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Method 1693: Cryptosporidium and Giardia in Disinfected Wastewater by Concentration/IMS/IFA

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Acknowledgments

This method is based on the December 2005 revision of EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (www.epa.gov/microbes/).

Jennifer Clancy and Randi McCuin, of Clancy Environmental Consultants, Inc. performed the research and developed the method procedures to adapt EPA Method 1623 for use in wastewater in a project funded by the Water Environment Research Foundation (WERF) and EPA. Both were consulted during the multiple laboratory validation of the method. Their contributions are gratefully acknowledged.

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Introduction

EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA, (Reference 20.1) was approved for monitoring ambient waters for *Cryptosporidium* and *Giardia* in the *Federal Register* final rule of July 21, 2003 (68 FR 43272). National Pollutant Discharge Elimination System (NPDES) permit holders and others have requested that Environmental Protection Agency (EPA) validate *Cryptosporidium/Giardia* methods for evaluation of disinfected wastewater effluents. Method 1693: *Cryptosporidium* and *Giardia* in Disinfected Wastewater by Concentration/IMS/IFA, a modification of Method 1623, includes two sample concentration options: 1) filtration using the Envirochek™ HV capsule (Section 1.1) and 2) direct centrifugation (Section 1.2). Both concentration options use immunomagnetic separation (IMS) and immunofluorescence assay (IFA) microscopy to isolate, detect, and quantify *Cryptosporidium* and *Giardia*.

Method 1693 describes two options (filtration and direct centrifugation) for the detection of *Cryptosporidium* and *Giardia* in disinfected wastewater. The filtration option is a modification of the filter elution and IMS procedures used in Method 1623 designed to optimize the concentration, isolation, and/or identification of *Cryptosporidium* and *Giardia* from disinfected wastewater. The filtration option includes a rinse of the filtered sample with sodium hexametaphosphate and reagent water prior to elution. The IMS procedure is modified by incorporating a phosphate buffered saline (PBS) rinse of the bead-sample complex prior to dissociation.

The direct centrifugation option eliminates the filtration/elution process and concentrates highly turbid or "unfilterable" matrices. This option includes the addition of Tween® 80 to the sample prior to centrifugation, kaolin during the IMS procedure, and a PBS rinse of the magnetic bead-sample complex prior to dissociation to mitigate the effects of sample turbidity and particulates on target organism recovery and detection.

Method 1693 was submitted to single laboratory validation in 2005. Results from this study indicated that the direct centrifugation of spiked reference matrices were not comparable to results observed by the method development laboratory (Reference 20.2). However, data generated using the filtration option indicated that method performance was acceptable in unspiked/spiked reference and wastewater matrices.

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.

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1.0 Scope and Application

1.1 Method 1693 describes a procedure for the detection of *Cryptosporidium* (CAS Registry number 137259-50-8) and *Giardia* (CAS Registry number 137259-49-5) in disinfected wastewater by concentration, immunomagnetic separation (IMS), and immunofluorescence assay (IFA) microscopy. Following detection using fluorescein isothiocyanate (FITC) for the IFA, *Cryptosporidium* and *Giardia* are examined and characterized using Texas Red fluorescence, 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy.

1.2 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.

1.3 This method assumes that the user is experienced in the determination of *Cryptosporidium* and *Giardia* by filtration, IMS, and IFA (*e.g.*, EPA Method 1623). It is suggested that laboratories unfamiliar with analyses of environmental samples by the techniques in this method gain experience using water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using fluorescence, bright-field, and DIC microscopy.

1.4 This method was validated using ColorSeed™, irradiated, pre-stained suspensions of flow cytometer-sorted *Cryptosporidium* (Iowa isolate; Bovine source) and *Giardia* (H3 isolate; Gerbil source) and live suspensions of flow cytometer-sorted *Cryptosporidium* (Harley Moon, Iowa isolate; Bovine source) and *Giardia* (H3 isolate; Gerbil source).

2.0 Summary of Method

2.1 Sample Concentration Options

2.1.1 Filtration option. A 10-L sample is filtered and the (oo)cysts and extraneous materials are retained on the filter. Prior to elution, materials on the filter are rinsed with sodium hexametaphosphate (NaHMP). The rinse prior to elution decreases the impact of turbidity caused by treatment chemicals and inorganic constituents on the recovery of the target organisms. Materials on the filter are then eluted and the eluate is centrifuged to concentrate the oocysts and cysts in the pellet.

2.1.2 Direct centrifugation option. A 1-L unfilterable sample is concentrated by direct centrifugation. Tween® 80 is added prior to centrifugation and the oocysts and cysts are concentrated in the pellet. Tween® 80 is included to help solubilize the fats, oils, and organics which may be present in a sample deemed to be unfilterable.

2.2 Sample separation (purification)

The concentrated oocysts and cysts are recovered by IMS using magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. The magnetic bead-sample complexes are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. For samples concentrated by direct centrifugation, this separation step includes the addition of kaolin to adsorb fats, oils, organics, and heavy particulates which may be present in a sample deemed to be unfilterable. The magnetic bead-sample complex is then rinsed prior to dissociation and recovery of the oocysts and cysts.

2.3 Enumeration

2.3.1 The purified oocysts and cysts are fixed on well slides and stained with FITC-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 well of the slide for objects that meet the size, shape, structural and fluorescence characteristics of *Cryptosporidium* oocysts or *Giardia* cysts.

2.3.3 Quantitative analysis is performed by counting the total number of fluorescent objects on the slide characterized as 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 analyses is provided in Section 9.0.

3.0 Definitions

3.1 *Cryptosporidium* is a protozoan parasite potentially found in water and other media. *Cryptosporidium* oocysts are defined in this method as objects exhibiting brilliant apple green fluorescence under ultraviolet (UV) light (IFA-positive), typical size (4 to 6 µm) and shape (round to oval), no atypical characteristics by IFA, DAPI fluorescence, or DIC microscopy, and if ColorSeed™ is used, no Texas Red fluorescence under UV light. Examination and characterization using fluorescence (IFA 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 protozoan parasite potentially found in water and other media. *Giardia* cysts are defined in this method as objects exhibiting brilliant apple green fluorescence under UV light (IFA-positive), typical size (8 to 18 µm long by 5 to 15 µm wide) and shape (oval to round), no atypical characteristics by IFA, DAPI fluorescence, or DIC microscopy, and if ColorSeed™ is used, no Texas Red fluorescence under UV light. Examination and characterization by fluorescence (IFA 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 can be found in the glossary (Section 21.0).

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.*, clay and algae), chemicals (*e.g.*, coagulants and polymers) added to wastewaters 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 IFA (Reference 20.3).

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 the 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 All equipment should be cleaned according to manufacturers' instructions. Disposable supplies should be used wherever possible.

5.0 Safety

5.1 The biohazard associated with, and the risk of infection from, oocysts and cysts are high in this method because the matrix may contain live organisms and live organisms may be used for sample spiking. In addition, other potentially pathogenic organisms may be found in the wastewater matrices. This method does not address all of the safety issues 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.

5.2 The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced 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. A reference file of material safety data sheets should be made available to all personnel involved in these analyses.

5.3 Samples may contain high concentrations of biohazards and toxic compounds, and should be handled with gloves to prevent exposure. Samples may be opened in a biological safety cabinet to prevent exposure. Reference materials and standards containing oocysts and cysts should also be handled with gloves and laboratory staff should never place gloves in or near the face after exposure to solutions known or suspected to contain oocysts and cysts. Do not mouth-pipette.

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

5.5 The U.S. Department of Transportation (DOT) Hazardous Material Regulations (49 CFR 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 20.4) 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 Regulations.

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 other than those specified here. Demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

6.1 General Supplies

6.1.1 Disposable serological pipets 10 and 25 mL

6.1.2 Disposable Pasteur pipets

6.1.3 Micropipettors with 10, 20, 200 and 1000 µL capacity

6.1.4 Micropipettor tips for 10, 20, 200 and 1000 µL capacity micropipettors

6.1.5 Graduated cylinders, autoclavable − 10, 100, and 1000 mL

6.1.6 10 to 15 L graduated containers − calibrate to 9.0, 9.5, 10.0, 10.5, and 11.0 L and mark levels with waterproof marker

6.1.7 Disposable collapsible 20 L Low-Density Polyethylene (LDPE) cubitainer (Fisher 05-721-122 or equivalent)

6.1.8 Cubitainer spigot to facilitate filtration of sample in the laboratory

6.1.9 Stir bars

6.1.10 Filters for filter-sterilizing reagents − Sterile Acrodisc, 0.45 µm pore size (Fisher 09-740-35K or equivalent)

6.1.11 Sample filtration capsule − Envirochek™ HV, Pall Corporation 12099 (individual filter) or 12098 (box of 25 filters)

6.1.12 Hemacytometer or Well slides (Dynal 740.04 or 12-mm diameter well slides, Meridian Diagnostics Inc. R2206 or equivalent)

6.1.13 Slide Cover slips 22 × 50 mm or 18 mm2 or appropriate size

6.1.14 Non-fluorescing immersion oil

6.1.15 Lens paper without silicone

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

6.1.17 Glass microanalysis filter holder − 25 mm diameter, with fritted glass support. (Note: Replace stopper with size 8, one-hole rubber stopper).

6.1.18 Cellulose acetate support membrane − 1.2 µm pore size, 25 mm diameter.

6.1.19 Polycarbonate track-etch hydrophilic membrane filter − 1µm pore size, 25 mm diameter.

6.1.20 100 × 15 mm polystyrene petri dishes

6.1.21 60 × 15 mm polystyrene petri dishes

6.1.22 Centrifuge tubes − Conical, graduated, plastic, 250 mL and other sizes as needed

6.1.23 Test tubes and rack

6.1.24 Flasks − Suction, Erlenmeyer, 2 or 4 L

6.1.25 Tubing − Glass, polytetrafluoroethylene (PTFE), high-density polyethylene (HDPE), or other tubing to which oocysts and cysts will not easily adhere. 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. Before use, the tubing should 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 when wear is evident.

6.1.26 Beakers − Glass or plastic, 5, 10, 50, 100, 500, 1000, and 2000 mL

6.1.27 Lint-free tissues

6.1.28 Wash bottles for buffer solutions

6.1.29 Dropper bottles for reagents

6.1.30 Powder-free latex gloves

6.2 Equipment

6.2.1 Stir plate

6.2.2 Pump − peristaltic, centrifugal, impeller, or diaphragm pump. 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.

6.2.3 Vacuum pump/source − Capable of maintaining 25 in. Hg, equipped with shutoff valve and vacuum gauge

6.2.4 Tubing − Glass, PTFE, HDPE, or other tubing to which oocysts and cysts will not easily adhere. 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. Before use, the tubing should 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 when wear is evident. Dispose of tubing after one use whenever possible.

6.2.5 Flow control valve − 0.5 gpm (2 Lpm) valve or 0.4 to 4 Lpm flow meter with valve

6.2.6 Flow meter − Alternatively, use a graduated container(s) (Section 6.1.6).

6.2.7 Laboratory shaker − Multi-Wrist® Lab Line model 3589 (VWR Scientific 57039 055, Fisher 14260 11, or equivalent)

6.2.8 Centrifuge − swinging bucket rotor capable of accepting 15 to 250 mL conical centrifuge tubes and achieving 1500 × g

6.2.9 Analytical balance capable of weighing 0.1 mg

6.2.10 Top loading balance capable of weighing 10 mg

6.2.11 pH meter

6.2.12 Incubator capable of operating at 36°C ± 1°C

6.2.13 Vortex mixer

6.2.14 Autoclave or sterilizer

6.3 Immunomagnetic separation (IMS) equipment and supplies

6.3.1 Sample mixer (Dynal Inc. 947.01 or equivalent)

6.3.2 Magnetic particle concentrator for 10 mL IMS sample tubes (Dynal MPC®-1 or MPC> -6, 120.01 or equivalent)

6.3.3 Magnetic particle concentrator for microcentrifuge tubes (Dynal MPC®-S, 120.20, or equivalent)

6.3.4 IMS sample tubes, 16 × 125 mm flat-sided glass tubes with 60 × 10 mm flat-sided magnetic capture area, (Dynal® L10 740.03, or equivalent)

6.3.5 Microcentrifuge tubes − 1.5 - 2.0 mL conical tubes

6.4 Microscope requirements

6.4.1 Epifluorescence/differential interference contrast (DIC) with stage and ocular micrometers and 20X (N.A.=0.4) to 100X (N.A.=1.3) objectives (Zeiss™ Axioskop, Olympus® BH, or equivalent)

6.4.2 Excitation/band-pass filters for FITC fluorochrome (Zeiss™ 487909 or equivalent) including, 450- to 490-nm exciter filter, 510-nm dichroic beam-splitting mirror, and 515- to 520-nm barrier or suppression filter

6.4.3 Excitation/band-pass filters for DAPI fluorochrome (Chroma Technology 11000 or equivalent) including 340-380 nm exciter filter, 400 nm dichroic beam-splitting mirror, and 420 nm barrier or suppression filter

6.4.4 Excitation/band-pass filters for Texas Red fluorochrome − (Chroma Technology 41004 or equivalent, including 530-585 nm exciter filter, 580 nm dichroic beam-splitting mirror, and 615 nm barrier or suppression filter. The ideal excitation wavelength is 580 nm; ideal barrier (emission) wavelength is 615 nm. Filter sets for all three fluorochromes should be optimized together on a particular microscope to ensure proper viewing of each fluorochrome.

7.0 Reagents and Standards

7.1 Reagents for adjusting pH

7.7.1 Sodium hydroxide (NaOH) − ACS reagent grade, 6.0N and 1.0N in reagent water

7.7.2 Hydrochloric acid (HCl) − ACS reagent grade, 6.0N, 1.0N, and 0.1N in reagent water

Note: Due to the low volumes of pH adjusting reagents used in this method, and the impact that changes in pH have on the immunofluorescence assay, the laboratory should purchase standards at the required normality directly from a vendor. Normality should not be adjusted by the laboratory.

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

7.3 Reagent-grade water − Water in which oocysts and cysts and interfering materials and substances, including magnetic minerals, are not detected by this method. See Reference 20.5 (Section 9020) for reagent-grade water requirements.

7.4 Reagents for rinsing filters prior to elution (Section 12.2.6.2) − Sodium Hexametaphosphate solution (NaHMP), 5% − Dissolve 50 g $(NaPO_3)_n$ Na_2O (where n>13) in 800 mL reagent water; dilute to 1000 mL. Store in glass or plastic at room temperature for up to one year. Discard when expiration date is reached or if microbial growth is apparent.

7.5 Reagents for eluting Envirochek™ HV sampling capsule

Note: Laboratories should store prepared eluting solution for no more than 1 week or when noticeably turbid, whichever is sooner.

7.5.1 Tween® 80 (Sigma P 4780 or equivalent)

7.5.2 Phosphate buffered saline (PBS), pH 7.4 − If using prepared PBS packets, add the contents of one sachet of PBS to 1.0 L of reagent water and dissolve by stirring for 30 minutes. Alternately, prepare PBS by adding the following to 1 L of reagent-grade water: 8 g NaCl; 0.2 g KCl; 1.15 g Na_2HPO_4, anhydrous; and 0.2 g KH_2PO_4.

7.5.3 Antifoam Y-30 (Sigma A6457 or equivalent)

7.5.4 Phosphate Buffered Saline with Tween® 80 (PBST) elution buffer − Add 0.75 mL of 20% Tween® 80 (Section 7.6) and 150 µL Antifoam Y-30 (Section 7.5.3) to 1 L of PBS (Section 7.5.2). Stir to mix for 5 minutes.

7.6 Reagent for sample concentration by direct centrifugation

Tween® 80 solution (20%) − Add 200 mL of Tween® 80 (Section 7.5.1) to 700 mL of reagent water and stir to mix thoroughly. Adjust final volume to 1 L with reagent water. Store at room temperature for up to 1 month. ***Do not use if solution appears cloudy or contains precipitates.***

7.7 Reagents for immunomagnetic separation (IMS)

7.7.1 Dynabeads® GC-Combo (Dynal 730.02, 730.12, or equivalent)

7.7.2 Phosphate buffered saline (PBS) − Section 7.5.2

7.7.3 Kaolin − Sigma Chemical Co. K7375, or equivalent

7.8 **Reagents for immunofluorescence assay (IFA) of oocysts and cysts** − Store reagents at 0°C to 10°C and return promptly to this temperature after each use. Do not allow any of the reagents to freeze. The FITC fluorochrome is light sensitive; therefore, IFA reagents should be protected from exposure to light. Diluted, unused working reagents should be discarded after 48 hours. Discard reagents after the expiration date is reached.

7.8.1 EasyStain™, BTF Pty Ltd, Sydney, Australia, or equivalent

7.8.2 Aqua-Glo™ G/C Direct FL, Waterborne A100FLR, New Orleans, LA, or equivalent

7.8.3 Crypt-a-Glo™ and Giardi-a-Glo™, Waterborne cat. nos. A400FLR and A300FLR, respectively, New Orleans, LA, or equivalent

7.8.4 Merifluor™ *Cryptosporidium*/*Giardia*, Meridian Bioscience, Inc. 250050, Cincinnati, OH or equivalent

7.8.5 Diluent for IFA-labeling reagents − PBS (Section 7.5.2).

Note: If a laboratory will use multiple types of labeling reagents, the laboratory should demonstrate acceptable performance through initial precision and recovery (IPR) analyses (Section 9.3) for each type, and should 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 also should be monitored in each wastewater or combined sewer overflow (CSO) water type. Careful observation should be made regarding the cross reactivity of the stains used in the matrices analyzed. The choice of stain should be based on the staining quality for the (oo)cysts as well as the cross reactivity with the matrices typically analyzed.

7.8.6 DAPI stain − Sigma Chemical Co. D9542, or equivalent

7.8.6.1 Stock solution − Dissolve 2 mg DAPI in 1 mL absolute methanol. Purchase in small quantities to eliminate weighing of powder. Prepare volume consistent with minimum use. Store at 0°C to 10°C in the dark (do not allow to freeze).

7.8.6.2 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 at 0°C to 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 should be tested first on expendable environmental samples to confirm that staining intensity is appropriate.

7.8.7 Slide mounting medium

7.8.7.1 DABCO/glycerol mounting medium (2%) − Dissolve 2 g of DABCO 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. Store in glass at room temperature for up to six months. Discard after expiration date.

7.8.7.2 Mounting medium supplied with EasyStain™ (Section 7.8.1)

7.8.7.3 Mounting medium supplied with Aqua-Glo™ G/C Direct FL kit (Section 7.8.2)

7.8.7.4 Mounting medium supplied with Merifluor™ *Cryptosporidium*/*Giardia* (Section 7.8.4)

7.8.7.5 Clear fingernail polish or equivalent

7.9 Reagents for spiking

7.9.1 Antifoam Y-30 (Sigma A6457 or equivalent)

7.9.1.1 Diluted Antifoam Y-30 Dissolve 400 µL of Antifoam Y-30 in 100 mL of reagent water and mix well to emulsify

7.9.2 Tween® 20 (Sigma P 2287 or equivalent)

7.9.2.1 Dilute in reagent water to 0.01%

7.9.3 Tween® 80 (Sigma P 4780 or equivalent)

7.9.3.1 Dilute in reagent water to 20%

7.9.4 Oocyst and cyst suspensions for spiking

7.9.5 Enumerated spiking suspensions prepared by flow cytometer – not formalin fixed

7.9.5.1 Pre-stained, irradiated, flow cytometer-sorted oocysts and cysts – BTF ColorSeed™, or equivalent

7.9.5.2 Live, flow cytometer-sorted oocysts and cysts – Wisconsin State Laboratory of Hygiene Flow Cytometry Unit (608-224-6260), or equivalent

7.9.5.3 Irradiated, flow cytometer-sorted oocysts and cysts – BTF EasySeed™, or equivalent

7.9.6 Purified stock suspensions − not formalin-fixed

7.9.6.1 Purified *Cryptosporidium* oocyst stock suspension for manual enumeration: Sterling Parasitology Laboratory, University of Arizona; Waterborne, Inc.; or equivalent. Use oocysts within 3 months of date shed.

7.9.6.2 Purified *Giardia* cyst stock suspension for manual enumeration: Waterborne, Inc., New Orleans, LA; Hyperion Research, Medicine Hat, Alberta, Canada; or equivalent. Use cysts within 2 weeks of date shed.

7.9.7 Storage procedure − Store oocyst and cyst suspensions at 0°C to 10°C, until ready to use; do not allow to freeze

7.9.8 Additional reagents for dilution, manual enumeration and use of spiking suspensions (See Appendix B)

8.0 Sample Collection and Storage

8.1 Sample collection, shipment, and receipt

8.1.1 Samples are collected as bulk samples and shipped to the laboratory for processing through the entire method.

8.1.2 Wastewater and CSO 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. Samples that are not analyzed the same day they are collected should be chilled to reduce biological activity, and preserve the state of wastewater and CSO samples between collection and analysis. Samples analyzed by an off-site laboratory should be shipped via overnight service on the day they are collected. Overnight service may not be necessary if the samples are maintained at <10°C (but not frozen) and holding times are met.

Note: See transportation precautions in Section 5.5.

8.1.2.1 If samples are collected early in the day, chill samples as much as possible between collection and shipment by storing in a refrigerator 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 held overnight in a refrigerator. This should be considered 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 >10°C and above freezing.

8.1.2.3 If samples are shipped after collection at >10°C, there should be enough ice in the cooler to chill the samples and maintain the temperature during shipment at <10°C and above freezing.

8.1.2.4 Public wastewater 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 10°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 should record the sample temperature. Samples that were not collected the same day they were received, and that are received at >10°C or frozen, or samples that the laboratory has determined did not maintain sample temperature <10°C after chilling or froze during shipment, should be rejected. After receipt, samples should be stored at the laboratory at <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 should establish acceptance criteria for receipt of

samples transported to their laboratory. Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment:

8.1.4.1 Thermocron® iButton. Measures the sample temperature during shipment and upon receipt. An iButton is a small, waterproof device that contains a computer chip that can be programmed to record temperature at different time intervals. The information is then downloaded from the iButton onto a computer. The iButton should be placed in a temperature sample, rather than placed loose in the cooler, or attached to the sample container. Information on Thermocron® iButtons is available at http://www.maxim-ic.com/products/ibutton/.

8.1.4.2 Stick-on temperature strips. Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but still mitigates the risk of sample contamination while providing an indication of sample temperature to verify that the sample temperature is acceptable. (Cole Parmer U-90316-00 or equivalent).

8.1.4.3 Infrared thermometers. Another option is to measure the temperature of the surface of the sample container or the sample using an infrared thermometer. The thermometer is pointed at the sample container or through the open lid, and measures the temperature without coming in contact with the sample volume. (Cole Parmer EW-39641-15 or equivalent).

8.2 Sample holding times. Samples should be processed or examined within each of the holding times specified in Sections 8.2.1 through 8.2.4. Sample processing should be completed as soon as possible by the laboratory. The laboratory should complete sample filtration, elution, concentration, purification, and staining the day the sample is received whenever possible. However, the laboratory should split up the sample processing steps if processing a sample completely in one day is not possible. If this is necessary, sample processing may be halted after filtration, application of the purified sample onto the slide, or staining. **Table 1** provides a breakdown of the holding times for each sample processing step.

Table 1. Method Holding Times

Sample Processing Step	Maximum Allowable Time between Breaks *(Samples should be processed as soon as possible)*
Collection	
Filtration	
Ə Up to 96 hours are acceptable between sample collection and initiation of concentration	
Concentration by filtration or direct centrifugation	Should be completed in one working day
Purification (IMS)	
Application of purified sample to slide	
Drying of sample	
Ə Up to 72 hours are acceptable from application of the purified sample to the slide to staining	
Staining	
Ə Up to 7 days are acceptable between sample staining and examination	
Examination	

8.2.1 Sample collection and concentration. Sample concentration should be initiated within 96 hours of sample collection.

8.2.2 Sample purification. The laboratory should complete the concentration (filtration or centrifugation), filter elution (if applicable), purification, and application to slide (Sections 12.2.6 through 13.3.3.12) 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 remains in eluate or concentrated matrix.

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

8.2.4 Examination. Although IFA, DAPI, and DIC microscopy examination and characterization should be performed immediately after staining is complete, laboratories may take up to 7 days (168 hours) from completion of sample staining to complete the examination and confirmation of samples. However, if fading/diffusion of the IFA or the DAPI fluorescence is noticed, the laboratory should reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Section 7.8.6.2) so that fading/diffusion does not occur.

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.

9.2 The minimum analytical requirements for Method 1693 include an initial demonstration of capability (IDC) through performance of IPR analyses (Section 9.3) and matrix spike/matrix spike duplicate (MS/MSD) analyses (Section 9.5), method blank (MB) analysis (Section 9.6), ongoing precision and recovery (OPR) analysis (Section 9.4), staining controls (Section 9.7), and

analyst verification (Section 9.13). A summary of Method 1693 quality control (QC) analyses is provided in **Table 2**.

Table 2. Summary of Required Quality Control

QC Sample or Procedure	Matrix	Number of Samples	Frequency	Purpose	Ongoing Statement of Accuracy Required
IDC	Reference matrix[1] and sample matrix	8	Initial use of method and each method modification; consists of IPR set, Method blank, and MS/MSD with field sample	To establish initial control over the analytical system	No
IPR	Reference matrix [1]	4	Initial use of method and each method modification	To establish initial control over the analytical system and demonstrate acceptable method performance	No
Method Blank	Reference matrix[1]	1	Each IPR set; each week samples are processed or every 20 samples	To demonstrate the absence of contamination throughout the analytical process	No
OPR	Reference matrix[1]	1	Each week samples are processed or every 20 samples	To demonstrate ongoing control of the analytical system and verify continuing method performance	Yes
MS	Sample matrix	1	Every sample if ColorSeed™ is used. First and every 20th sample per matrix if ColorSeed™ is not used	To determine the effect of the matrix on (oo)cyst recoveries	Yes - per matrix
MSD	Sample matrix	1	Recommended for method modifications	To demonstrate consistency of (oo)cyst recoveries in a matrix	No
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
Negative staining control	None	1	Each time samples are stained	To demonstrate the absence of contamination through staining process	No

[1] The filtration option uses reagent water and the centrifugation option uses PBS as reference matrix.

9.2.1 Laboratory performance is evaluated by comparing the results of all required QC analyses to established performance criteria, **Tables 3** and **4**, to verify that the laboratory's analytical results meet the method performance requirements.

9.2.2 If more than 1 concentration option or method variation is used, separate IPR analyses, method blanks, and OPR analyses should be prepared and completed for each concentration option and method variation. Adjustment and/or recalibration of the analytical system should be performed until all performance criteria are met. Only after all method performance criteria are met should samples be analyzed.

9.3 Initial precision and recovery (IPR). The IPR analysis is used to demonstrate acceptable method performance (recovery and precision) before the method is used for monitoring samples. Each analyst should complete and document the procedures in Sections 9.3.1 through 9.3.8. EPA recommends but does not require that IPR analyses be conducted by each analyst.

9.3.1 Using enumerated spiking suspensions (Section 7.9.1 or Appendix B), spike between 100-500 oocysts and 100-500 cysts into each of four reference matrix samples, reagent water for the filtration option and PBS for the centrifugation option, following the spiking procedure in Section 11.2 or 11.3. Concentrate, separate (purify by IMS), stain, and examine the samples according to the procedures in Sections 12 - 15. The volume of each spiked sample should be 10 L for the filtration option or 1 L for the direct centrifugation option.

9.3.2 Identify and enumerate each organism using epifluorescence microscopy. The first three *Cryptosporidium* oocysts and three *Giardia* cysts identified in each IPR sample should be examined using fluorescence (IFA and DAPI stain) and DIC microscopy, as per Section 15. If pre-stained spiking suspensions, such as ColorSeed™, are used, the first three oocysts and cysts should also be examined using the Texas Red filter, as per Section 15. The detailed characteristics (size, shape, DAPI category, and DIC category) are reported on the *Cryptosporidium* and *Giardia* examination form, as well as any additional comments on organism appearance, if notable. If ColorSeed™ is used, the presence/absence of red fluorescence is also reported on the examination form.

9.3.3 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 (oo)cysts detected
T = the number of (oo)cysts spiked

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

9.3.5 Compare the mean recovery and RSD to the corresponding method performance criteria limits for IPR in Tables 3 and 4. If the mean and RSD for recovery meet the method performance criteria, system performance is acceptable and analysis of samples may begin. If the mean or the RSD fall outside the acceptable range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, starting from the end of the method (see guidance in Section 9.8), correct the problem and repeat the IPR analyses.

9.3.6 The laboratory should analyze the four IPR samples on the same day or on as many as four different days, and may use different analysts and/or reagent lots for each sample (however, the procedures used for all analyses should be identical). Laboratories should 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 analyses are performed across multiple days, analysts, and /or reagent lots.

9.3.7 The processing and analysis of the four IPR samples should be accompanied by an acceptable method blank (Section 9.6).

9.3.8 Examine the slides from the IPR analyses using DIC microscopy and 400-1000X magnification. More than 50% of the oocysts or cysts should 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 sample 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 spiking organisms appear undamaged and morphologically intact under DIC microscopy, determine the processing step or reagent that is causing damage to the organisms. Correct the problem (see Section 9.8 for guidelines) and repeat the IPR analyses.

Table 3. Calculated Initial and Ongoing Precision and Recovery Criteria for the Filtration Option using 10 L of Reference Matrix (Reagent Water)

Organism	Performance Test	Section	ColorSeed™ Spike Acceptance Criteria	WSLH Spike Acceptance Criteria
Cryptosporidium	Initial precision and recovery (IPR) • Mean percent recovery • Precision[1]	9.3	49% – 100% 22%	55% – 100% 17%
	Ongoing precision and recovery (OPR)[2]	9.7	46% – 100%	52% – 100%
Giardia	Initial precision and recovery (IPR) • Mean percent recovery • Precision[1]	9.3	26% – 100% 29%	33% – 100% 23%
	Ongoing precision and recovery (OPR)[2]	9.7	25% – 100%	31% – 100%

[1] Precision as maximum relative standard deviation
[2] Ongoing precision and recovery (OPR) as percent recovery

Table 4. Calculated Initial and Ongoing Precision and Recovery Acceptance Criteria for the Centrifugation Option Using 1 L of Reference Matrix (PBS)

Organism	Performance Test	Section	ColorSeed™ Spike Acceptance Criteria	WSLH Spike Acceptance Criteria
Cryptosporidium	Initial precision and recovery (IPR) • Mean percent recovery • Precision[1]	9.3	16% – 100% 36%	12% – 100% 37%
	Ongoing precision and recovery (OPR)[2]	9.7	14% – 100%	11% – 100%
Giardia	Initial precision and recovery (IPR) • Mean percent recovery • Precision[1]	9.3	24% – 100% 47%	15% – 100% 44%
	Ongoing precision and recovery (OPR)[2]	9.7	18% – 100%	12% – 100%

[1] Precision as maximum relative standard deviation
[2] Ongoing precision and recovery (OPR) as percent recovery

9.4 Ongoing precision and recovery (OPR; positive control sample; laboratory control sample) The OPR analysis is performed to demonstrate ongoing control of the analytical system and verify continuing method performance (recovery and precision). The laboratory should analyze one OPR sample each week (7 day time period which begins with processing the OPR) in which samples are analyzed if 20 or fewer field samples are analyzed during this period. The laboratory should analyze one OPR sample for every 20 samples if more than 20 samples are analyzed in a one week (7 day) period.

9.4.1 Using enumerated spiking suspensions (Section 7.9.1 or Appendix B), spike between 100-500 oocysts and 100-500 cysts into each sample following the spiking procedure in Section 11.2 or 11.3. Concentrate, separate (purify by IMS), stain, and examine a reference matrix sample, reagent water for the filtration option or PBS for the centrifugation option, according to the procedures in Sections 12 through 15. The volume of each spiked sample should be 10 L for the filtration option or 1 L for the direct centrifugation option.

9.4.2 Identify and enumerate each organism using epifluorescence microscopy. The first three *Cryptosporidium* oocysts and three *Giardia* cysts identified in the OPR sample should be examined using fluorescence (IFA and DAPI stain) and DIC microscopy as per Section 15. If ColorSeed™ is used, the first three oocysts and cysts should also be examined using the Texas Red filter, as per Section 15. The detailed characteristics (size, shape, DAPI category, and DIC category) are reported on the *Cryptosporidium* and *Giardia* examination form, as well as any additional comments on organism appearance, if notable. If ColorSeed™ is used, the presence/absence of red fluorescence is also reported on the examination form.

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

$$N = 100 \times \left(\frac{\mathrm{N_R}}{\mathrm{T}}\right)$$

where:
R = the percent recovery
N_R = the number of (oo)cysts detected

T = the number of (oo)cysts spiked

9.4.4 Compare the recovery with the method performance criteria for ongoing precision and recovery in Tables 3 and 4.

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

9.4.4.2 If the recoveries for *Cryptosporidium* or *Giardia* fall outside of the method performance criteria, system performance is unacceptable. Any sample associated with an unacceptable OPR sample is unacceptable. Analysis of additional samples is halted until the analytical system is brought under control. Identify the problem using the procedures in Section 9.8 as a guide. After assessing the issue, perform another OPR analysis and verify that *Cryptosporidium* and *Giardia* recoveries meet the acceptance criteria.

9.4.5 The processing and analysis of the OPR sample should be accompanied by an acceptable method blank (Section 9.6).

9.4.6 Examine the slide from the OPR test using DIC microscopy and 400-1000X magnification. More than 50% of the oocysts or cysts should 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 OPR slide are 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 spiking organisms appear undamaged and morphologically intact under DIC microscopy, determine the processing step or reagent that is causing damage to the organisms. Correct the problem (see Section 9.8 for guidelines) and repeat the OPR analysis.

9.5 Matrix spike/Matrix Spike Duplicate (MS/MSD). The MS analysis is performed to determine the effect of the matrix on oocyst and cyst recoveries. A MS sample is analyzed as part of the initial demonstration of capability. The laboratory performs the analysis of the MS and unspiked field sample using the same sample if ColorSeed™ is used as the spiking suspension. Alternatively, the laboratory should analyze an additional sample as the MS at a frequency of 1 every 20 samples from a specific site if pre-stained (oo)cysts are not used. It is recommended that the unspiked field sample be analyzed prior to the matrix spike sample to determine the ambient concentrations of (oo)cysts and the appropriateness of the spiked sample volume.

9.5.1 Spike a field sample using the enumerated spiking suspension (Section 7.9.1 or Appendix B) and the spiking procedure in Section 11.2 or 11.3. Concentrate (filtration or direct centrifugation), separate (purify by IMS), stain, and examine the spiked field sample (MS) according to Sections 12 through 15.

9.5.1.1 The MS/MSD sample should be prepared following the same processing option as the field sample. The processing and analysis of all MS/MSD and field samples should be consistent with the associated IPR, OPR, and MB procedures.

9.5.1.2 One sample should be collected from the sampling location, if ColorSeed™ is used as the spiking suspension. The MS and unspiked field sample should be collected from the same sampling location as split samples or as samples sequentially collected immediately after one another if ColorSeed™ is not used as the spiking suspension. The MS sample volume analyzed should be within 10% of the unspiked field sample volume.

9.5.2 Examine the slides from the MS sample and the unspiked field sample, if analyzed. Identify and enumerate each organism by FITC and Texas Red using epifluorescence microscopy if ColorSeed™ is used. The first three *Cryptosporidium* oocysts and three *Giardia* cysts identified in the MS sample should be examined using fluorescence (IFA and DAPI stain) and DIC microscopy, as per Section 15. The detailed characteristics (size, shape, DAPI category, and DIC category) are reported on the *Cryptosporidium* and *Giardia* examination form, as well as any additional comments on organism appearance, if notable. If ColorSeed™ is used, the presence/absence of red fluorescence is also reported on the examination form.

9.5.3 For each organism, calculate the percent recovery (R) using one of the following equations.

For ColorSeed™,

$$N = 100 \times \left(\frac{N_R}{T}\right)$$

where:
R = the percent recovery
N_R = the number of oocysts or cysts detected with red fluorescence
T = the true value of the oocysts or cysts spiked

For non pre-stained organisms,

$$N = 100 \times \left(\frac{N_s - N_u}{T}\right)$$

where:
R = the percent recovery
N_s = the number of oocysts or cysts detected in the spiked sample
N_u = the number of oocysts or cysts detected in the unspiked sample
T = the true value of the oocysts or cysts spiked

9.4.5 Compare the recovery for each organism with the corresponding criteria for MS recovery in Tables 3 and 4.

9.5.5 As part of the QA program for the laboratory, method precision for samples should be assessed and records maintained. After the analysis of five MS samples from a particular source, the laboratory should calculate the average percent recovery (P) and the standard deviation of the percent recovery (s_r). Express the precision assessment as a percent recovery interval from P - 2 s_r to P + 2 s_r for each matrix. For example, if P = 80% and s_r = 30%, the accuracy interval is expressed as 20% to 140%. The precision assessment should be updated regularly for each MS sample source.

Table 5. Calculated MS or MS/MSD Criteria for the Filtration Option

Organism	Performance Test	WSLH Spike Acceptance Criteria	ColorSeed™C&G Spike Acceptance Criteria
Cryptosporidium	Percent recovery for MS or MS/MSD	18% – 100%	29% – 100%
	Precision [a]	70%	56%
Giardia	Percent recovery for MS or MS/MSD	16% – 100%	21% – 100%
	Precision [a]	75%	55%

[a] Precision as maximum RSD of MS or MS/MSD

9.6 **Method blank (negative control sample, laboratory blank).** Reference matrix (reagent water or PBS) blanks are routinely analyzed to demonstrate the absence of contamination throughout the analytical process. The laboratory should analyze one method blank each week (7 day time period that begins with processing the OPR) during which samples are analyzed if 20 or fewer field samples are analyzed during this period. The laboratory should analyze one method blank for every 20 samples if more than 20 samples are analyzed in a 1 week (7 day) period.

9.6.1 Concentrate, separate (purify by IMS), stain, and examine a reference matrix sample, reagent water using the filtration option or PBS using the direct centrifugation option, according to the procedures in Sections 12 through 15. The volume of each method blank should be 10 L for the filtration option or 1 L for the direct centrifugation option.

9.6.2 Processing and analysis of method blanks should accompany the processing and analysis of the IPR (Section 9.3) and OPR sample (Section 9.4), and should be performed immediately after the IPR samples and OPR sample but prior to the processing and analysis of samples.

9.6.3 If *Cryptosporidium* oocysts, *Giardia* cysts or potentially interfering organisms or materials that may be misidentified as (oo)cysts are **NOT** found in the method blank, the method blank analysis is acceptable and analysis of samples may proceed.

9.6.4 If *Cryptosporidium* oocysts or *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 analysis is unacceptable. Any field sample associated with an unacceptable method blank is assumed to be contaminated and should be recollected and analyzed. Further analysis of samples is halted until the source of contamination is eliminated (see guidance in Section 9.8), the method blank analysis is performed again, and no evidence of contamination is detected.

9.7 **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 should prepare a positive and negative staining control (Section 14.1) each time samples are stained. Positive and negative staining controls should be acceptable before proceeding with sample examination.

9.7.1 Prepare positive and negative immunofluorescent, Texas Red, and DAPI staining controls as recommended by the specific reagent manufacturer or supplier (Section 14.1).

9.7.2 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.

9.7.3 Examine the positive staining control(s) to confirm that it contains oocysts and cysts with the appropriate fluorescence for IFA, DAPI stain, and Texas Red, if ColorSeed™ is used (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.

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

9.8 **Method troubleshooting.** If any of the required QC analyses (IPR, OPR, MB, MS/MSD) fail to meet the specified method performance criteria, the laboratory should correct the problem. The laboratory should identify the problem by working backward in the analytical process from the final microscopic examination to initial sample concentration.

9.8.1 Microscope system and antibody stain: To determine if the failure of the QC analysis is due to changes in the microscope or problems with the antibody stain, re-examine the positive staining control(s) (Section 9.7) associated with the QC failure, check Köhler illumination (Appendix A), and the fluorescence of the fluorescein-labeled monoclonal antibodies (Mabs), DAPI, and Texas Red, if ColorSeed™ is used. 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. Refer to Appendix A for microscopic adjustments.

9.8.2 Quality of spiked 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 damaged under DIC microscopy, 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.

9.8.3 Separation (purification) system: To determine if the failure of the QC test is attributable to the IMS purification system, check system performance by spiking a 10-mL volume of reference matrix with 100 - 500 oocysts and cysts and processing the sample through the IMS, staining, and examination procedures in Sections 13.3 through 15.0. Recoveries should be greater than 70%.

9.8.4 Filtration/elution/concentration system: To determine if the failure of the QC test is attributable to the initial concentration step (filtration/elution or direct centrifugation), check system performance by processing spiked reagent water or PBS according to the procedures in Section 12.2 through 13.2, and filter, stain, and examine the sample concentrate according to Appendix B.

9.9 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.

9.10 The laboratory should maintain records to define the quality of data that are generated. Development of accuracy statements is described in Sections 9.5.5 and 9.10.1.

9.10.1 The laboratory should add results that pass specifications to initial and ongoing data and update the control chart to form a graphic representation of continued laboratory performance. The laboratory should develop a statement of laboratory accuracy for each matrix type (reagent water, PBS, disinfected wastewater, or CSO) by calculating the average 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. For example, if R = 95% and s_r = 25%, the accuracy is 45% to 145%.

9.10.2 The laboratory should periodically analyze an external QC sample, such as a performance testing (PT) sample or standard reference material, when available. The laboratory also should periodically participate in interlaboratory comparison studies using the method.

9.11 The IPR (Section 9.3), OPR (Section 9.4), and MS/MSD procedures, reagents, and equipment should be identical, so that the most precise results will 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.

9.12 A test of the microscope used for detection of oocysts and cysts should be performed prior to examination of slides. Adjust the microscope as specified in Section 10.0 and Appendix A. All of the requirements in Section 10.0 and Appendix A should be met prior to analysis of samples (IPR, method blanks, OPR, field samples, and MS/MSD) in order to obtain reliable results.

9.13 **Verification of analyst performance.** This method relies upon the ability of the analyst for correct identification and enumeration of oocysts and cysts. Until standard reference materials, such as National Institute of Standards and Technology standard reference materials, are available that contain a reliable number of DAPI- and DIC-positive or negative oocysts and cysts, the procedures below are required of each analyst to verify performance.

9.13.1 At least monthly when microscopic examinations are being performed, the analyst should prepare a slide containing 40 to 100 oocysts and 40 to 100 cysts. More than 50% of the oocysts and cysts should be DAPI-positive and undamaged under DIC microscopy.

9.13.2 The analyst should determine the total number of immunofluorescent (IFA-positive) oocysts and cysts for the entire slide. If ColorSeed™ is used, the analyst should also determine the total number of Texas Red positive oocysts and cysts for the entire slide. For 10 oocysts and 10 cysts that are IFA-positive, the analyst should determine the DAPI category (positive or negative), number of nuclei by DAPI (if DAPI-positive) and the

DIC category (empty, containing amorphous structures, or containing identifiable internal structures) of each.

9.13.3 Laboratories with a single analyst. The analyst should perform repetitive counts (immunofluorescent organisms) of a single verification slide. The total number of oocysts and cysts counted (IFA-positive) should be within ±10% for the repetitive counts. Laboratories with a single analyst should also coordinate with other laboratories to share slides and compare counts.

9.13.4 Laboratories with multiple analysts

9.13.4.1 Each analyst determines the total number of oocysts and cysts by fluorescence, IFA-positive and Texas Red-positive (ColorSeed™), for the same slide. Each analyst determines the DAPI category (positive or negative), number of nuclei by DAPI (if DAPI-positive), and the DIC category for the same 10 oocysts and 10 cysts. The total IFA-positive count, Texas Red-positive count, and DAPI-positive and negative counts should be within ±10% of each other. If the number is not within this range, the analysts should identify the source of any variability between analysts' examination criteria, prepare a new slide, and repeat the performance verification. Differences in the number of nuclei by DAPI fluorescence and in DIC categorizations among analysts should be discussed and resolved.

9.13.4.2 Document the date, name(s) of analyst(s), number of total, Texas Red-positive, DAPI- positive or negative oocysts and cysts determined by each analyst, whether the test was passed/failed and the results of attempts before the test was passed. Document the date, name(s) of analyst(s), number of nuclei by DAPI fluorescence, DIC categories, and differences that were resolved, if applicable.

9.13.4.3 Analysts should pass the criteria before identifying and enumerating oocysts and cysts in QC and field samples.

9.13.5 Protozoan photographic library. Laboratories are encouraged to develop libraries of photographs and drawings for identification of protozoa from photographs in books, publications, and websites to aid in proper identification of target and non-target differentiation.

9.13.5.1 Take color photographs of *Cryptosporidium* oocysts and *Giardia* cysts identified by fluorescence (IFA, Texas Red, and DAPI) and DIC microscopy that the analyst(s) determine are accurate (Section 15.2).

9.13.5.2 Similarly, take color photographs of interfering organisms and materials identified by fluorescence (IFA, Texas Red, and DAPI), and DIC microscopy 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.

9.13.5.3 The laboratory should compare the results of the examinations performed in Section 9.13.2 to photographs of oocysts and cysts and interfering organisms to verify that examination results are consistent with photographic references.

10.0 Microscope Calibration

This method requires the proper and consistent use of the microscope. All analysts should be properly trained in the operation of the microscope. Microscope procedures and requirements including bulb adjustment (mercury and transmitted), ocular adjustments, ocular micrometer calibration, Köhler illumination, and cleaning techniques are detailed in Appendix A. The frequency of each procedure is as follows: bulb adjustment - at time of changing of bulb or if problems noted; ocular adjustment - each microscope session; ocular micrometer calibration - initial use or when oculars or objectives are replaced; Köhler illumination - each microscope session; cleaning techniques - each microscope session and weekly.

11.0 Sample Spiking Using Enumerated Suspensions of Oocysts and Cysts

11.1 This method requires the analysis of spiked QC samples to demonstrate acceptable IPR samples, Section 9.3; MS/MSD samples, Section 9.5, and OPR samples, Section 9.4. Either irradiated or live organisms may be used for QC samples. The organisms used for spiking these samples should be accurately enumerated in order to calculate target recoveries (and precision) and monitor method performance. EPA recommends that flow cytometry be used for enumerating spiking suspensions, rather than manual techniques. Flow cytometer-sorted spikes generally are characterized by a relative standard deviation of ≤ 2.5%, versus greater variability for manual enumeration techniques (Reference 20.6). Guidance on preparing spiking suspensions using a flow cytometer is provided in Appendix B. Manual enumeration procedures are provided in Appendix B. Procedures for spiking bulk samples are provided below in Sections 11.2 and 11.3.

11.2 Procedure for spiking bulk samples for concentration by filtration

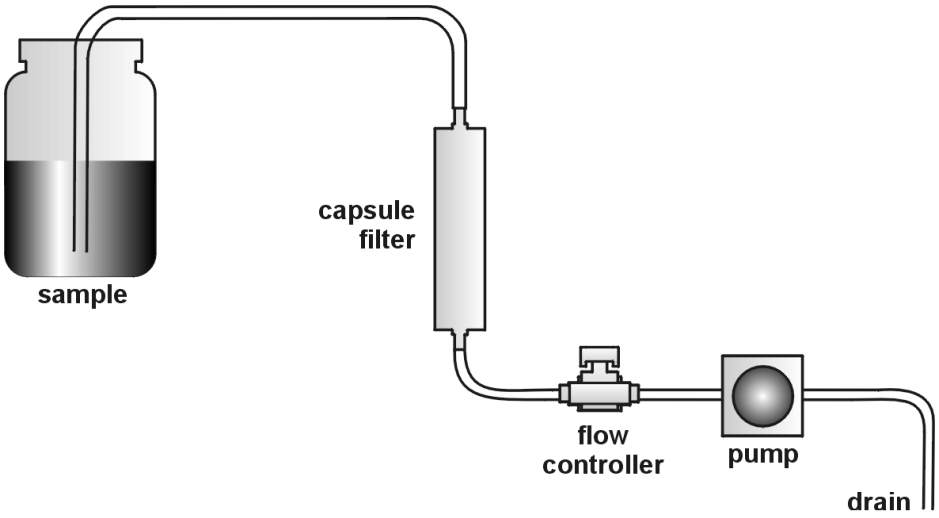
11.2.1 Flow rate adjustment

11.2.1.1 Connect the sampling system, minus the capsule, to a cubitainer filled with reagent water (**Figure 1**).

11.2.1.2 Turn on the pump; confirm that the flow rate is 2 L/min. Adjust flow control valve (if available) to achieve proper flow rate.

11.2.1.3 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.

Figure 1. Laboratory Filtration System



11.2.2 Arrange a cubitainer with a spigot to feed the filter or insert the influent end of the tube connected to the filter through the top of a cubitainer to allow siphoning of the sample. Install the capsule filter in the line, securing the **inlet** and **outlet** ends with the appropriate clamps/fittings.

11.2.3 For IPR (Section 9.3) and OPR (Section 9.4) samples, fill the cubitainer with 10 L of reagent water. For MS/MSD (Section 9.5), fill the cubitainer with 10 L (or appropriate volume) of the field sample to be spiked or spike into the collection container, as appropriate. Continuously mix the sample (using a stir bar and stir plate for smaller-volume samples and alternate means for larger-volume samples).

11.2.4 Follow the procedures in Section 11.2.4.1 for flow cytometer-enumerated suspensions and Section 11.2.4.2 for manually enumerated spiking suspensions.

11.2.4.1 For flow cytometer↗enumerated suspensions (where the entire volume of a spiking suspension tube, ~50 mL, will be used), follow manufacturer's instructions or the following:

11.2.4.1.1 Add 500 µL of diluted Antifoam Y-30 (Section 7.9.1.1) to the tube containing the spiking suspension and vortex for 30 seconds.

11.2.4.1.2 Pour the suspension into the sample container.

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

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

11.2.4.1.5 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.2.5.

11.2.4.2 For manually enumerated spiking suspensions:

11.2.4.2.1 Vortex the spiking suspension(s) (Appendix B) for a minimum of 30 seconds.

11.2.4.2.2 Rinse a pipette tip with 0.01% Tween® 20 once, then repeatedly pipette the well-mixed spiking suspension a minimum of five times before withdrawing an aliquot to spike the sample.

11.2.4.2.3 Add the spiking suspension(s) to the sample container, delivering the aliquot below the surface of the sample.

11.2.4.2.4 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.2.5.

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

11.2.6 Turn on the pump and allow the flow rate to stabilize. Adjust and maintain flow rate at 2 L/min. As the sample container is depleted, check the flow rate and adjust if necessary.

11.2.7 When the sample level approaches the discharge port of the container, tilt the container so that it is completely emptied. Turn off the pump and add 1 L of reagent water to the 10 L container to rinse. Swirl the contents to rinse down the sides. Additional rinses may be performed.

11.2.8 Turn on the pump. Allow all of the rinse to flow through the filter and turn off the pump.

11.2.9 Proceed to filter disassembly (Section 12.2.5).

11.3 Procedure for spiking samples for concentration by direct centrifugation

11.3.1 Add 1 L (or appropriate volume) of sample to a clean 2 L beaker containing a stir bar and stir to mix.

11.3.2 While stirring, slowly add 50 mL of 20% Tween® 80 (1% final concentration) and continue mixing for 15 minutes.

11.3.3 Spike sample(s) using flow cytometer-enumerated or manually enumerated spiking suspensions according to Section 11.2.4.1 or Section 11.2.4.2, respectively.

11.3.4 Proceed to Section 12.3.3 for concentration.

12.0 Sample Concentration

12.1 Determination of sample volume and concentration option

Determine if 10 L is filterable based on: amount and type of pellet concentrated from 250 mL, turbidity or total suspended solids (TSS) of sample, knowledge of the specific sample matrix,

analytical volume requirement, and experience of the technician/analyst. A preliminary evaluation of sample volume/packed pellet ratios can be rapidly obtained by centrifuging 250 mL of bulk sample and evaluating the packed pellet volume. If the sample appears to be filterable proceed to Section 12.2 and follow the filtration option. The target sample volume for examination by the filtration option is 10 L or a maximum packed pellet volume of 2 mL. If the sample is not filterable (i.e., contains large particulates, clogs the filter prior to 10 L being filtered), proceed to Section 12.3 and follow the direct centrifugation option. The target sample volume for examination by the direct centrifugation option is 1 L or a maximum packed pellet volume of 2 mL.

12.2 Filtration option

A wastewater or CSO sample is filtered (capsule filtration adapted from References 20.1, 20.2, and 20.7) according to the procedures in Section 12.2.1 through 12.2.5. Alternative (>10 L) sample volumes may be used, provided the laboratory demonstrates acceptable performance through an IDC and ongoing laboratory and method performance tests (Table 3).

Note: Sample elution should be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample).

12.2.1 Flow rate adjustment

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

12.2.1.2 Turn on the pump, confirm that the flow rate is 2 L/min. Adjust flow control valve (if available) to achieve proper flow rate.

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 type, and sample filtration start date and time 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 the stir plate to lowest setting needed to keep sample thoroughly mixed. Collect a subsample of the sample matrix, measure the sample turbidity, and record on the bench sheet. Connect the sampling system to the sample cubitainer. The use of a spigot (Section 6.1.8) with the sample collection cubitainer will facilitate sample filtration.

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 (Section 6.1.6). This container will be used to determine the sample volume filtered. Alternately, connect a flow

meter (Section 6.2.6) downstream of the filter, and record the initial meter reading.

12.2.4.3 Allow the cubitainer discharge tube and capsule to fill with sample 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 sample 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. Allow the pressure to decrease until flow stops.

12.2.4.5 Turn off the stir plate; add 1 L of reagent water to rinse. Swirl or shake the cubitainer to rinse down the side walls.

12.2.4.6 Reconnect to the pump, turn on the pump and allow the pump to pull all the rinse water through the filter; turn off the 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. Restart the pump and allow as much rinse water to drain as possible. Turn off the pump.

12.2.5.2 Based on the sample level in the graduated container (Section 6.1.6) or final meter reading, estimate the volume filtered to the nearest quarter liter and record on the bench sheet. Do not include rinse volume. Discard the contents of the graduated container.

12.2.5.3 Loosen the **outlet** fitting, then cap the **inlet** and **outlet** fittings.

12.2.6 Elution

Note: The laboratory should complete the elution, concentration, and purification (Sections 12.2.6.1 through 13.3.3.12) 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 Setup

12.2.6.1.1 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.

12.2.6.1.2 Prepare sufficient 5% NaHMP (Section 7.4) and PBST (Section 7.5.4) elution buffer so that all filters to be rinsed and eluted in one QC batch can be rinsed and eluted with the same batch of buffers used for the OPR/MB. The pre-elution filter rinse may

require up to 150 mL of NaHMP buffer per sample. Sample elution may require up to 250 mL of PBST buffer per sample.

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

12.2.6.2 Pre-elution filter rinse

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

12.2.6.2.2 If excess sample remains in the filter capsule, pull the remaining sample volume through the filter.

12.2.6.2.3 Pour 5% NaHMP buffer through the **inlet** fitting after removing the inlet cap. Allow the liquid level to stabilize. Sufficient NaHMP buffer should be added to cover the pleated white membrane with buffer solution. Replace the **inlet** cap.

12.2.6.2.4 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 900 rpm). Agitate the capsule for 5 minutes. Time the agitation using a lab timer, rather than the timer on the shaker to ensure accurate time measurement.

12.2.6.2.5 Remove the capsule from the shaker, remove the **outlet** cap, open bleed valve, and remove NaHMP buffer from the capsule by attaching the **outlet** to a vacuum source with a fluid disposal flask. Replace the **outlet** cap and close bleed valve when the buffer has been completely removed.

12.2.6.2.6 Clamp the capsule vertically with the **inlet** end up and fill the capsule with reagent water through the **inlet** port. Immediately remove the reagent water through the **outlet** by attaching to a vacuum source as in Section 12.2.6.2.5. Replace both **inlet** and **outlet** caps.

12.2.6.3 Sample elution

12.2.6.3.1 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.

12.2.6.3.2 Pour PBST elution buffer through the inlet fitting after removing the inlet cap. Allow the liquid level to stabilize. Sufficient elution buffer should be added to cover the pleated white membrane with buffer solution. Replace the inlet cap.

12.2.6.3.3 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 900 rpm). Agitate the capsule for 5 minutes. Time the agitation using a lab timer, rather than the timer on the shaker to ensure accurate time measurement.

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

12.2.6.3.5 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.

12.2.6.3.6 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 5 minutes.

12.2.6.3.7 Loosen clamp, rotate the capsule in the shaker to the 8 o'clock position, and re-tighten clamp. Turn on the shaker and agitate the capsule for a final 5 minutes.

12.2.6.3.8 Remove the filter from the shaker 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 directed through the **inlet** end of the capsule. Invert the capsule filter over the centrifuge tube or shake down the capsule filter to ensure that as much of the eluate as possible has been transferred.

12.2.6.3.9 Centrifuge the 250-mL centrifuge tube containing the capsule filter eluate at 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 if only one 250 mL centrifuge tube was used.

Note: Recoveries may be improved if centrifugation force is increased to 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.

12.2.6.3.10 Using a Pasteur pipette attached to a vacuum source, carefully aspirate the supernatant leaving 5 mL of supernatant for every 0.5 mL of pellet. Extra care should be taken to avoid aspirating oocysts and cysts during this step, particularly if the sample is reference matrix (*e.g.*, IPR or OPR sample).

12.2.6.3.11 Quantitatively transfer the pellets, combine into 1, 250 mL centrifuge tube, and repeat Section 12.2.6.3.9 and 12.2.6.3.10 if

more than one 250 mL centrifuge tube was used. Record total sample pellet volume on the appropriate bench sheet.

12.2.7 Proceed to Section 13.0 for sample separation (purification).

12.3 Direct Centrifugation Option. A wastewater or CSO sample that cannot be **concentrated** by filtration due to high turbidity or excessive particulate material may be concentrated by direct centrifugation (adapted from Reference 20.2). Procedures and guidelines for determining sample volume are described in Section 12.1. Alternative (>1 L) sample volumes may be used, provided the laboratory demonstrates acceptable performance through an IDC and ongoing laboratory and method performance analyses (**Table 4**).

Note: Sample concentration should be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample).

12.3.1 Add 1 L (or minimum required volume) of sample to a clean 2 L beaker containing a stir bar and stir to mix.

12.3.2 While stirring, slowly add 50 mL of 20% Tween® 80 (1% final concentration) (Section 7.6) and continue mixing for 15 minutes.

12.3.3 Quantitatively transfer the required sample volume to one or more 250 mL conical centrifuge tubes, as necessary.

12.3.4 Centrifuge the sample tube(s) at 1500 × g for 15 minutes. Allow the centrifuge rotor to coast to a stop without using the brake. Record total sample pellet volume on the appropriate bench sheet if only one 250 mL centrifuge tube was used.

12.3.5 Using a Pasteur pipette attached to a vacuum source, carefully aspirate the supernatant leaving 5 mL of supernatant for every 0.5 mL of pellet. Extra care should be taken to avoid aspirating oocysts and cysts during this step, particularly if the sample is reference matrix (*e.g.*, IPR or OPR sample).

12.3.6 If more than one 250 mL centrifuge tube was used, quantitatively transfer and combine pellets from all 250 mL tubes, repeating Sections 12.3.4 and 12.3.5. Record total sample volume concentrated and total sample pellet volume on the appropriate bench sheet.

Note: Recoveries may be improved if centrifugation force is increased to 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.

12.3.7 Proceed to Section 13.0 for sample separation (purification).

13.0 Sample Separation (Purification)

13.1 Prior to sample separation (purification) by IMS, filter eluates are concentrated by centrifugation (Section 12.2.6.3.9 through 12.2.6.3.10), resulting pellets are resuspended (and combined, if necessary) and processed according to Section 13.2. Sample pellets derived from concentration using the direct centrifugation option are processed according to Section 13.2.

13.2 Preparation and adjustment of sample pellet volume. The target sample volume for examination by the filtration option is 10 L or a maximum packed pellet volume of 2 mL; target sample volume for examination by the direct centrifugation option is 1 L or a maximum packed pellet volume of 2 mL. The recommended maximum amount of particulate material to process through IMS (purification) and examination steps in the method is 0.5 mL; therefore, a subsample is equivalent to no greater than 0.5 mL of packed pellet material suspended in 5 mL of fluid and processed in one flat-sided tube.

Note: Extra care should 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. Recommend decreasing vortex speed, decreasing time of vortex, substitute pipette mixing, swirling or rocking tube 180°.

13.2.1 Total packed pellet volume is ≤0.5 mL. The entire pellet is transferred and analyzed in one IMS sample tube. Vortex the 250-mL centrifuge tube vigorously until pellet is completely resuspended in the 5 mL of fluid remaining after aspiration. Swirl the centrifuge tube gently to reduce any foaming after vortexing.

13.2.2 Total packed pellet volume is >0.5 mL but ≤2 mL. The entire pellet is analyzed by separating into up to 4 subsamples. Resuspend the entire pellet in up to 20 mL of fluid (5 mL of fluid for every 0.5 mL pellet) by vortexing the 250-mL centrifuge tube vigorously. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Each subsample is equivalent to 5 mL of resuspended pellet and transferred to individual IMS sample tubes.

13.2.3 Total packed pellet volume is >2 mL. Analysis of a partial sample is required. Process at least 2 mL of pellet or sample volume requested. Resuspend every 0.1 mL of pellet in 1 mL of fluid remaining after aspiration. For example, aspirate to 27 mL for a pellet size of 2.7 mL; 5 mL of resuspended pellet will be transferred to each of 4 tubes for a total of 20 mL or 2 mL of pellet. Vortex the 250-mL centrifuge tube vigorously; swirl the centrifuge tube gently to reduce any foaming after vortexing. Each 5 mL subsample of resuspended pellet is transferred to individual IMS sample tubes.

13.2.4 Use Equation #1 to determine the sample volume analyzed

Equation #1

$$\text{Volume analyzed} = \frac{\text{Packed pellet volume processed through IMS}}{\text{Total pellet volume}} \times \text{Total volume filtered or concentrated}$$

For example, 500 mL of sample was concentrated by centrifugation resulting in a 2.5 mL pellet; 2 mL of pellet were processed through IMS. Volume analyzed is equal to 0.4 L.

13.2.5 Record the total volume of resuspended concentrate on the bench sheet. Also record the volume transferred for each subsample and the number of subsamples processed independently through the method on the bench sheet.

13.2.6 Process subsamples through IMS. Proceed immediately to Section 13.3, and transfer aliquots of the resuspended concentrate as determined in Sections 13.2.1-13.2.3 to IMS sample tube(s) 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

13.3 IMS procedure (adapted from References 20.1, 20.2, and 20.8)

Note: The IMS procedure should be performed on a bench top with all materials 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 (Section 7.3) as the diluent. For every 1 mL of 1X SL-buffer-A required, dilute 100 µL of 10X SL-buffer-A to 1 mL with the diluent. 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 IMS sample (flat-sided) tube (Section 6.3.4).

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

13.3.2 Oocyst and cyst capture

13.3.2.1 Use a graduated, 10-mL pipette that has been pre-rinsed with elution buffer to transfer the sample concentrate from Section 13.2 to the IMS sample tube 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 IMS sample tube containing the concentrate (or to the tube containing the first subsample, if multiple subsamples will be processed). Each of the two rinses should be half the volume needed to bring the volume transferred to 10 mL. (For example, if 5 mL of sample was transferred after resuspension of the pellet, the centrifuge tube would be rinsed twice with 2.5 mL of reagent water to bring the total volume in the IMS sample tube to 12 mL which includes 1 mL of SL-buffer-A, 1 mL of SL-buffer-B, and 10 mL sample and rinsate.) Visually inspect the centrifuge tube after completing the transfer to ensure that no concentrate remains. If multiple subsamples will be processed, bring the total volume (which includes 1 mL of SL-buffer-A and 1 mL of SL-buffer-B) in the remaining IMS sample tubes to 12 mL with reagent water. Label the IMS sample 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 inverting the vial and making sure that there are no residual beads 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 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 inverting the vial and making sure that there are no residual beads at the bottom.

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

13.3.2.6 If the sample concentrate was prepared by the direct centrifugation option, add 0.25 g kaolin to the sample tube(s) containing the sample concentrate, Dynabeads® Crypto-Combo, Dynabeads® *Giardia*-Combo and SL-buffer. Gently rock the sample tube to mix the kaolin.

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

13.3.2.8 After rotating for 1 hour, remove one sample at a time from the mixer leaving the remaining samples rotating. Place the tube in the magnetic particle concentrator (MPC®-1 or MPC®-6) with flat side of the tube toward the magnet.

13.3.2.9 Without removing the sample tube from the MPC®-1, place the magnet side of the MPC®-1 downwards, so the tube is horizontal and the flat side of the tube is facing down. If sample(s) was concentrated by the filtration option, continue with 13.3.2.10. If sample(s) was concentrated by the direct centrifugation option, proceed to Section 13.3.2.12.

13.3.2.10 Gently rock the sample tube by hand end-to-end through approximately 90°, 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.

13.3.2.11 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 is allowed to stand motionless for more than 10 seconds, remove the IMS sample tube from the MPC®-1, gently resuspend all material, replace the sample tube in the MPC®-1 and repeat Section 13.3.2.10 before continuing to Section 13.3.2.13.

13.3.2.12 For samples concentrated by the direct centrifugation option, continue by gently rocking the sample tube by hand end-to-end through approximately 90°, tilting the cap-end and base-end of the tube up and down in turn. Continue the tilting action for 1 minute with approximately one tilt per second. Return the MPC®-1 to the upright position, sample tube vertical, with the cap at the top. Allow sample tube to stand motionless for 3 minutes. At the end of 3 minutes, gently rock the tube for 1 minute. Allowing the sample to stand motionless contributes to the adsorption of fats, oils, particulates, and debris to the kaolin.

13.3.2.13 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 into a

suitable waste container. Do not shake the tube and do not remove the tube from the MPC®-1 during this step. Allow more supernatant to settle; aspirate the additional supernatant with a pipette.

13.3.2.14 Without removing the tube from the MPC®-1, add 10 mL PBS down the side of the tube away from the beads. Remove the tube from the magnet and very gently tilt the tube five times to resuspend the beads. Place the tube back in the MPC®-1 and repeat Sections 13.3.2.8 through 13.3.2.13. The PBS rinse of the sample-bead complex removes additional debris before transferring to the microcentrifuge tube.

13.3.2.15 Remove the sample tube from the MPC®-1 and resuspend the sample in 0.5 mL 1X SL-buffer-A (prepared from 10X SL-buffer-A stock − supplied). Mix very gently to resuspend all the material in the tube. Do not vortex.

13.3.2.16 Quantitatively transfer (transfer followed by two rinses) all the liquid from the sample tube to a labeled, 1.5-mL microcentrifuge tube. Use 0.5 mL of 1X SL-buffer-A to perform each rinse. Allow the IMS 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.

13.3.2.17 Place the microcentrifuge tube into the second magnetic particle concentrator (MPC®-S), with magnetic strip in the vertical position.

13.3.2.18 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. At the end of this step, the beads should produce a distinct brown dot at the back of the tube.

13.3.2.19 Immediately open the microcentrifuge tube and aspirate the supernatant from the cap and the tube while still in position in the MPC®-S. If more than one sample is being processed, conduct three 90° rock/roll actions before removing to the wall of the tube adjacent to the magnet.

13.3.3 Dissociation of beads/oocyst/cyst complex

Note: Two acid dissociations are required.

13.3.3.1 Label a well slide for each sample or subsample and allow the slide(s) to warm to room temperature.

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

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

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

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

13.3.3.5 Vortex vigorously for approximately 30 seconds.

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

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

13.3.3.8 Add 5 µL of 1.0N NaOH to the sample wells of two well slides or 2 sample wells on the same well slide (if the volume from the two required dissociations will be added to the same slide, then add 10 µL to the sample well of one well slide prior to the first dissociation or 5 µL prior to each dissociation twice).

Note: The laboratory should 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 13.3.3.2 through 13.3.3.9 a second time. The volume from the second dissociation may be added to the slide containing the volume from the first dissociation, or can be applied to a second slide or a second well on the same slide.

13.3.3.11 Record the date and time the purified sample was applied to the 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 should take care to ensure that the sample has dried completely before staining to prevent losses during the rinse steps. Other options include: drying the slides on a slide warmer set at 35°C to 42°C or drying the slides overnight in the refrigerator.

13.3.4 Tips for minimizing carry-over of debris onto microscope slides after IMS

- Make sure the pellet is fully resuspended before placing the tube in the MPC®-1 or MPC®-S to avoid trapping "clumps" or a dirty layer between the beads and the side of the tube.

- When using the MPC®-1 magnet, make sure that the tube is flat against the magnet. Push the tube flat if necessary. Sometimes the magnet is not flush with the outside of the holder and, therefore, the attraction between the beads and the magnet is not as

strong as it should be. However, it can be difficult to determine this if you do not have more than one MPC®-1 to make comparisons.

- After the supernatant has been poured off (Section 13.3.2.13), leave the tube in the MPC®-1 and allow time for any supernatant remaining in the tube to settle down to the bottom. Then aspirate the settled supernatant and associated particles from the bottom of the tube. The same procedure can be performed with the MPC®-S and the microcentrifuge tube (Section 13.3.2.19).

- After the supernatant has been aspirated from the microcentrifuge tube (Section 13.3.2.19), add 1 mL of PBS, remove the tube from the MPC®-S, and resuspend. Repeat Sections 13.3.2.17 through 13.3.2.19.

- Use a slide with the largest diameter well available to spread out the sample as much as possible.

- When using the MPC®-S with 2 magnet positions, use the vertical placement (Section 13.3.2.17 through 13.3.2.19) with larger volume(s) of liquid (1.5 mL) and the slanted placement (Section 13.3.3.4 through 13.3.3.10) with smaller volume(s) of liquid (0.05 mL) to ensure the best bead pellet formation.

14.0 Sample Staining

Note: The sample should be stained within 72 hours of application of the purified sample to the slide.

14.1 Prepare positive and negative staining controls.

14.1.1 For the positive staining control, pipette 10 µL of positive antigen or 200 to 400 oocysts and 200 to 400 cysts to the center of a well. If ColorSeed™ is used as the spiking suspension, positive control organisms should be ColorSeed™ organisms; request concentrated positive control from BTF, Pty or use another ColorSeed™ vial.

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

14.1.3 Positive and negative staining controls should be prepared when samples are applied to slides

14.1.4 Dry the staining control slides with the sample slides using the same drying technique described in Section 13.3.3.12.

Note: If the 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, the laboratory should prepare multiple control slides with the batch of field slides and alternate between the positive controls when performing the positive control check.

14.2 Follow manufacturer's instructions in applying IFA stain to slides. Exceptions are noted in Sections 14.2.1 through 14.2.2.

14.2.1 Delete the methanol step when using EasyStain™.

Note: DAPI (*Section* 7.8.6.2) *staining* is performed prior to IFA staining and the working DAPI solution is prepared by adding 50 µL of DAPI stock (Section 7.8.6.1) to 50 mL PBS.

14.2.2 When using Meridian Merifluor™ *Cryptosporidium*/*Giardia* or Waterborne Aqua-Glo™ G/C (or Crypt-a-Glo™ and Giardi-a-Glo™), perform DAPI staining (Sections 14.3 through 14.5) before adding mounting medium. Continue with Section 14.6.

14.3 Apply 50 µL of DAPI staining solution (Section 7.8.6.2) to each well. Allow to stand at room temperature for a minimum of 1 minute. (The solution concentration may be increased up to 1 g/mL if fading/diffusion of DAPI staining is encountered, but the staining solution should be tested first on expendable environmental samples to confirm that staining intensity is appropriate.)

14.4 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. Use a new pipette for each sample. Ensure that the vacuum source is at the absolute minimum (<2 in Hg).

14.5 Apply one drop of wash buffer (prepared according to the manufacturer's instructions) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess wash buffer 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 Merifluor™ *Cryptosporidium*/*Giardia* (Section 7.8.4), do not allow slides to dry completely.

14.6 Continue with manufacturer's instructions by adding mounting medium (Section 7.8.7) to each well, apply cover slip and seal (depending on mounting medium used).

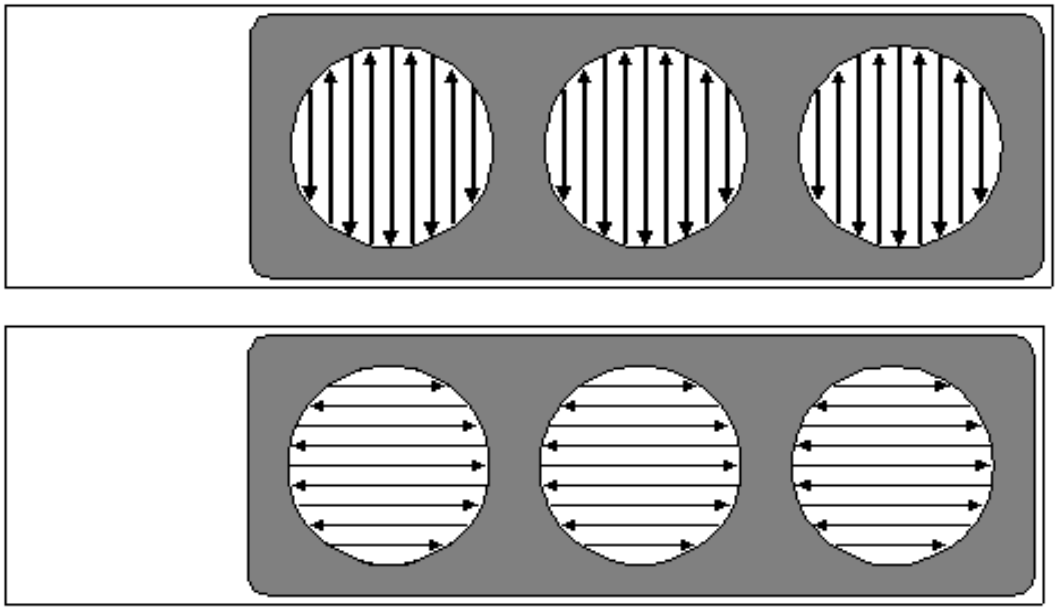
14.7 Record the date and time that staining was completed on the bench sheet. If slides will not be read immediately, store in a humid chamber in the dark at <10°C (but not frozen) until ready for examination.

15.0 Examination

Note: Although immunofluorescence assay (IFA), Texas Red, DAPI and DIC microscopy examination should be performed immediately after staining is complete, laboratories have up to 7 days (168 hours) from completion of sample staining to complete the examination and characterization of samples. However, if fading/diffusion of the IFA or the DAPI fluorescence is noticed, the laboratory should reduce this holding time. In addition, the laboratory may adjust the concentration of the DAPI staining solution (Section 7.8.6.2) 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 2**).

Figure 2. Methods for Scanning a Well Slide



15.2 Examination using IFA, Texas Red, DAPI staining characteristics, and DIC microscopy. The minimum magnification requirements for each type of examination are noted below. Record examination results for *Cryptosporidium* oocysts on a *Cryptosporidium* examination form; record examination results for *Giardia* cysts on a *Giardia* examination form. All organisms that meet the criteria specified in Sections 15.2.2 and 15.2.3, less atypical organisms, should be reported. IFA-positive organisms should also be examined using DAPI fluorescence and DIC microscopy to determine if the organisms exhibit the correct fluorescence, the correct size and shape and possess no atypical characteristics (Sections 15.2.2 and 15.2.3). A positive result includes the number and description of organisms that meet the criteria in Sections 15.2.2 and 15.2.3. A negative result means no organisms meeting these criteria were detected. If ColorSeed™ is used, examine each IFA-positive organism using the Texas Red filter and record the presence/absence of red fluorescence on the examination form.

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

15.2.1 Staining control examination − Perform staining control examinations prior to sample examination (Section 9.7)

15.2.2 Sample examination – *Cryptosporidium*

15.2.2.1 **IFA examination** (use a minimum of 200X total magnification for scanning). Use epifluorescence to scan the entire well for apple-green fluorescence of oocyst shape. 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 or 1000X and switch the

microscope to the UV filter block for DAPI fluorescence examination (Section 15.2.2.2), then to DIC microscopy (Section 15.2.2.3) at 1000X. If ColorSeed™ is used, switch the microscope to the Texas Red filter block for red fluorescence examination (Section 15.2.2.4).

15.2.2.2 **DAPI fluorescence examination** [use a minimum of 400X total magnification for observation, 1000X total magnification (oil immersion lens) for characterization]. 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. Record oocysts in category (a) as DAPI-negative; record oocysts in categories (b) and (c) as DAPI-positive.

15.2.2.3 **DIC examination** [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, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from References 20.1 and 20.9). If atypical structures are not observed, then categorize each apple-green fluorescing object as:

(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 category (a, b, or c above) on the *Cryptosporidium* examination form. Record number of sporozoites (if applicable) for each apple-green fluorescing object assigned to category c. Although not a defining characteristic, surface oocyst folds may be observed in some specimens.

15.2.2.4 **Texas Red examination** (use a minimum of 400X total magnification). Using the Texas Red filter block, look for red fluorescence and any atypical characteristics. Record the presence/absence of red fluorescence for every IFA-positive oocyst on the examination form.

15.2.2.5 A positive result is a *Cryptosporidium* oocyst which exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA,

DAPI fluorescence, or DIC microscopy. A positive result should be characterized and assigned to one of the DAPI and DIC categories in Sections 15.2.2.2 and 15.2.2.3. If ColorSeed™ is used, a positive result should be characterized by the presence/absence of red fluorescence.

15.2.3 Sample examination – *Giardia*

15.2.3.1 **IFA examination** (use a minimum of 200X total magnification for scanning). 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 or 1000X and switch the microscope to the UV filter block for DAPI fluorescence examination (Section 15.2.3.2) then to DIC microscopy (Section 15.2.3.3) at 1000X. If ColorSeed™ is used, switch the microscope to the Texas Red filter block for red fluorescence examination (Section 15.2.3.4).

15.2.3.2 **DAPI fluorescence examination** [use a minimum of 400X total magnification for observation, 1000X total magnification (oil immersion lens) for characterization]. 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) Two to four sky-blue nuclei

Look for atypical DAPI fluorescence, *e.g.*, more than four stained nuclei, size of stained nuclei, and wall structure and color. Record cysts in category (a) as DAPI-negative; record cysts in categories (b) and (c) as DAPI- positive.

15.2.3.3 **DIC examination** [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, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from References 20.6 and 20.10). If atypical structures are not observed, categorize each object meeting the criteria specified in Sections 15.2.3.1 through 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 the category (a, b, c, or d above) on the *Giardia*

examination form. Record the number of nuclei and presence of median body or axonemes (if applicable) for each apple-green fluorescing object assigned to categories c and d.

15.2.3.4 **Texas Red examination** (use a minimum of 400X total magnification). Using the Texas Red filter block, look for red fluorescence and any atypical characteristics. Record the presence/absence of red fluorescence for every IFA-positive cyst on the examination form.

15.2.3.5 A positive result is a *Giardia* cyst which exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy. A positive result is also characterized and assigned to one of the DAPI and DIC categories in Section 15.2.3.2 and 15.2.3.3. If ColorSeed™ is used, a positive result should be characterized by the presence/absence of red fluorescence.

15.2.4 Record analyst name and 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.

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 Some samples may adhere to the centrifuge tube walls. The use of siliconized or low-adhesion centrifuge tubes (siliconized/low retention microcentrifuge tubes) may reduce adhesion. Alternately, rinse centrifuge tubes with PBST elution buffer or Sigmacote® prior to use.

16.3 For samples that clog the filter or cannot be filtered, concentrate a smaller sample volume (1 L target volume) using the direct centrifugation option.

16.4 If sample processing holding times listed in Table 1 are exceeded, the site should be re-sampled. If re-sampling is not an option, the results should be qualified accordingly.

16.5 The ambient levels of (oo)cysts may dictate that each organism be detected and enumerated in separate subsamples representing different sample volumes. Analyses may be performed using different concentrated sample volume equivalents for *Cryptosporidium* and for *Giardia* if the appropriate (oo)cyst-specific IMS beads and stains are employed for sample purification and staining. Matrix spikes (MS) for each organism may also be performed on different sample volume equivalents if (oo)cyst- specific reagents are employed (as above). However, equivalent sample volumes of each field sample and corresponding MS sample should be processed and analyzed.

17.0 Method Performance

Method acceptance criteria are shown in Tables 3 and 4. The initial and ongoing precision and recovery criteria are based on the results of spiked reference matrix samples analyzed during the interlaboratory validation study of Method 1693 involving 6 laboratories. The MS/MSD criteria for the filtration option are based on data from spiked disinfected wastewater samples generated during the interlaboratory validation. (Reference 20.10).

Note: Some sample matrices may prevent the MS acceptance criteria in Table 3 and Table 4 to be met.

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 Solutions and reagents should be prepared 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 should 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

20.1 US EPA. 2006. *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule), Appendix B: Method 1622: Cryptosporidium in Water by Filtration/IMS/FA and Appendix C: Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA*, EPA 815-R06-006, Office of Water, Office of Ground Water and Drinking Water Technical Support Center, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268.

20.2 McCuin, R.M. and J.L. Clancy. 2005. *Cryptosporidium in Wastewater: Occurrence, Removal and Inactivation*. Final Report: Water Environment Research Foundation, Project 98-HHE-1, Alexandria, VA.

20.3 Rodgers, M.R., D.J. Flanigan, and W. Jakubowski. 1995. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method of alleviating this interference. Applied and Environmental Microbiology **61(10)**: 3759-3763.

20.4 *Federal Register*, 71 FR 32244-63. June 2, 2006.

20.5 APHA, AWWA, and WEF. 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th edition, APHA, Washington, D.C.

20.6 Connell, K., C.C. Rodgers, H.L. Shank-Givens, J. Scheller, M.L. Pope, and K. Miller. 2000. Building a Better Protozoa Data Set. Journal AWWA **92**:10-30.

20.7 Envirochek™ HV Sampling Capsule Protocol, Pall Corporation, Ann Arbor, MI, product no. 12099 or 12098.

20.8 Dynabeads® GC-Combo, 2003, Revision no. 011. Dynal Biotech, Inc. (Invitrogen Corporation), Lake Success, NY.

20.9 US EPA. 1996. *ICR Microbial Laboratory Manual*, EPA/600/R-95/178, National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268.

20.10 USEPA. 2014. *Interlaboratory Validation of EPA Method 1693: Cryptosporidium and Giardia in Disinfected Wastewater by Concentration/IMS/IFA*, EPA 821-R-14-008, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington, DC 20460.

21.0 Glossary

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

21.1 Units of weight and measure and their abbreviations

21.1.1 Symbols

°C	degrees Celsius
µL	microliter
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
%	percent

21.1.2 Alphabetical characters

cm	centimeter
g	gram
× g	acceleration due to gravity
hr	hour
ID	inside diameter
in.	inch
L	liter
m	meter
MCS	microscope cleaning solution
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
TSS	total suspended solids
s_r	standard deviation of recovery
X	average percent recovery

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

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 *Trichonmonas*.

Combined Sewer Overflow (CSO) − A combination of sewage (raw or partially treated) and storm water runoff.

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.

Disinfected wastewater − Secondary or tertiary treated wastewater, disinfected by chlorine or UV; meets NPDES permit specifications for discharge.

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

Immunofluorescent assay (IFA) − An assay using a specific fluorochrome (FITC) to label the monoclonal antibodies targeted against the cell wall antigens of both *Giardia* and *Cryptosporidium*. The antibody bond results in a fluorescein compound attached to the cell wall and fluoresces a brilliant apple-green.

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 precision and recovery (IPR) − A sample prepared by adding a known quantity of organisms to a specified amount of reference matrix and analyzed to establish the ability to generate acceptable precision and accuracy. An IPR is performed prior to the first time this method is used and any time the method or instrumentation is modified.

Laboratory blank − See Method blank

Laboratory control sample (LCS) − 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.

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 crescent-shaped or round.

Method blank − 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.

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.

(Oo)cyst − Collective term for both oocysts and cysts.

Ongoing precision and recovery (OPR) standard − A reference matrix 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

Oocyst and cyst stock suspension − See Stock suspension

Positive control − See Ongoing precision and recovery standard

Principal analyst − The principal analyst should have a BS/BA in microbiology or closely related field and a minimum of 1 year of continuous bench experience with *Cryptosporidium* and IFA microscopy.

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 - stock suspension containing the 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.

Stock suspension - A concentrated suspension containing the organism(s) of interest that is obtained from a source that will attest to the host source, purity, authenticity, and viability of the organism(s).

Appendix A
Microscope Protocols

1.0 This method requires the proper and consistent use of the microscope. All analysts must should be properly trained in the operation of the microscope. Microscope procedures and requirements including bulb adjustment (mercury and transmitted), ocular adjustments, ocular micrometer calibration, Köhler illumination, and cleaning techniques are detailed in this appendix.

2.0 In a room capable of being darkened to near-complete darkness, assemble the microscope, all filters, and attachments. The microscope should be placed on a solid surface free from vibration. Adequate workspace should be provided on either side of the microscope for taking notes and placement of slides and ancillary materials.

3.0 Microscope adjustment and calibration (see References 20.6 and 20.10 of Method 1693)

3.1 Preparations for adjustment

3.1.1 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 maximum 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 are properly adjusted.

3.1.2 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 work for a particular instrument.

3.1.3 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.

3.1.4 Persons with astigmatism should always wear contact lenses or glasses when using the microscope.

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.

3.2 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.

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

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

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

3.2.4 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.

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

3.2.6 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.

3.2.7 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.

3.2.8 Reattach the objective to the nosepiece.

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

3.2.10 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 3.2.7 above may be required.

3.2.11 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. Follow bulb manufacturer's recommendations for number of hours of use.

3.3 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.

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

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

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

3.3.4 Focus the lamp filament image with the appropriate adjustment on the lamp house.

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

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

3.4 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 3.4.2 assumes use of a microscope with both oculars adjustable; Section 3.4.3 assumes use of a microscope with a single adjustable ocular. The procedure must be followed each time an analyst uses the microscope.

3.4.1 Interpupillary distance

3.4.1.1 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.

3.4.1.2 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.

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

3.4.2.1 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.

3.4.2.2 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.

3.4.3 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.

3.4.3.1 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.

3.4.3.2 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.

3.5 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 must be reestablished for the new objective lens. Previous sections have adjusted oculars and 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 maximum 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 and with each objective used for DIC microscopy.

3.5.1 Turn on both the UV and the transmitted light. Adjust the microscope to brightfield microscopy settings. Using a prepared slide, locate a *Giardia* cyst or *Cryptosporidium* oocyst using IFA on 200X magnification. Add oil and switch to 100X oil objective. Switch from UV light to transmitted light and focus the specimen using the fine adjustment knob.

3.5.2 Both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Close the radiant field diaphragm in the microscope base until the lighted field is reduced to a small opening.

3.5.3 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.

3.5.4 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 oculars. 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.

3.5.5 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 closing the aperture diaphragm iris leaves until they are visible just inside the rear plane of the objective.

3.5.6 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.

3.6 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, the ocular reticle should be left in place. The more an ocular is manipulated the greater the probability is for it to become contaminated with dust particles. This calibration should be done for each objective in use on the microscope. 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, each time an objective is replaced, and each time microscope modifications are made.

3.6.1 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.

3.6.2 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.

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

3.6.4 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.

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

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

3.6 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, the ocular reticle should be left in place. The more an ocular is manipulated the greater the probability is for it to become contaminated with dust particles. This calibration should be done for each objective in use on the microscope. 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, each time an objective is replaced, and each time microscope modifications are made.

3.6.1 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.

3.6.2 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.

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

3.6.4 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.

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

3.6.6 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:

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

3.6.7 Follow the procedure above for each objective. Record the information in the example table below and keep the information available at the microscope.

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 x [1000 µm/mm]) ÷ no. ocular micrometer spaces
[3] N.A. refers to numerical aperature. The numerical aperature value is engraved on the barrel of the objective.

4.0 Microscope cleaning procedure

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

4.2 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.

4.3 Protocol for cleaning oculars and condenser

4.3.1 Use a new, clean cotton swab dampened with MCS to clean each lens. Start at the center of the lens and spiral the cotton swab outward using little to no pressure. Rotate the cotton swab while spiraling to ensure a clean surface is always contacting the lens.

4.3.2 Repeat the procedure using a new, dry cotton swab.

4.3.3 Repeat Sections 4.3.1 and 4.3.2.

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

4.4 Protocol for cleaning objective lenses

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

4.4.2 Hold a new cotton swab dampened with MCS at a 45° angle on the objective and twirl.

4.4.3 Repeat Sections 4.4.2 with a new, dry cotton swab.

4.4.4 Repeat Sections 4.4.2 and 4.4.3.

4.4.5 Clean all objectives whether they were/are used or not.

4.5 Protocol for cleaning light source lens and filters

4.5.1 Using a piece of lens paper dampened with microscope cleaning solution, wipe off the surface of each lens and filter.

4.5.2 Repeat the procedure using a dry piece of lens paper.

4.5.3 Repeat Sections 4.5.1 and 4.5.2.

4.6 Protocol for cleaning microscope stage

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

4.7 Use household cleaner and a paper towel to clean the bench top surrounding the microscope.

4.8 Frequency

4.8.1 Perform Sections 4.1, 4.3, 4.4, and 4.6 after each microscope session.

4.8.2 Perform complete cleaning each week at a minimum or after each microscope session.

5.0 Filter block evaluation to confirm use with ColorSeed™. Follow manufacturer instructions to determine if the proper Texas Red and FITC filter sets are installed and optimized for use.

Appendix B
Spike Suspension Enumeration Procedures

1.0 Method 1693 requires the analysis of spiked quality control (QC) samples to demonstrate acceptable initial (IPR samples, MS/MSD samples), and ongoing (OPR samples, MS samples) laboratory and method performance. The organisms used for spiking these samples must be accurately enumerated in order to calculate target recoveries (and precision) and monitor method performance. EPA recommends that flow cytometry be used for enumerating spiking suspensions, rather than manual techniques. Guidance on preparing spiking suspensions using a flow cytometer or manual enumeration procedures is provided in this appendix.

2.0 Equipment required for manual enumeration of spiking suspensions

2.1 Enumeration using a hemacytometer chamber

2.1.1 Hemacytometer

2.1.2 Hemacytometer coverslip

2.2 Enumeration using well slides

2.2.1 Well slides - Spot-On well slides, Dynal cat. no. 740.04 or 12-mm diameter well slides, Meridian Diagnostics Inc., Cincinnati, OH, cat. no. R2206 or equivalent

2.2.2 Glass coverslips – 18 × 18 mm or 22 × 50 mm

2.3 Enumeration using membrane filters

2.3.1 Glass microanalysis filter holder - 25-mm-diameter, with fritted glass support. Replace stopper with size 8, one-hole rubber stopper.

2.3.2 Three-port vacuum filtration manifold and vacuum source

2.3.3 Cellulose acetate support membrane - 1.2-µm-pore-size, 25-mm-diameter.

2.3.4 Polycarbonate track-etch hydrophilic membrane filter - 1-µm-pore-size, 25-mm-diameter.

2.3.5 100 × 15 mm polystyrene petri dishes

2.3.6 60 × 15 mm polystyrene petri dishes

2.3.7 Glass microscope slides - 1” 3” or 2” × 3”

2.3.8 Coverslips - 25 × 25 mm

2.4 Reagents for dilution, manual enumeration and use of spiking suspensions

2.4.1 Tween® 20, 0.01% - Dissolve 1.0 mL of a 10% solution of Tween® 20 in 1 L of reagent water

2.4.2 Diluted Antifoam Y-30 - Dissolve 400 µL of Antifoam Y-30 in 100 mL of reagent water and mix well to emulsify

2.4.3 Additional reagent for enumeration of spiking suspensions using membrane filtration (Section 4.6) - Sigmacote® Sigma Company product no. SL-2 or equivalent

3.0 Flow cytometry enumeration guidelines

Although it is unlikely that many laboratories performing Method 1693 will have direct access to a flow cytometer for preparing spiking suspensions, flow-sorted suspensions are available from commercial vendors and other sources. The information provided in Sections 3.1 through 3.6 is simply meant as a guideline for preparing spiking suspensions using a flow cytometer. Laboratories performing flow cytometry must develop and implement detailed standardized protocols for calibration and operation of the flow cytometer.

3.1 Spiking suspensions should be prepared using unstained organisms that have not been formalin-fixed.

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

3.3 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 should be ≤2.5%. If the RSD is >2.5%, the laboratory should perform the initial calibration again, until the RSD of the 10 counts is ≤2.5%. In addition to counting the organisms, the laboratory also should evaluate the quality of the organisms using DAPI fluorescence and DIC microscopy to confirm that the organisms are in good condition.

3.4 Ongoing calibration. When sorting the spiking suspensions for use in QC samples, the laboratory should perform ongoing calibration samples at a 10% frequency, at a minimum. The laboratory 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 RSD of the ongoing calibration counts is ≤2.5%. If the RSD is >2.5%, the laboratory should discard the batch.

3.5 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 3.4).

3.6 Holding time criteria. Flow-cytometer-sorted spiking suspensions used for spiked QC samples must be used within the expiration date noted on the suspension. The holding time specified by the flow cytometry laboratory should be determined based on a holding time study.

4.0 Manual enumeration procedures

Two sets of manual enumerations are required per organism before purified *Cryptosporidium* oocyst and *Giardia* cyst stock suspensions received from suppliers can be used to spike samples in the laboratory. First, the stock suspension must be diluted and enumerated (Section 4.3) to yield a suspension at the appropriate oocyst or cyst concentration for spiking (spiking suspension). Then, 10 aliquots of spiking suspension must be enumerated to calculate a mean spike dose. Spiking suspensions can be enumerated using hemacytometer chamber counting (Section 4.4), well slide counting (Section 4.5), or membrane filter counting (Section 4.6).

4.1 Precision criteria. The RSD of the calculated mean spike dose for manually enumerated spiking suspensions must be ≤16% for *Cryptosporidium* and ≤19% for *Giardia* before proceeding (these criteria are based on the pooled RSDs of 105 manual *Cryptosporidium* enumerations and 104 manual *Giardia* enumerations submitted by 20 different laboratories under the EPA Protozoa Performance Evaluation Program).

4.2 Holding time criteria. Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 4.4); or within 24 hours of application of the spiking suspension or membrane filter to the slides if the well slide or membrane filter enumeration technique is used (Sections 4.5 and 4.6).

4.3 Enumerating and diluting stock suspensions

4.3.1 Purified, concentrated stock suspensions must be diluted and enumerated before the diluted suspensions are used to spike samples in the laboratory. Stock suspensions should be diluted with reagent water/Tween® 20, 0.01% (Section 2.4.1), to a concentration of 20 to 50 organisms per large hemacytometer square before proceeding to Section 4.3.2.

4.3.2 Apply a clean hemacytometer coverslip (Section 2.1.2) to the hemacytometer and load the hemacytometer chamber with 10 µL of vortexed suspension per chamber. If this operation has been properly executed, the liquid should amply fill the entire chamber without bubbles or overflowing into the surrounding moats. Repeat this step with a clean, dry hemacytometer and coverslip if loading has been incorrectly performed. See Section 4.3.13, below, for the hemacytometer cleaning procedure.

4.3.3 Place the hemacytometer on the microscope stage and allow the oocysts or cysts to settle for 2 minutes. Do not attempt to adjust the coverslip, apply clips, or in any way disturb the chamber after it has been filled.

4.3.4 Use 200X magnification.

4.3.5 Move the chamber so the ruled area is centered underneath the objective.

4.3.6 Move the objective close to the coverslip while watching it from the side of the microscope, rather than through the microscope.

4.3.7 Focus up from the coverslip until the hemacytometer ruling appears.

4.3.8 At each of the four corners of the chamber is a 1-square-mm area divided into 16 squares in which organisms are to be counted (Figure 1). Beginning with the top row of four squares, count with a hand-tally counter in the directions indicated in Figure 2. Avoid counting organisms twice by counting only those touching the top and left boundary lines. Count each square millimeter in this fashion.

Figure 1. Hemacytometer Platform Ruling. Squares 1, 2, 3, and 4 are used to count stock suspensions of *Cryptosporidium* oocysts and *Giardia* cysts (after Miale, 1967)

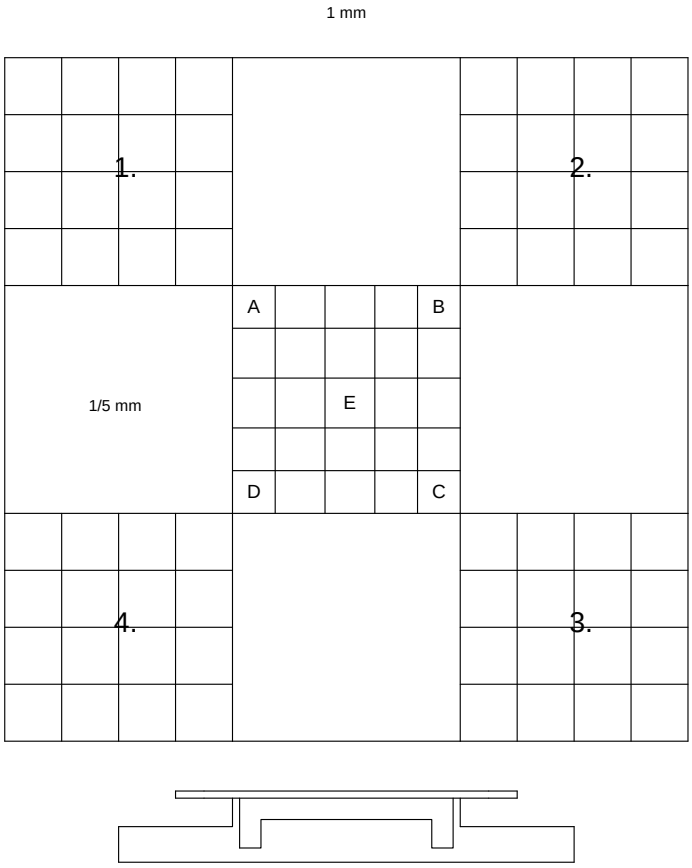
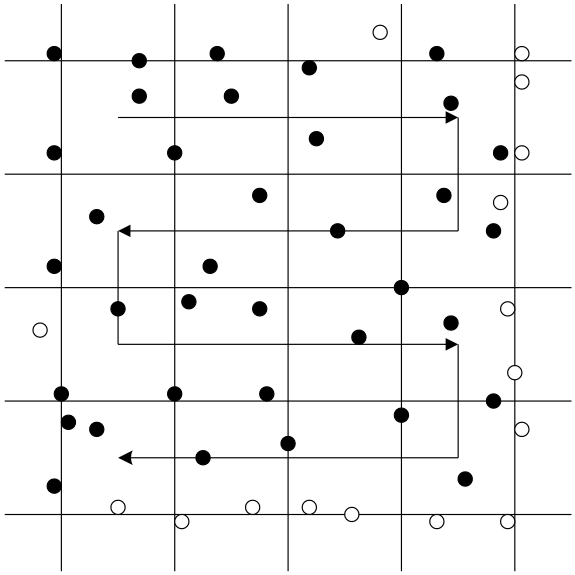


Figure 2. Manner of Counting Oocysts and Cysts in 1 Square mm. Dark organisms are counted and light organisms are omitted (after Miale, 1967).



4.3.9 Use the following formula to determine the number of organisms per µL of suspension:

$$\frac{\text{number of organisms counted}}{\text{number of mm}^2\text{ counted}} \; x \; \frac{10}{1\,mm} \; x \; \frac{dilution\ factor}{1} \; x \; \frac{1\,mm^3}{1\ \mu\text{L}} = \frac{\text{number of organisms}}{\mu}$$

4.3.10 Record the result on a hemacytometer data sheet.

4.3.11 A total of six different hemacytometer chambers must be loaded, counted, and averaged for each suspension to achieve optimal counting accuracy.

4.3.12 Based on the hemacytometer counts, the stock suspension should be diluted to a final concentration of between 8 to 12 organisms per µL; however, ranges as great as 5 to 15 organisms per µL can be used.

Note: If the diluted stock suspensions (the spiking suspensions) will be enumerated using hemacytometer chamber counts (Section 4.4) or membrane filter counts (Section 4.6), then the stock suspensions should be diluted with 0.01% Tween® 20. If the spiking suspensions will be enumerated using well slide counts (Section 4.5), then the stock suspensions should be diluted in reagent water.

To calculate the volume (in µL) of stock suspension required per µL of reagent water (or reagent water/Tween® 20, 0.01%), use the following formula:

$$\text{volume of stock suspension (µL)required} = \frac{\text{required number of organisms}}{\text{number of organisms/µL of stock suspension}}$$

If the volume is less tan 10 µL, an additional dilution of the stock suspension is recommended before proceeding.

To calculate the dilution factor needed to achieve the required number of organisms per 10 µL, use the following formula:

$$\text{total volume (µL)} = \frac{\text{number of organisms required x 10 µL}}{\text{predicted number of organisms per 10 µL (8 to 12)}}$$

To calculate the volume of reagent water (or reagent water/Tween® 20, 0.01%) needed, use the following formula:

$$\text{reagent water volume (µL)} = \text{total volume (µL)} - \text{stock suspension volume required (µL)}$$

4.3.13 After each use, the hemacytometer and coverslip must be cleaned immediately to prevent the organisms and debris from drying on it. Since this apparatus is precisely machined, abrasives cannot be used to clean it, as they will disturb the flooding and volume relationships.

4.3.13.1 Rinse the hemacytometer and cover glass first with tap water, then 70% ethanol, and finally with acetone.

4.3.13.2 Dry and polish the hemacytometer chamber and cover glass with lens paper. Store it in a secure place.

4.3.14 Several factors are known to introduce errors into hemacytometer counts, including:

- Inadequate mixing of suspension before flooding the chamber
- Irregular filling of the chamber, trapped air bubbles, dust, or oil on the chamber or coverslip
- Total number of organisms counted is too low to provide statistical confidence in the result
- Error in recording tally
- Calculation error; failure to consider dilution factor, or area counted
- Inadequate cleaning and removal of organisms from the previous count
- Allowing filled chamber to sit too long, so that the chamber suspension dries and concentrates.

4.4 Enumerating spiking suspensions using a hemacytometer chamber

Note: Spiking suspensions enumerated using a hemacytometer chamber must be used within 24 hours of enumeration.

4.4.1 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 4.3) for a minimum of 2 minutes. Gently invert the tube three times.

4.4.2 To an appropriate-size beaker containing a stir bar, add enough spiking suspension to perform all spike testing and the enumeration as described. The liquid volume and beaker relationship should be such that a spinning stir bar does not splash the sides of the beaker, the stir bar has unimpeded rotation, and there is enough room to draw sample from the beaker with a 10-µL micropipette without touching the stir bar. Cover the beaker with a watch glass, petri dish, or parafilm to prevent evaporation between sample withdrawals.

4.4.3 Allow the beaker contents to stir for a minimum of 30 minutes before beginning enumeration.

4.4.4 While the stir bar is still spinning, remove a 10-µL aliquot and carefully load one side of the hemacytometer. Count all organisms on the platform, at 200X magnification using phase-contrast or darkfield microscopy. The count must include the entire area under the hemacytometer, not just the four outer 1-mm2 squares. Repeat this procedure nine times. This step allows confirmation of the number of organisms per µL (Section 4.3.12). Based on the 10 counts, calculate the mean, standard deviation, and RSD of the counts. Record the counts and the calculations on a spiking suspension enumeration form. The relative standard deviation (RSD) of the calculated mean spike dose must be ≤16% for

Cryptosporidium and ≤19% for *Giardia* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts. Refer to Section 4.3.14 for factors that may introduce errors.

4.5 Enumerating spiking suspensions using well slides

Note: Spiking suspensions enumerated using well slides must be used within 24 hours of application of the spiking suspension to the slides.

4.5.1 Prepare well slides for sample screening and label the slides.

4.5.2 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 4.3) for a minimum of 2 minutes. Gently invert the tube three times.

4.5.3 Remove a 10-µL aliquot from the spiking suspension and apply it to the center of a well.

4.5.4 Before removing subsequent aliquots, cap the tube and gently invert it three times to ensure that the oocysts or cysts are in suspension.

4.5.5 Ten wells must be prepared and counted, and the counts averaged, to sufficiently enumerate the spike dose. Air-dry the well slides. Because temperature and humidity varies from laboratory to laboratory, no minimum time is specified. However, the laboratory must take care to ensure that the sample has dried completely before staining to prevent losses during the rinse steps. A slide warmer set at 35°C to 42°C also can be used.

4.5.6 Positive and negative controls must be prepared.

4.5.6.1 For the positive control, pipette 10 µL of positive antigen or 200 to 400 intact oocysts or cysts to the center of a well and distribute evenly over the well area.

4.5.6.2 For the negative control, pipette 50 µL of PBS onto the center of a well and spread it over the well area with a pipette tip.

4.5.6.3 Air-dry the control slides.

4.5.7 Follow the manufacturer's instructions in applying the stain, mounting medium, and cover slip to the slide.

Note: If using Merifluor™ *Cryptosporidium*/*Giardia*, do not allow slides to dry completely.

4.5.8 Record the date and time that staining was completed. If slides will not be read immediately, store in a humid chamber in the dark at 0°C to <10°C and not frozen until ready for examination.

4.5.9 After examination of the 10 wells, calculate the mean, standard deviation, and RSD of the 10 replicates. Record the counts and the calculations on a spiking suspension enumeration form. The RSD of the calculated mean spike dose must be ≤16% for *Cryptosporidium* and ≤19% for *Giardia* before proceeding. If the RSD is unacceptable, or the mean is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts.

4.6 Enumeration of spiking suspensions using membrane filters

Note: Spiking suspensions enumerated using membrane filters must be used within 24 hours of application of the filters to the slides.

4.6.1 Precoat the glass funnels with Sigmacote® by placing the funnel in a large petri dish and applying 5-mL of Sigmacote® to the funnel opening using a pipette and allowing it to run down the inside of the funnel. Repeat for all funnels to be used. The pooled Sigmacote® may be returned to the bottle for re-use. Place the funnels at 35°C or 41°C for approximately 5 minutes to dry.

4.6.2 Place foil around the bottoms of the 100 x 15 mm petri dishes.

4.6.3 Filter-sterilize approximately 10 mL of PBS. Dilute detection reagent as per manufacturer's instructions using sterile PBS. Multiply the anticipated number of filters to be stained by 100 µL to calculate total volume of stain required. Divide the total volume required by 5 to obtain the microliters of antibody necessary. Subtract the volume of antibody from the total stain volume to obtain the required microliters of sterile PBS to add to the antibody.

4.6.4 Label the tops of foil-covered, 60 × 15 mm petri dishes for 10 spiking suspensions plus positive and negative staining controls and multiple filter blanks controls (one negative control, plus a blank after every five sample filters to control for carry-over). Create a humid chamber by laying damp paper towels on the bottom of a stain tray (the inverted foil-lined Petri dishes will protect filters from light and prevent evaporation during incubation).

4.6.5 Place a decontaminated and cleaned filter holder base (Section 2.3.1) into each of the three ports of the vacuum manifold (Section 2.3.2).

4.6.6 Pour approximately 10 mL of 0.01% Tween® 20 into a 60 × 15 mm petri dish.

4.6.7 Using forceps, moisten a 1.2-µm cellulose-acetate support membrane (Section 2.3.3) in the 0.01% Tween® 20 and place it on the fritted glass support of one of the filter bases. Moisten a polycarbonate filter (Section 2.3.4) the same way and position it on top of the cellulose-acetate support membrane. Carefully clamp the glass funnel to the loaded filter support. Repeat for the other two filters.

4.6.8 Add 5 mL of 0.01% Tween® 20 to each of the three filtration units and allow to stand.

4.6.9 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 4.3) for a minimum of 2 minutes. Gently invert the tube three times.

4.6.10 Using a micropipettor, sequentially remove two, 10-µL aliquots from the spiking suspension and pipet into the 5 mL of 0.01% Tween® 20 standing in the unit. Rinse the pipet tip twice after each addition. Apply 10 µL of 0.01% Tween® 20 to the third unit to serve as the negative control. Apply vacuum at 2" Hg and allow liquid to drain to miniscus, then close off vacuum. Pipet 10 mL of reagent water into each funnel and drain to miniscus, closing off the vacuum. Repeat the rinse and drain all fluid, close off the vacuum.

4.6.11 Pipet 100 µL of diluted antibody to the center of the bottom of a 60 × 15 mm petri dish for each sample.

4.6.12 Unclamp the top funnel and transfer each cellulose acetate support membrane/ polycarbonate filter combination onto the drop of stain using forceps (apply each membrane/filter combination to a different petri dish containing stain). Roll the filter into the drop to exclude air. Place the small petri dish containing the filter onto the damp towel and cover with the corresponding labeled foil-covered top. Incubate for approximately 45 minutes at room temperature.

4.6.13 Reclamp the top funnels, apply vacuum and rinse each three times, each time with 20 mL of reagent water.

4.6.14 Repeat Sections 4.6.4 through 4.6.10 for the next three samples (if the diluted spiking suspension has sat less than 15 minutes, reduce the suspension vortex time to 60 seconds). Ten, 10-µL spiking suspension aliquots must be prepared and counted, and the counts averaged, to sufficiently enumerate the spike dose. Include a filter blank sample at a frequency of every five samples; rotate the position of filter blank to eventually include all three filter placements.

4.6.15 Repeat Sections 4.6.4 through 4.6.10 until the 10-µL spiking suspensions have been filtered. The last batch should include a 10-µL 0.01% Tween® 20 blank control and 20 µL of positive control antigen as a positive staining control.

4.6.16 After incubation is complete, for each sample, transfer the cellulose acetate filter support and polycarbonate filter from drop of stain and place on fritted glass support. Cycle vacuum on and off briefly to remove excess fluid. Peel the top polycarbonate filter off the supporting filter and place on labeled slide. Discard cellulose acetate filter support. Mount and apply coverslips to the filters immediately to avoid drying.

4.6.17 To each slide, add 20 µL of mounting medium.

4.6.18 Apply a coverslip. Seal the edges of the coverslip onto the slide using clear nail polish. (Sealing may be delayed until cover slips are applied to all slides.)

4.6.19 Record the date and time that staining was completed. If slides will not be read immediately, store sealed slides in a closed container in the dark at <10°C (but not frozen) until ready for examination.

4.6.20 After examination of the 10 slides, calculate the mean, standard deviation, and RSD of the 10 replicates. Record the counts and the calculations on a spiking suspension enumeration form. The RSD of the calculated mean spike dose must be ≤16% for *Cryptosporidium* and ≤19% for *Giardia* before proceeding. If the RSD is unacceptable, or the mean is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts.

4.6.21 If oocysts or cysts are detected on the filter blanks, modify the rinse procedure to ensure that no caryover occurs and repeat enumeration.