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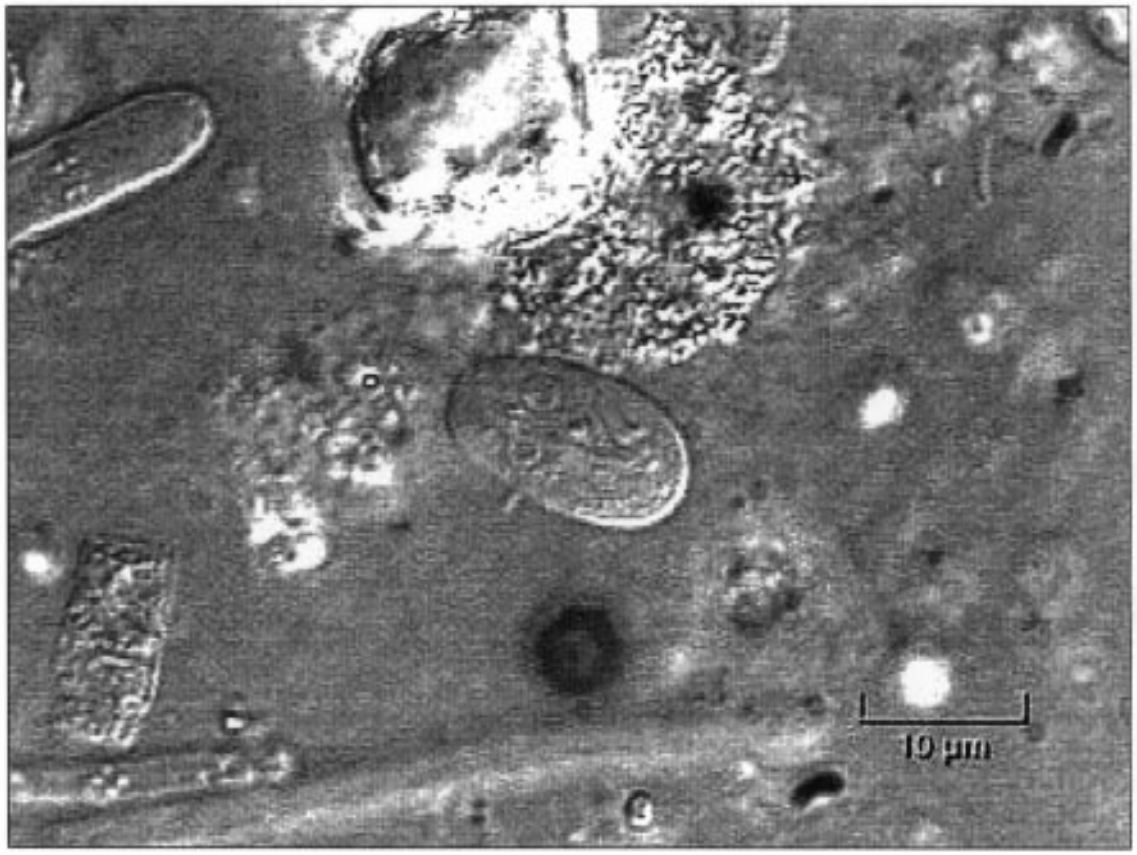
EPA

ICR Protozoan Method for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procedure



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United States Environmental Protection Agency
OGWDW/TSD/WSTB
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Cincinnati, OH 45268

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On the cover: A classic *Giardia* cyst photographed at 1,000X magnification showing nuclei, axonemes, and median bodies.

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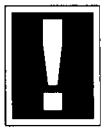
About This Document

This bench-top guide is intended for use by laboratory personnel analyzing water samples for the presence of *Giardia* cysts and *Cryptosporidium* oocysts in accordance with the Information Collection Requirements Rule. It provides details of the procedure presented in the accompanying video. To further associate the steps of the procedure with the training video, this manual is illustrated with stills taken directly from the video.

Several graphic conventions are used throughout the manual to differentiate steps or draw attention to important information:

A step icon is used to denote each step in the protozoan monitoring protocol. These steps also are illustrated in the training video.



Important information, and valuable tips which are not part of the published protocol, are denoted by this icon.

EPA recommends that laboratory analysts and technicians watch the training video and read this manual in order to become familiar with the method for detecting cysts and oocysts by this fluorescent antibody procedure. The video concludes with a summary of the method. Analysts and technicians also can use the manual as a bench-top reference during the execution of the method.

Acknowledgments

This bench-top guide and the accompanying training video were developed by the U.S. Environmental Protection Agency (EPA), Office of Water, Office of Ground Water and Drinking Water (OGWDW), Cincinnati, Ohio. Jim Walasek of OGWDW's Technical Support Division (TSD) was the project manager, and provided the aerial photography used in the video. Frank Schaefer, Ph.D. of the Office of Research and Development's National Exposure Research Laboratory (NERL) wrote the text of the guide. He was the principal technical advisor in the preparation of the video, and was responsible for the photomicrography used in both the guide and the video. Shay Fout, Ph.D. of NERL also served as technical advisor on the project. The exhibits found in Appendix E were prepared by Frederick Williams of NERL. Ken Mayo of The Cadmus Group, Inc. in Waltham, Massachusetts edited, designed, and coordinated production of the guide.

The still photos used in this guide come from the accompanying training video. The video was taped at the U.S. EPA laboratories in Cincinnati, Ohio. Melissa Godoy of Impact Video Productions in Cincinnati was the producer, and Graham Spencer and Steve Willis were the editors. Donna Jensen of Cadmus acted as analyst during the taping, and Michelle Thomas, an Oak Ridge DOE (Department of Energy) Fellow at EPA, was the technician.

A Note About Safety

Neither this bench-top manual, nor the training video which accompanies it, address all of the safety and quality assurance needs of the analytical method. The materials used are of a potentially biohazardous nature, and it is the responsibility of the users of this method to establish appropriate safety and health practices.

In particular, the analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of sample concentrates, reagents and materials, and while operating sterilization equipment. **Do not mouth pipet in any portion of this procedure.**

Chapter 1
Introduction to the Method

Scope of the Method

This test method describes the detection and enumeration of *Giardia* cysts and *Cryptosporidium* oocysts in ground, surface, and finished waters by a fluorescent antibody procedure. These pathogenic intestinal protozoa occur in domestic and wild animals as well as in humans. The environment may become contaminated through direct deposit of human and animal feces or through sewage and wastewater discharges to receiving waters. Ingestion of water containing these organisms may cause disease.

Results obtained by this method should be interpreted with extreme caution. High turbidity, as well as turbidities less than 1 NTU, can affect the recovery efficiency of this method. Failure to detect organisms of interest and/or a low detection limit does not ensure that the water tested is pathogen-free.

This method does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this method to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

Summary of Test Method

In this test method, pathogenic intestinal protozoa are concentrated from a large volume water sample by retention on a yarn-wound filter. Retained particulates are eluted from the filter with an eluting solution and are concentrated by centrifugation. *Giardia* cysts and *Cryptosporidium* oocysts are separated to some extent from other particulate debris by flotation on a Percoll-sucrose solution with a specific gravity of 1.1. A monolayer of the water layer/Percoll-sucrose interface is placed on a membrane filter, indirectly stained with fluorescent antibody, and examined under a microscope.

Cysts and oocysts are classified according to specific criteria (immunofluorescence, size, shape, and internal morphological characteristics), and the results are reported in terms of the categories per 100 L. The reporting categories include cysts and oocysts that are empty, that have amorphous structure, and that have internal structure. A sum of the cysts and oocysts that fall into each of these categories is also reported as the total Indirect Fluorescent Antibody (IFA) count.

Significance and Use

This test method will provide a quantitative indication of the level of contamination in raw and treated drinking waters with the environmentally resistant stages of two genera of pathogenic intestinal protozoa: *Giardia* and *Cryptosporidium*.

The method will not identify the species of protozoa, or the host species of origin. It cannot determine the viability status or the infectivity status of detected cysts and oocysts.

This test method may be useful in determining the source or sources of contamination of water sup-

plies, as well as the occurrence and distribution of protozoa in water supplies. The method also may be useful in evaluating the effectiveness of treatment practices.

Interferences

Turbidity due to inorganic and organic debris and other organisms, can interfere with the concentration, purification and examination of the sample for *Giardia* cysts and *Cryptosporidium* oocysts.

Inorganic and organic debris may be naturally occurring (e.g., clays and algae) or may be added to water in the treatment process (e.g., iron and alum coagulants and polymers).

Organisms and debris that autofluoresce or demonstrate nonspecific fluorescence when examined by epifluorescent microscopy (e.g., algal and yeast cells and *Spironucleus* (*Hexamita)* sp.[1]) could interfere with the detection of cysts and oocysts and contribute to false positive values.

Chlorine compounds, and perhaps other chemicals used to disinfect or treat drinking water and wastewater, may interfere with the visualization of internal structures of *Giardia* cysts and *Cryptosporidium* oocysts.

Freezing filter samples, eluates, or concentrates could interfere with the detection and/or identification of cysts and oocysts originally present in the sample.

Education, Training and Proficiency

Minimal personnel requirements include the following:

Principal Analyst/Supervisor

To be qualified for approval, a laboratory must have a principal analyst, who may also serve as a supervisor if an additional analyst(s) is to be involved. The principal analyst/supervisor oversees the entire analysis and carries out QC performance checks on technicians and/or other analysts. This person must confirm all protozoan internal structures that are demonstrated at the microscope by subordinates.

The principal analyst/supervisor must be an experienced microbiologist with at least a B.A./B.S. degree in microbiology or a closely related field. The principal analyst also must have at least 1 year of continuous bench experience with immunofluorescent antibody (IFA) techniques and microscopic identification and have analyzed at least 100 water and/or wastewater samples for *Giardia* and/or *Cryptosporidium*. In addition, PE samples must be analyzed using the ICR protozoan method, and the results must fall within acceptance limits. The principal analyst/supervisor must demonstrate acceptable performance during an on-site evaluation.

Analyst

This person(s) performs at the bench level under the supervision of a principal analyst/supervisor and is involved in all aspects of the analysis, including preparation of sampling equipment, filter extraction, sample processing, microscopic protozoan identification, and data handling. Recording presence or absence of morphological characteristics may be done by the analyst but must be confirmed by the principal analyst.

The analyst must have two years of college lecture and laboratory course work in microbiology or a closely related field. The analyst also must have at least 6 months bench experience, must have at least 3 months experience with IFA techniques, and must have successfully analyzed at least 50 water and/or wastewater samples for *Giardia* and/or *Cryptosporidium*. Six months of additional bench

experience in the above areas may be substituted for two years of college. In addition, PE samples must be analyzed using the ICR protozoan method and results must fall within acceptance limits. The analyst must also demonstrate acceptable performance during an on-site evaluation.

Technician

This person extracts filters and processes the samples under the supervision of an analyst, but does not perform microscopic protozoan detection and identification. The technician must have at least three months of experience in filter extraction and processing of protozoa samples.

Terminology

- **Axoneme.** An internal flagellar structure which occurs in some protozoa, e.g., *Giardia*, *Spironucleus*, and *Trichomonas*.

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

- **Median bodies.** Prominent, dark-staining, paired organelles consisting of microtubules and found in the posterior half of *Giardia*. In *G. lamblia* (from humans), these structures often have a claw-hammer shape, while in *G. muris* (from mice) the median bodies are round.

- **Oocyst.** The encysted zygote of some Sporozoa, e.g., *Cryptosporidium*. This is a phase or a form of the 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.

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

- **Nucleus.** A prominent internal structure seen in *Giardia* cysts and *Cryptosporidium* oocysts. Sometimes 2 to 4 nuclei can be seen in *Giardia* cysts. In *Cryptosporidium* oocysts, one nucleus per sporozoite sometimes can be seen.

Key Words

Antibody, *Cryptosporidium parvum*, cysts, fluorescence, *Giardia*, immunoassay, oocysts, protozoa.

End Notes

[1] Januschka, M.M., et al. 1988. A comparison of *Giardia microti* and *Spironucleus muris* cysts in the vole: an immunocytochemical, light, and electron microscopic study. Journal of Parasitology 74(3):452-458.

Chapter 2
Equipment

Sample Processing Apparatus

- Centrifuge, with swinging bucket rotors having a capacity of 15 to 250 mL or larger per conical tube or bottle.
- Mixer, vortexer.
- Vacuum source.
- Membrane filter holder, Hoefer manifold, model FH 225V[1], 10 place holder for 25 mm diameter filters.
- Slide warming tray, or incubator, 37°C.
- pH meter.
- Rubber policeman.
- Stomacher Lab Blender, model 3500 (BA 7022)[2] (optional).

Sample Examination Apparatus

- Microscope, capable of epifluorescence and differential interference contrast (D.I.C.) or Hoffman modulation® optics, with stage and ocular micrometers and 20X (N.A. = 0.6) to 100X (N.A. = 1.3) objectives. Equip the microscope with appropriate excitation and band pass filters for examining fluorescein isothiocyanate-labeled specimens (exciter filter: 450-490 nm; dichroic beam-splitting mirror: 510 nm; barrier or suppression filter: 515-520 nm).

Reagents and Materials

Purity of Reagents

Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the committee on Analytical Reagents of the American Chemical Society where such specifications are available[3].

Preparation of Reagents

Prepare reagents as specified by the formulations.

Purity of Water

Use distilled deionized, double distilled, or reagent grade water.

Sample Collection Reagents

- Sodium Thiosulfate Solution (2.0%) - Dissolve 2.0 g of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in 50 mL water and then adjust to a final volume of 100 mL.

Sample Processing Reagents

- Neutral Buffered Formalin Solution (10%) - Dissolve 0.762 g disodium hydrogen phosphate (Na_2HPO_4), 0.019 g sodium dihydrogen phosphate (NaH_2PO_4), and 100 mL formalin in water to a final volume of 1 L.

- Phosphate Buffered Saline (PBS) - Prepare a 10X stock solution by dissolving 80 g sodium chloride (NaCl), 2 g potassium dihydrogen phosphate (KH_2PO_4), 29 g hydrated disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12\ H_2O$) and 2 g potassium chloride (KCl) in water to a final volume of 1 L. The 10X solution is used to prepare 1X PBS by diluting one volume of the 10X solution with 9 volumes of water and adjust the pH with a pH meter to 7.4 with 0.1 N HCl or 0.1 N NaOH before use.

- Sodium Dodecyl Sulfate Stock Solution (1%) - Prepare solution by dissolving 1.0 g of sodium dodecyl sulfate (SDS) in water to a final volume of 100 mL.

- Tween 80 Stock Solution (1%) - Mix 1.0 mL of polyoxyethylenesorbitan monooleate 80 (Tween 80) stock solution with 99 mL of water.

- Eluting Solution (Buffered Detergent Solution) - Prepare solution by mixing 100 mL 1% SDS, 100 mL 1% Tween 80, 100 mL 10X PBS, and 0.1 mL Sigma Antifoam A (Cat. # A 5758) with 500 mL water. Adjust the pH to 7.4 using a pH meter. Adjust the final volume to 1 L with additional water. Use within one week of preparation.

- Sucrose Solution (2.5 M) - Dissolve 85.58 g of sucrose in 40 mL prewarmed water then adjust the final volume to 100 mL with water.

- Percoll-Sucrose Flotation Solution, Sp. Gr. 1.10 - Mix 45 mL Percoll (sp. gr. 1.13; Sigma Cat. # P 1644), 45 mL water and 10 mL 2.5 M sucrose solution. Check the specific gravity with a hydrometer. The specific gravity should be between 1.09 and 1.10 (do not use if less than 1.09). Store at 4°C and use within a week. Allow to reach room temperature before use.

Sample Examination Reagents

- Ensys Hydrofluor-Combo kit[4] for detecting *Giardia* cysts and *Cryptosporidium* oocysts in water samples. The expiration date for the reagents is printed on the Hydroflour-Combo kit label. Discard the kit once the expiration date is reached. Store the kit at 2-8°C and return it promptly to this temperature range after each use. The labeling reagent should be protected from exposure to light. Do not freeze any of the reagents in this kit. Diluted, unused working reagents should be discarded after 48 hours.

- Ethanol, (95%).

- Glycerol.

- Ethanol/Glycerol Series - Prepare a series of solutions according to the following table:

95% Ethanol	Glycerol	Reagent Water	Final Volume	Final % Ethanol
10mL	5 mL	80 mL	95 mL	10
20 mL	5 mL	70 mL	95 mL	20
40 mL	5 mL	50 mL	95 mL	40
80 mL	5 mL	10 mL	95 mL	80
95 mL	5 mL	0 mL	100 mL	90.2

- DABCO-Glycerol Mounting Medium (2%) - Prewarm 95 mL glycerol using a magnetic stir bar on a heating stir plate. Add 2 g 1,4 diazabicyclo [2.2.2] octane (DABCO, Sigma #D-2522) to the warm glycerol with continuous stirring until it dissolves. (**CAUTION: hygroscopic; causes burns; avoid inhalation, as well as skin and eye contact.**) Adjust the final volume to 100 mL with additional glycerol. Store at room temperature and discard after 6 months.

- Bovine Serum Albumin (1%) - Sprinkle 1.0 g bovine serum albumin (BSA) crystals over 85

mL 1X PBS, pH 7.4. Allow crystals to fall before stirring into solution with a magnetic stir bar. After the BSA is dissolved, adjust the volume to 100 mL with PBS. For prolonged storage, sterilize by filtering through a 0.22 µm membrane filter into a sterile tube or bottle. Store at 4°C and discard after 6 months.

Sample Collection Materials

- Filters and filter holder, either a 25.4 cm (10 in.) long 1 µm nominal porosity, yarn-wound polypropylene cartridge Commercial honeycomb filter tube (M39R10A; Commercial Filters Parker Hannifin Corp., P.O. Box 1300, Lebanon, IN) with a Commercial LT-10 filter holder or a 25.4 cm (10 in.) long 1 µm nominal porosity Filterite polypropylene cartridge (U1A10U; Filterite Corporation, Timmonium, MD), with a Filterite LMO10U-3/4 filter holder must be used.
- Garden hose or PVC tubing and connectors.
- Pressure regulator.
- Pressure gauge(s).
- Proportioner.
- Plastic sample bags, double-track, zipper-lock or equivalent, approximately 15 in. (38 cm) x 15 in (38 cm).
- Cold packs or wet ice.

Sample Processing Materials

- Pans or trays, stainless steel or glass trays, approx. 16.5 in. (41.91 cm) x 10 in. (25.4 cm) x 2 in. (5.08 cm) deep.
- Disposable knife/cutting tool, for cutting the polypropylene filter fibers off filter core.
- Hydrometer, for liquids heavier than water (range: 1.000-1.225), for adjusting specific gravity of flotation solutions.

Sample Examination Materials

- Slides, glass microscope, 1 in. (2.54 cm.) x 3 in. (7.62 cm) or 2 in. (5.08 cm.) x 3 in. (7.62 cm.).
- Cover slips, 25 mm^2, No. 1 1/2.
- Filters, Sartorius brand[5] cellulose acetate, 0.22 µm pore size, 25 mm diameter.
- Support Filters, ethanol-compatible membrane, any pore size, 25 mm.
- Fingernail polish, clear, or clear fixative (Cat. # 60-4890; PGC Scientifics[6]).
- Splinter forceps, fine tip.
- Blunt-end filter forceps.

End Notes

[1] Hoefer Scientific Instruments, 654 Minnesota Street, Box 77387, San Francisco, CA 94107

[2] Tekmar Company, P.O. Box 371856, Cincinnati, OH 45222-1856

[3] "Reagent Chemicals, American Chemical Society Specifications", American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Annular Standards for Laboratory Chemicals," BDH, Poole, Dorset, U.K. and the "United States Pharmacopeia."

[4] Ensys Environmental Products, Inc., P.O. Box 14063, Research Triangle Park, NC 27709

[5] Sartorius Corp., Filter Div., 30940 San Clemente, Hayward, CA 94544

[6] PGC Scientifics, P.O. Box 7277, Gaithersburg, MD 20898-7277

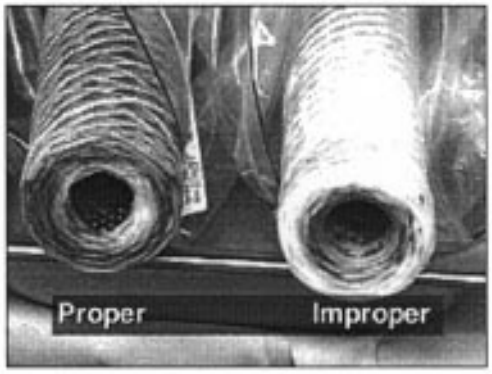
Chapter 3
Filter Elution

Before beginning the assay procedure, check the validity and completeness of the data provided with the sample. Essential information includes the:

- Name of the source water sampled.
- Number of gallons filtered.
- Turbidity of the sampled source water.
- Time and date when the sample collection began.

This information is required to properly report the results of the analysis, and to ensure that the filter is eluted within 96 hours of the start of sampling.

! Check the filter to ensure that the sample was collected properly. If the fibers closest to the filter core appear to be the dirtiest, the sampled water may have flowed through the filter in the wrong direction. If improper sampling is suspected, investigate the sampling procedures. An improperly collected sample must not be analyzed.



The initiation of sample collection and elution from the collection filter must be performed within 96 hours. Two approaches to eluting the particulates from the filter may be used: either washing by hand or using a stomacher.

! The materials used from this point on are potentially of a biohazardous nature, and must be treated and discarded appropriately. Before starting the analysis, make sure you are wearing safety glasses and latex gloves.

Filter Washing

Step 1 Pour the residual solution in the bag into a beaker, rinse the bag with eluting solution, add the rinse solution to the beaker and discard the bag.

Handwashing

Step 1 Using a razor knife or other appropriate disposable cutting instrument, cut the filter fibers lengthwise down to the core. Discard the blade after the fibers have been cut.

Rinse the filter core with eluating solution into the beaker containing residual solution from the filter bag.

Divide the filter fibers into a minimum of six equal portions with one-sixth consisting of those cleanest fibers nearest the core; the second one-sixth being the second, slightly dirtier, layer of fibers, and so on to the final one-sixth consisting of the outer-most filter fibers (the dirtiest fibers).

Step 2 Beginning with the cleanest fibers (the one-sixth nearest the core), hand wash the fibers in three consecutive 1.0 L volumes of eluting solution.

Wash the fibers by kneading them in the eluting solution contained either in a beaker or a plastic bag.

Wring the fibers to express as much of the liquid as possible before discarding.

Maintain the three 1.0 L volumes of eluate separate throughout the washing procedure.

Step 3 Using the same three 1.0 L volumes of eluate used in Step 2, repeat the washing procedure on the second one-sixth layer of fibers and then on sequentially to the final outer one-sixth layer of fibers. The minimum total wash time of fibers should be 30 minutes.

! For extremely dirty filters, additional beakers of eluting solution may be required.

Step 4 After all the fibers have been washed, combine the three 1.0 L volumes of eluate with the residual filter water collected when the filter was unwrapped. Discard the filter fibers before preparing the eluate and residual water for concentration in a centrifuge.

Stomacher Washing

Step 1 Using a razor knife or other appropriate disposable cutting instrument, cut the filter fibers lengthwise down to the core. Discard the blade after cutting the fibers.

Rinse the filter core with eluating solution into the beaker containing residual solution from the filter bag.

Step 2 After loosening the fibers, place all the filter fibers in a stomacher bag that has a 3,500 mL capacity. To ensure against bag breakage and sample loss, place the stomacher bag that contains the filter fibers into a second stomacher bag.

Step 3 Add 1.75 L of eluting solution to the fibers, and homogenize for two 5-minute intervals. Between each homogenization period, knead the filter material by hand to redistribute the fibers in the bag.

Step 4 At the end of the second homogenization period, pour the eluted particulate suspension into a 4-liter pooling beaker. Wring the fibers out to express as much of the liquid as possible before.

Step 5 Return the fibers to the stomacher bag and add 1.0 L of eluting solution. Homogenize, as in Step 3, for two 5-minute periods. Between each homogenization period, knead the fiber materials by hand to redistribute them
in the bag.

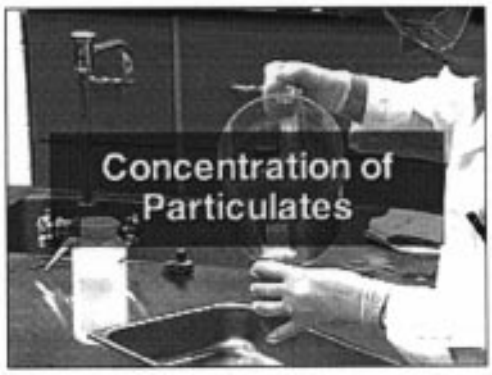
Step 6 At the end of the second homogenization period, add the eluted particulate suspension to the 4-liter pooling beaker. Wring the fibers out to express as much of the liquid as possible into the beaker. Discard the fibers, and rinse the stomacher bag with eluting solution into the pooling beaker.

Concentration of Particulates

After the filter fibers have been washed, and the eluate and residual water has been pooled, the particulates in suspension must be recovered using a centrifuge.

Step 1 Pour the liquid into conical centrifuge bottles, then balance the bottles in their shields.

! Make sure to use the appropriate cushion for each bottle in the shield. Otherwise, the conical bottle will collapse during centrifugation.



Step 2 Concentrate the combined eluate and residual water into a single pellet by centrifugation at 1,050 x g for 10 minutes using a swinging bucket rotor and plastic conical centrifuge bottles.

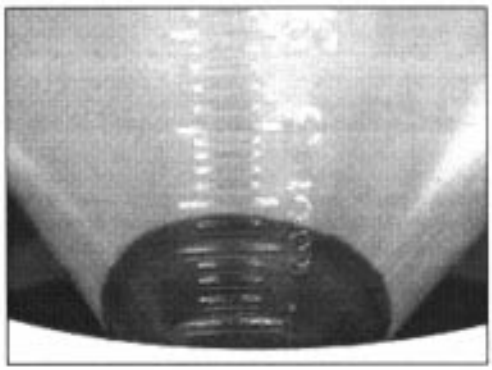
! You must use a swinging bucket rotor for this operation.

Step 3 At the end of 10 minutes, carefully aspirate and discard the supernatant fluid. Resuspend the pellet in sufficient elution solution by vortexing.

! Vortexing is done between centrifugations to prevent excessive packing of the particulates that form the pellet.

Step 4 Continue centrifugations of the pooled eluate and residual water at 1,050 x g for 10 minutes until all the particulates are concentrated in one conical bottle.

Step 5 At the end of this period, remove the bottle from the centrifuge and record the packed pellet volume.



Step 6 Carefully aspirate and discard the supernatant fluid. Resuspend the pellet by vortexing in an equal volume of 10% neutral buffered formalin solution. If the packed pellet volume is less than 0.5 mL, bring the pellet and solution volume to 0.5 mL with eluting solution before adding enough 10% neutral buffered formalin solution to bring the resuspended pellet volume to 1.0 mL.

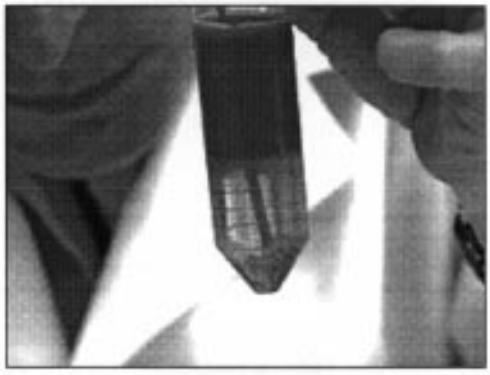
Step 7 At this point, a break of up to 72 hours may be inserted if the procedure is not going to progress immediately to the Flotation Purification procedure described in chapter 4. If a break is inserted at this point, be sure to store the formalin-treated sample at 4°C for not more than 72 hours.

Chapter 4
Flotation Purification

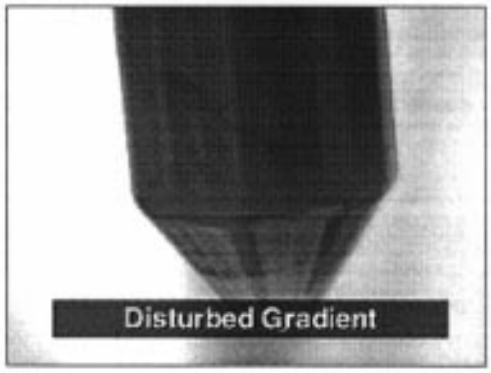
Flotation Purification is used to separate any *Giardia* cysts and *Cryptosporidium* oocysts present in the sample from certain other particulates and other debris which have a greater specific gravity.

Step 1 In one or more clear plastic 50 mL conical centrifuge tubes, as necessary, vortex a volume of resuspended pellet equivalent to not more than 0.5 mL of packed pellet volume with enough eluting solution to make a final volume of 20 mL.

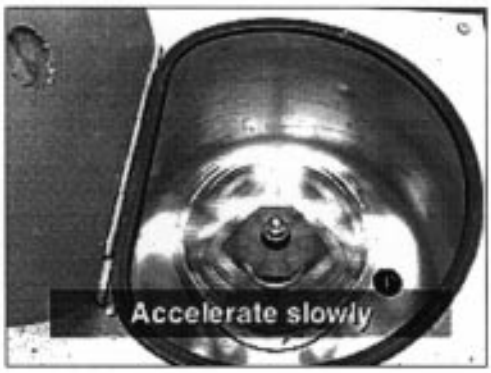
Step 2 Using a 50 mL syringe and 14 gauge cannula, underlay the 20 mL vortexed suspension of particulates with 30 mL Percoll-sucrose flotation solution (sp. gr. 1.1).



! Some water samples will have heavier particulates that penetrate the gradient before centrifugation begins in Step 3. This is normal. However, if for any reason the interface is disturbed and mixed, then another sample aliquot must be used.

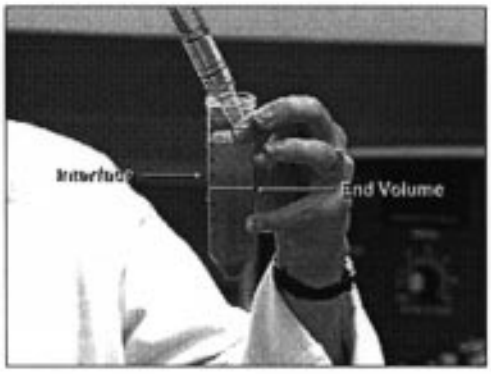


Step 3 Without disturbing the pellet suspension/Percoll-sucrose interface, centrifuge the preparation at 1,050 x g for 10 minutes using a swinging bucket rotor.



! To avoid disrupting the interface, slowly accelerate the centrifuge over a 30-second interval up to the speed where the tubes are horizontal. Similarly, at the end of centrifugation, decelerate slowly. **Do not use the brake!**

Step 4 Using a polystyrene 25 mL pipet rinsed with eluting solution, draw off the top 20 mL particulate suspension layer, the interface, and 5 mL of the Percoll-sucrose below the interface.



Place all these volumes in a plastic 50 mL conical centrifuge tube.

Step 5 Add additional eluting solution to the plastic conical centrifuge tube (Step 4) to bring the final volume to 50 mL. Centrifuge at 1,050 x g for 10 minutes.

Step 6 Aspirate and discard the supernatant fluid down to 5 mL (plus pellet).

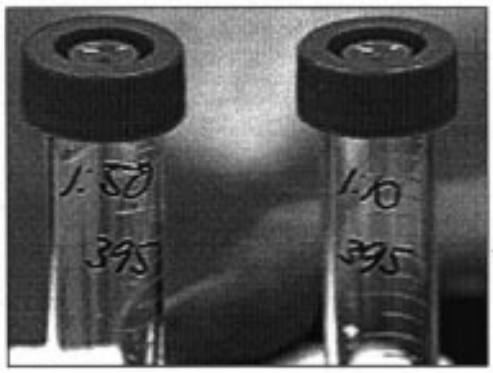
Resuspend the pellet by vortexing, and save this suspension for further processing with fluorescent antibody reagents.

Chapter 5
Indirect Fluorescent Antibody Procedure

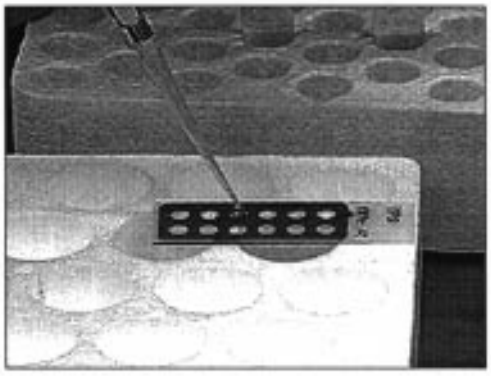
The Indirect Fluorescent Antibody, or IFA, Procedure stains *Giardia* cysts and *Cryptosporidium* oocysts so they can be seen with epifluorescence microscopy.

Determining Sample Volume per Filter

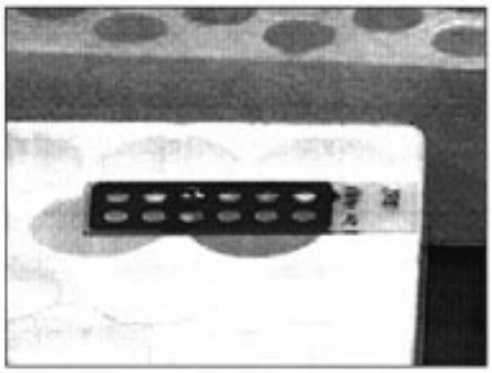
Step 1 Determine the volume of sample concentrate from the Flotation Purification procedure just completed that may be applied to each 25-mm diameter membrane filter used in the IFA assay.



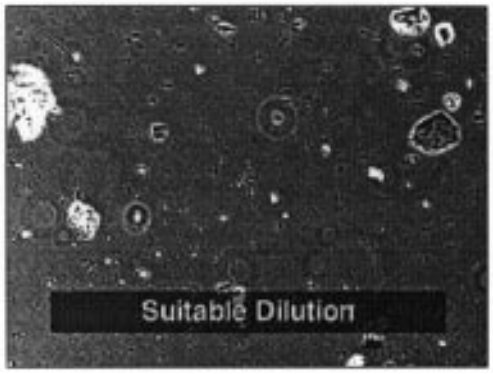
Step 2 Vortex the sample concentrate and apply 40 µL to one 5-mm diameter well of a 12-well red heavy teflon-coated slide[1].



Step 3 Allow the sample to sit approximately 2 minutes at room temperature.

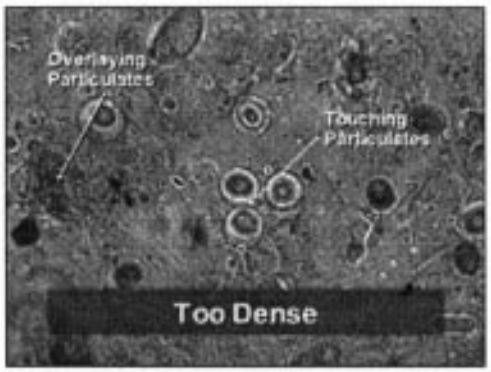


Step 4 Examine the flooded well microscopically at 200X total magnification. If the particulates are distributed evenly over the well surface area, and are not crowded or touching, apply 1 mL of the undiluted sample to a 25-mm diameter membrane filter in Step 6 of Sample Application below.

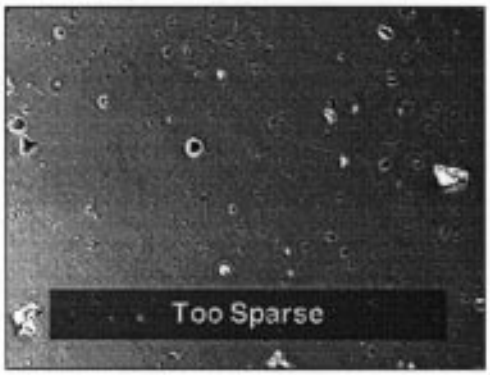


! When examining the flooded well, you must use an objective lens with a very high working distance so the meniscus does not touch or wet it.

Step 5 If the particulates are too dense or too widely spread, adjust the volume of the sample accordingly. Retest on another well.

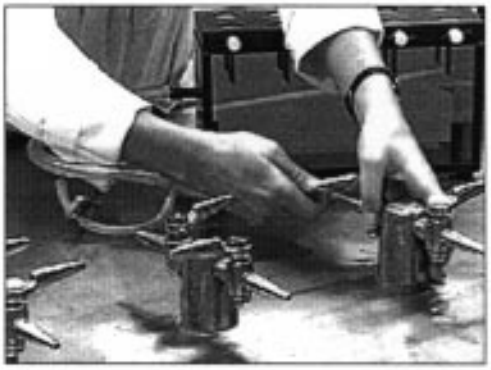


! Always adjust the sample concentrate volume so that the density of the particulates is just a little sparse. If the layer of sample particulates on the membrane filters is too dense, any cysts or oocysts present in the sample may be obscured during microscopic examination. Make sure the dilution factor, if any, from this step is recorded so that the number of parasite categories per 100 liters of sampled water can be determined.

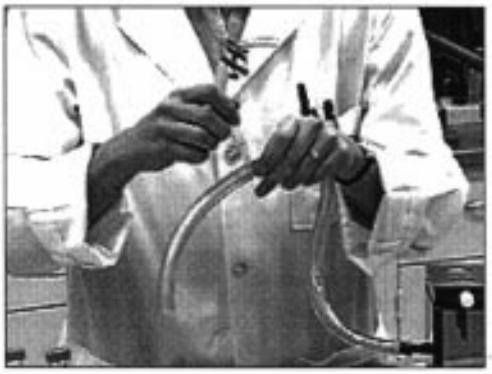


Preparing the Filtration Manifold

Step 1 Connect the filtration manifold to the vacuum supply using a vacuum tube that has a "T"-shaped tubing connector at one end.



Step 2 Attach a Hoffman screw clamp to 4-6 cm of latex tubing and then attach the latex tubing to the stem of the "T" connector. The screw clamp is used as a bleeder valve to regulate the vacuum to 2-4 inches (5-10 cm) of Hg.



Step 3 Close all the manifold valves and open the vacuum all the way.

Using the bleeder valve on the vacuum tubing, adjust the applied vacuum to 2-4 inches (5-10 cm) of Hg.

! Once adjusted, do not readjust the bleeder valve during filtration. If necessary, turn the vacuum on and off during filtration at the vacuum source.

Membrane Filter Preparation

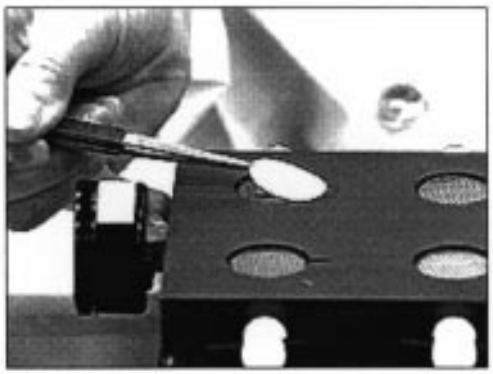
One Sartorius 25 mm diameter cellulose acetate filter, 0.22 µm pore size, and one 25-mm diameter ethanol-compatible membrane support filter[2], any porosity, are required for each 1 mL of adjusted suspension obtained previously. (See Determining Sample Volume per Filter, above.)

Step 1 Soak the required number of each type of filter separately in Petri dishes filled with 1X PBS. Using blunt-end filter forceps to handle the filters, drop them, one by one, flat on the surface of the buffer.

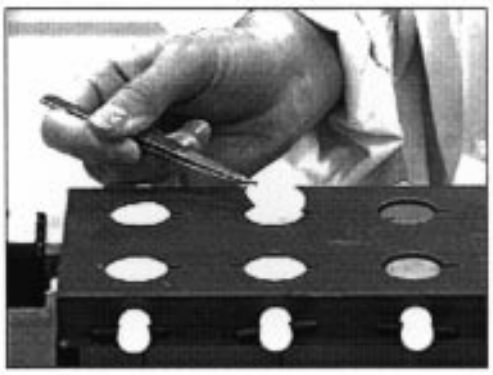
! Be sure that the colored filter dividers provided in the packaging have been removed.

Once the filters are wet, push the filters under the fluid surface with the forceps. Allow filters to soak for a minimum of 1 minute before use.

Step 2 Turn on the filtration manifold vacuum source. Leaving all the manifold well support valves closed, place one support filter on each manifold support screen. This filter ensures even distribution of sample.



Step 3 Place one Sartorius 25-mm diameter cellulose acetate filter on top of each support filter. Use a rubber policeman to adjust the cellulose acetate filter, if necessary. Open the manifold well support valves to flatten the filter membranes. Make sure that no bubbles are trapped and that there are no creases or wrinkles on any of the filter membranes.



Step 4 Use as many filter positions as there are sample volumes to be assayed. Record the number of sample 25-mm membrane filters prepared and the volume of floated pellet (See Determining Sample Volume per Filter, above) represented by these membranes.

In addition, include at least one positive control for *Giardia* cysts and *Cryptosporidium* oocysts and one negative control each time the manifold is used.

Step 5 Position a 1 lb (454 g) stainless steel well firmly over each filter.

! To ensure a good seal, use only wells with smooth rims that are free of nicks or scratches.

Step 6 Label each sample and control well appropriately with little pieces of tape on the top of the stainless steel wells and/or use manifold membrane labeling diagram (see Appendix D, Sample Forms) to keep track of each sample and control.

Sample Application

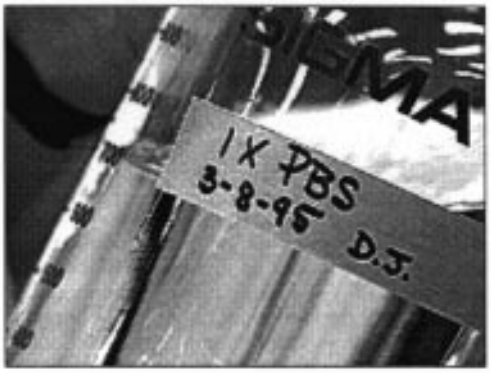
Step 1 Open the manifold support valve for each well containing filters.

Step 2 Rinse the inside of each stainless steel well and membrane filter with 2 mL 1% BSA. Drain the BSA solution completely from the membrane.

Step 3 Close the manifold valve under each membrane filter.

Step 4 For the positive control, add to the appropriately labelled well 500-1,000 *Giardia lamblia* cysts and 500-1,000 *Cryptosporidium parvum* oocysts, or use the Ensys positive control antigen as specified in the kit.

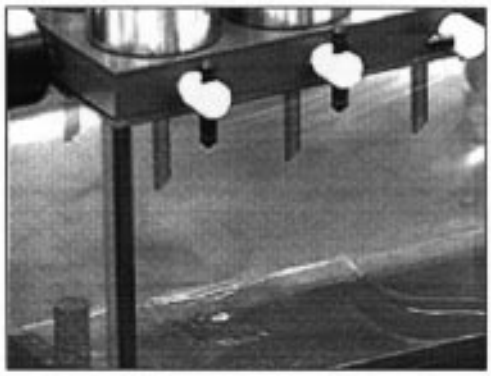
Step 5 For a negative control, add to the appropriately labelled well 1.0 mL 1X PBS.



Step 6 Add 1.0 mL of the vortexed, adjusted water sample (See Determining Sample Volume per Filter, above) to an appropriately labelled well.

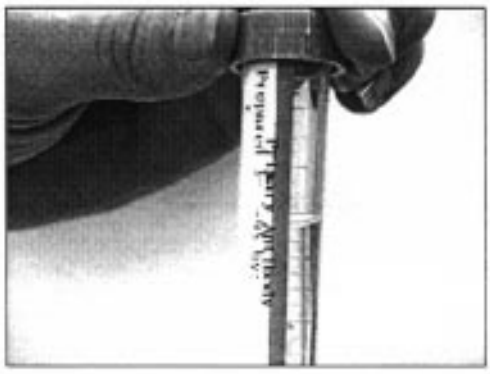
Step 7 Open the manifold valve under each membrane filter to drain the wells Rinse each stainless steel well with 2 mL 1% BSA, then close the manifold valve under each membrane filter.

! Do not touch the pipet to the membrane filter or to the well.

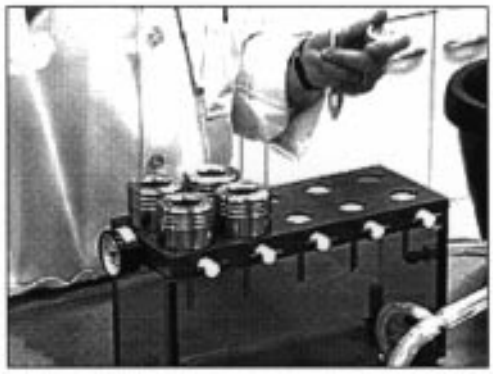


Indirect Fluorescent Antibody Staining

Step 1 Dilute the primary antibody mixture and labeling reagent according to the manufacturer's instructions using 1X PBS.



Step 2 Pipet 0.5 mL of the diluted primary antibody mixture onto each membrane and allow to remain in contact with the filter for 25 minutes at room temperature.



Step 3 At the end of the contact period, open the manifold valve to drain the antisera.

Step 4 Rinse each well and filter 5 times with 2 mL 1X PBS. Close all manifold valves after the last wash is completed.

! Do not touch the tip of the pipet to the membrane filter or to the stainless steel wells.

Step 5 Pipet 0.5 mL of the labeling reagent onto each membrane and allow it to remain in contact with the filter for 25 minutes at room temperature.

! Cover all wells with aluminum foil to shield the reagents from light and to prevent dehydration and crystallization of the fluorescein isothiocyanate dye during the contact period.

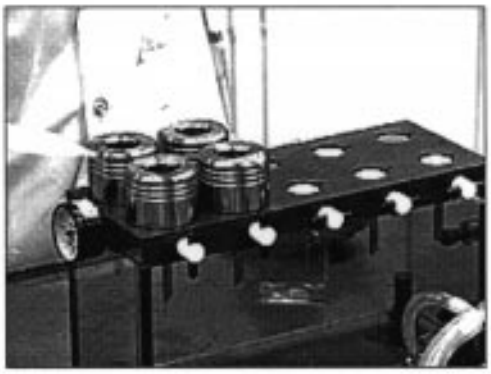
Step 6 At this point, start the Filter Mounting procedure presented in the following section.

Step 7 At the end of the contact period, open the manifold valves to drain the labeling reagent.

Step 8 Rinse each well and filter 5 times with 2 mL 1X PBS. Close all manifold valves after the last wash is completed.

! Do not touch the tip of the pipet to the membrane filter or to the stainless steel wells.

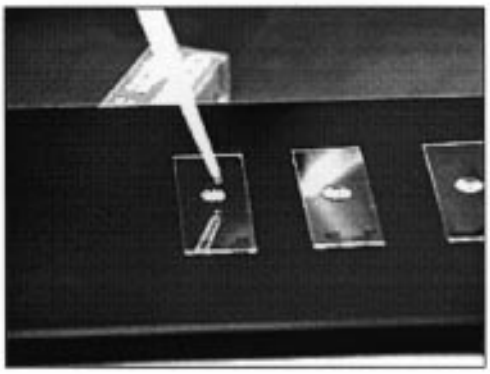
Step 9 Dehydrate the membrane filters in each well by sequentially applying 1.0 mL of 10, 20, 40, 80 and 90.2% ethanol solutions containing 5% glycerol. Allow each solution to drain thoroughly before applying the next in the series.



Filter Mounting

Step 1 Label glass slides for each filter and place them on a slide warmer or in an incubator calibrated to 37°C.

Step 2 Add 75 µL 2% DABCO-glycerol mounting medium to each slide on the slide warmer or in the incubator and allow to warm for 20-30 minutes.

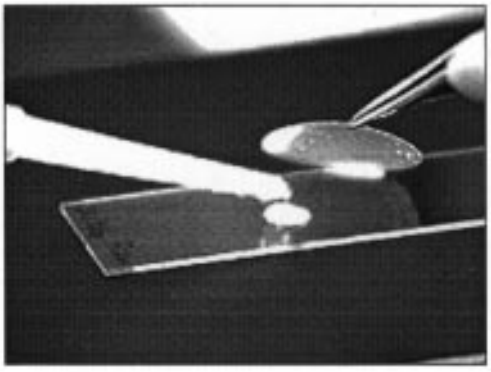


Step 3 Remove the top cellulose acetate filter with fine-tip forceps and layer it over the correspondingly labeled slide prepared with DABCO-glycerol mounting medium. **Make sure the sample application side is up.**

! If the entire filter is not wetted by the DABCO-glycerol mounting medium, pick up the membrane filter with the same forceps and add a little more DABCO-glycerol mounting medium under the slide filter.

Step 4 Repeat Step 3 for each filter. Use a clean pair of forceps to handle each membrane filter. Soak the used forceps in diluted detergent cleaning solution.

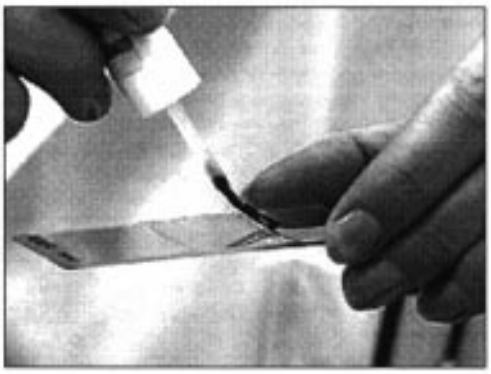
Step 5 Allow the slides to remain on the slide warmer or in the incubator for 20 minutes in order to clear.



! If the membrane starts to turn white after clearing, apply a small amount of DABCO-glycerol mounting medium <u>under</u> the filter.

Step 6 After the 20-minute clearing period, apply 20 µL DABCO-glycerol mounting medium to the center of each membrane filter and cover with a 25 mm x 25 mm cover glass. Tap out air bubbles with the handle end of a pair of forceps. Wipe off excess DABCO-glycerol mounting medium from the edge of each cover glass with a slightly moistened Kimwipe.

Step 7 Seal the edge of each cover glass to the slide with clear fingernail polish.



Step 8 Store the slides in a covered "dry box" at 4°C.

! A dry box can be constructed from a covered Tupperware®-type container to which a thick layer of anhydrous calcium sulfate has been added. Cover the desiccant with paper towels, and lay the slides flat on the top of the paper towels.

Step 9 Examine the slides microscopically as soon as possible but within 5 days of preparation. They may become opaque if stored longer, and D.I.C. or Hoffman modulation® optical examination would then no longer be possible.

Chapter 6
Microscopic Examination

The microscopic portion of this procedure depends on very sophisticated optics. Without proper alignment and adjustment, the microscope will not function at peak efficiency. Therefore, it is imperative that all portions of the microscope, from the light sources to the oculars, are adjusted properly. These adjustments should be practiced until they become second nature. The procedures for adjusting the epifluorescent mercury bulb and the transmitted light filament are presented in Appendix B.

Adjustment of Interpupillary Distance and Oculars for Each Eye

These adjustments are necessary so that eye strain is reduced to a minimum. These adjustments must be made for each individual using the microscope. This section assumes the use of a binocular microscope.

Interpupillary Distance

The spacing between the eyes varies from person to person and must be adjusted for each individual using the microscope.

Step 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.

Step 2 Using both hands, adjust the oculars in and out until a single circle of light is observed while looking through the two oculars with both eyes.

Ocular Adjustment for Each Eye.

This section assumes a focusing ocular(s). This adjustment can be made two ways, depending upon whether or not the microscope is capable of photomicrography and whether it is equipped with a photographic frame which can be seen through the binoculars.

! Persons with astigmatic eyes should always wear their contact lenses or glasses when using the microscope.

For microscopes not capable of photomicrography

This section assumes only the right ocular is capable of adjustment.

Step 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.

Step 2 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.

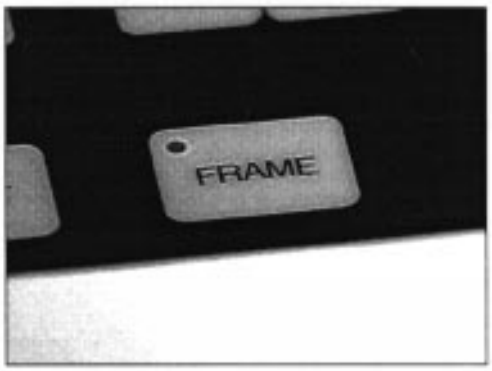
Step 3 Now transfer the card to between the left eye and ocular. **Without touching the coarse or fine adjustment,** and with keeping both eyes open, bring the image for the right eye into sharp focus by adjusting the ocular collar at the top of the ocular.

For microscopes capable of viewing a photographic frame through the viewing binoculars

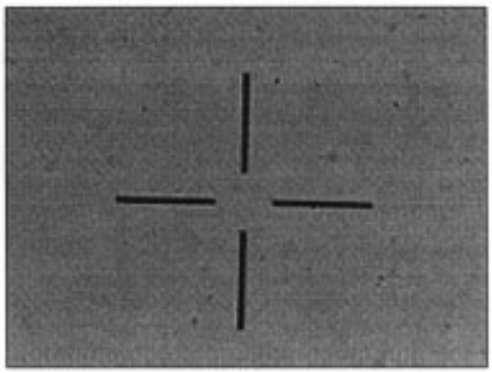
This section assumes both oculars are adjustable.

Step 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.

Step 2 After activating the photographic frame, place a card between the right ocular and eye, keeping both eyes open. Using the correction (focusing) collar on the left ocular, focus the left ocular until the double lines in the center of the frame are as sharply focused as possible.



Step 3 Now transfer the card to between the left eye and ocular. Again keeping both eyes open, bring the image of the double lines in the center of the photographic frame 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.

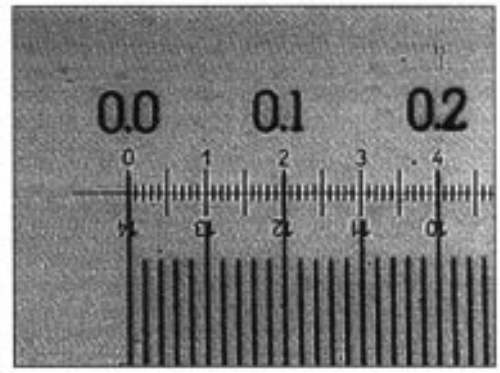


Calibration of an Ocular Micrometer[1]

This section assumes that an ocular 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 an optivar[2] on the microscope, then the calibration procedure must be done for the respective objective at each optivar setting.

Step 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.

Step 2 Adjust the alignment of the stage and ocular micrometers so the 0 line on the ocular micrometer is exactly superimposed on the 0 line on the stage micrometer.



Step 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.

Step 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.

Step 5 Calculate the number of mm/ocular micrometer space.

Calculating Ocular Micrometer Space

$$\frac{0.6 \text{ mm}}{48 \text{ o.m. spaces}} = 0.0125 \text{ mm/o.m. space}$$

Step 6 Since most measurements of microorganisms are given in µm rather than mm, the value calculated above must be converted to µm by multiplying it by 1,000 µm/mm.

Calculating Ocular Micrometer Space

$$\frac{0.6 \text{ mm}}{48 \text{ o.m. spaces}} = 0.0125 \text{ mm/o.m. space}$$

0.0125 mm/o.m. space x 1,000 µm/mm
= 12.5 µm/o.m. space

Step 7 Follow steps 1 through 6 for each objective. It is helpful to record this information in a table, like the example at right, which can be kept near the microscope.

Item #	Objective Power	Description	No. of Ocular Micrometer Spaces	No. of Stage Micrometer millimeters[a]	µm/Ocular Micrometer Space[b]
1	10X	N.A.[c] =			
2	20X	N.A. =			
3	40X	N.A. =			
4	100X	N.A. =			

[a] 1,000 µm/mm

[b] (Stage Micrometer length in mm x (1,000 µm/mm)) ÷ No. Ocular Micrometer Spaces

[c] N.A. stands for numerical aperture. The numerical aperture value is engraved on the barrel of the objective.

Köhler Illumination

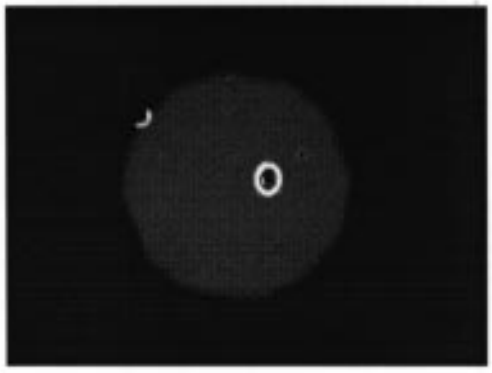
This section assumes that Köhler illumination will be established for only the 100X oil D.I.C. or Hoffman modulation® objective, which will be used to identify internal morphological characteristics in *Giardia* cysts and *Cryptosporidium* oocysts. If by chance more than one objective is to be used for either D.I.C. or Hoffman modulation® optics, then each time the objective is changed, Köhler illumination must be re-established 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 D.I.C. or Hoffman modulation® optics will not work to their maximal potential. These steps need to become second nature and must be practiced regularly until they are a matter of reflex rather than a chore.

Step 1 Place a prepared slide on the microscope stage, place oil on the slide, move the 100X oil objective into place, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

Step 2 At this point both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Now close down the radiant field diaphragm in the microscope base until the lighted field is reduced to a small opening.

Step 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.

Step 4 Now look to see whether the leaves of the iris field diaphragm are sharply defined (focused) or not.



If they are not sharply defined, they can be focused distinctly by changing the height of the condenser up and down with the condenser focusing knob while you are looking through the binoculars. Once you have accomplished the precise focusing of the radiant field diaphragm leaves, open the radiant field diaphragm until the leaves just disappear from view.

Step 5 The aperture diaphragm of the condenser should be adjusted now to make it compatible with the total numerical aperture of the optical system. This is done by removing an ocular, looking into the tube at the rear focal plane of the objective, and stopping down the aperture diaphragm iris leaves until they are visible just inside the rear plane of the objective.

Step 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 either D.I.C. or Hoffman modulation® optics.

Microscopic Examination

Microscopic work performed by a single analyst should not exceed 4 hours/day nor more than 5 consecutive days/week. Intermittent rest periods during the 4 hours/day are encouraged.

Step 1 Remove the dry box from 4°C storage and allow it to warm to room temperature before opening.

Step 2 Adjust the microscope to ensure that the epifluorescence and Hoffman modulation® or D.I.C. optics are in optimal working order.

Make sure that the fluorescein isothiocyanate cube is in place in the epifluorescent portion of the microscope (see the section on Sample Examination below.) Detailed procedures required for adjusting and aligning the microscope are found in Appendix B.

Assay Controls

The purpose of these controls is to ensure that the assay reagents are functioning, that the assay procedures have been properly performed, and that the microscope has been adjusted and aligned properly.

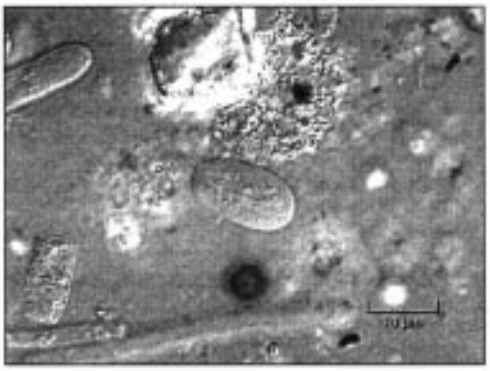
Assay Giardia/Cryptosporidium Control

Step 1 Using epifluorescence, scan the positive control slide at no less than 200X total magnification for apple-green fluorescence of *Giardia* cyst and *Cryptosporidium* oocyst shapes. Background fluorescence of the membrane should be either very dim or nonexistent.

Cryptosporidium oocysts may or may not show evidence of oocyst wall folding, which is characterized under epifluorescence by greater concentrations of FITC along surface fold lines. Whether the oocysts show this characteristic depends on the manner in which the oocysts have been treated and the amount of turgidity they have been able to maintain[3].

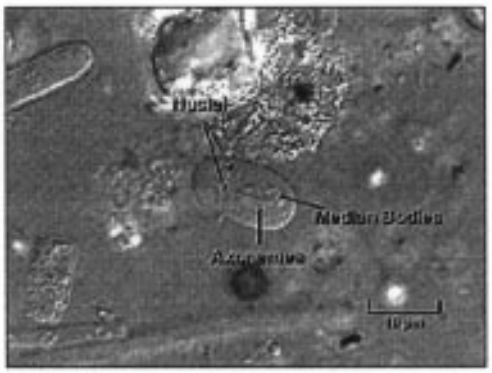
! If no apple-green fluorescing *Giardia* cyst or *Cryptosporidium* oocyst shapes are observed, then the fluorescent staining did not work or the positive control cyst preparation was faulty. Do not examine the water sample slides for *Giardia* cysts and *Cryptosporidium* oocysts. Recheck reagents and procedures to determine the problem.

Step 2 If apple-green fluorescing cyst and oocyst shapes are observed, change the microscope from epifluorescence to the 100X oil immersion Hoffman modulation® or differential interference contrast objective.

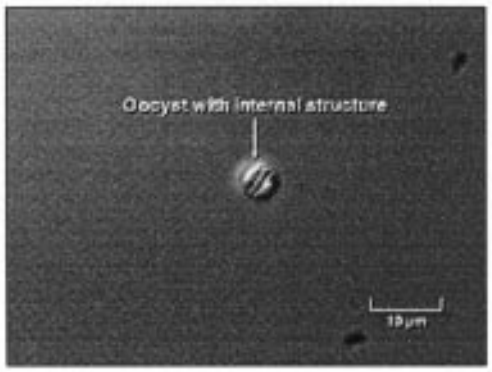


At no less than 1,000X total oil immersion magnification, examine *Giardia* cyst shapes and *Cryptosporidium* oocyst shapes for internal morphology.

The *Giardia* cyst internal morphological characteristics include 1-4 nuclei, axonemes, and median bodies. *Giardia* cysts should be measured to the nearest 0.5 µm with a calibrated ocular micrometer. Record the length and width of cysts. Also record the morphological characteristics observed. Continue until at least 3 *Giardia* cysts have been detected and measured in this manner.



The *Cryptosporidium* oocyst internal morphological characteristics include 1-4 sporozoites. Examine the *Cryptosporidium* oocyst shapes for sporozoites and measure the oocyst diameter to the nearest 0.5 µm with a calibrated ocular micrometer. Record the size of the oocysts. Also record the number, if any, of sporozoites observed. Sometimes a single nucleus is observed per sporozoite. Continue until at least 3 oocysts have been detected and measured in this manner.



Assay Negative Control

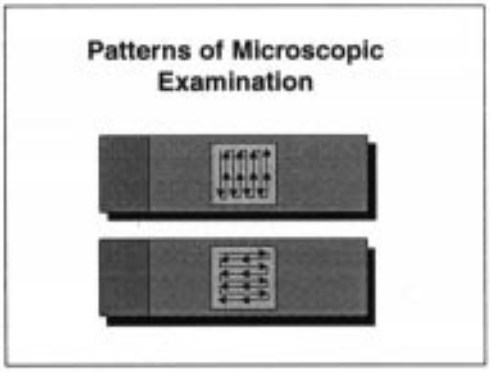
Step 1 Using epifluorescence, scan the negative control membrane at no less than 200X total magnification for apple-green fluorescence of *Giardia* cyst and *Cryptosporidium* oocyst shapes.

Step 2 If no apple-green fluorescing cyst or oocyst shapes are found, and if background fluorescence of the membrane is very dim or nonexistent, continue with examination of the water sample slides.

! If apple-green fluorescing cyst or oocyst shapes are found, discontinue examination since possible contamination of the other slides is indicated. Clean the equipment (see Appendix A), recheck the reagents and procedure, and repeat the assay using additional aliquots of the sample.

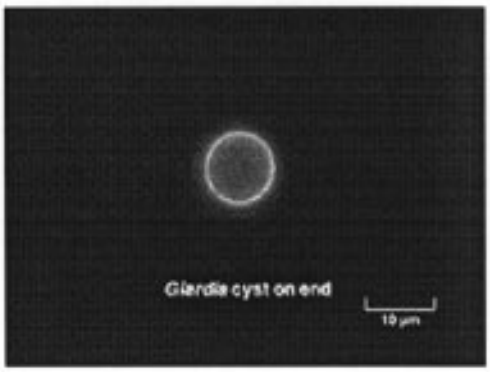
Sample Examination

Scan each slide in a systematic fashion, beginning with one edge of the mount and covering the entire coverslip. An up-and-down or a side-to-side scanning pattern may be used. The diagram at the right illustrates two alternative patterns for systematic slide scanning.

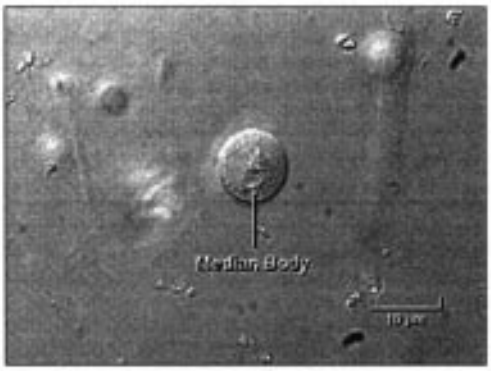


Empty Counts, Counts with Amorphous Structure, Counts with Internal Structure, and Total IFA Count

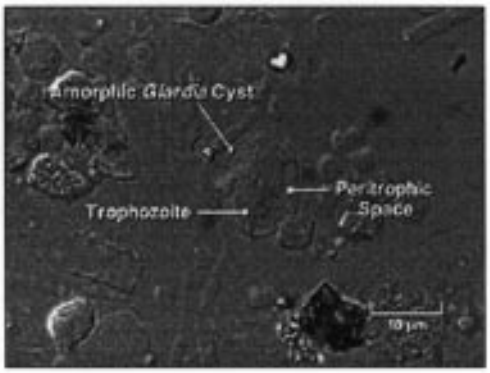
Step 1 When appropriate responses have been obtained for the positive and negative controls, use epifluorescence to scan the entire coverslip from each sample at not less than 200X total magnification for apple-green fluorescence of cyst and oocyst shapes.



Step 2 When brilliant, apple-green fluorescing round-to-oval objects (8 to 18 µm long by 5 to 15 µm wide) with brightly highlighted edges are observed, switch the microscope to either Hoffman modulation® or D.I.C. optics.

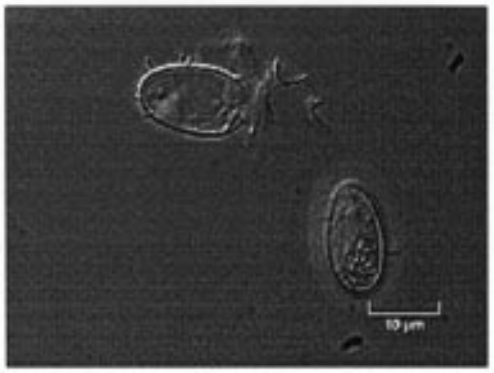


Look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). If these atypical structures are not observed, then categorize such apple-green fluorescing objects of the aforementioned size and shape as either empty *Giardia* cysts, *Giardia* cysts with amorphous structure, or *Giardia* cysts with internal structures (nuclei, axonemes, and median bodies).



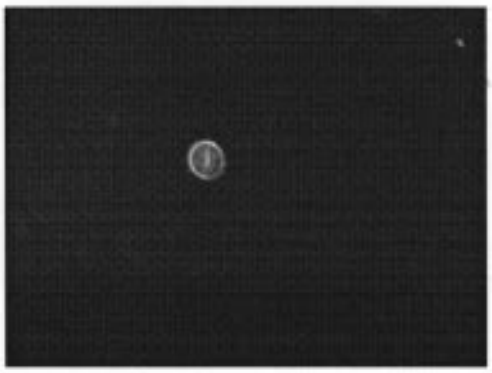
Record the shape and measurements (to the nearest 0.5 µm at 1,000X total magnification) for each such object. Record the internal structures observed.

Sum the counts of empty *Giardia* cysts, *Giardia* cysts with amorphous structure, and *Giardia* cysts with internal structures. Report this sum as the total *Giardia* IFA count (see Report Forms in Appendix zebbo).



! *Giardia* cysts with internal structures must be confirmed by a senior analyst.

Step 3 When brilliant, apple-green fluorescing ovoid or spherical objects (3 to 7 µm in diameter) with brightly highlighted edges are observed, switch the microscope to either Hoffman modulation® or D.I.C. optics. 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.). If these atypical structures are not observed, then categorize such apple-green fluorescing



objects of the aforementioned size and shape as either empty *Cryptosporidium* oocysts, *Cryptosporidium* oocysts with amorphous structure, or *Cryptosporidium* oocysts with internal structure (1 to 4 sporozoites/oocyst).

Although not a defining characteristic, surface oocyst folds may be observed in some specimens.

Record the shape and measurements (to the nearest 0.5 µm at 1,000X total magnification) for each such object. Record the number of sporozoites observed. Sum the counts of empty *Cryptosporidium* oocysts, *Cryptosporidium* oocysts with amorphous structure, and *Cryptosporidium* oocysts with internal structure. Report this sum as the total *Cryptosporidium* IFA count (see Appendix D, Sample Forms).

Cryptosporidium oocysts with sporozoites must be confirmed by a senior analyst.

End Notes

[1] Melvin, D.M. and M.M. Brooke. 1982. Laboratory Procedures for the Diagnosis of Intestinal Parasites. U.S. Department of Health and Human Services, HHS Publication No. (CDC) 82-8282.

[2] A device between the objectives and the oculars that is capable of adjusting the total magnification.

Chapter 7
Calculations and Reporting

Calculations

It is necessary to calculate the percentage of floated sediment examined microscopically. This percentage is calculated from:

- The total volume of the floated pellet obtained through filter elution;
- The number of 25-mm membrane filters prepared together with the volume of floated pellet represented by these membrane filters; and
- The number of membrane filters examined.

Although the calculations can be performed with a hand calculator, EPA strongly recommends that they be done with the computer spreadsheet provided with the ICR Guidance Manual.

Step 1 Calculate and record the percentage of floated sediment examined microscopically.

Calculate this value from the total volume of floated pellet obtained (see Chapter 3, Filter Elution), the number of 25-mm membrane filters prepared together with the volume of floated pellet represented by these membrane filters (see the section on Determining Sample Volume per Filter in Chapter 5), and the number of membrane filters examined.

The following values are used in calculations:

V = Volume (liters) of original water sample.

P = Eluate packed pellet volume.

F = Fraction of eluate packed pellet volume (P) subjected to flotation. It is determined by this equation:

$$F = \frac{\text{mL P subjected to flotation}}{P}$$

R = Percentage (expressed as a decimal) of floated sediment examined.

TG = Total *Giardia* IFA cyst count (Empty Count, Count with Amorphous Structure, and Count with Internal Structure).

EG = Count of *Giardia* cysts which are empty.

AG = Count of *Giardia* cysts with amorphous internal structure.

GW1S = Count of *Giardia* cysts with 1 internal structure.

GW2S = Count of *Giardia* cysts with more than 1 internal structure.

TC = Total *Cryptosporidium* IFA oocyst count (Empty Count, Count with Amorphous Structure, and Count with Internal Structure).

EC = Count of *Cryptosporidium* oocysts which are empty.

AC = Count of *Cryptosporidium* oocysts with amorphous internal structure.

CWS = Count of *Cryptosporidium* oocysts with internal structure.

Step 2 For positive samples, calculate the number of cysts or oocysts per 100 liters of sample as follows:

$$\frac{X}{100L} = \frac{(TG, EG, AG, GW1S, GW2S, TC, EC, AC, \textit{or}\ CWS)(100)}{FVR}$$

A sample calculation is shown in Appendix C.

Step 3 For samples in which no cysts or oocysts are detected (for example, TG or TC or GWS or CWS <1), calculate the detection limit as follows:

$$\frac{<X}{100L} = \frac{(<1)(100)}{FVR}$$

A sample calculation is shown in Appendix C.

Reporting

Step 1 Report *Giardia* results as empty *Giardia* cysts, *Giardia* cysts with amorphous structure, *Giardia* cysts with 1 internal structure, and/or *Giardia* cysts with 2 or more internal structures, and total *Giardia* IFA count per 100 L of sample.

Report *Cryptosporidium* results as empty *Cryptosporidium* oocysts, *Cryptosporidium* oocysts with amorphous structure, and/or *Cryptosporidium* oocysts with internal structure and total *Cryptosporidium* IFA count per 100 L of sample.

Report negative results in terms of the detection limit. Representative reporting forms are given in Appendix D.

Step 2 Enter all data into the computer spreadsheet provided with this protocol.

Chapter 8
Water Sample Controls

Water Sample Negative Control

This control is a check on equipment, materials, reagents and technique. It involves processing a 1 µm nominal porosity cartridge filter as if it were an unknown. All samples analyzed over the course of a week are considered to be a **batch**. For each batch, there must be a negative control.

Step 1 Cut the fibers from an unused 1 µm nominal porosity cartridge filter and process the fibers using the Filter Elution, Concentration, Flotation Purification, and Indirect Fluorescent Antibody procedures presented in this guide.

Step 2 Examine the entire concentrate from this sample, using the Microscopic Examination procedures presented in Chapter 6.

! If any cysts or oocysts are detected, do not process any unknown samples until the source of the contamination is located and corrected. The results from samples in the batch analyzed prior to the finding of a positive in a negative control will be excluded from the ICR Data Base.

Water Sample Positive Control

The purpose of this control is to ensure that the laboratory can recover cysts and oocysts when they are spiked into a sample at a known level. All samples analyzed over the course of a week are considered a **batch**.

Step 1 Using the sample collection procedures (see Appendix E) and the assay and microscopic examination procedures presented in Chapters 3 through 6, collect, process, and examine a 40 L (10 gal) or larger sample of reagent water or tap water that has been spiked with 1,000 *Giardia* cysts and 2,000 *Cryptosporidium* oocysts.

Step 2 Process the filtered water using the Filter Elution, Concentration, Flotation Purification, and Indirect Fluorescent Antibody Procedures presented in this guide.

Step 3 Examine the entire concentrate from this sample, using the Microscopic Examination procedures presented in Chapter 6. **It is not necessary to identify internal morphological characteristics using differential interference contrast microscopy.**

! If cysts and oocysts are not detected, do not process any more unknown samples until the reason for not recovering cysts and oocysts is determined and corrected. The results from samples in the batch analyzed prior to not finding cysts and oocysts in a positive control will be excluded from the ICR Data Base.

Appendix A
Cleaning the Manifold and Stainless Steel Wells

Manifold

Step 1
After all the membrane filters have been mounted on glass slides, discard the support filters.

Step 2
Open all the manifold valves and increase the vacuum pressure to the manifold by closing the bleeder valve associated with the vacuum tubing.

Step 3
Rinse each manifold filter support screen with 10-20 mL of 0.01% Tween 80 solution.

Step 4
Rinse each manifold filter support screen with 10-20 mL reagent water.

Step 5
Disconnect the manifold from the vacuum and wash the cover and fluid collection box in warm detergent solution. Rinse with tap water and reagent water.

Stainless Steel Wells

Step 1
Place a cloth on the bottom of an autoclavable container which is large enough to accommodate all 10 stainless steel wells in a single layer.

Step 2
Put the stainless steel wells top side down on the cloth. The rim on the underside of the well is fragile. Care must be taken to avoid scratching and denting the rim.

Step 3
Add enough reagent water containing detergent to cover the stainless steel wells by an inch or more.

Step 4
Autoclave the stainless steel container with the stainless steel wells for 15 minutes at 15 lbs/in^2 and 121°C. Use the slow exhaust mode at the completion of the autoclave cycle.

Step 5
Transfer the wells to a pan of hot detergent cleaning solution.

Step 6
Individually scrub the inside and bottom of stainless steel wells with a sponge.

Step 7
Rinse each well with tap water followed by reagent water. Drain and air dry the wells.

Step 8
Always check the bottom ridge of each stainless steel well for dents and scratches.

Step 9
If dents or scratches are found on the bottom of a stainless steel well, do not use it until it is properly reground.

Appendix B
Microscope Adjustments[1]

The microscopic portion of this procedure depends upon very sophisticated optics. Without proper alignment and adjustment of the microscope the instrument will not function at maximal efficiency and the probability of obtaining the desired image (information) will not be possible. Consequently, it is imperative that all portions of the microscope from the light sources to the oculars are properly adjusted.

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 these guidelines work for the particular instrument at hand.

Adjustment of the Epifluorescent Mercury Bulb and Transmitted Light Bulb Filament.

The sole purpose of these procedures is to ensure even field illumination.

Mercury Bulb Adjustment.

This section assumes that you have successfully replaced the mercury bulb in your particular lamp socket and reconnected the lamp socket to the lamp house. These instructions also assume the condenser has been adjusted to produce Köhler illumination. **Make sure that you have not touched any glass portion of the mercury bulb with your bare fingers while installing it.**

WARNING: Never look at the ultraviolet light coming out of the mercury lamp house or the ultraviolet light image without a barrier filter in place.

Step 1
Usually there is a diffuser lens between the lamp and the microscope which either must be removed or swung out of the light path.

Step 2
Using a prepared microscope slide, adjust the focus so the image in the oculars is sharply defined.

Step 3
Replace the slide with a business card or a piece of lens paper.

Step 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 tells you where the center of the field of view is.

Step 5
Mount the mercury lamp house on the microscope without the diffuser lens in place and turn on the mercury bulb.

Step 6
Remove the objective in the light path from the nosepiece. You should see a primary (brighter) and secondary image (dimmer) of the mercury bulb arc on the card after focusing the image with the appropriate adjustment.

Step 7
Using the other 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.

Step 8
Reattach the objective to the nosepiece.

Step 9
Insert the diffuser lens into the light path between the mercury lamp house and the microscope.

Step 10
Turn off the transmitted light, remove the card from the stage, and replace it with a slide of fluorescent material. Check the field for even fluorescent illumination. Adjustment of the diffuser lens will most likely be required. Additional slight adjustments as in step 6 above may be required.

Step 11
Maintain a log of the number of hours the U.V. bulb has been used. Never use the bulb for longer than it has been rated. Fifty watt bulbs should not be used longer than 100 hours; 100 watt bulbs should not be used longer than 200 hours.

Transmitted Bulb Adjustment

This section assumes that you have successfully replaced the transmitted bulb in your particular lamp socket and reconnect the lamp socket to the lamp house. **Make sure that you have not touched any glass portion of the transmitted light bulb with your bare fingers while installing it.** These instructions also assume the condenser has been adjusted to produce Köhler illumination.

Step 1
Usually there is a diffuser lens between the lamp and the microscope which either must be removed or swung out of the light path. Reattach the lamp house to the microscope.

Step 2
Using a prepared microscope slide and a 40X (or similar) objective, adjust the focus so the image in the oculars is sharply defined.

Step 3
Without the ocular or Bertrand optics in place the pupil and filament image inside can be seen at the bottom of the tube.

Step 4
Focus the lamp filament image with the appropriate adjustment on your lamp house.

Step 5
Similarly, center the lamp filament image within the pupil with the appropriate adjustment(s) on your lamp house.

Step 6
Insert the diffuser lens into the light path between the transmitted lamp house and the microscope.

End Notes

[1] Smith, R.F. 1982. Microscopy and Photomicrography: A Practical Guide. Appleton-Century-Crofts, New York.

Appendix C
Sample Calculations

Positive Samples

Assume that a 100 gallon (380 L) water sample was collected. The sample was eluted, resulting in 5 mL of sediment. Fifty percent (2.5 L) of the sediment was purified by Percoll-surcrose flotation. Forty percent of the floated material was examined microscopically. Eight empty *Giardia* cysts and 3 *Giardia* cysts with internal structure were found. No *Cryptosporidium* oocysts were observed.

Using the formula in Chapter 7:

V = 380 L

P = 5 mL

F = 2.5/5 = 0.5

R = 40% = 0.4

TG = 11

GW1S = 3

$$\frac{\textit{Giardia}\text{ cysts with structures}}{100\text{ L}} = \frac{(\text{GW1S})(100)}{\text{FVR}}$$

$$= \frac{(3)(100)}{(0.05)(380)(0.4)}$$

$$= 4\,;$$

and

$$\frac{\text{Total IFA }\textit{Giardia}\text{ cysts}}{100\text{ L}} = \frac{(\text{TG})(100)}{\text{FVR}}$$

$$= \frac{(11)(100)}{(0.5)(380)(0.4)}$$

$$= 14$$

Negative Samples

Using the description given in the previous example, no Cryptosporidium oocysts were observed. The calculated detection limit per 100 liters would be:

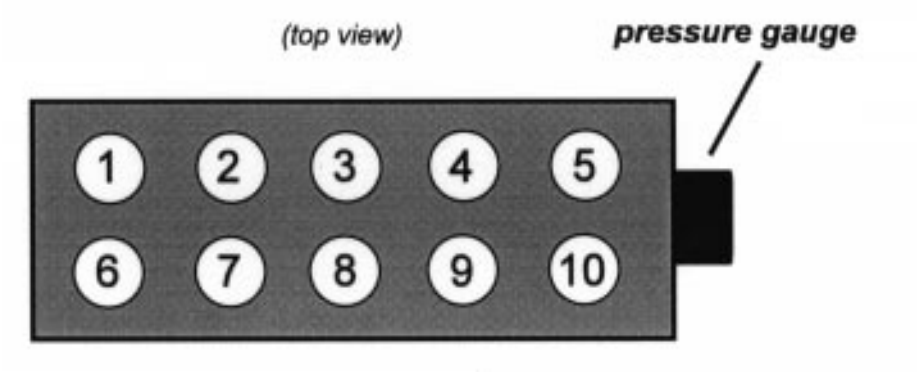
$$\frac{\text{Total IFA }\textit{Cryptosporidium}\text{ oocysts}}{100\text{ L}} = \frac{(\text{TC})(100)}{\text{FVR}}$$

$$= \frac{(<1)(100)}{(0.5)(380)(0.4)}$$

$$= <1.3$$

Appendix D
Sample Forms

Ten-Place Hoefer Manifold Membrane Labeling Diagram



1.	_____	6.	_____
2.	_____	7.	_____
3.	_____	8.	_____
4.	_____	9.	_____
5.	_____	10.	_____

Giardia Report Form

Slide prepared by: _____ Date prepared: _____

Analyst: _____ Date Analyzed: _____

Object Located by IFA No.	Shape (oval or round)	Size LxW (µm)	Empty *Giardia* Cysts (✔) (A)	*Giardia* Cysts with Internal Structure (C)			Total IFA *Giardia* Count (✔) (D = A+B+C)
				Morphological Characteristics			
				Nucleus (#)	Median Body (✔)	Axonemes (✔)	
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
Total				# with 1 morph. char.			
				# with >1 morph. char.			

A. Calculated number of Empty *Giardia* Cysts/100 liters _____

B. Calculated number of *Giardia* Cysts with Amorphous Structure/100 liters _____

C. Calculated number of *Giardia* Cysts with 1 Internal Structure/100 liters _____

C. Calculated number of *Giardia* Cysts with more than 1 Internal Structure/100 liters _____

D. Calculated Total IFA *Giardia* Count/100 liters _____

Cryptosporidium Report Form

Slide prepared by: _____ Date prepared: _____

Analyst: _____ Date Analyzed: _____

Object Located by IFA No.	Shape (oval or round)	Size LxW (µm)	Empty *Cryptosporidium* Oocysts (✔) (A)	*Cryptosporidium* Oocysts with Amorphous Structure (✔) (B)	*Cryptosporidium* Oocysts with Internal Structure (C)		Total IFA *Cryptosporidium* Count (✔) (D = A+B+C)
					Morphological Characteristics	Sporozoite (#)	
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
		Total					

A. Calculated number of Empty *Cryptosporidium* Oocysts/100 liters _____

B. Calculated number of *Cryptosporidium* Oocysts with Amorphous Structure/100 liters _____

C. Calculated number of *Cryptosporidium* Oocysts with Internal Structure/100 liters _____

D. Calculated Total IFA *Cryptosporidium* Count/100 liters _____

Appendix E
Sampling

Sampling Apparatus Preparation and Assembly

Sampling Apparatus

The sampling apparatus (see Figure 1) used for raw water consists of a female hose connector, an inlet hose, pressure regulator, pressure gauge, filter holder, a 1 µm nominal porosity filter, an outlet hose, a water meter, and a 1 gallon per minute flow control valve or device (4 L/min). A pump will be needed for unpressurized sources and a fluid proportioner or proportioning injector and pressure gauge will be needed before the filter for chlorinated or other disinfectant treated waters (see Figure 2 on the next page).

Filter Holder

Thoroughly wash the filter holder with a stiff brush in hot water containing detergent, when sampling is completed.

Rinse the filter holder with tap water until the soap residue is gone. Follow with a thorough rinse in reagent water and air dry.

Label

Attach a water-resistant label containing the following information to the filter holder:

Start Time:_____ Meter Reading:_____ Turbidity:

Stop Time:_____ Meter Reading:_____ Turbidity:

Operator's Name:_____ Total Volume Filtered:

Date:_____ Sampling Location:

Hoses

Inlet and outlet hoses for the filter holder consist of standard garden hoses and fittings. If desired

Figure 1: Raw-Water Sampling Apparatus

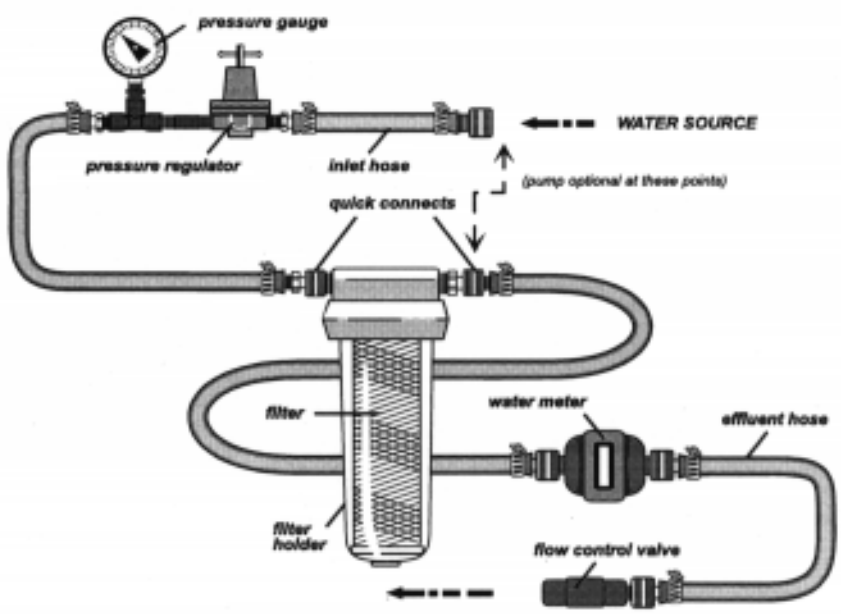
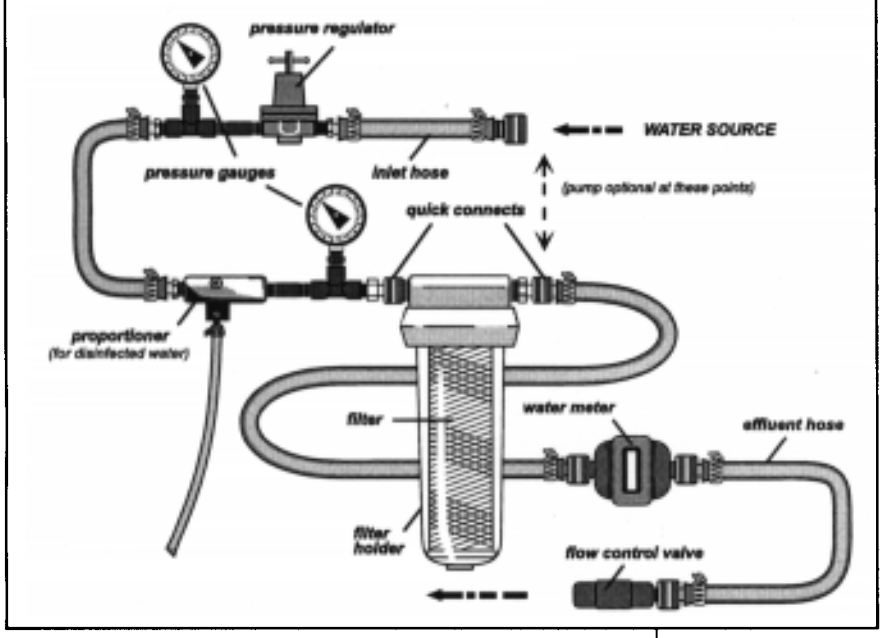


Figure 2: Treated-Water Sampling Apparatus



pressure, PVC tubing (1/2-inch I.D., 3/4-inch O.D., 1/8-inch wall) and/or quick connects may be substituted for the standard garden hose and/or hose clamps.

Hoses may be used repeatedly provided they are rinsed with at least 20 gallons (76 liters) of the water to be sampled prior to starting the sampling.

Pump

A pump is needed, when an unpressurized source is being sampled.

Fluid Proportioner or Proportioning Injector

If the water to be sampled is chlorinated or disinfected by any other chemicals, the disinfectant must be neutralized during sample collection. While the assay system allows detection of disinfected cysts and oocysts, exposure to disinfectant may interfere with the visualization of internal morphologies of these organisms.

Use sodium thiosulfate solution to neutralize the disinfectant in water samples. Add the sodium thiosulfate solution to the water during sample collection with a mechanical fluid proportioner pump or an in-line injector.[1]

Raw Water Sample Collection

Step 1
Put on a pair of the latex gloves.

Step 2
Before connecting the sampling apparatus (see Figure 1) to the tap or source to be sampled, turn on the tap and allow the water to purge residual debris from the line for 2 to 3 minutes, or until the turbidity of the water becomes uniform.

Step 3
Connect the apparatus minus the filter to the tap and allow 20 gallons (76 liters) to flush the system. If a pressurized source is not available, use a pump, following the manufacturer's instructions, to get water through the sampling apparatus. While the flushing of the apparatus is being done, adjust the pressure regulator so the adjacent pressure gauge reads no more than 30 pounds per square inch (PSI).

Step 4
Turn off the water flow, when the flushing of the apparatus is complete. Open the filter housing and pour all the water out. Put the filter in, close, and tighten the filter housing.

Step 5
Use a water-resistant marking pen to record the start time, meter reading, name of person collect-

ing the sample, turbidity, date and sampling location on the filter holder label.

Step 6
Start water flow through the filter. Check the pressure gauge after the pressure regulator to make sure the reading is no more than 30 PSI. Readjust the regulator, if necessary.

Step 7
After the 100 L (27 gallons) of raw water has passed through the filter, shut off the water flow, record the stop time, final meter reading and turbidity of the water at the end of filtration on the filter holder label.

Step 8
Disconnect the sampling apparatus while maintaining the inlet hose level above the level of the opening on the outlet hose in order to prevent backwashing and the loss of particulate matter from the filter.

Step 9
After allowing the apparatus to drain, open the filter housing and pour the residual water remaining in the filter holder into a plastic sample bag.

Step 10
Aseptically remove the filter from the holder and transfer the filter to the plastic sample bag containing the residual water.

Step 11
Seal the bag and place it inside a second plastic sample bag. Transfer the label or label information from the filter holder to the outside of this second (outer) bag.

Step 12
Transport the sample to the laboratory on wet ice or with but not on cold packs. When the filter(s) arrive at the laboratory, they should be immediately stored at 2-5°C. **Do not freeze the filter during transport or storage.**

Finished Water Sample Collection

If the water must be neutralized, add sodium thiosulfate solution via the proportioner system. For each 100 L of finished water sampled, 250 ml of 2.0% sodium thiosulfate solution will be needed.

Step 1
Put on a pair of the latex gloves.

Step 2
Before connecting the sampling apparatus (see Figure 2) to the tap or source to be sampled, turn on the tap and allow the water to purge residual debris from the line for 2 to 3 minutes, or until the turbidity of the water becomes uniform.

Step 3
Connect the apparatus minus the filter to the tap and allow 20 gallons (76 liters) to flush the system. If a pressurized source is not available, use a pump, following the manufacturer's instructions, to get water through the sampling apparatus.

While the flushing is being done, adjust the pressure regulator, so the adjacent pressure gauge reads no more than 30 PSI. Pour the 2% sodium thiosulfate solution into a graduated cylinder. Place the injector tube into the solution and adjust the larger top (vacuum) screw on the injector, so the pressure on the pressure gauge following the injector reads no more than 19 PSI.

Now adjust the smaller bottom (flow) screw on the injector, so the flow rate of the thiosulfate solution is 10 ml per minute. A hose cock clamp on the injector tube may be required to achieve the correct thiosulfate flow rate. After this adjustment is complete, transfer the injector tube to a graduated carboy of thiosulfate solution.

Step 4
Turn off the water flow, when the flushing of the apparatus is complete. Open the filter housing and pour all the water out. Put the filter in, close, and tighten the filter housing.

Step 5
Use a water-resistant marking pen to record the start time, meter reading, name of person collecting the sample, turbidity, date and sampling location on the filter holder label.

Step 6
Start water flow through the filter. Check the pressure gauge after the pressure regulator to make sure the reading is no more than 30 PSI. Also check to make sure the thiosulfate solution is being drawn into the sampling apparatus. Readjust the regulator and injector, if necessary.

Step 7
After the 1,000 L (264 gallons) of finished water has passed through the filter, shut off the water flow, record the stop time, final meter reading and turbidity of the water at the end of filtration on the filter holder label.

Step 8
Disconnect the sampling apparatus while maintaining the inlet hose level above the level of the opening on the outlet hose in order to prevent backwashing and the loss of particulate matter from the filter.

Step 9
After allowing the apparatus to drain, open the filter housing and pour the residual water remaining in the filter holder into a plastic sample bag.

Step 10
Aseptically remove the filter from the holder and transfer the filter to the plastic sample bag containing the residual water.

Step 11
Seal the bag and place it inside a second plastic sample bag. Transfer the label or label information from the filter holder to the outside of this second (outer) bag.

Step 12
Transport the sample to the laboratory on wet ice or with but not on cold packs and refrigerate at 2-5°C. **Do not freeze the filter during transport or storage.**

End Notes

[1] Details on the operation and use of proportioner pumps and injectors can be found in *Standard Methods for the Examination of Water and Wastewater*, Section 9510C, "Virus Concentration from Large Sample Volumes by Adsorption to and Elution from Microporous Filters (PROPOSED)," 18th ed., 1989, pp. 9-105 to 9-109.