equivalent) in 600 mL of reagent water. Dilute to final volume of 1000 mL using reagent water. Store in a plastic or glass container at room temperature for up to 3 months. Discard when expiration date is reached or sooner if microbial growth is apparent.

7.6.1.2 Laureth-12—Fisher cat. no. NC9253856, or equivalent. Store Laureth-12 as a 10% solution in reagent water. Weigh 10 g of Laureth-12 and dissolve using a microwave or hot plate in 90 mL of reagent water. Dispense 10-mL aliquots into sterile vials and store at room temperature for up to 2 months, or in the freezer for up to a year.

7.6.1.3 1 M Tris, pH 7.4—Dissolve 121.1 g Tris (Fisher cat. no. BP152) in 700 mL of reagent water and adjust pH to 7.4 with HCl or NaOH. Dilute to a final 1000 mL with reagent water and adjust the final pH. Filter-sterilize through a 0.2-µm membrane into a sterile plastic container and store at room temperature. Alternatively, use prepared Tris (Sigma T2194 or equivalent) or prepared Tris-EDTA (Sigma T9285 or equivalent).

7.6.1.4 0.5 M EDTA, 2 Na, pH 8.0—Dissolve 186.1 g ethylenediamine tetraacetic acid, disodium salt dihydrate (Fisher cat. no. S311) in 800 mL of reagent water and adjust pH to 8.0 with HCl or NaOH. Dilute to a final volume of 1000 mL with reagent water and confirm pH. Alternatively, use prepared

EDTA (Sigma E7889 or equivalent) or prepared Tris-EDTA (Sigma T9285 or equivalent).

7.6.1.5 Antifoam A—Sigma Chemical Co. cat. no. 10794, or equivalent

7.6.1.6 Preparation of elution buffer solution—Add the contents of a pre-prepared Laureth-12 vial (Section 7.6.1.2) to a 1000-mL graduated cylinder. Rinse the vial several times to ensure the transfer of the detergent to the cylinder. Add 10 mL of Tris solution (Section 7.6.1.3), 2 mL of EDTA solution (Section 7.6.1.4), (alternatively, if using a manufacturer-combined Tris-EDTA [100X, Sigma T9285], add 10 mL total), and 150 µL Antifoam A (Section 7.6.1.5). Dilute to 1000 mL with reagent water.

7.6.2 Reagents for eluting Filta-Max® foam filters (Section 6.2.9)

7.6.2.1 PBS, pH 7.4 (Section 7.5)

7.6.2.2 TWEEN® 20—Sigma Chemical Co. cat. no. P-7949, or equivalent

7.6.2.3 High-vacuum grease—Fisher cat. no. 14-635-5D, or equivalent

7.6.2.4 Preparation of phosphate buffered saline with TWEEN® (PBST) elution buffer. Add 100 µL of TWEEN® 20 to prepared PBS (Section 7.5). Alternatively, add the contents of one packet of PBS to 1.0 L of reagent water. Dissolve by stirring for 30 minutes. Add 100 µL of TWEEN® 20. Mix by stirring for 5 minutes.

7.6.3 Reagents for Portable Continuous-Flow Centrifuge (Section 6.2.10)

7.6.3.1 Sodium dodecyl sulfate—Sigma Chemical Co. cat. no. 62862 or equivalent

7.6.3.2 TWEEN® 80—Sigma Chemical Co. cat. no. P1754 or equivalent

7.6.3.3 Antifoam A—Sigma Chemical Co. cat. no. 10794, or equivalent

7.6.3.4 Preparation of concentrated elution buffer. Add above reagents to obtain a final concentration of 1% sodium dodecyl sulfate, 0.01% TWEEN® 80, and 0.001% Antifoam A in concentrated sample volume of ~250mL

7.7 Reagents for IMS

7.7.1 Dynabeads® GC-Combo—Available through IDEXX, Westbrook, ME, Dynal cat. no.730.12, or equivalent

7.7.2 NaOH—ACS reagent grade, 1.0 N, Sigma Chemical Co. cat. no. S2770, or equivalent

7.7.3 HCl—ACS reagent grade, 0.1 N, Sigma Chemical Co. cat. no. 84428, or equivalent

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NOTE: Due to the low volumes of pH-adjusting reagents used during IMS, and the impact that changes in pH have on the FA, the laboratory must purchase standards at the required normality directly from a vendor. Normality must not be adjusted by the laboratory.

7.8 Direct antibody labeling reagents for detection of oocysts and cysts. Store reagents between 1°C and 10°C and return promptly to this temperature after each use. Do not allow any of the reagents to freeze. Protect the reagents from exposure to light. Discard diluted, unused working reagents

after 48 hours or following manufacturer's instructions. Discard reagents after the expiration date is reached. The labeling reagents in Sections 7.8.1-7.8.4 have been approved for use with this method.

7.8.1 MeriFluor® *Cryptosporidium/Giardia*, Meridian Diagnostics cat. no. 250050, Cincinnati, OH, or equivalent

7.8.2 Aqua-Glo™ G/C Direct FL, Waterborne, Inc. cat. no. A100FLK, New Orleans, LA, or equivalent

7.8.3 Crypt-a-Glo™ and Giardi-a-Glo™, Waterborne, Inc. cat. nos. A400FLK and A300FLK, respectively, New Orleans, LA, or equivalent

7.8.4 EasyStain™C&G, BTF Pty Limited, Sydney, Australia or equivalent

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NOTE: If a laboratory will use multiple types of labeling reagents, the laboratory must demonstrate equivalent or superior performance through an IDC per Section 9.2 for each type, and must perform positive and negative staining controls for each batch of slides stained using each product. However, the laboratory is not required to analyze additional ongoing precision and recovery samples or method blank samples for each type. Monitor the performance of each labeling reagent used in each source water type through MS samples (Section 9.6.1).

7.9 DAPI stain (4',6-diamidino-2-phenylindole)—Sigma Chemical Co. cat. no. D9542, or equivalent

7.9.1 Stock solution—Purchase smallest amount possible (typically 1 mg vials) to eliminate weighing a portion of the powder. Dissolve 2 mg/mL DAPI in absolute methanol by adding 0.5 mL of methanol to 1 mg vial of DAPI. Store between 1°C and 10°C in the dark. Do not allow to freeze. Discard unused solution when positive staining control fails or after specified time determined by laboratory.

7.9.2 Staining solution—Follow antibody kit manufacturer's instructions. Add 10 µL of 2 mg/mL DAPI stock solution to 50 mL of PBS for use with Aqua-Glo™ G/C Direct FL or MeriFluor® *Cryptosporidium/Giardia*. Add 50 µL of 2 mg/mL DAPI stock solution to 50 mL of PBS for use with EasyStain™. Prepare working solution daily and store between 1°C and 10°C (do not allow to freeze). DAPI is light sensitive; therefore, store in the dark except when staining. The DAPI concentration may be increased if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.

7.10 Mounting medium

7.10.1 DABCO/glycerol mounting medium (2%)—Dissolve 2 g of DABCO (Sigma Chemical Co. cat. no. D27802, or equivalent) in 95 mL of warm glycerol/PBS (60% glycerol, 40% PBS). After the DABCO has dissolved completely, adjust the solution volume to 100 mL by adding an appropriate volume of glycerol/PBS solution. Alternately, dissolve the DABCO in 40 mL of PBS, then add azide (1 mL of 100X, or 10% solution), then 60 mL of glycerol.

7.10.2 Mounting medium supplied with MeriFluor® *Cryptosporidium/Giardia*, Meridian Diagnostics cat. no. 250050, or equivalent (Section 7.8.1)

7.10.3 Mounting medium supplied with Aqua-Glo™ G/C Direct FL kit, Waterborne, Inc. cat. no. A100FLK, cat. no. M101, or equivalent (Section 7.8.2)

7.10.4 Mounting medium supplied with EasyStain™C&G, BTF Pty Limited or equivalent (Section 7.8.4)

7.10.5 Elvanol® or equivalent permanent, non-fade archiving mounting medium

7.11 Clear fingernail polish or clear fixative, Fisher, cat. no. NC0154994, or equivalent

7.12 Oocyst and cyst suspensions for spiking

7.12.1 Enumerated spiking suspensions prepared by flow cytometer

7.12.1.1 Live, flow cytometer–sorted oocysts and cysts—Wisconsin State Laboratory of Hygiene Flow Cytometry Unit, or equivalent

7.12.1.2 Irradiated, flow cytometer-sorted oocysts and cysts –EasySeed™ BTF Pty Limited, or AccuSpike™-IR Waterborne, Inc., or equivalent.

7.12.2 Storage procedure—Store oocyst and cyst suspensions between 1°C and 10°C, until ready to use or follow manufacturer's instructions; do not allow to freeze

8.0 Sample Collection and Storage

8.1 Sample collection, shipment, and receipt

8.1.1 **Sample collection**—Samples are collected as bulk samples and shipped to the laboratory on ice for processing through the entire method, or are filtered in the field and shipped to the laboratory on ice for processing from elution (Section 12.2.6 or 12.3.2) onward.

8.1.2 **Sample shipment**—Ambient water samples are dynamic environments and, depending on sample constituents and environmental conditions, *Cryptosporidium* oocysts or *Giardia* cysts present in a sample can degrade, potentially biasing analytical results. Chill all samples to reduce biological activity, and preserve the state of source water samples between collection and analysis. Samples analyzed by an off-site laboratory should be shipped on ice via overnight service on the day they are collected.

NOTE: See transportation precautions in Section 5.5.

8.1.2.1 If samples are collected early in the day, chill samples by storing in a refrigerator between 1°C and 10°C or pre-icing the sample in a cooler. If the sample is pre-iced before shipping, replace with fresh ice immediately before shipment.

8.1.2.2 If samples are collected later in the day, these samples may be chilled overnight in a refrigerator between 1°C and 10°C. Consider overnight refrigeration for bulk water samples that will be shipped off-site, as this minimizes the potential for water samples collected during the summer to melt the ice in which they are packed and arrive at the laboratory at >20°C.

8.1.2.3 If samples are shipped after collection at >20°C with no chilling, the sample will not maintain the temperature during shipment at ≤20°C.

8.1.2.4 Public water systems shipping samples to off-site laboratories for analysis should include in the shipping container a means for monitoring the temperature of the sample during shipping to verify that the sample did not

freeze or exceed 20°C. Suggested approaches for monitoring sample temperature during shipping are discussed in Section 8.1.4.

8.1.3 **Sample receipt**—Upon receipt, the laboratory must record the sample temperature. Samples that were not collected the same day they were received, and that are received at >20°C or frozen, or samples that the laboratory has determined exceeded >20°C or froze during shipment, must be rejected. After receipt, samples must be stored at the laboratory between 1°C and 10°C, and not frozen, until processed.

8.1.4 **Suggestions on measuring sample temperature**—Given the importance of maintaining sample temperatures for *Cryptosporidium* and *Giardia* determination, laboratories performing analyses using this method must establish acceptance criteria for receipt of samples transported to their laboratory. As with other laboratory equipment, all temperature measurement devices must be calibrated routinely to ensure accurate measurements. See EPA *Manual for the Certification of Laboratories Analyzing Drinking Water* (Reference 20.10) for more information. Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment:

8.1.4.1 **Temperature sample**—One option, for filtered samples only (not for 10-L bulk samples), is for the sampler to fill a small, inexpensive sample bottle with water and pack this "temperature sample" next to the filtered sample. The temperature of this extra sample volume is measured upon receipt to estimate the temperature of the filter. Temperature sample bottles are not appropriate for use with bulk samples because of the potential effect that the difference in sample volume may have in temperature equilibration in the sample cooler. *Example product:* Cole Parmer cat. no. U-06252-20.

8.1.4.2 **Thermometer vial**—A similar option is to use a thermometer that is securely housed in a liquid-filled vial. Unlike temperature samples, the laboratory does not need to perform an additional step to monitor the temperature of the vial upon receipt, but instead just needs to read the thermometer. The thermometer vial is appropriate for use with filtered samples not bulk samples. *Example product:* Eagle-Picher Sentry Temperature Vial 3TR-40CS-F or 3TR-40CS.

8.1.4.3 **Temperature Recorders**—Option allows the measurement and recording of sample temperature during shipment and upon receipt. These small, waterproof devices contain a computer chip that can be programmed to record temperature at different time intervals. The information is then downloaded from the temperature recorder onto a computer. Place the temperature recorder in a temperature sample, rather than loose in the cooler, or attached to the sample container. This option is appropriate for use with both filtered and bulk samples. *Example products:* Fisherbrand Exact-Temp Temperature Datalogger 15-059-201, Thermocron® iButtons, distributors at http://www.maxim-ic.com/products/ibutton/ and http://www.scigiene.com/.

8.1.4.4 **Stick-on temperature strips**—Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container (cubitainer or filter) upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but provides an

indication of sample temperature to verify that the sample temperature is acceptable. This option is appropriate for use with both filtered and bulk samples. *Example product:* Cole Parmer cat. no. U-90316-00.

8.1.4.5 **Infrared thermometers**—The thermometer is pointed at the sample, and measures the temperature without coming in contact with the sample volume. This option is appropriate for use with both filtered and bulk samples. *Example product*: Cole Parmer cat. no. EW-39641-00.

8.2 **Sample holding times**—Samples must be processed or examined within each of the holding times specified in Sections 8.2.1 through 8.2.4. Complete sample processing as soon as possible. The laboratory should complete sample filtration, elution, concentration, purification, and staining the day the sample is received whenever possible. However, the laboratory is permitted to split up the sample processing steps if processing a sample completely in one day is not possible. If this is necessary, sample processing can be halted after filtration, application of the purified sample onto the slide, or staining. **Table 5**, in Section 21.0 provides a breakdown of the holding times for each set of steps. Sections 8.2.1 through 8.2.4 provide descriptions of these holding times.

8.2.1 **Sample collection and filtration**—Sample elution must be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field).

8.2.2 **Sample elution, concentration, and purification**—The laboratory must complete elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) 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. This process ends with the application of the purified sample on the slide for drying.

8.2.3 **Staining**—The sample must be stained within 72 hours of application of the purified sample to the slide.

8.2.4 **Examination**—Although it is preferable to perform FA and DAPI and DIC microscopy examination and characterization immediately after staining is complete, laboratories have up to 168 hours (7 days) from the completion of sample staining to perform the examination and verification of samples. However, if fading/diffusion of FITC or DAPI staining is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.9.2) so that fading/diffusion does not occur.

8.3 **Spiking suspension enumeration holding times**—Flow cytometer-sorted spiking suspensions (Sections 7.12.1 and Appendix C) used for spiked QC samples (Section 9) must be used within the expiration date noted on the suspension. Oocyst and cyst suspensions must be stored between 1°C and 10°C or following manufacturer's instructions, until ready to use; do not allow to freeze.

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program that addresses and documents data quality, instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. General requirements and recommendations for QA and QC procedures for microbiology laboratories are provided in References 20.9 and 20.10. The minimum analytical requirements of this program consist of an IDC through performance of the initial precision and

recovery (IPR) test (Section 9.5), matrix spike/matrix spike duplicate (MS/MSD) test along with unspiked field sample (Section 9.6) and method blank (MB, Section 9.7), and ongoing demonstration of laboratory capability and method performance through the MS test along with unspiked field sample (Section 9.6.1), the MB test (Section 9.7), the ongoing precision and recovery (OPR) test (Section 9.8), staining controls (Sections 9.9, 14.1, and 15.2.1), and analyst verification tests (Section 9.10). A principal analyst (Section 22.2) verifies the quality and accuracy of all sample results. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method. Table 2 summarizes the minimum QC requirements.

9.2 Prior to first use of the method, or if a laboratory changes to another option for a method procedural component, the laboratory must demonstrate acceptable performance through an IDC (Table 2) which consists of acceptable performance in four IPR samples (Section 9.5), a MS/MSD (Section 9.6), an unspiked field sample, and one MB (Section 9.7). The laboratory is permitted to choose options for each procedural component as listed in Table 1.

NOTE: Only consider method procedural component changes to improve method performance, reduce cost, or reduce sample processing time. Method modifications that reduce cost or sample processing time, but that result in poorer method performance must not be used.

9.3 The laboratory is required to maintain records of modifications or procedural component changes made to this method. These records include the following, at a minimum:

9.3.1 The names, titles, addresses, and telephone numbers of the analyst(s) who performed the analyses and modification, and of the quality control officer who witnessed and will verify the analyses and modification.

9.3.2 A listing of the analyte(s) measured (*Cryptosporidium* and *Giardia*).

9.3.3 A narrative stating reason(s) for the modification.

9.3.4 Results from all QC tests comparing the modified method to this method, including: IPR (Section 9.5), MS/MSD (Section 9.6), and analysis of method blanks (Section 9.7).

9.3.5 Data that will allow an independent reviewer to validate each determination by tracing the following processing and analysis steps leading to the final result:

(a) Sample numbers and other identifiers
(b) Source of spiking suspensions, as well as lot number and date received (Section 7.12)
(c) Spike enumeration date and time
(d) Spiking suspension values (Section 11.0 and Appendix C)
(e) Sample spiking dates and times
(f) Volume filtered (Section 12.2.4.5 and 12.3.1.5.5)
(g) Filtration and elution dates and times (Section 12.2.3, 12.2.7.2, 12.3.1.4, 12.3.2.1)
(h) Pellet volume, resuspended concentrate volume, resuspended concentrate volume transferred to IMS, and all calculations required to verify the percent of concentrate examined (Section 13.2)
(i) Purification completion dates and times (Section 13.3.3.11)
(j) Staining completion dates and times (Section 14.10)

(k) Staining control results (Section 15.2.1)
(l) All required examination information (Sections 15.2.2 and 15.2.3)
(m) Examination completion dates and times (Section 15.2.4)
(n) Analysis sequence/run chronology
(o) Lot numbers of elution, IMS, and staining reagents
(p) Copies of bench sheets, logbooks, and other recordings of raw data
(q) Data system outputs, and other data to link the raw data to the results reported

9.4 **Microscope adjustment and calibration**—Adjust the microscope as specified in Section 10.0 and Appendix B. All of the requirements in Section 10.0 and Appendix B must be met prior to analysis of IPRs, method blanks, OPRs, field samples, and MS/MSDs.

9.5 **Initial precision and recovery (IPR)**—To establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery through an IDC or if equipment/supplies are changed, the laboratory must perform the following operations:

9.5.1 Using the spiking procedure in Section 11.2 and flow cytometer-enumerated spiking suspensions (Section 7.12.1), spike, filter, elute, concentrate, separate (purify), stain, and examine the four reagent water samples spiked with ~100-500 oocysts and ~100-500 cysts.

9.5.1.1 The laboratory is permitted to analyze the four spiked reagent samples on the same day or on as many as four different days (provided that the spiked reagent samples are analyzed consecutively), and also may use different analysts and/or reagent lots for each sample (however, the procedures used for all analyses must be identical). Note that the variability of four measurements performed on multiple days or using multiple analysts or reagent lots may be greater than the variability of measurements performed on the same day with the same analysts and reagent lots. As a result, the laboratory is at a greater risk of generating unacceptable IPR results if the test is performed across multiple days, analysts, and /or reagent lots.

9.5.1.2 If more than one option will be used for a procedural component, a separate set of IPR samples must be prepared for each option as part of an IDC.

9.5.1.3 The set of four IPR samples must be accompanied by analysis of an acceptable method blank (Section 9.7).

9.5.2 For each organism, calculate the percent recovery (R) using the following equation:

$$R = 100 \times \frac{N}{T}$$

where:

R = the percent recovery
N = the number of oocysts or cysts counted
T = the number of oocysts or cysts spiked

This calculation assumes that the total volume spiked was processed and examined.

9.5.3 Using percent recovery (R) of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries for *Cryptosporidium* and for *Giardia*. The RSD is the standard deviation divided by the mean, times 100.

9.5.4 Compare the mean and RSD to the corresponding method performance acceptance criteria for initial precision and recovery in Tables 3 and 4 in Section 21.0. If the mean and RSD for recovery meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may begin. If the mean or RSD falls outside the range for recovery, system performance is unacceptable. In this event, troubleshoot the problem by starting at the end of the method (see guidance in Section 9.8.7), correct the problem and repeat the IPR test (Section 9.5.1).

9.5.5 Examine and document the IPR slides following the procedure in Section 15.0. The first three *Cryptosporidium* oocysts and first three *Giardia* cysts identified in each IPR sample must be characterized (size, shape, DAPI category, and DIC category) and documented on the examination form, as well as any additional comments on organisms appearance, if notable.

9.5.6 Using 200X to 400X magnification, more than 50% of the oocysts or cysts must appear undamaged and morphologically intact; otherwise, the organisms in the spiking suspension may be of unacceptable quality or the analytical process may be damaging the organisms. If the quality of the organisms on the IPR test slides is unacceptable, examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the unprocessed organisms appear undamaged and morphologically intact under DIC, determine the step or reagent that is causing damage to the organisms. Correct the problem (see Section 9.8.7) and repeat the IPR test.

9.6 Matrix spike (MS) and matrix spike duplicate (MSD)

9.6.1 **MS—**The laboratory must spike a separate sample aliquot from the same source to determine the effect of the matrix on the method's oocyst and cyst recovery. The laboratory must analyze a MS sample when samples are first received from a PWS for which the laboratory has never before analyzed samples to identify potential method performance issues with the matrix (Tables 3 and 4). If an MS sample cannot be analyzed on the first sampling event, the first MS sample must be analyzed as soon as possible to identify potential method performance issues with the matrix. The laboratory must analyze MS samples at a minimum frequency of 1 MS sample per 20 field samples, or portions thereafter, for each individual source analyzed.

9.6.2 The MS and field sample must be collected from the same sampling location as split samples or as samples sequentially collected immediately after one another. The MS sample volume analyzed must be within 10% of the field sample volume. Every MS must be associated with an acceptable OPR and MB.

9.6.2.1 Analyze an unspiked field sample according to the procedures in Sections 12.0 to 15.0. Using the spiking procedure in Section 11.2 and flow cytometry-enumerated spiking suspensions (Section 7.12.1), spike, filter, elute, concentrate, separate (purify), stain, and examine a second field sample aliquot with a similar number of organisms as that used in the IPR or OPR tests (Sections 9.5 and 9.8).

9.6.2.2 For each organism, calculate the percent recovery (R) using the following equation.

$$R = 100 \times \frac{N_{sp} - N_s}{T}$$

where:

R is the percent recovery
N_{sp} is the number of oocysts or cysts counted in the spiked sample
N_s is the number of oocysts or cysts counted in the unspiked sample
T is the true value of the oocysts or cysts spiked

9.6.2.3 Compare the recovery for each organism with the acceptance criteria in Tables 3 and 4 in Section 21.0. Add results to the MS control chart (Section 9.12.2).

*NOTE: Some sample matrices may prevent the MS acceptance criteria in Tables 3 and 4 from being met. Close evaluation of mean MS/MSD recovery (Section 9.6.3) and RSD should be performed if the MS recovery is below acceptance criteria. Repeated failure to meet the MS quality control acceptance criteria, when all other QC passes, warrants corrective action from quality conscience laboratories to systematically review control charts and modify analytical protocols to improve matrix spike recoveries. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in **Table 6**.*

9.6.3 MSD analysis is required for: 1) an IDC; and 2) as part of multi-laboratory validation study using multiple matrices (Section 1.6) to demonstrate that the modified version of this method produces results equal or superior to results produced by the method as written. As noted above, an MSD is also performed as a corrective action when quality control criteria are not met. The laboratory spikes and analyzes another field sample aliquot (MSD) at the same time the laboratory spikes and analyzes the MS field sample aliquot (Section 9.6.2.1). The MS, MSD and field sample must be collected from the same sampling location as split samples or as samples sequentially collected immediately after one another. The MS and MSD sample volumes analyzed must be within 10% of the field sample volume.

9.6.3.1 For each organism, calculate the percent recovery (R) in the MSD using the equation in Section 9.6.2.2.

9.6.3.2 Calculate the mean of the number of oocysts or cysts in the MS and MSD as follows: $(X_{mean}) = ([MS+MSD]/2)$.

9.6.3.3 Calculate the relative percent difference (RPD) of the recoveries using the following equation:

$$RPD = 100 \times \frac{\left| N_{MS} - N_{MSD} \right|}{X_{MEAN}}$$

where

RPD is the relative percent difference
N_{MS} is the number of oocysts or cysts counted in the MS
N_{MSD} is the number of oocysts or cysts counted in the MSD
X_{MEAN} is the mean number of oocysts or cysts counted in the MS and MSD

9.6.3.4 Compare the mean MS/MSD recovery and RPD with the acceptance criteria in Tables 3 and 4 in Section 21.0 for each organism. Add results to the MS control chart (Section 9.12.2).

9.7 **Method blank (MB)**—Method blanks serve as the negative control sample, as well as the laboratory blank. Reagent water blanks are routinely analyzed to demonstrate the absence of contamination throughout the analytical process. Analyze the blank immediately after analysis of the IPR test (Section 9.5) and OPR test (Section 9.8) and prior to analysis of samples for the week to demonstrate freedom from contamination. A method blank must be analyzed each week (7 day or 168 hours time period that begins with processing the OPR) in which samples are analyzed if 20 or fewer field samples are analyzed during this period. If more than 20 samples are analyzed in a week (7 days or 168 hours), process and analyze one reagent water method blank for every 20 samples or portions thereafter.

9.7.1 Filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water method blank per week according to the procedures in Sections 12.0 to 15.0.

9.7.2 If *Cryptosporidium* oocysts, *Giardia* cysts, or potentially interfering organisms or materials that may be misidentified as oocysts or cysts are not found in the method blank, the method blank test is acceptable and analysis of samples may proceed.

9.7.3 If *Cryptosporidium* oocysts, *Giardia* cysts (as defined in Section 3), or any potentially interfering organism or materials that may be misidentified as oocysts or cysts are found in the method blank, the method blank test is unacceptable. Analysis of additional samples is halted until the source of contamination is eliminated, the method blank test is performed again, and no evidence of contamination is detected.

9.7.4 Every field sample and MS must be associated with an acceptable method blank.

9.8 **Ongoing precision and recovery (OPR)**—The OPR serves as the positive control sample, as well as the laboratory control sample. Using the spiking procedure in Section 11.2 and flow cytometry-enumerated spiking suspensions (Section 7.12.1), filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water sample spiked with ~100 to 500 oocysts and ~100 to 500 cysts each week samples are analyzed to verify all performance criteria. The laboratory must analyze one OPR sample for every 20 samples, or portions thereafter, if more than 20 samples are analyzed in a week. If multiple method variations are used, separate OPR samples must be prepared for each method variation. Adjustment and/or recalibration of the analytical system must be performed until all performance criteria are met.

9.8.1 Examine the slide from the OPR prior to analysis of samples from the same batch.

9.8.1.1 Using 200X to 400X magnification, more than 50% of the oocysts or cysts must appear undamaged and morphologically intact; otherwise, the organisms in the spiking suspension may be of unacceptable quality or the analytical process may be damaging the organisms. Examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the organisms appear undamaged and morphologically intact under DIC, determine the step or reagent that is causing damage to the organisms. Correct the problem and repeat the OPR test.

9.8.1.2 Identify and enumerate each organism using epifluorescence microscopy. The first three *Cryptosporidium* oocysts and three *Giardia* cysts identified in the OPR sample must be examined using FITC, DAPI, and DIC, as per Section 15.2, and the detailed characteristics (size, shape, DAPI category, and DIC category) reported on the *Cryptosporidium* and *Giardia* examination form, as well as any additional comments on organism appearance, if notable.

9.8.2 For each organism, calculate the percent recovery (R) using the following equation:

$$R = 100 \times \frac{N}{T}$$

where:

R = the percent recovery
N = the number of oocysts or cysts detected
T = the number of oocysts or cysts spiked

9.8.3 Compare the recovery with the acceptance criteria for ongoing precision and recovery in Tables 3 and 4 in Section 21.0 for each organism. Add results to the OPR control chart (Section 9.12.1).

9.8.4 If the recoveries for *Cryptosporidium* and *Giardia* meet the acceptance criteria, system performance is acceptable and analysis of samples may proceed.

9.8.5 If the recovery for *Cryptosporidium* or *Giardia* falls outside of the criteria, system performance is unacceptable. Analysis of additional samples must be halted until the analytical system is brought under control. Troubleshoot the problem using the procedures at Section 9.8.7 as a guide. After assessing the issue, perform another OPR test and verify that *Cryptosporidium* and *Giardia* recoveries meet the acceptance criteria.

9.8.6 Every field sample must be associated with a MS, an acceptable OPR and MB.

9.8.7 **Troubleshooting**—If an OPR sample has failed, and the cause of the failure is not known, then identify the problem by working backward in the analytical process from the microscopic examination to filtration.

9.8.7.1 **Quality of spiked organisms**—Examine the flow cytometry-enumerated spiking suspension organisms (Section 7.12.1) directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the organisms appear damaged under DIC, obtain fresh spiking materials. If the organisms appear undamaged and morphologically intact, determine whether the problem is associated with the microscope system or antibody stain (Section 9.8.7.2).

9.8.7.2 **Microscope system and antibody stain**—To determine if the failure of the OPR test is due to changes in the microscope or problems with the antibody stain, re-examine the positive staining control (Section 15.2.1), check Köhler illumination, and check the fluorescence of the fluorescein-labeled monoclonal antibodies and DAPI. If results are unacceptable, re-examine a previously-prepared positive staining control to determine whether the problem is associated with the microscope or the antibody stain.

9.8.7.3 **Separation (purification) system**—To determine if the failure of the OPR test is attributable to the separation system, check system performance by adding a flow cytometry-enumerated spiking suspension of ~100 oocysts and cysts (Section 7.12.1) along with the appropriate rinses (reagent water or elution buffer) to a flat-sided tube and processing the sample through the IMS, staining, and examination procedures in Sections 13.3 through 15.0.

If recoveries are less than 70%, further troubleshooting of the IMS system may be necessary.

9.8.7.4 **Filtration/elution/concentration system**—If the failure of the OPR test is attributable to the filtration/elution/concentration system, check system performance by processing spiked reagent water according to the procedures in Section 12.2 through 13.2.3, and stain the oocysts in suspension, filter through a 0.8 µm porosity, 13 mm diameter polycarbonate filter, and enumerate by microscopy.

9.9 **Staining controls—**These controls are performed to confirm appropriate performance of the immunofluorescent and DAPI staining reagents and the microscope. The controls also demonstrate proper staining technique and the absence of contamination through the staining process. The laboratory must prepare a positive and negative staining control (Section 14.1) each time samples are stained. Positive and negative staining controls must be acceptable before proceeding with sample examination.

9.9.1 Examine the negative staining control to confirm that it does not contain any fluorescent oocysts or cysts (Section 15.0). Indicate on each sample examination form whether the negative staining control was acceptable. If the negative staining control is acceptable, examination of samples may proceed.

9.9.2 Examine the positive staining control(s) to confirm that it contains oocysts and cysts with the appropriate fluorescence for FA and DAPI (Section 15.0). Indicate on each sample examination form whether the positive staining control(s) was acceptable. If the positive staining control is acceptable, examination of samples may proceed.

9.9.3 Each analyst must characterize a minimum of three *Cryptosporidium* oocysts and three *Giardia* cysts on the positive staining control slide before examining field sample slides. This characterization must be performed by each analyst at the beginning of each microscope examination session. FA examination must be conducted at a minimum of 200X total magnification, DAPI examination must be conducted at a minimum of 400X, and DIC examination and size measurements must be conducted at a minimum of 1000X. Size, shape, and DIC and DAPI characteristics of three *Cryptosporidium* oocysts and three *Giardia* cysts must be recorded by the analyst on a microscope log.

9.10 **Verification of analyst performance**—Until standard reference materials, such as National Institute of Standards and Technology standard reference materials, are available that contain a reliable number of DAPI positive or negative oocysts and cysts, this method relies upon the ability of the analyst for identification and enumeration of oocysts and cysts. The goal of analyst verification is to encourage comparison and discussion among analysts to continually refine the consistency of characterizations between analysts.

9.10.1 At least monthly when microscopic examinations are being performed, the laboratory must prepare or purchase a slide containing 40 to 200 oocysts and 40 to 200 cysts. More than 50% of the oocysts and cysts must be DAPI positive and undamaged under DIC.

9.10.2 Each analyst must determine the total number of oocysts and cysts detected by FITC on the entire slide meeting the criteria in Section 9.10.1. For the same 10 oocysts and 10 cysts, each analyst must determine the DAPI category (DAPI negative, DAPI positive internal intense blue and DAPI positive number of nuclei) and the DIC category (empty, containing amorphous structures, or containing identifiable internal structures)

of each. The DAPI/DIC comparisons may be performed on the slide prepared or purchased in Section 9.10.1, OPR slide, MS slide, or a positive staining control slide.

9.10.3 Requirements for laboratories with multiple analysts

9.10.3.1 The total number of oocysts and cysts determined by each analyst (Section 9.10.2) must be within ±10% of each other. If the number is not within this range, the analysts must identify the source of any variability between analysts' examination criteria, prepare a new slide, and repeat the performance verification (Sections 9.10.1 to 9.10.2). It is recommended that the DAPI and DIC categorization of the same 10 oocysts and 10 cysts occur with all analysts at the same time, i.e. each analyst determines the categorizations independently, then the differences in the DAPI and DIC categorizations among analysts are discussed and resolved, and these resolutions documented. Alternatively, organism coordinates may be recorded for each analyst to locate and categorize the organisms at different times. Differences among analysts must be discussed and resolved.

9.10.3.2 Document the date, name(s) of analyst(s), number of total oocysts and cysts, and DAPI and DIC categories determined by the analyst(s), whether the test was passed / failed and the results of attempts before the test was passed.

9.10.3.3 Only after an analyst has passed the criteria in Section 9.10.3 may oocysts and cysts in QC samples and field samples be identified and enumerated.

9.10.4 Laboratories with only one analyst should maintain a protozoa library (Section 9.11) and compare the results of the examinations performed in Sections 9.10.1 and 9.10.2 to photographs of oocysts and cysts and interfering organisms to verify that examination results are consistent with these references. This analyst must perform repetitive counts of a single verification slide for FITC demonstrating counts within ±10% of each other. Alternatively, laboratories may coordinate with other laboratories to share slides and compare counts or purchase blind spiked slides from vendor.

9.11 **Protozoa libraries**—Each laboratory is encouraged to develop libraries of photographs and drawings for identification of protozoa.

9.11.1 Take color photographs of *Cryptosporidium* oocysts and *Giardia* cysts by FA, DAPI, and DIC that the analysts (Section 22.2) determine are accurate (Section 15.2).

9.11.2 Similarly, take color photographs of interfering organisms and materials by FA, DAPI, and DIC that the analysts believe are not *Cryptosporidium* oocysts or *Giardia* cysts. Quantify the size, shape, microscope settings, and other characteristics that can be used to differentiate oocysts and cysts from interfering debris and that will result in accurate identification of positive or negative organisms.

9.12 **Control charts**-- The laboratory must maintain graphic records to define quality of OPR and MS data generated over time. Control Charts confirm high and improving performance but also alert laboratories of trends toward failing quality control acceptance criteria limits and the potential for sample analysis failures when performance degrades. Failure to meet OPR quality control acceptance criteria (Tables 3 and 4) indicates systemic problems the laboratory must address prior to processing any samples. Repeated failure to meet the MS/MSD quality control acceptance criteria (Tables 3 and 4), when all other QC passes, warrants corrective action from quality

conscience laboratories to systematically review control charts and modify analytical protocols to improve matrix spike recoveries.

9.12.1 As part of the QA program for the laboratory, laboratory precision must be assessed, records maintained, and typical control charting procedures followed. The laboratory must add OPR results to initial and/or previous ongoing data and update the QC chart to form a graphic representation of continued laboratory performance. The laboratory should develop a statement of accuracy by calculating the mean percent recovery (R) and the standard deviation of percent recovery (s_r). Express the accuracy as a recovery interval from R - 2 s_r to R + 2 s_r using the most recent 20 - 30 data points. For example, if R = 95% and s_r = 25%, the accuracy is 45% to 145%.

9.12.2 As part of the QA program for the laboratory, method accuracy and precision for MS samples must be assessed and records maintained.

9.12.2.1 After the analysis of five MS samples, the laboratory must calculate the mean percent recovery (P) and the standard deviation of the percent recovery (s_r). The precision assessment should be updated regularly (e.g., monthly, after each sample, etc.) across all MS samples and stratified by MS samples for each source.

9.12.2.2 In addition the laboratory must develop a frequency distribution of MS recoveries as a table or graph (histogram) using intervals of 10%. The laboratory must chart their distribution of MS recoveries compared with Table 6. Overall MS recoveries are anticipated to follow a similar pattern as the frequency distribution of recoveries shown in Table 6.

9.13 The laboratory may routinely process an IMS control (e.g. each lot of beads, each proficiency testing round) to monitor the recovery of the separation (purification) system (Section 9.8.7.3). IMS control results may be added to the QC chart (Section 9.12.1) to monitor laboratory performance.

9.14 External QC samples, such as proficiency testing or standard reference material are to be analyzed when available. Laboratory participation in interlaboratory comparison studies using the method is encouraged to permit laboratories to gain, maintain and demonstrate proficiency with the method.

9.15 The specifications contained in this method can be met if the analytical system is under control. Use of identical standards for initial (Section 9.5) and ongoing (Section 9.8) precision and recovery samples will permit the most precise results to be obtained. The microscope in particular will provide the most reproducible results if dedicated to the settings and conditions required for the determination of *Cryptosporidium* and *Giardia* by this method.

9.16 Depending on specific program requirements, field replicates may be collected to determine the precision of the sampling technique, and duplicate spiked samples may be required to determine the precision of the analysis.

10.0 Microscope Calibration

10.1 In a room capable of being darkened to near-complete darkness, assemble the microscope, all filters, and attachments. Place the microscope on a solid surface free from vibration. Provide adequate workspace on either side of the microscope for taking notes and placement of slides and ancillary materials.

10.2 Using the manuals provided with the microscope, all analysts must familiarize themselves with operation of the microscope. Write SOPs for the specific microscopes in use and make these available to analysts.

10.3 The microscopy portion of this procedure depends upon proper alignment and adjustment of very sophisticated optics. Without proper alignment and adjustment, the microscope will not function at maximal efficiency, and reliable identification and enumeration of oocysts and cysts will not be possible. Consequently, it is imperative that all portions of the microscope from the light sources to the oculars must be properly adjusted.

10.4 While microscopes from various vendors are configured somewhat differently, they all operate on the same general physical principles. Therefore, slight deviations or adjustments may be required to make the procedures below and Appendix B work for a particular instrument.

10.5 The sections below and Appendix B assume that the mercury bulb has not exceeded time limits of operation, that the lamp socket is connected to the lamp house, and that the condenser is adjusted to produce Köhler illumination.

10.6 When using the microscope, corrective lenses must be worn by people with astigmatism.

WARNING: Never look at the ultraviolet (UV) light from the mercury lamp, lamp house, or the UV image without a barrier filter in place. UV radiation can cause serious eye damage.

10.7 **Adjustment of the interpupillary distance and oculars for each eye**—These adjustments are necessary so that eye strain is reduced to a minimum, and must be made for each individual using the microscope. Section 10.7.2 assumes use of a microscope with both oculars adjustable; Section 10.7.3 assumes use of a microscope with a single adjustable ocular. The procedure must be followed each time an analyst uses the microscope.

10.7.1 Interpupillary distance

10.7.1.1 Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

10.7.1.2 Using both hands, move the oculars closer together or farther apart until a single circle of light is observed while looking through the oculars with both eyes. Note interpupillary distance.

10.7.2 Ocular adjustment for microscopes capable of viewing a photographic frame through the viewing binoculars: This procedure assumes both oculars are adjustable.

10.7.2.1 Place a card between the right ocular and eye keeping both eyes open. Adjust the correction (focusing) collar on the left ocular by focusing the left ocular until it reads the same as the interpupillary distance. Bring an image located in the center of the field of view into as sharp a focus as possible.

10.7.2.2 Transfer the card to between the left eye and ocular. Again keeping both eyes open, bring the same image into as sharp a focus for the right eye as possible by adjusting the ocular correction (focusing) collar at the top of the right ocular.

10.7.3 Ocular adjustment for microscopes without binocular capability: This procedure assumes a single focusing ocular. The following procedure assumes that only the right ocular is capable of adjustment.

10.7.3.1 Place a card between the right ocular and eye keeping both eyes open. Using the fine adjustment, focus the image for the left eye to its sharpest point.

10.7.3.2 Transfer the card to between the left eye and ocular. Keeping both eyes open, bring the image for the right eye into sharp focus by adjusting the ocular collar at the top of the ocular without touching the coarse or fine adjustment.

10.8 **Köhler illumination**—This section assumes that Köhler illumination will be established for only the 100X oil DIC objective that will be used to identify internal morphological characteristics in *Cryptosporidium* oocysts and *Giardia* cysts. If more than one objective is to be used for DIC, then each time the objective is changed, Köhler illumination should be reestablished for the new objective lens. Previous sections addressed ocular adjustment and Appendix B addressed adjusting light sources. This section aligns and focuses the light going through the condenser underneath the stage at the specimen to be observed. If Köhler illumination is not properly established, then DIC will not work to its maximal potential. These steps need to become second nature and must be practiced regularly until they are a matter of reflex rather than a chore. The procedure must be followed each time an analyst uses the microscope.

10.8.1 Place a prepared slide on the microscope stage, place oil on the slide, move the 100X oil objective into place, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

10.8.2 At this point both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Now close down the radiant field diaphragm in the microscope base until the lighted field is reduced to a small opening.

10.8.3 Using the condenser centering screws on the front right and left of the condenser, move the small lighted portion of the field to the center of the visual field.

10.8.4 Now look to see whether the leaves of the iris field diaphragm are sharply defined (focused) or not. If they are not sharply defined, then they can be focused distinctly by changing the height of the condenser up and down with the condenser focusing knob while you are looking through the binoculars. Once you have accomplished the precise focusing of the radiant field diaphragm leaves, open the radiant field diaphragm until the leaves just disappear from view.

10.8.5 The aperture diaphragm of the condenser should now be adjusted to make it compatible with the total numerical aperture of the optical system. This is done by removing an ocular, looking into the tube at the rear focal plane of the objective, and stopping down the aperture diaphragm iris leaves until they are visible just inside the rear plane of the objective.

10.8.6 After completing the adjustment of the aperture diaphragm in the condenser, return the ocular to its tube and proceed with the adjustments required to establish DIC.

10.9 **Microscope cleaning procedure**

10.9.1 Use canned air to remove dust from the lenses, filters, and microscope body.

10.9.2 Use a lint-free tissue dampened with a microscope cleaning solution (MCS) (consisting of 2 parts 90% isoproponal and 1 part acetone) to wipe down all surfaces of the microscope body. Dry off with a clean, dry lint-free tissue.

10.9.3 Protocol for cleaning oculars and condenser

10.9.3.1 Use a new, clean, dust-free cotton-tipped stick dampened with MCS to clean each lens. Start at the center of the lens and spiral the cotton tip outward using little to no pressure. Rotate the cotton tip while spiraling to ensure a clean surface is always contacting the lens.

10.9.3.2 Repeat the procedure using a new, dry cotton-tipped stick.

10.9.3.3 Repeat Sections 10.9.3.1 and 10.9.3.2.

10.9.3.4 Remove the ocular and repeat the cleaning procedure on the bottom lens of the ocular.

10.9.4 Protocol for cleaning objective lenses

10.9.4.1 Wipe 100X oil objective with lens paper to remove the bulk of the oil from the objective.

10.9.4.2 Hold a new clean, dust-free cotton-tipped stick dampened with MCS at a 45° angle on the objective and twirl.

10.9.4.3 Repeat Section 10.9.4.2 with a new, dry cotton-tipped stick.

10.9.4.4 Repeat Sections 10.9.4.2 and 10.9.4.3.

10.9.4.5 Clean all objectives whether they are used or not.

10.9.5 Protocol for cleaning light source lens and filters

10.9.5.1 Using lens paper dampened with MCS, wipe off the surface of each lens and filter.

10.9.5.2 Repeat the procedure using dry lens paper.

10.9.5.3 Repeat Sections 10.9.5.1 and 10.9.5.2.

10.9.6 Protocol for cleaning microscope stage

10.9.6.1 Using a lint-free tissue dampened with microscope cleaning solution, wipe off the stage and stage clip.

10.9.6.2 Be sure to clean off any residual immersion oil or fingernail polish. Remove the stage clip if necessary to ensure that it is thoroughly cleaned.

10.9.7 Use commercial multi-surface cleaner and a paper towel to clean the bench top surrounding the microscope.

10.9.8 Frequency: Clean microscope after every session

11.0 Sample Spiking

11.1 This method requires routine analysis of spiked QC samples to demonstrate acceptable initial and ongoing laboratory and method performance (IPR samples [Section 9.5], MS/MSD [Section 9.6], OPR samples [Section 9.8], and IMS controls [Section 9.13]). The organisms used for these samples must be enumerated to calculate recoveries and precision, and monitor method

performance. Laboratories must use flow cytometry-enumerated spiking suspensions, rather than manually enumerated suspensions. Guidance on preparing spiking suspensions using a flow cytometer is provided in Appendix C. The procedure for spiking bulk samples in the laboratory is provided in Section 11.2.

11.2 **Procedure for spiking samples in the laboratory with flow cytometry-enumerated spiking suspensions**—Three optional spiking suspensions have been demonstrated to be acceptable for use with Method 1623.1: 1) Wisconsin State Laboratory of Hygiene, 2) BTF EasySeed™, and 3) Waterborne AccuSpike™-IR.

11.2.1 Arrange a disposable cubitainer or bottom-dispensing container with a spigot to feed the filter or insert a pipette connected to the influent end of the tube attached to the filter through the top of a carboy to allow siphoning of the sample.

11.2.2 For initial precision and recovery (Section 9.5) and ongoing precision and recovery (Section 9.8) samples, fill the container with 10 L of reagent water or a volume of reagent water equal to the volume of the field samples analyzed in the analytical batch. For MS samples (Section 9.6), fill the container with the field sample to be spiked. Continuously mix the sample (using a stir bar and stir plate for smaller-volume samples and alternate means for larger-volume samples).

11.2.3 Follow the procedures in Section 11.2.3.1 or manufacturer's instructions for flow cytometer–enumerated suspensions. Adjust volume of antifoam and rinses in proportion to volume of spiking suspension; instructions below assume ~45 mL of spiking suspension in 50-mL tube.

11.2.3.1 Add 400 µL of Antifoam A to 100 mL of reagent water, and mix well to emulsify.

11.2.3.2 Add 500 µL of the diluted antifoam to the tube containing the spiking suspension and vortex for 30 seconds.

11.2.3.3 Pour the suspension into the sample container.

11.2.3.4 Add 20 mL of reagent water to the empty tube, cap, vortex 10 seconds to rinse, and add the rinsate to the carboy.

11.2.3.5 Repeat this rinse using another 20 mL of reagent water.

11.2.3.6 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet.

11.2.4 Allow the spiked sample to mix for approximately 1 minute in the container.

11.2.5 Turn on the pump and allow the flow rate to stabilize. Set flow at the rate designated for the filter being used. As the carboy is depleted, check the flow rate and adjust if necessary.

11.2.6 When the water level approaches the discharge port of the carboy, tilt the container so that it is completely emptied. At that time, turn off the pump and add 1 L of reagent water to the 10-L carboy to rinse (5 L of reagent water rinse to 50-L carboy). Swirl the contents to rinse down the sides. Additional rinses may be performed.

11.2.7 Turn on the pump. Allow all of the water to flow through the filter and turn off the pump.

11.2.8 Proceed to filter disassembly.

12.0 Sample Filtration and Elution

NOTE: Sample elution must be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field).

12.1 **Three optional filtration/elution procedures have been demonstrated to be acceptable for use with Method 1623.1—**1) Envirochek® HV Sampling Capsule (Section 12.2), 2) Filta-Max® Foam Filter (Section 12.3), and 3) Portable Continuous Flow Centrifuge (PCFC, Section 12.4) (Table 1). Laboratories may use a different optional procedure if the laboratory first demonstrates that the optional procedure provides equivalent or superior performance per Section 9.2. Alternate procedures and products may be added to the optional procedures in Table 1 only after demonstrating equivalent or superior performance through a multi-laboratory validation study using multiple matrices, as per Reference 20.1.

12.2 **Filtration/Elution Option 1—**Capsule filtration using the Envirochek® HV (adapted from Reference 20.11). This procedure was validated using 50-L sample volumes and historically with 10-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates equivalent or superior performance through an IDC per Section 9.2.

12.2.1 Flow rate adjustment

12.2.1.1 Connect the sampling system, minus the capsule, to a carboy filled with reagent water (Figure 1).

12.2.1.2 Turn on the pump and adjust the flow rate to 2.0 L/min.

12.2.1.3 Allow 2 to 10 L of reagent 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.

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

12.2.3 Record the sample number, sample turbidity (if not provided with the field sample), sample type, sample filtration start date and time, and name of analyst filtering the sample on a bench sheet.

12.2.4 Filtration

12.2.4.1 Mix the sample well by shaking, add stir bar and place on stir plate. Turn on stir plate to lowest setting needed to keep sample thoroughly mixed. Connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.2.1.1. If the sample will be filtered from a field carboy, a spigot (Section 6.2.1) can be used with the carboy to facilitate sample filtration.

NOTE: If the bulk field sample is transferred to a laboratory carboy, the laboratory carboy must be cleaned and disinfected before it is used with another field sample.

12.2.4.2 Place the drain end of the sampling system tubing into an empty graduated container with a capacity of 10 to 15 L, calibrated at 9.0, 9.5, 10.0, 10.5, and 11.0 L (Section 6.16). This container will be used to determine the

sample volume filtered. Alternately, determine the volume by weight or connect a flow meter (Section 6.2.5) downstream of the filter, and record the initial meter reading.

12.2.4.3 Allow the carboy discharge tube and capsule to fill with sample water by gravity. 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.

12.2.4.4 After the entire sample has passed through the filter, turn off the pump and stir plate. Allow the pressure to decrease until flow stops. (If the sample was filtered in the field, and excess sample remains in the filter capsule upon receipt in the laboratory, pull the remaining sample volume through the filter before eluting the filter [Section 12.2.6].)

12.2.4.5 Based on the water level in the graduated container and ½-L hash marks, the volume determined by weight, or meter reading, record the volume filtered on the bench sheet to the nearest quarter liter. Discard the contents of the graduated container.

12.2.4.6 Add 1 L reagent water rinse (to 10-L carboy) or 5 L reagent water rinse (to 50-L carboy). Swirl or shake the carboy to rinse down the side walls.

12.2.4.7 Reconnect to pump, turn on pump and allow pump to pull all water through filter; turn off pump.

12.2.5 Disassembly

12.2.5.1 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 and cysts from the filter. If needed, restart the pump and allow as much water to drain as possible. Turn off the pump.

12.2.5.2 Loosen the outlet fitting, then cap the inlet and outlet fittings.

12.2.6 Elution Setup

NOTE: The laboratory must complete the elution, concentration, and purification (Sections 12.2.7 through 13.3.3.11) 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. This process ends with the application of the purified sample on the slide for drying.

12.2.6.1 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.

12.2.6.2 Prepare sufficient quantity of elution buffer to elute all samples that are associated with the OPR/MB which used that batch of elution buffer. Elution may require up to 275 mL of buffer per sample.

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

12.2.6.4 Prepare sufficient quantity of 5% sodium hexametaphosphate (NaHMP) solution to pre-treat all of the designated filters associated with the OPR/MB used for that batch of elution buffer. Pre-treatment may require up to 150 mL of NaHMP per sample.

12.2.7 Dispersant Addition

12.2.7.1 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). Record on the bench sheet that the sampling capsule is clogged, and proceed to Section 12.2.8.2.

12.2.7.2 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.

12.2.7.3 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.

12.2.7.4 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.

12.2.7.5 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.

12.2.7.6 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.

12.2.8 Elution

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

12.2.8.2 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.

12.2.8.3 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.

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

12.2.8.5 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.

12.2.8.6 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.

12.2.8.7 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.

12.2.8.8 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.

12.2.9 Proceed to Section 13.0 for concentration and separation (purification).

12.3 **Filtration/Elution Option 2—**Sample filtration using the Filta-Max® foam filter. This procedure was validated using 50-L sample volumes and historically with 10-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates equivalent or superior performance through an IDC per Section 9.2.

NOTE: The filtration procedures specified in Sections 12.3.1.2 - 12.3.1.6.2 are specific to laboratory filtration of a bulk sample. These procedures may require modification if samples will be filtered in the field.

12.3.1 Filtration Preparation

12.3.1.1 Flow rate adjustment

12.3.1.1.1 Connect the sampling system, minus the filter housing, to a carboy filled with reagent water (Figure 2).

12.3.1.1.2 Place the peristaltic pump upstream of the filter housing.

12.3.1.1.3 Turn on the pump and adjust the flow rate to 1 to 4 L per minute.

NOTE: A head pressure of 0.5 bar (7.5 psi) is required to create flow through the filter, and the recommended pressure of 5 bar (75 psi) should produce the flow rate of 3 to 4 L per minute. The maximum operating pressure of 8 bar (120 psi) should not be exceeded.

12.3.1.1.4 Allow 2 to 10 L of reagent water to flush the system. Adjust the pump speed as necessary during this period. Turn off the pump when the flow rate has been adjusted.

12.3.1.2 Place filter module into the filter housing bolt head down and secure lid, hand tighten housings, apply gentle pressure to create the seal between the module and the 'O' rings in the base and the lid of the housing. Excessive tightening is not necessary, and may shorten the life of the 'O' rings. Tools may be used to tighten housing to the alignment marks (refer to manufacturer's instructions). Lightly grease the 'O' rings before use (refer to manufacturer's instructions).

12.3.1.3 Install the filter housing in the line, securing the inlet and outlet ends with the appropriate clamps/fittings. Verify that the filter housing is installed so that the end closest to the screw top cap is the inlet and the opposite end is the outlet.

12.3.1.4 Record the sample number, sample turbidity (if not provided with the field sample), sample type, sample filtration start date and time, and the name of the analyst filtering the sample on a bench sheet.

12.3.1.5 Filtration

NOTE: If the bulk field sample is transferred to a laboratory carboy, the laboratory carboy must be cleaned and disinfected before it is used with another field sample.

12.3.1.5.1 Mix the sample well by shaking, add stir bar and place on stir plate. Turn on stir plate to lowest setting needed to keep sample thoroughly mixed. Connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.3.1.1.1. If the sample will be filtered from a field carboy, a spigot can be used with the carboy to facilitate sample filtration.

12.3.1.5.2 Place the drain end of the sampling system tubing into an empty graduated container with a capacity greater than or equal to the volume to be filtered. This container will be used to determine the sample volume filtered. Alternately, connect a flow meter downstream of the filter, and record the initial meter reading.

12.3.1.5.3 Allow the carboy discharge tube and filter housing to fill with sample water. Turn on the pump to start water flowing through the filter. Verify that the flow rate is between 1 and 4 L per min.

12.3.1.5.4 After the entire sample has passed through the filter, turn off the pump and stir plate. Allow the pressure to decrease until flow stops.

12.3.1.5.5 Based on the water level in the graduated container and ½ -L marks, the volume determined by weight, or the meter reading, record the volume filtered on a bench sheet to the nearest quarter liter. Discard the contents of the graduated container.

12.3.1.5.6 Add 1 L reagent water rinse (to 10-L carboy) or 5 L reagent water to rinse (to 50-L carboy). Swirl or shake the carboy to rinse down the side walls.

12.3.1.5.7 Reconnect to pump, turn on pump and allow pump to pull all water through filter; turn off pump.

12.3.1.6 Disassembly

12.3.1.6.1 Disconnect the inlet end of the filter housing assembly while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the loss of oocysts and cysts from the filter. If needed, restart the pump and allow as much water to drain as possible. Turn off the pump.

12.3.1.6.2 Loosen the outlet fitting and seal the filter housing with rubber plugs.

NOTE: Prevent filters from drying out, as this can impair their ability to expand when decompressed.

12.3.2 Elution

12.3.2.1 The filter is eluted to wash the oocysts from the filter using the Filta-Max® wash station (manual or automatic), which moves a plunger up and down a tube containing the filter and eluting solution (Section 12.3.2.2). If the Filta-Max® automatic wash station is used please see the manufacturer's operator's guide for instructions on its use. If Filta-Max® Quick Connect kit is used please follow manufacturer's instructions. Record the elution date and time on the bench sheet.

12.3.2.2 Filta-Max® wash station elution procedure

12.3.2.2.1 First wash

(a) Detach the removable plunger head using the tool provided, and remove the splash guard.

(b) Place the filter membrane flat in the concentrator base with the rough side up. Locate the concentrator base in the jaws of the wash station and screw on the concentrator tube (the longer of the two tubes), creating a tight seal at the membrane. Take the assembled concentrator out of the jaws and place on the bench.

(c) Replace the splash guard and temporarily secure it at least 15 cm above the end of the rack. Secure the plunger head with the tool provided ensuring that the lever is fully locked down.

(d) Remove the filter module from the filter housing or transportation container. Pour excess liquid into the assembled concentrator, then rinse the housing or container with PBST and add the rinse to the concentrator tube. Screw the filter module onto the base of the plunger. Locate the elution tube base in the jaws of the wash station and screw the elution tube (the shorter of the two tubes) firmly in place.

(e) Pull the plunger down until the filter module sits at the bottom of the elution tube; the locking pin (at the top left of the wash station) will "click" when the plunger is correctly locked in position.

(f) Remove the filter module bolt by turning the adapted allen key (provided) in a clockwise direction (as seen from above). Attach the steel tube to the elution tube base.

(g) Add 600 mL of PBST to the assembled concentrator. If more than 50 mL of liquid has been recovered from the shipped filter module, reduce the volume of PBST accordingly. Screw the concentrator tube onto the base beneath the elution tube. Release the locking pin.

NOTE: When functioning correctly, the pin is easily released by gentle pressure on the lever, coupled with a pulling action on the locking pin.

(h) Wash the foam disks by moving the plunger up and down 20 times. Gentle movements of the plunger are recommended to avoid generating excess foam.

NOTE: The plunger has an upper movement limit during the wash process to prevent it popping out of the top of the chamber.

(i) Detach the concentrator and hold it such that the stainless steel tube is just above the level of the liquid. Purge the remaining liquid from the elution tube by moving the plunger up and down 5 times, then lock the plunger in place. To prevent drips, place the plug provided in the end of the steel tube.

(j) Prior to the second wash, concentrate the eluate from the first wash using the Filta-Max® apparatus according to Section 12.3.3.2.1.

12.3.2.2.2 Second wash

(a) Add an additional 600 mL of PBST to the concentrator module, remove the plug from the end of the steel tube and screw the concentrator tube back onto the elution module base. Release the locking pin.

(b) Wash the foam disks by moving the plunger up and down 10 times. Gentle movements of the plunger are recommended to avoid generating excess foam.

(c) Concentrate the eluate using the Filta-Max® apparatus according to Section 12.3.3.2.2.

12.3.3 Concentration

12.3.3.1 The eluate is concentrated using the Filta-Max® concentrator apparatus, which pulls most of the eluate through a membrane filter leaving the oocysts concentrated in a small volume of the remaining eluting solution.

12.3.3.2 The Filta-Max® concentrator procedure

12.3.3.2.1 Concentration of first wash

(a) Stand the concentrator tube on a magnetic stirring plate and attach the lid (with magnetic stirrer bar). Connect the waste bottle trap and hand or electric vacuum pump to the valve on the concentrator base. Begin stirring and open the tap. Increase the vacuum using the hand pump.

NOTE: The force of the vacuum should not exceed 30 cm Hg (11.8 in Hg).

(b) Allow the liquid to drain until it is approximately level with the middle of the stirrer bar then close the valve. Remove the magnetic stirrer, and rinse it with PBST or reagent water to recover all oocysts. Decant the concentrate into a 50-mL tube, then rinse the sides of the concentration tube and add the rinsate to the 50-mL tube.

12.3.3.2.2 Concentration of second wash

(a) Add the concentrate, in the 50-mL tube, retained from the first concentration (Section 12.3.3.2.1 (b)) to the 600 mL of eluate from the second wash, then repeat concentration steps in Section 12.3.3.2.1. The final sample can be poured into the same 50-mL tube used to retain the first concentrate. Rinse the sides of the concentrator tube with PBST and add the rinse to the 50-mL tube.

(b) Remove the magnetic stirrer. Insert the empty concentrator module into the jaws of the wash station and twist off the concentrator tube.

(c) Transfer the membrane from the concentrator base to the bag provided using membrane forceps.

12.3.3.2.3 Membrane elution

(a) Add 5 mL of PBST to the bag containing the membrane. Rub the surface of the membrane through the bag until the membrane appears clean.

(b) Using a pipette, transfer the eluate to a 50-mL tube

(c) Repeat the membrane wash with another 5 mL of PBST and transfer the eluate to the 50-mL tube. (Optional: Perform a third wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

NOTE: Mark the bag with an "X" to note which side of the membrane has the oocysts to encourage the hand-kneading to focus on the appropriate side of the membrane.

12.3.3.2.4 If the membrane filter clogs before concentration is complete, replace the membrane as often as necessary. Filter membranes may be placed smooth side up during the second concentration step. Separate 50-mL tubes may be used for the eluate of each membrane or the eluate from multiple membranes may be combined into one 50-mL tube.

(a) Disassemble the concentrator tube and pour any remaining eluate back into a pooling beaker. Remove the membrane using membrane forceps, placing it in the bag provided. Place a new membrane in the concentrator tube smooth side up, reassemble, return the eluate to the concentrator tube, rinse the pooling beaker and add rinse to the eluate, and continue the concentration. Replace the membrane as often as necessary.

12.3.3.2.5 If multiple 50-mL tubes have been used, the sample can be further concentrated by centrifugation or each 50-mL tube may be processed separately following Section 13.0.

(a) Make sure that the centrifuge tubes are balanced. Centrifuge the tubes containing the eluate at 1500 × G for 15 minutes. Allow the centrifuge to coast to a stop.

(b) Using a Pasteur pipette, carefully aspirate off the supernatant to 5 mL above the pellet. If the sample is reagent water (e.g., initial or ongoing precision and recovery sample) extra care must be taken to avoid aspirating oocysts and cysts during this step.

(c) Vortex each 50-mL tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Combine the contents of each 50-mL centrifuge tube into one 50-mL

centrifuge tube. Rinse each of the 50-mL centrifuge tubes with PBST and add the rinse to the final 50-mL centrifuge tube.

12.3.3.3 Proceed to Section 13.0 for centrifugation and separation (purification) of the eluate.

12.3.4 Maintenance and cleaning

12.3.4.1 Maintenance of O-rings

12.3.4.1.1 Check all rubber O-rings for wear or deterioration prior to each use and replace as necessary.

12.3.4.1.2 Lubricate the plunger head O-ring inside and out with silicon before each use.

12.3.4.1.3 Lubricate all other O-rings (concentrator tube set, filter housing) regularly in order to preserve their condition.

12.3.4.2 Cleaning

12.3.4.2.1 All components of the Filta-Max® system can be cleaned using warm water and laboratory detergent. After washing, rinse all components with oocyst and cyst free reagent water and dry them. Re-lubricate all O-rings. Alternatively a mild (40°C) dishwasher cycle without bleach or rinse aid can be used.

12.3.4.2.2 To wash the detachable plunger head slide the locking pin out and wash the plunger head and locking pin in warm water and laboratory detergent. Rinse the plunger head and locking pin with oocyst and cyst free reagent water and dry. Lightly lubricate the locking pin and reassemble the plunger head.

12.4 **Filtration/Elution Option 3—**Sample collection (filtration and concentration) using portable continuous-flow centrifugation. Please follow manufacturer's instructions. This procedure was validated for the detection of *Cryptosporidium* using 50-L sample volumes and historically for the detection of *Cryptosporidium* and *Giardia* using 10-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates equivalent or superior performance through IDC per Section 9.2.

13.0 Sample Concentration and Separation (Purification)

13.1 During concentration and separation, the filter eluate is concentrated through centrifugation, and the oocysts and cysts in the sample are separated from other particulates through IMS. Optional procedures listed in Table 1 may be used if the laboratory first demonstrates that the optional procedure provides equivalent or superior performance per Section 9.2. Alternate procedures and products may be added to the optional procedures in Table 1 only after demonstrating equivalent or superior performance through a multi-laboratory validation study using multiple matrices, as per Reference 20.1.

13.2 Adjustment of pellet volume

13.2.1 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.

NOTE: Historical demonstration at multiple laboratories indicates that recoveries may be improved if centrifugation force is increased to 1800-2000 × G. However, do not use this higher force if the sample contains sand or other gritty material that may degrade the condition of any oocysts and/or cysts in the sample.

13.2.2 **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 (e.g., initial or ongoing precision and recovery sample).

NOTE: Recoveries may be improved if aspiration is performed using a Pasteur pipette or serological pipette with an internal diameter of orifice ranging from 1.2 to 1.5 mm.

13.2.2.1 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, volume transferred to IMS, and the number of any subsamples on the bench sheet. Proceed to Section 13.3. 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.

NOTE: Care must be taken with samples containing sand or other gritty material when vortexing to ensure that the condition of any oocysts and/or cysts in the sample is not compromised.

13.2.3 **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. Use the following formula to determine the total volume required in the centrifuge tube before separating the concentrate into two or more subsamples, rounding the result up to the nearest multiple of 5:

$$\text{total volume (mL) required} = \frac{\text{pellet volume}}{0.5\ \text{mL}} \times 5\ \text{mL}$$

Record total volume in centrifuge tube, which includes the pellet and supernatant, on the bench sheet.

NOTE: Care must be taken with samples containing sand or other gritty material when vortexing to ensure that the condition of any oocysts in the sample is not compromised.

13.2.3.1 **Analysis of entire sample.** If analysis of the entire sample is required, determine the number of subsamples to be processed independently through the remainder of the method:

13.2.3.1.1 **Calculate number of subsamples:** Divide the total volume in the centrifuge tube by 5 mL. Record the number of subsamples on the bench sheet.

13.2.3.1.2 **Process subsamples through IMS.** Vortex the tube vigorously for 10 to 15 seconds and/or pipette mix to completely resuspend the pellet. Proceed immediately to Section 13.3, and transfer aliquots of the resuspended concentrate equivalent to the volume in the previous step to multiple, flat-sided sample tubes in Section 13.3.2.1. Process the sample as multiple, independent subsamples from Section 13.3 onward, including the preparation and examination of separate slides for each aliquot. Record the volume of resuspended concentrate transferred to all flat-sided tubes on the bench sheet. 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.

13.2.3.2 **Analysis of partial sample.** If not all of the concentrate will be examined, vortex the tube vigorously for 10 to 15 seconds and/or pipette mix to completely resuspend the pellet. Proceed immediately to Section 13.3, and transfer one or more 5-mL aliquots of the resuspended concentrate to one or more flat-sided sample tubes in Section 13.3.2.1. Record the volume of resuspended concentrate transferred to all flat-sided tubes and the number of subsamples on the bench sheet. 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. To determine the volume analyzed, calculate the percent of the concentrate examined using the following formula:

$$\text{percent examined} = \frac{\text{total volume of resuspended concentrate transferred to IMS}}{\text{total volume of resuspended concentrate in Section 13.2.3}} \times 100\%$$

Then multiply the volume filtered (Section 12.2.4.5 or 12.3.1.5.5) by this percentage to determine the volume analyzed.

13.3 IMS procedure (adapted from Reference 20.12)

NOTE: The IMS procedure should be performed with samples and IMS buffers at room temperature, ranging from 15°C to 25°C.

13.3.1 Preparation and addition of reagents

13.3.1.1 Prepare a 1X dilution of SL-buffer-A from the 10X SL-buffer-A (clear, colorless solution) supplied. Use reagent water (demineralized; Section 7.3) as the diluent. For every 1 mL of 1X SL-buffer-A required, mix 100 µL of 10X SL-buffer-A and 0.9 mL diluent water. A volume of 1.5 mL of 1X SL-buffer-A will be required per sample or subsample on which the Dynal IMS procedure is performed.

13.3.1.2 For each sample or subsample (Section 13.2) to be processed through IMS, add 1 mL of the 10X SL-buffer-A (supplied—not the diluted 1X SL-buffer-A) to a flat-sided tube (Section 6.3.4).

13.3.1.3 For each sample or subsample, add 1 mL of the 10X SL-buffer-B (supplied— magenta solution) to the flat-sided tube containing the 10X SL-buffer-A.

NOTE: The volumes of IMS reagents listed above are a requirement for this method. Changes in reagent concentrations and volumes are not allowed.

13.3.2 Oocyst and cyst capture

13.3.2.1 Use a graduated, 5- or 10-mL pipette that has been pre-rinsed with elution buffer to measure and transfer the water sample concentrate from Section 13.2 to the flat-sided tube(s) containing the SL-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). Rinse twice with half the volume needed to bring the total volume in the flat-sided sample tube to 12 mL (including the buffers added in Sections 13.3.1.2 and 13.3.1.3). (For example, if the tube contained 1 mL of SL-buffer-A and 1 mL of SL-buffer-B, and 5 mL of sample was transferred after resuspension of the pellet, for a total of 7 mL, the centrifuge tube would be rinsed twice with 2.5 mL of reagent water to bring the total volume in the flat-sided tube to 12 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 12 mL with reagent water. Label the flat-sided tube(s) with the sample number (and subsample letters).

13.3.2.2 Vortex the Dynabeads®Crypto-Combo vial from the IMS kit for approximately 10 seconds to suspend the beads. Ensure that the beads are fully resuspended by tilting the sample tube and making sure that there is no residual pellet at the bottom.

13.3.2.3 Add 100 µL of the resuspended Dynabeads®Crypto-Combo (Section 13.3.2.2) to the sample tube(s) containing the water sample concentrate and SL-buffers.

13.3.2.4 Vortex the Dynabeads®*Giardia*-Combo vial from the IMS kit for approximately 10 seconds to suspend the beads. Ensure that the beads are fully resuspended by tilting the tube and making sure that there is no residual pellet at the bottom.

13.3.2.5 Add 100 µL of the resuspended Dynabeads®*Giardia*-Combo (Section 13.3.2.4) to the sample tube(s) containing the water sample concentrate, Dynabeads®Crypto-Combo, and SL-buffers.

13.3.2.6 Affix the sample tube(s) to a rotating mixer and rotate at approximately 18 rpm for 1 hour at room temperature.

13.3.2.7 After rotating for 1 hour, remove each sample tube from the mixer and place the tube in the magnetic particle concentrator (MPC®-1 or MPC®-6) with flat side of the tube toward the magnet.

13.3.2.8 Make sure that the tube is snug and flat against the magnet; push the tube flat and hold there if necessary.

13.3.2.9 Gently rock the sample tube by hand end-to-end through approximately 90° (180° for MPC®-6), tilting the cap-end and base-end of the tube up and down in turn. Continue the tilting action for 2 minutes with approximately one tilt per second.

13.3.2.10 Ensure that the tilting action is continued throughout this period to prevent binding of low-mass, magnetic or magnetizable material. If the sample in the MPC®-1 or MPC®-6 is allowed to stand motionless for more than 10 seconds, remove the flat-sided tube from the MPC®-1 or MPC®-6, gently resuspend all material, replace the sample tube in the MPC®-1 or MPC®-6 and repeat Section 13.3.2.9 before continuing to Section 13.3.2.11.

13.3.2.11 Return the MPC®-1 or MPC®-6 to the upright position, sample tube vertical, with cap at top. Immediately remove the cap and, keeping the flat side of the tube on top, pour off all of the supernatant from the tube held in the MPC®-1 or MPC®-6 into a suitable container. Allow more supernatant to settle; aspirate additional supernatant with Pasteur pipette. Do not shake the tube and do not remove the tube from MPC®-1 or MPC®-6 during this step. Use a clean, lint-free tissue to blot the end of flat-sided tube after decanting to remove more matrix debris. With the MPC®-6, the supernatant may be decanted from 1 to 3 flat-sided tubes at one time; maximum 3 tubes at once. Rock each side through a 90° angle 3 times before decanting the remaining tubes.

13.3.2.12 Remove the sample tube from the MPC®-1 or MPC®-6 and resuspend the sample in 0.5 mL 1X SL-buffer-A (prepared from 10X SL-buffer-A stock—supplied). Add 1X SL Buffer A directly to the flat side of the tube; avoid any debris present on the round side of the tube. Mix very gently (using a 1.0 mL pipette) to resuspend bead pellet in the tube; release the liquid down the flat side of the tube to further rinse the tube. Do not vortex.

13.3.2.13 Place a labeled, 1.5-mL microcentrifuge tube into the second magnetic particle concentrator, MPC®-S, with its magnetic strip in the vertical (back) position.

13.3.2.14 Quantitatively transfer (transfer followed by two rinses) all the liquid from the sample tube to the labeled, 1.5-mL microcentrifuge tube in the MPC®-S. Use 0.5 mL of 1X SL-buffer-A to perform the first rinse and 0.5 mL of 1X SL-buffer-A for the second rinse. Mix each rinse gently (using a 1.0 mL pipette) and release the liquid down the flat side of the tube to further rinse the flat side of the tube. Avoid transferring debris present on the round side of the tube. Allow the flat-sided sample tube to sit for a minimum of 1 minute after transfer of the second rinse volume, then use a pipette to collect any residual volume that drips down to the bottom of the tube to ensure that as much sample volume is recovered as possible. Ensure that all of the liquid and beads are transferred.

13.3.2.15 Without removing the microcentrifuge tube from MPC®-S, gently rock/roll the tube through 180° by hand. Continue for approximately 1 minute with approximately one 180° roll/rock per second. The magnet is rocked 180° in one second in one direction and then rocked back the following second. At the end of this step, the beads will produce a distinct brown dot at the back of the tube. If this brown dot is not visible, check the magnet and microcentrifuge tube placement in the MPC®-S to assure that it has been assembled correctly and repeat this step.

13.3.2.16 Immediately aspirate the supernatant from the tube and cap held in the MPC®-S. If more than one sample is being processed, conduct three 90° rock/roll actions before removing the supernatant from each tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. *Do not shake the tube. Do not remove the tube from MPC®-S while conducting these steps.*

13.3.2.17 Rinse the beads inside the microcentrifuge tube held in the MPC®-S.

13.3.2.17.1 Gently add 1.0 mL of 1X PBS to the tube. Take care not to disturb the bead pellet attached to the wall of the tube adjacent to the magnet.

13.3.2.17.2 Remove the magnet from the MPC®-S and gently rock the sample 8 – 10 times 180˚ until the beads are resuspended.

13.3.2.17.3 Replace the magnetic strip in the vertical (back) position in the MPC®-S.

13.3.2.17.4 Repeat Sections 13.3.2.15 and 13.3.2.16.

13.3.2.18 Let the tube stand undisturbed for 1 minute, allowing any residual liquid to flow to the bottom of the tube. Using a Pasteur pipette, gently mix residual liquid/debris and aspirate. *Do not shake the tube. Do not remove the tube from MPC®-S while conducting these steps.*

13.3.3 Dissociation of beads/oocyst/cyst complex

NOTE: Two acid dissociations are required.

13.3.3.1 Remove the magnetic strip from the MPC®-S.

13.3.3.2 Add 50 µL of 0.1 N HCl, then vortex at the highest setting for approximately 50 seconds.

NOTE: The laboratory must use 0.1-N standards purchased directly from a vendor, rather than adjusting the normality in-house.

13.3.3.3 Keep the tube in the MPC®-S without the magnetic strip in place and allow it to stand in a vertical position for at least 10 minutes at room temperature.

13.3.3.4 Vortex vigorously for approximately 30 seconds.

13.3.3.5 Ensure that the entire sample is at the base of the tube. Place the microcentrifuge tube in the MPC®-S.

13.3.3.6 Replace magnetic strip in the slanted (front) position of the MPC®-S and allow the tube to stand undisturbed for a minimum of 10 seconds.

13.3.3.7 Prepare a well slide for sample screening and label the slide. See Section 13.4.5 for suggestions for dirty samples.

13.3.3.8 Add 5 µL of 1.0 N NaOH to two sample wells on same or separate well slides (add 10 µL to one sample well if the volume from the two required dissociations will be added to the same well).

NOTE: The laboratory must use 1.0-N standards purchased directly from a vendor rather than adjusting the normality in-house.

13.3.3.9 Without removing the microcentrifuge tube from the MPC®-S, transfer the entire sample from the microcentrifuge tube in the MPC®-S to the sample well with the NaOH. Do not disturb the beads at the back wall of the tube. Ensure that all of the fluid is transferred.

13.3.3.10 Do not discard the beads or microcentrifuge tube after transferring the volume from the first acid dissociation to the well slide. Perform the steps in Sections 13.3.3.1 through 13.3.3.9 a second time. The volume from the second dissociation can be added to the well containing the volume from the first dissociation, or can be applied to a second well on the same slide or a separate slide.

13.3.3.11 Record the date and time the purified sample was applied to the well slide(s).

13.3.3.12 Air-dry the sample on the well slide(s). Because temperature and humidity vary from laboratory to laboratory, no minimum time is specified. However, the laboratory must take care to ensure that the sample has dried completely and to stain manufacturers' instructions before staining to prevent losses during the rinse steps. A slide warmer set at 35°C to 42°C also can be used.

13.4 Additional IMS techniques for use with complex samples**.**

13.4.1 **Removing Magnetic Materials** - 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 1623.1 through Section 13.2.3.2; 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). Rinse with two rinses each of which is comprised of 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 (Section 13.3.1.3). 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 may 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 SOP.

13.4.2 Adjusting pH - 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 1623.1 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 (Sections 13.2.2.1, 13.2.3.1.2, or 13.2.3.2), 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. Ensure technique used prevents cross-contamination of samples.

13.4.3 Additional Rinse of Flat-sided Tube - 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. Process sample(s) according to Method 1623.1 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 to almost horizontal with bead pellet and magnet on top.

13.3.2.11.2 Gently add between 2.5 and 10 mL of reagent water, PBS 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.

13.4.4 Heat Dissociation - The addition of acid to some source water matrices for dissociation may drive chemical reactions that interfere with the method, e.g., bead clumping. 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 1623.1 through Section 13.3.3.1; then revise 13.3.3.2 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"

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.

13.4.5 Increasing Surface Area for Sample Application to Slide - 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 wells.

To split each dissociation volume, process sample(s) according to Method 1623.1 through Section 13.3.3.6 (sample is in the microcentrifuge tube in the MPC®-S); then proceed at Section 13.3.3.7 with the following substitutions and additions:

13.3.3.7 Prepare two separate wells (on same or separate slides) 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 A**; 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 wells as before (modified 13.3.3.9 above, See **Figure A**).

13.3.3.11 Add 5 µL of 1.0 N NaOH to each of the two wells after applying the samples. Record the date and time the purified sample was applied to the well slide(s).

13.3.3.12 Same as method, to completion of the method.

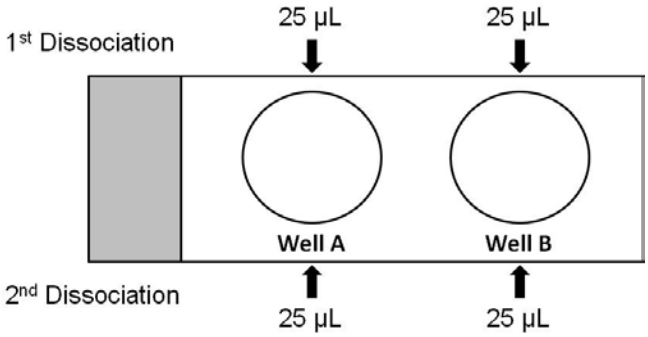

Figure A.

14.0 Sample Staining

NOTE: The sample must be stained within 72 hours of application of the purified sample to the slide.

NOTE: If a laboratory will use multiple types of labeling reagents, the laboratory must demonstrate equivalent or superior performance through an IDC per Section 9.2 for each type, and must perform positive and negative staining controls for each batch of slides stained using each product. However, the laboratory is not required to analyze additional ongoing precision and recovery samples or method blank samples for each type. The performance of each labeling reagent used is monitored in each source water type through MS samples (Section 9.6.1).

14.1 Prepare positive and negative controls

14.1.1 For the positive control, pipette 10 µL of positive antigen or >200 intact oocysts and >200 cysts to the center of a well.

14.1.2 For the negative control, pipette 50 µL of PBS (Section 7.5) into the center of a well and spread it over the well area with a pipette tip.

14.1.3 Air-dry the control slides at the same time and following the same procedure as used for field samples (see Section 13.3.3.12 for guidance).

NOTE: Multiple control slides may be prepared if a laboratory has a large batch of slides that will be examined over several days, and is concerned that a single positive control may fade, due to multiple examinations. These control slides are prepared along with the field slides and examined, alternating between the positive controls when performing the positive control check.

14.2 **Four optional stains have been demonstrated to be acceptable for use with Method 1623.1**— 1) MeriFluor® *Cryptosporidium/Giardia*, 2) Aqua-Glo™G/C Direct FL, 3) Crypt-a-Glo™ and Giardi-a-Glo™, and 4) EasyStain™C&G. Follow manufacturer's instructions in applying stain to slides.

14.3 Place the slides in a humid chamber in the dark and incubate at room temperature for approximately 30 minutes. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed.

14.4 Remove slides from humid chamber and allow condensation to evaporate, if present.

14.5 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.8]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

14.6 Apply 50 µL of DAPI staining solution (Section 7.9.2) to each well. Allow to stand at room temperature for a minimum of 1 minute. (The solution concentration may be increased up to 1 µg/mL if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.)

14.7 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.8]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess DAPI staining solution from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

NOTE: If using the MeriFluor® Cryptosporidium/Giardia (Section 7.8.1), do not allow slides to dry completely.

14.8 Add mounting medium (Section 7.10) to each well.

14.9 Apply a cover slip. Use a tissue to remove excess mounting fluid from the edges of the coverslip. Seal the edges of the coverslip onto the slide using clear nail polish.

14.10 Record the date and time that staining was completed on the bench sheet. If slides will not be read immediately, store in a humid chamber in the dark between 1°C and 10°C until ready for examination.

15.0 Examination

NOTE: Although it is preferable to perform FA and DAPI and DIC microscopy examination and characterization immediately after staining is complete, laboratories have up to 168 hours (7 days) to complete the examination and verification of samples. However, if fading/diffusion of FITC or DAPI fluorescence is noticed, the laboratory must reduce this holding time. In addition, the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.9.2) so that fading/diffusion does not occur.

15.1 Scanning technique: Scan each well in a systematic fashion. An up-and-down or a side-to-side scanning pattern may be used (Figure 3).

15.2 Examination using FA, DAPI staining characteristics, and DIC microscopy. The minimum magnification requirements for each type of examination are noted below.

NOTE: All characterization (DAPI and DIC) and size measurements must be determined using 1000X magnification and reported to the nearest 0.5 µm.

Record examination results for *Cryptosporidium* oocysts on a *Cryptosporidium* examination form; record examination results for *Giardia* cysts on a *Giardia* examination results form. All organisms that meet the criteria specified in Sections 15.2.2 and 15.2.3, less atypical organisms specifically identified as non-target organisms by DIC or DAPI (e.g., possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc), must be reported.

15.2.1 Positive and negative staining control. Positive and negative staining controls must be acceptable before proceeding with examination of field sample slides.

15.2.1.1 Each analyst must characterize a minimum of three *Cryptosporidium* oocysts and three *Giardia* cysts on the positive staining control slide before examining field sample slides. This characterization must be performed by each analyst during each microscope examination session. FITC examination must be conducted at a minimum of 200X total magnification, DAPI examination must be conducted at a minimum of 400X, and DIC examination and size measurements must be conducted at a minimum of 1000X. Size, shape, and DIC and DAPI characteristics of three *Cryptosporidium* oocysts and three *Giardia* cysts must be recorded by the analyst on a microscope log. The analyst also must indicate on each sample examination form whether the positive staining control was acceptable.

15.2.1.2 Examine the negative staining control to confirm that it does not contain any oocysts or cysts (Section 14.1). Indicate on each sample examination form whether the negative staining control was acceptable.

15.2.1.3 If the positive staining control contains oocysts and cysts within the expected range and at the appropriate fluorescence for both FA and DAPI, and the negative staining control does not contain any oocysts or cysts (Section 14.1), proceed to Sections 15.2.2 and 15.2.3.

15.2.2 Sample examination—*Cryptosporidium*

15.2.2.1 A positive result is a *Cryptosporidium* oocyst which exhibits all of the following: 1) typical FA fluorescence, 2) typical size and shape, 3) nothing atypical on DAPI fluorescence, and 4) nothing atypical on DIC microscopy

(Figure 4). **Each positive result must be characterized and assigned to one of the DAPI and DIC categories in Sections 15.2.2.3 and 15.2.2.4 and recorded on an examination form**.

15.2.2.2 **FITC examination** (the analyst must use a minimum of 200X total magnification). Use epifluorescence to scan the entire well for apple-green fluorescence of oocyst and cyst shapes. When brilliant apple-green fluorescing ovoid or spherical objects 4 to 6 µm in diameter are observed with brightly highlighted edges, increase magnification to 400X and switch the microscope to the UV filter block for DAPI (Section 15.2.2.3), then to DIC (Section 15.2.2.4) at 1000X.

15.2.2.3 **DAPI fluorescence examination** (the analyst must use a minimum of 400X total magnification). Using the UV filter block for DAPI, the object will exhibit one of the following characteristics:

(a) Light blue internal staining (no distinct nuclei) with a green rim
(b) Intense blue internal staining
(c) Up to four distinct, sky-blue nuclei

Look for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color. If atypical structures are not observed, then categorize each object meeting the criteria defined as a positive result in Section 15.2.2.1 and record oocysts in category (a) as DAPI-negative; record oocysts in categories (b) and (c) as DAPI-positive.

15.2.2.4 **DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC microscopy, look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, crystals, spores, etc.). If atypical structures are not observed, then categorize each object meeting the criteria defined as a positive result in Section 15.2.2.1 and specified in Sections 15.2.2.2 and 15.2.2.3 as one of the following, based on DIC examination:

(a) An empty *Cryptosporidium* oocyst
(b) A *Cryptosporidium* oocyst with amorphous structure
(c) A *Cryptosporidium* oocyst with internal structure (one to four sporozoites/oocyst)

Using 1000X total magnification, record the shape, measurements (to the nearest 0.5 µm), and number of sporozoites (if applicable) for each apple-green fluorescing object meeting the size and shape characteristics. Although not a defining characteristic, surface oocyst folds may be observed in some specimens.

15.2.3 Sample examination—*Giardia*

15.2.3.1 A positive result is a *Giardia* cyst which exhibits all of the following: 1) typical FA fluorescence, 2) typical size and shape, 3) nothing atypical on DAPI fluorescence, and 4) nothing atypical on DIC microscopy (Figure 4). **Each positive result must be characterized and assigned to one of the DAPI and DIC categories in Sections 15.2.3.3 and 15.2.3.4 and recorded on an examination form.**

15.2.3.2 **FITC examination** (the analyst must use a minimum of 200X total magnification). When brilliant apple-green fluorescing round to ovoid objects (8 - 18 µm long by 5 - 15 µm wide) are observed with brightly highlighted edges, increase magnification to 400X and switch the microscope to the UV filter block for DAPI (Section 15.2.3.3) then to DIC (Section 15.2.3.4) at 1000X.

15.2.3.3 **DAPI fluorescence examination** (the analyst must use a minimum of 400X total magnification). Using the UV filter block for DAPI, the object will exhibit one or more of the following characteristics:

(a) Light blue internal staining (no distinct nuclei) and a green rim
(b) Intense blue internal staining
(c) Up to four distinct, sky-blue nuclei

Look for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color. If atypical structures are not observed, then categorize each object meeting the criteria defined as a positive result in Section 15.2.3.1 and record cysts in category (a) as DAPI negative; record cysts in categories (b) and (c) as DAPI positive.

15.2.3.4 **DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC microscopy, look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, crystals, spores, etc.). If atypical structures are not observed, then categorize each object meeting the criteria defined as a positive result in Section 15.2.3.1 and specified in Sections 15.2.3.2 and 15.2.3.3 as one of the following, based on DIC examination:

(a) An empty *Giardia* cyst
(b) A *Giardia* cyst with amorphous structure
(c) A *Giardia* cyst with one type of internal structure (nuclei, median body, or axonemes), or
(d) A *Giardia* cyst with more than one type of internal structure

Using 1000X total magnification, record the shape, measurements (to the nearest 0.5 µm), and number of nuclei and presence of median body or axonemes (if applicable) for each apple-green fluorescing object meeting the size and shape characteristics.

15.2.4 Record the date and time that sample examination was completed on the examination form.

15.2.5 Report *Cryptosporidium* and *Giardia* concentrations as oocysts/L and cysts/L, respectively. If no oocysts or cysts, as defined in Section 15.2.2.1 and 15.2.3.1, are detected, report zero organisms.

15.2.6 Record analyst name

16.0 Analysis of Complex Samples

16.1 Some samples may contain high levels (>1000/L) of oocysts and cysts and/or interfering organisms, substances, or materials. Some samples may clog the filter (Section 12.0); others will not allow separation of the oocysts and cysts from the retentate or eluate; and others may contain materials that preclude or confuse microscopic examination.

16.2 If the sample holding time has not been exceeded and a full-volume sample cannot be filtered, dilute an aliquot of sample with reagent water and filter this smaller aliquot (Section 12.0). This dilution must be recorded and reported with the results.

16.3 See the Training modules available for downloading at http://water.epa.gov/lawsregs/rulesregs/sdwa/mdbp/training.cfm for further suggestions.

17.0 Method Performance

Method acceptance criteria are shown in Tables 3 and 4 in Section 21.0. The criteria listed for *Cryptosporidium* in Table 3 and *Giardia* in Table 4 were based on results generated from 56 spiked reagent and 53 spiked raw surface water samples from 14 sites across the U.S. analyzed during the inter-laboratory validation of Method 1623.1 involving 14 laboratories (Reference 20.13).

NOTE: Some sample matrices may prevent the MS acceptance criteria in Tables 3 and 4 to be met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 6.

18.0 Pollution Prevention

18.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

18.2 Prepare solutions and reagents in volumes consistent with laboratory use to minimize the volume of expired materials that need to be discarded.

19.0 Waste Management

19.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required. An overview of these requirements can be found in the *Environmental Management Guide for Small Laboratories* (EPA 233-B-98-001).

19.2 Samples, reference materials, and equipment known or suspected to have viable oocysts or cysts attached or contained must be sterilized prior to disposal.

19.3 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* and *Less is Better: Laboratory Chemical Management for Waste Reduction*, both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

20.0 References

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20.4 Fleming, Diane O., et al.(eds.), *Laboratory Safety: Principles and Practices,* 2nd edition.1995. ASM Press, Washington, DC

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20.12 "Dynabeads® GC-Combo," 730.02, Invitrogen Dynal, Oslo, Norway (2007, Revision no. 014).

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20.15 Connell, K., J. Scheller, K. Miller, and C.C. Rodgers, 2000. *Performance of Methods 1622 and 1623 in the ICR Supplemental Surveys.* Proceedings, American Water Works Association Water Quality Technology Conference, November 5 - 9, 2000, Salt Lake City, UT.

21.0 Tables and Figures

Table 1. Method 1623.1 Procedural Component Options

Method 1623.1 Procedural Component	Optional Component or Processing Step	Protocol (Section references to Method 1623.1)	Reference*
Sample Collection	Field filter – Envirochek® HV; 10 L or 50 L	Section 12.2 – 12.2.5; LT2 Rule *Cryptosporidium* and *E. coli* Sample Collection Recommendations Training Module and Pocket Guide	NA
	Field filter – Filta-Max®; 10 L or 50 L	Section 12.3 – 12.3.1.6; LT2 Rule *Cryptosporidium* and *E. coli* Sample Collection Recommendations Training Module and Pocket Guide	NA
	Bulk; 10 L	Section 8.1.1; LT2 Rule *Cryptosporidium* and *E. coli* Sample Collection Recommendations Training Module and Pocket Guide	Validation study
Spiking Suspension	Wisconsin State Laboratory of Hygiene	Section 11.0; Manufacturer's instructions	Validation study
	BTF EasySeed™	Section 11.0; Manufacturer's instructions	Historically documented SOP; Validation study
	Waterborne AccuSpike™-IR	Section 11.0; Manufacturer's instructions	Validation study
Filtration/ Elution	Envirochek® HV/shaker (700-900 opm)/NaHMP/LA-12	Section 12.2	Historically documented SOP; Validation study
	Filta-Max®/manual wash station/PBST/concentrator tube	Section 12.3-12.3.1.6.2; 12.3.2.1 - 12.3.2.2, 12.3.4	Validation study
	Filta-Max®/automatic wash station/PBST/concentrator tube	Manufacturer's instructions	refer to manual wash validation study
	PCFC	Manufacturer's instructions	Validation study
Concentration /Aspiration	Centrifugation (minimum 1500 × G ; 1800-2000 × G)	Section 13.2.1	Historically documented SOP; Validation study
	Concentrator Membrane/Centrifugation	Section12.3.3; 13.2.1	Validation study
	Aspiration	Sections 13.2.2-13.2.3	Validation Study

Method 1623.1 Procedural Component	Optional Component or Processing Step	Protocol (Section references to Method 1623.1)	Reference*
IMS	Dynabeads® GC Combo with MPC®-1 or MPC®-6 and MPC®-S	Sections 13.3 – 13.4	Historically documented SOP; Validation study
	Transfer to microcentrifuge tube - 0.7, 0.3, 0.3-mL	Sections 13.3.2.12 and 13.3.2.14	Historically documented SOP; Validation study
	Multiple wells; on same or separate slides	Section 13.3.3.10	Validation study
Staining	MeriFluor® *Cryptosporidium*/*Giardia*	Section 14.0, Manufacturer's instructions	Validation study
	Aqua-Glo™G/C Direct FL	Section 14.0, Manufacturer's instructions	Validation study
	Crypt-a-Glo™ and Giardi-a-Glo™	Section 14.0, Manufacturer's instructions	Validation study
	EasyStain™C&G	Section 14.0, Manufacturer's instructions	Historically documented SOP; Validation study
Equipment or supply changes	Using different equipment/supplies within procedural component, such as • pumps • lab shaker • IMS magnets • flat-sided tubes • microcentrifuge tubes • slides • mounting medium • microscope	Follow same procedure in Method 1623.1, manufacturer's instructions or historically documented SOP	NA

*Multi-laboratory validation studies or historical demonstration of accuracy and precision at multiple laboratories.

Table 2. Summary of Routine QC Requirements

Method 1623.1 Reference	QC sample or procedure	Matrix	Number of samples	Frequency	Purpose	Control Charts
Section 9.5, 9.6, 9.7	IDC	Reagent water and water matrix of interest	8	Initial use of method and each procedural component change	To demonstrate control over the analytical system with new or different procedural component; consists of IPR set, Method blank, field sample and MS/MSD	No
Section 9.5	IPR	Reagent water	4	Each equipment/supply change	To establish control over the analytical system and demonstrate acceptable method performance (recovery and precision) as part of IDC and with new equipment or supplies	No
Section 9.7	Method Blank	Reagent water	1	Each IPR and OPR set	To demonstrate the absence of contamination throughout the analytical process	No
Section 9.8, 9.12.1	OPR	Reagent water	1	At least each week samples are processed or every 20 samples, whichever is more frequent	To demonstrate ongoing control of the analytical system and verify continuing method performance (recovery and precision)	Required
Section 9.6.1	MS	Water matrix of interest	1, plus 1 unspiked field sample	For each water matrix - initial sampling and every 20 samples	To determine the effect of the matrix on (oo)cyst recoveries; must be accompanied by an unspiked field sample collected at the same time as the MS sample	Required
Section 9.6.3	MS/MSD	Water matrix of interest	2, plus 1 unspiked field sample	Each IDC, and multi-laboratory validation of modification	To estimate the (oo)cyst recovery precision with the effect of the matrix; must be accompanied by an unspiked field sample collected at the same time as MS/MSD samples	Required
Sections 9.9, 14.1 and 15.2.1	Positive staining control	none	1	Process each time samples are stained; examine each microscope session	To demonstrate ongoing control of the staining process and performance of reagents and microscope	No
Sections 9.9, 14.1 and 15.2.1	Negative staining control	none	1	Each time samples are stained	To demonstrate the absence of contamination through staining process	No
Section 9.10	Verification of Analyst Performance	Reagent Water	N/A	Monthly	Refine consistency of organism enumeration and characterizations between analysts	No

Table 3. Quality Control Acceptance Criteria for *Cryptosporidium*

Performance test	Section	Acceptance criteria
Initial precision and recovery[1] (IPR)	9.5	
Mean recovery (percent)	9.5.2	38 – 100
Precision (as maximum relative standard deviation)	9.5.3	37
Ongoing precision and recovery[1] (OPR as percent)	9.8	33 - 100
MS/MSD (for method modifications)	9.6	
Mean recovery[2,3] (as percent)	9.6.3.2	32 - 100
Precision (as maximum relative percent difference)	9.6.3.3	46

(1) Failure to meet IPR or OPR quality control acceptance criteria indicates systemic problems the laboratory must address prior to processing any samples.

(2) The acceptance criteria for mean MS/MSD recovery serve as the acceptance criteria for MS recovery during routine use of the method (Section 9.6.1).

(3) Some sample matrices may prevent the acceptance criteria from being met. Repeated failure to meet the MS/MSD quality control acceptance criteria, when all other QC passes, warrants corrective action from quality conscience laboratories to systematically review control charts and modify analytical protocols to improve matrix spike recoveries. An assessment of the distribution of MS recoveries from multiple MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 6. The laboratory must chart their distribution of MS and MSD recoveries compared with Table 6 (Section 9.12.2.2).

Table 4. Quality Control Acceptance Criteria for *Giardia*

Performance test	Section	Acceptance criteria
Initial precision and recovery[1] (IPR)	9.5	
Mean recovery (percent)	9.5.2	27 – 100
Precision (as maximum relative standard deviation)	9.5.3	39
Ongoing precision and recovery[1] (OPR as percent)	9.8	22 - 100
MS/MSD (for method modifications)	9.6	
Mean recovery [2, 3] (as percent)	9.6.3.2	8 - 100
Precision (as maximum relative percent difference)	9.6.3.3	97

Footnotes 1, 2, and 3 are the same as Table 3.

NOTE: The criteria listed for Cryptosporidium *and* Giardia *were based on data generated from 56 spiked reagent and 53 spiked raw surface water samples from 14 sites across the U.S. analyzed during the interlaboratory validation of Method 1623.1 involving 14 laboratories (Reference 20.13.).*

Table 5. Method Holding Times (See Section 8.2 for details)

Maximum Allowable Time for Sample Processing Steps
➢ 4 days (96 hours) between collection/filtration and elution
➢ 1 working day between elution and application of sample to slide
➢ 3 days (72 hours) between application to slide and staining
➢ 7 days (168 hours) between staining and examination

Table 6. Distribution of MS Recoveries from Multiple Samples Collected from 87 Source Waters During the ICR Supplemental Surveys (Adapted from References 20.14 and 20.15)

MS Recovery Range	Percent of 430 *Cryptosporidium* MS Samples in Recovery Range	Percent of 270 *Giardia* MS Samples in Recovery Range
<10%	6.7%	5.2%
>10% - 20%	6.3%	4.8%
>20% - 30%	14.9%	7.0%
>30% - 40%	14.2%	8.5%
>40% - 50%	18.4%	17.4%
>50% - 60%	17.4%	16.3%
>60% - 70%	11.2%	16.7%
>70% - 80%	8.4%	14.1%
>80% - 90%	2.3%	6.3%
>90%	0.2%	3.7%

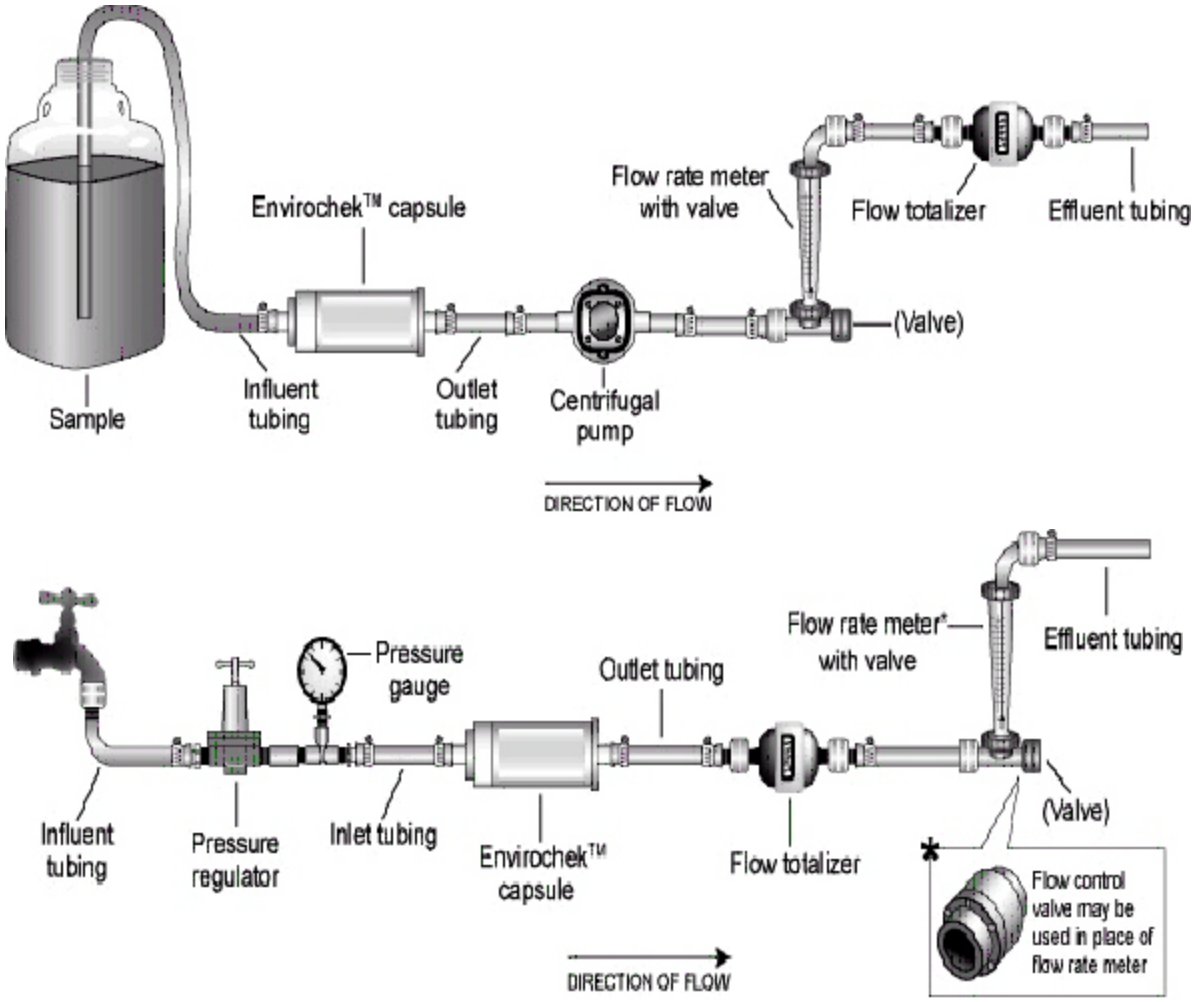

Figure 1. Filtration Systems for Envirochek® HV Capsule (unpressurized source - top, pressurized source - bottom)

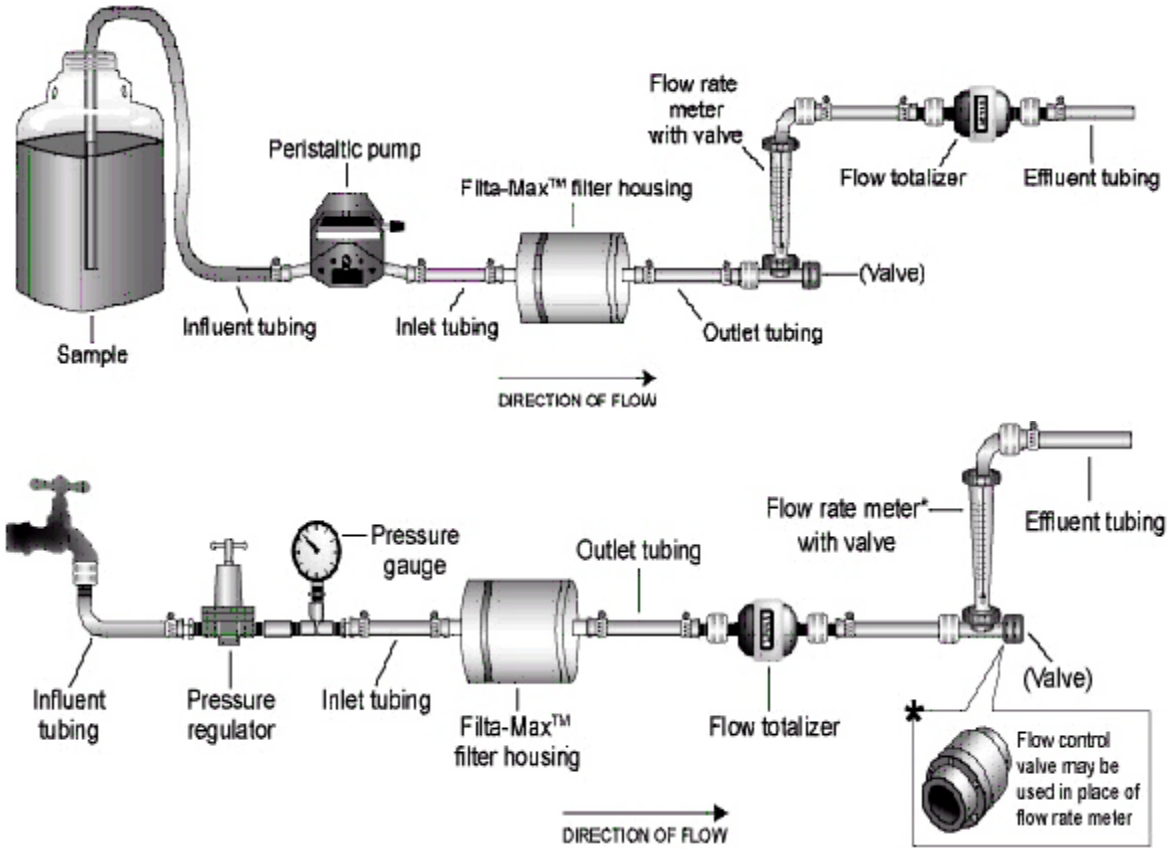

Figure 2. Filtration Systems for Filta-Max® filters (unpressurized source - top, pressurized source - bottom)

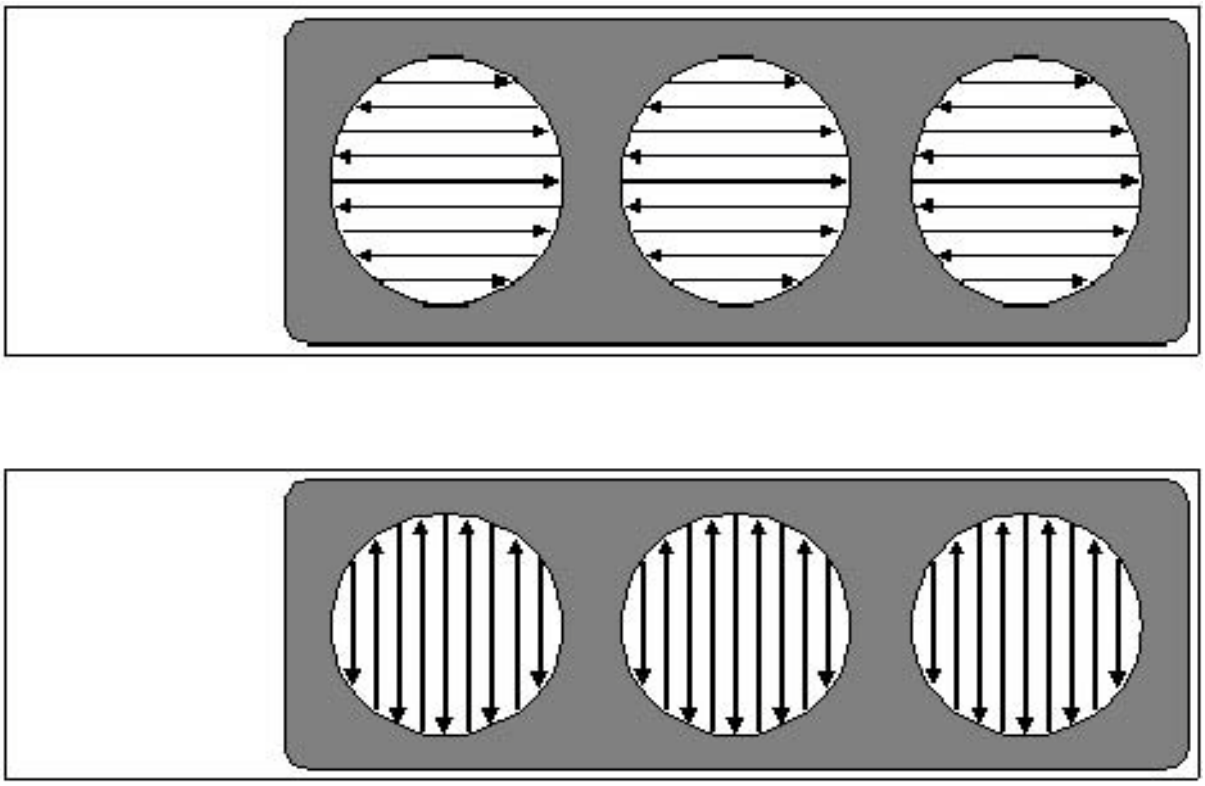

Figure 3. Methods for Scanning a Well Slide

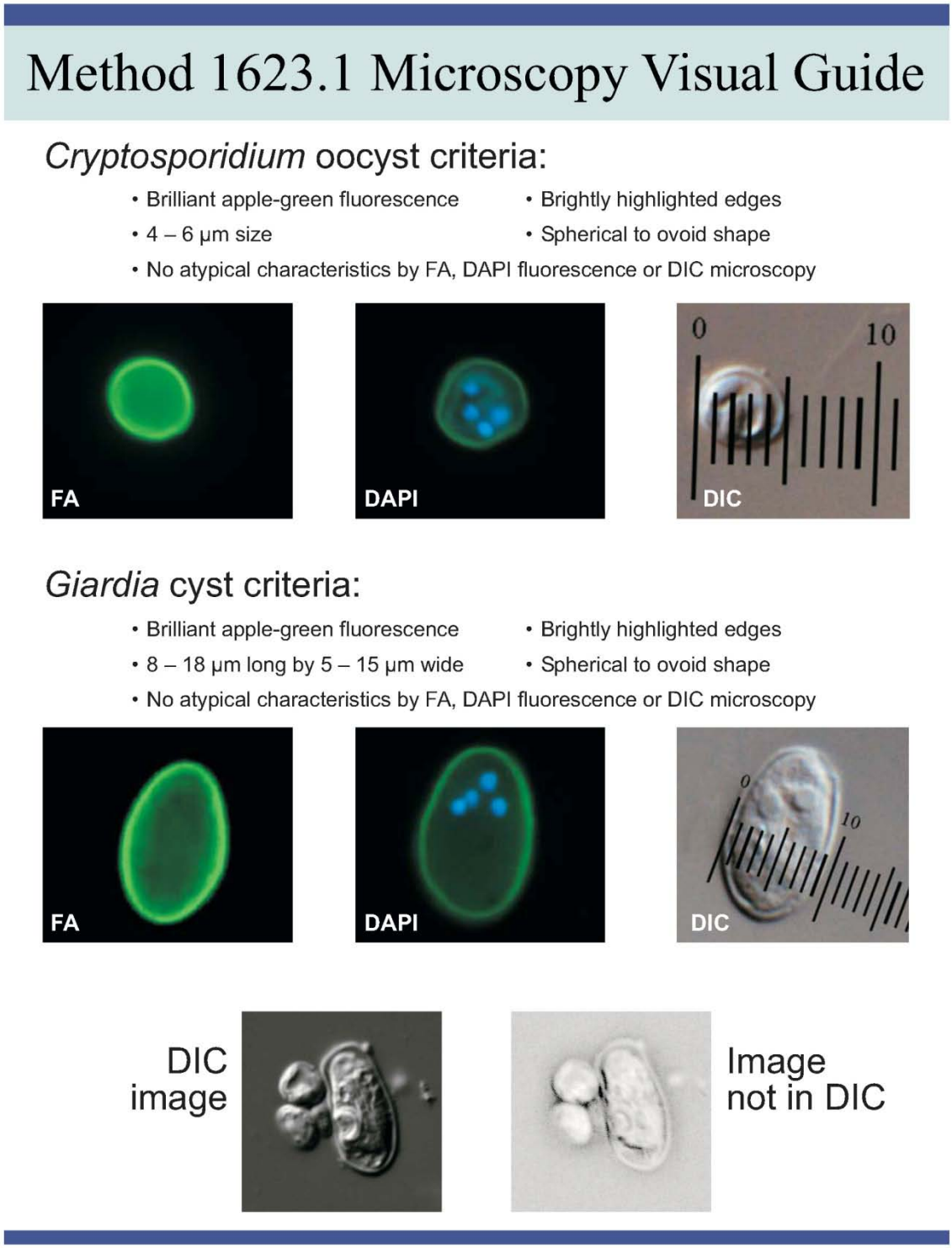

Figure 4. Method 1623.1 Microscopy Visual Guide - Photographs courtesy of The City of San Diego Water Quality Laboratory; Texas AgriLife Research Center at El Paso, Texas A&M University System; University of Iowa Hygienic Laboratory

22.0 Glossary of Definitions and Purposes

These definitions and purposes are specific to this method but have been conformed to common usage as much as possible.

22.1 Units of weight and measure and their abbreviations

22.1.1 Symbols

°C	degrees Celsius
µL	microliter
<	less than
>	greater than
%	percent

22.1.2 Alphabetical characters

cm	centimeter
g	gram
G	acceleration due to gravity
hr	hour
ID	inside diameter
in.	inch
L	liter
m	meter
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
N	normal; gram molecular weight of solute divided by hydrogen equivalent of solute, per liter of solution
RSD	relative standard deviation
s_r	standard deviation of recovery
X	mean percent recovery

22.2 Definitions, acronyms, and abbreviations (in alphabetical order)

4',6-diamidino-2-phenylindole (DAPI) – A nucleic acid stain which fluoresces blue and can show the position and number of nuclei present in the cyst/oocyst.

Analyst—The analyst participates in a monthly analyst verification (Section 9.10), establishes Köhler illumination for the microscope, may perform the same duties as a technician, and is able to examine samples using the microscope. An analyst has at least 2 years of college education in microbiology, or an equivalent field and a minimum of 6 months of continuous bench experience with *Cryptosporidium* and FA microscopy. In addition, an analyst must have a minimum of 3 months experience using EPA Method 1623 or 1623.1 analyzing a minimum of 50 samples using either method. "Grandfathering" analysts with >10 years experience of continuous protozoan identification duties may be substituted for college education.

Analyte—A protozoan parasite tested for by this method. The analytes in this method are *Cryptosporidium* and *Giardia*.

Axoneme—An internal flagellar structure that occurs in some protozoa, such as *Giardia*, *Spironucleous*, and *Trichomonas*.

Cyst—A phase or a form of an organism produced either in response to environmental conditions or as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant cell wall.

Flow cytometer—A particle-sorting instrument capable of counting protozoa.

Fluorescein isothiocyanate (FITC) – The fluorochrome used to label the monoclonal antibodies targeted against the cell wall antigens of both *Giardia* and *Cryptosporidium* which fluoresces a brilliant apple green color.

Immunofluorescence assay (FA) – A microscopic assay technique in which a fluorochrome is conjugated to an antibody molecule that selectively binds to the organism of interest which is detected using fluorescent microscopy.

Immunomagnetic separation (IMS)—A purification procedure that uses microscopic, magnetically responsive particles coated with an antibodies targeted to react with a specific pathogen in a fluid stream. Pathogens are selectively removed from other debris using a magnetic field.

Initial demonstration of capability (IDC)—Four IPR samples, one MB, and MS/MSD/unspiked field sample analyzed to establish the ability to generate acceptable precision and accuracy. An IDC is performed prior to the first time this method is used and any time the method is modified.

Initial precision and recovery (IPR)—Four aliquots of spiking suspension analyzed to establish the ability to generate acceptable precision and accuracy. An IPR is performed as part of the IDC or when equipment/supply changes are made to this method.

Laboratory blank—See Method blank

Laboratory control sample —See Ongoing precision and recovery (OPR) standard

Matrix spike (MS)—A sample prepared by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used to determine the effect of the matrix on a method's recovery efficiency.

Matrix spike duplicate (MSD)—A second sample prepared by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike duplicate is used to determine the precision of a method's recovery efficiency in a particular matrix type.

May—This action, activity, or procedural step is neither required nor prohibited.

May not—This action, activity, or procedural step is prohibited.

Median bodies—Prominent, dark-staining, paired organelles consisting of microtubules and found in the posterior half of *Giardia*. These structures can be a crescent-shaped or round.

Method blank (MB)—An aliquot of reagent water that is treated exactly as a sample, including exposure to all glassware, equipment, solvents, and procedures that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

Must—This action, activity, or procedural step is required.

Negative control—See Method blank

Nucleus—A membrane-bound organelle containing genetic material. Nuclei are a prominent internal structure seen both in *Cryptosporidium* oocysts and *Giardia* cysts. In *Cryptosporidium* oocysts, there is one nucleus per sporozoite. One to four nuclei can be seen in *Giardia* cysts.

Oocyst—The encysted zygote of some sporozoa; e.g., *Cryptosporidium*. The oocyst is a phase or form of the organism produced as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant outer wall.

Ongoing precision and recovery (OPR) standard—A method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Oocyst and cyst spiking suspension—See Spiking suspension

Positive control—See Ongoing precision and recovery (OPR) standard

Principal analyst—The principal analyst participates in a monthly analyst verification (Section 9.10), supervises and verifies the processing and microscopy in the laboratory, and may perform the same duties as an analyst. A principal analyst has a BS/BA in microbiology or closely related field and a minimum of 1 year of continuous bench experience with *Cryptosporidium* and FA microscopy. In addition to formal training in microbiology, the principal analyst must also have a minimum of 6 months experience using EPA Method 1623 or 1623.1 and have analyzed a minimum of 100 samples using EPA Method 1623 or 1623.1. “Grandfathering” principal analysts with >10 years experience of continuous protozoan identification duties may be substituted for college education.

PTFE—Polytetrafluoroethylene

Quality control (QC) – Operational techniques and activities used for maintaining standards.

Quantitative transfer—The process of transferring a solution from one container to another using a pipette in which as much solution as possible is transferred, followed by rinsing of the walls of the source container with a small volume of rinsing solution (e.g., reagent water, buffer, etc.), followed by transfer of the rinsing solution, followed by a second rinse and transfer.

Reagent water—Water demonstrated to be free from the analytes of interest and potentially interfering substances at the method detection limit for the analyte.

Reagent water blank—see Method blank

Relative standard deviation (RSD)—The standard deviation divided by the mean times 100.

Should—This action, activity, or procedural step is suggested but not required.

Spiking suspension—Flow cytometry-enumerated organism(s) of interest at a concentration appropriate for spiking samples.

Sporozoite—A motile, infective stage of certain protozoans; e.g., *Cryptosporidium.* There are four sporozoites in each *Cryptosporidium* oocyst, and they are generally banana-shaped.

Technician—The technician filters samples, performs centrifugation, elution, concentration, and purification using IMS, and prepares purified samples on slides for microscopic examination, but does not perform microscopic protozoan detection and identification. No minimum education or experience requirements with *Cryptosporidium* and IFA microscopy apply to the technician. At least 3 months of experience in filter extraction and processing of protozoa samples by EPA Method 1623 or 1623.1 and successful processing of a minimum of 50 samples using EPA Method 1623 or 1623.1 are required to become a technician

Appendix A
Micropipette Calibration

A.0 Micropipette Calibration

A.1 Micropipettes must be sent to the manufacturer for calibration annually. Alternately, a qualified independent technician specializing in micropipette calibration can be used, or the calibration can be performed by the laboratory, provided the laboratory maintains a detailed procedure that can be evaluated by an independent auditor. Documentation on the precision of the recalibrated micropipette must be obtained from the manufacturer or technician.

A.2 Internal and external calibration records must be kept on file in the laboratory's QA logbook.

A.3 If a micropipette calibration problem is suspected, the laboratory should tare an empty weighing boat on the analytical balance and pipette the following volumes of reagent water into the weigh boat using the pipette in question: 100% of the maximum dispensing capacity of the micropipette, 50% of the capacity, and 10% of the capacity. Ten replicates should be performed at each weight. Record the weight of the water (assume that 1.00 mL of reagent water weighs 1.00 g) and calculate the relative standard deviation (RSD) for each. If the weight of the reagent water is within 1% of the desired weight (mL) and the RSD of the replicates at each weight is within 1%, then the pipette remains acceptable for use.

A.4 If the weight of the reagent water is outside the acceptable limits, consult the manufacturer's instruction manual troubleshooting section and repeat steps described in Section A.3. If problems with the pipette persist, the laboratory must send the pipette to the manufacturer for recalibration.

Appendix B
Microscope Protocols

B.0 Microscope Protocols

CAUTION: In the procedures below, do not touch the quartz portion of the mercury bulb with your bare fingers. Finger oils can cause rapid degradation of the quartz and premature failure of the bulb.

WARNING: Never look at the ultraviolet (UV) light from the mercury lamp, lamp house, or the UV image without a barrier filter in place. UV radiation can cause serious eye damage.

Section 10 and the sections below assume that the mercury bulb has not exceeded time limits of operation, that the lamp socket is connected to the lamp house, and that the condenser is adjusted to produce Köhler illumination.

B.1 **Epifluorescent mercury bulb adjustment**—The purpose of this procedure is to ensure even field illumination. This procedure must be followed when the microscope is first used, when replacing bulbs, and if problems such as diminished fluorescence or uneven field illumination are experienced.

B.1.1 Remove the diffuser lens between the lamp and microscope or swing it out of the transmitted light path.

B.1.2 Using a prepared microscope slide, adjust the focus so the image in the oculars is sharply defined.

B.1.3 Replace the slide with a business card or a piece of lens paper.

B.1.4 Close the field diaphragm (iris diaphragm in the microscope base) so only a small point of light is visible on the card. This dot of light indicates the location of the center of the field of view.

B.1.5 Mount the mercury lamp house on the microscope without the UV diffuser lens in place and turn on the mercury bulb.

B.1.6 Remove the objective in the light path from the nosepiece. A primary (brighter) and secondary image (dimmer) of the mercury bulb arc should appear on the card after focusing the image with the appropriate adjustment.

B.1.7 Using the lamp house adjustments, adjust the primary and secondary mercury bulb images so they are side by side (parallel to each other) with the transmitted light dot in between them.

B.1.8 Reattach the objective to the nosepiece.

B.1.9 Insert the diffuser lens into the light path between the mercury lamp house and the microscope.

B.1.10 Turn off the transmitted light and replace the card with a slide of fluorescent material. Check the field for even fluorescent illumination. Adjustment of the diffuser lens probably will be required. Additional slight adjustments as in Section B.1.7 may be required.

B.1.11 Maintain a log of the number of hours the UV bulb has been used. Never use the bulb for longer than it has been rated by the manufacturer.

B.2 **Transmitted bulb adjustment**—The purpose of this procedure is to center the filament and ensure even field illumination. This procedure must be followed when the bulb is changed.

B.2.1 Remove the diffuser lens between the lamp and microscope or swing it out of the transmitted light path.

B.2.2 Using a prepared microscope slide and a 40X (or similar) objective, adjust the focus so the image in the oculars is sharply defined.

B.2.3 Without the ocular or Bertrand optics in place, view the pupil and filament image at the bottom of the tube.

B.2.4 Focus the lamp filament image with the appropriate adjustment on the lamp house.

B.2.5 Similarly, center the lamp filament image within the pupil with the appropriate adjustment(s) on the lamp house.

B.2.6 Insert the diffuser lens into the light path between the transmitted lamp house and the microscope.

B.3 **Calibration of an ocular micrometer**—This section assumes that a reticle has been installed in one of the oculars by a microscopy specialist and that a stage micrometer is available for calibrating the ocular micrometer (reticle). Once installed, leave the ocular reticle in place. The more an ocular is manipulated the greater the probability is for it to become contaminated with dust particles. Calibrate each objective in use on the microscope following this procedure. If there is a top lens on the microscope, the calibration procedure must be done for the respective objective at each top lens setting. The procedure must be followed when the microscope is first used and each time the objective is changed.

B.3.1 Place the stage micrometer on the microscope stage, turn on the transmitted light, and focus the micrometer image using the coarse and fine adjustment knobs for the objective to be calibrated. Continue adjusting the focus on the stage micrometer so you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.

B.3.2 Adjust the stage and ocular with the micrometer so the “0” line on the ocular micrometer is exactly superimposed on the “0” line on the stage micrometer.

B.3.3 Without changing the stage adjustment, find a point as distant as possible from the two “0” lines where two other lines are exactly superimposed.

B.3.4 Determine the number of ocular micrometer spaces as well as the number of millimeters on the stage micrometer between the two points of superimposition. For example: Suppose 48 ocular micrometer spaces equal 0.6 mm.

B.3.5 Calculate the number of mm/ocular micrometer space. For example:

$$\frac{0.6\ \text{mm}}{48\ \text{ocular micrometer spaces}} = \frac{0.0125\ \text{mm}}{\text{ocular micrometer space}}$$

B.3.6 Because most measurements of microorganisms are given in µm rather than mm, the value calculated above must be converted to µm by multiplying it by 1000 µm/mm. For example:

$$\frac{0.0125\ \text{mm}}{\text{ocular micrometer space}} \times \frac{1{,}000\ \mu\text{m}}{\text{mm}} = \frac{12.5\ \mu\text{m}}{\text{ocular micrometer space}}$$

B.3.7 Follow the procedure for each objective. Record the information as shown in the example below. Make this information available at the microscope. One way of making the information available is to print the chart below on plain paper, fill it in, and affix the chart to the microscope or table with tape.

Item no.	**Objective power**	**Description**	No. of ocular micrometer spaces	No. of stage micrometer mm[1]	µm/ocular micrometer space[2]
1	10X	N.A.[3] =			
2	20X	N.A.=			
3	40X	N.A.=			
4	100X	N.A.=			

[1] 1000 µm/mm

[2] (Stage micrometer length in mm × (1000 µm/mm)) ÷ no. ocular micrometer spaces

[3] N.A. refers to numerical aperture. The numerical aperture value is engraved on the barrel of the objective.

Appendix C
Flow Cytometry-Enumeration Guidelines

C.0 Flow Cytometry-Enumeration Guidelines

Flow cytometry-sorted suspensions are available from commercial vendors and other sources (Section 7.12.1). The information provided in Sections C.1 through C.5 is simply meant as a guideline for the preparation of spiking suspensions using a flow cytometer. Laboratories or vendors performing flow cytometry must develop and implement detailed standardized protocols for calibration and operation of the flow cytometer.

C.1 Spiking suspensions should be prepared using *Cryptosporidium* oocysts <3 months old, and *Giardia* cysts <2 weeks old.

C.2 **Initial calibration.** Immediately before sorting spiking suspensions, an initial calibration of the flow cytometer should be performed by conducting 10 sequential sorts directly onto membranes or well slides. The oocyst and cyst levels used for the initial calibration should be the same as the levels used for the spiking suspensions. Each initial calibration sample should be stained and manually counted microscopically and the manual counts used to verify the accuracy of the system. The relative standard deviation (RSD) of the 10 counts must be documented and should be ≤ 2.5%. If the RSD is > 2.5%, the laboratory or vendor should perform the initial calibration again, until the RSD of the 10 counts is ≤ 2.5%. In addition to counting the organisms, the laboratory or vendor should evaluate the quality of the organisms using DAPI fluorescence and DIC to confirm that the organisms are in good condition. Oocyst or cyst preparations with many (>10%) atypical organisms must be discarded.

C.3 **Ongoing calibration.** When sorting the spiking suspensions for use in QC samples, the laboratory or vendor should perform ongoing calibration samples at a 10% frequency, at a minimum. The laboratory or vendor should sort the first run and every eleventh sample directly onto a membrane or well slide. Each ongoing calibration sample should be stained and manually counted microscopically and the manual counts used to verify the accuracy of the system. The mean of the ongoing calibration counts also should be used as the estimated spike dose, if the relative standard deviation (RSD) of the ongoing calibration counts is ≤ 2.5%. If the RSD is > 2.5%, the laboratory or vendor should discard the batch.

C.4 **Method blanks.** Depending on the operation of the flow cytometer, method blanks should be prepared and examined at the same frequency as the ongoing calibration samples (Section C.3).

C.5 **Holding time criteria.** Flow cytometer-sorted spiking suspensions (Section 7.12.1) used for spiked QC samples (Section 9) must be used within the expiration date noted on the suspension. The holding time specified by the flow cytometry laboratory or vendor should be determined based on a holding time study.