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Standard Operating Procedure for the Detection of *Cryptosporidium* and *Giardia* in Water by Filtration, IMS, and FA (Based on EPA Method 1623, December 2005)

Access to this SOP shall be available within the laboratory for reference purposes; the official copy of this SOP resides on the official Georgia EPD website at https://epd.georgia.gov/about-us/epd-laboratory-operations. Printed copies of this SOP will contain a watermark indicating the copy is an uncontrolled copy.

1.0 Scope and Application

1.1 This SOP is based on Method 1623, the procedure used to detect the presence of *Giardia* and *Cryptosporidium* in water. Using this procedure enables the lab to detect and determine the concentration (organisms/ liter) of the target organism(s) found in a field sample. It also enables the lab to determine if the matrix has any effect on recovery. It does not, however, enable the lab to determine the species, viability or infectivity of the organism(s) present. The general procedure is as follows: A 10 L volume of water is filtered at a rate of 1-4 liters per minute. The filter is then eluted and the filtrate concentrated. The organisms are extracted from the concentrate via immunomagnetic separation (IMS). The sample is placed on an adhesive well slide and stained with a fluorescent labeled monoclonal antibody and 4', 6-diamindino-2'phenylindole (DAPI). The slide is then examined with an epifluorescent microscope. All organisms that fit the size, shape, fluorescence, and morphology requirements of either *Giardia* or *Cryptosporidium* are recorded and quantitated. Method 1623, while specific, does allow procedural modifications in specific areas provided that the lab can substantiate the success of said modifications with the required documentation. The laboratory employs this method to analyze those water systems affected by the Long Term 2 Enhanced Surface Water Treatment Rule.

2.0 Definitions

2.1 Refer to Section 3 and Section 4 of the Georgia EPD Laboratory Quality Assurance Manual for Quality Control Definitions. (See SOP reference 12.8)

3.0 Interferences

3.1 The turbidity of different matrices caused by inorganic and organic debris can affect recoveries of *Cryptosporidium* oocysts and *Giardia* cysts. Other interferences can occur during microscopy in differentiating between auto-fluorescing and cross-reacting organisms that may be mistaken for *Cryptosporidium* oocysts and *Giardia* cysts.

4.0 Safety

4.1 Analysts should be aware of the biohazard risks that are associated with this method and take adequate precautions. There is a high risk of infection from oocysts because live organisms are handled. Laboratory staff should 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.

4.2 Each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to the lowest possible level. Refer to material safety data, which are available on all chemicals and reagents used in the Microbiology laboratory.

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

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

4.5 For additional safety points, please refer to Laboratory Chemical Hygiene Plan & Fire Safety Plan, online revision. (See SOP reference 12.9)

5.0 Apparatus and Equipment

5.1.1 Equipment for Filtration of Samples

5.1.2 10 L Carboy with 2 port vented cap
5.1.3 Graduated Carboy for Filtered Sample calibrated 9.0, 9.5, 10.0, 10.5 and 11.0
5.1.4 Stir Bar
5.1.5 High Volume Magnetic Stir Plate
5.1.6 High Pressure Tubing
5.1.7 Air Pressure Gauge
5.1.8 House Air Source
5.1.9 Filta-Max® Foam Filter Module
5.1.10 Filta-Max ®Housing Unit
5.1.11 Screw Clamps
5.1.12 Plastic Bags for the refrigeration of filters
5.1.13 Peristaltic Pump (Emergency Substitute)

5.1.14 Equipment for Elution and Concentration of Samples

5.1.15 Filta-Max® Wash Station
5.1.16 Filta-Max® Elution Tubing Set
5.1.17 Filta-Max® Membrane Filters
5.1.18 Filta-Max® Magnetic Stirrer

5.1.19 House Vacuum Source
5.1.20 Hand Pump (Emergency Substitute)
5.1.21 Waste Bottle
5.1.22 Centrifuge
5.1.23 50 ml Centrifuge Tubes
5.1.24 Small Pasteur pipettes
5.1.25 Pipette bulb
5.1.26 Forceps
5.1.27 High-Vacuum Grease
5.1.28 Small Plastic Bags for membrane wash
5.1.29 Magnetic Stir Plate
5.1.30 Equipment for Immuno-Magnetic Separation
5.1.31 ® MPC-1 or MPC-6
5.1.32 ®MPC-S
5.1.33 Various Micropipettes 100 µl – 1000 µl
5.1.34 Various Micropipette Tips 100 µl – 1000 µl
5.1.35 Vortex Mixer
5.1.36 Micro Centrifuge Tubes
5.1.37 Timers
5.1.38 ®Leighton Tubes (flat-sided)
5.1.39 Beaker for waste
5.1.40 Transfer Pipettes
5.1.41 Equipment for Slide Application and Staining
5.1.42 Well Slides
5.1.43 Slide Warmer
5.1.44 Various Micropipettes 5 µl – 100 µl
5.1.45 Various Micropipette Tips 5 µl – 100 µl
5.1.46 Plastic Container with wet paper towels in bottom
5.1.47 Paper Towels
5.1.48 Cover slips
5.1.49 Clear Fixative
5.1.50 Equipment for Slide Reading
5.1.51 Epiflourecent Microscope (magnification capabilities of 200X, 400X and 1000X oil immersion lens)
5.1.52 Immersion Oil
5.1.53 Computer Camera (for video library)
5.1.54 Equipment for Reagent Preparation
5.1.55 pH Meter
5.1.56 Analytical Balance
5.1.57 Desiccator
5.1.58 Desiccant
5.1.59 Filter Sterilization Bottle
5.1.60 Carboy

5.1.61 Graduated Cylinders
5.1.62 Scoops
5.1.63 Weigh Boats
5.1.64 Small Beakers for pH Meter
5.1.65 Set of certified ASTM Class 1 Weights
5.1.66 Stir Plates
5.1.67 Miscellaneous
5.1.68 Powder-Free Latex Gloves
5.1.69 Turbidity Meter
5.1.70 Equipment Sterilization
5.1.71 Carboys that contain field samples are washed in the automated washer and air-dried. If field sample is gritty, soapy water is added to the carboy and it is scrubbed with a brush, rinsed and then washed in the automated washer and air-dried.
5.1.72 Discards from OPRs and Matrix Spikes are poured into dirty carboys after which the carboys are autoclaved. The autoclaved discard is poured down the drain and the carboy washed in the automated washer and air-dried.
5.1.73 The FiltaMax wash station plunger is manually washed with soap and water and rinsed with distilled water in between each use.
5.1.74 The FiltaMax parts, elution tubes, tubing, filter housing, etc. are washed in a mild detergent, soaked in bleach for 30 minutes, rinsed in deionized water and allowed to air dry. Glassware is sterilized in the hot air oven.

6.0 Reagents

6.1.1 Vendor Purchased Reagents
6.1.2 Acetone
6.1.3 DAPI (4',6- diamidino- 2-phenylindole)
6.1.4 Hydrochloric Acid 0.1N and1.0N Reagent Grade
6.1.5 Methanol, Absolute
6.1.6 EasyStain®
6.1.7 Potassium Chloride (KCl)
6.1.8 Sodium Phosphate, anhydrous (Na_2HPO_4) Potassium Phosphate(KH_2PO_4)
6.1.9 Sodium Chloride (NaCl)
6.1.10 Sodium Hydroxide, 0.1N (NaOH)
6.1.11 ® Dynabeads GC Combo Kit
6.1.12 Tween® 20 polyoxyethylenesorbitan monolaureate
6.1.13 Easy Seed® for OPR, Matrix Spike, Verification of Analyst Per., IMS control

6.2 Prepared Lab Reagents

Note: Label all prepared reagent with the name of reagent, date of preparation, expiration date, and technician's initials.

6.2.1 Purified Cryptosporidium oocysts and *Giardia* cysts, Univ. of Wisc. (PT samples)
6.2.2 *Phosphate Buffer (PBS)*: Add 8 g of NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4

anhydrous, and 0.2 g KH_2 PO_4 to 1.0 L of water. Stir to dissolve and check pH. Adjust to 7.4 (+/-0.2) with NaOH or HCl. Sterilize by using a filtration. This is stored at room temperature for six months or until growth is observed. Record in all reagent log books.

6.2.3 *Phosphate Buffer with Tween (PSBT)*: Add 8 g of NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 anhydrous, and 0.2 g KH_2PO_4 to 1.0 L of water. Add 100 µl of Tween 20. Stir to dissolve and check pH. Adjust the pH to 7.4(+/-0.2) with NaOH or HCl. Buffer is stored at room temperature for one week. Record in all reagent log books.

6.2.4 *DAPI Stock Solution*: Dissolve 2 mg/ml DAPI in absolute methanol. Prepare volume consistent with minimum use. Store at 1°C to 10°C in the dark. Do not allow freezing. Prepare a stock solution every two weeks or when a positive staining control fails for DAPI. Record in all reagent log books.

6.2.5 *DAPI Staining Solution*: Add 30 µl of 2 mg/ml DAPI stock solution to 50 ml of PBS. If a higher intensity is desired, increase the original amount of 30 µl by increments of 10 µl. Test the new concentration of DAPI on expendable samples prior to using on field samples. Prepare daily. Store at 1°C to 10°C in the dark except when staining. Do not allow freezing. Record in reagent log books.

7.0 Sample Collection

7.1 Samples are filtered at the respective water systems using the Idexx Filta-Max products and shipped to the laboratory on ice.

7.2 Matrix Spike samples are collected in filled cubitainers and are sent to the lab on ice.

7.3 Systems are referred to Instructional Sheet sent in their coolers as well as to collection training module on EPA website.

7.4 Sample volume analysis requirements: For LT2 Rule compliant samples, the minimum sample volumes are 10 L of sample, 2 ml of packed pellet volume, or as much as two filters can accommodate before clogging.

8.0 Calibrations

8.1 Consult the microscope SOP for calibrations. (SOP reference 12.10)

9.0 Quality Control

9.1 Refer to Table 14.1 for Reporting Limits.

9.2 Refer to Tables 14.2 and Appendix A, Table A.1 for other Quality Control Acceptance Criteria associated with this method.

9.3 Initial Demonstration of Capability recovery and precision for *Cryptosporidium*: Mean recovery range is 36-100 and precision is 34. Refer to Initial Demonstration of capability SOP 6-001, Rev. 5 or later. (See SOP reference 12.6)

9.4 Initial Demonstration of Capability recovery and precision for *Giardia*: Mean recovery range is 32-100 and precision is 37. Refer to Initial Demonstration of capability SOP 6-001, Rev. 5 or later. (See SOP reference 12.6)

9.6 Troubleshooting Low Recoveries

9.6.1 As noted in Appendix A, no recovery rates that fall outside of the acceptable ranges are used however, if a low recovery pattern develops as noted by the control charts, certain procedures are used to try to pinpoint the problem area.

9.6.2 Determine if the low recovery is lab-wide or an individual by comparing the control charts and OPR recoveries among staff members.

9.6.3 If determined to be lab wide, use the same lot numbered reagents and same analyst to prepare a slide and stain, analyze an IMS spike (Immunomagnetic Separation) and then analyze a sample spiked at filtration to see if any processes are particularly low.

9.6.4 If all are low, the problem points to the organisms that were used. Check with supplying company and other labs to see if other problems have been reported with this particular lot #. Use or order another lot number.

9.6.5 If low recoveries are present at individual steps, repeat analyses at that determined point of analysis to deduce which steps specifically may be the area of concern (poorly performing equipment or reagents) and correct it by repairing/ replacing equipment or requesting another lot number of reagents if that is the problem.

9.5.6 If determined to be that of an individual, have a senior analyst watch the individual analyst run a spiked sample to determine if there is an obvious technique problem to be corrected.

9.6.7 If no cause for the low recoveries was determined by the above, then have the senior analyst prepare a slide and an IMS control to be stained and analyzed, respectively by the individual analyst to assist in pinpointing the problem.

9.7 IMS Quality Control

9.7.1 An IMS Quality Control is performed on all lot numbers of Dynabeads GC Combo, Easyseed, Easystain, and the results are recorded in the Quality Control book.

9.7.2 Expected recovery rates are around 70% and will be used to troubleshoot significant drops in OPR's and Matrix Spikes associated with the lot.

9.7.3 Follow procedure listed 10.2.14.

10.0 Procedure

10.1 **NOTE:** Prior to analysis, wipe down bench top and set up equipment for filtration elution to allow for quicker transitions between steps.

10.2 QC and Field Sample Preparation

10.2.1 **NOTE**: **Analyze the OPR first and the Method Blank second. Do not begin any part of field sample analysis before acceptable QC results have been achieved and documented**.

10.2.2 **OPR:** Randomly select a clean carboy and fill it with 10 L of reagent water. Sit the vessel on a large stir plate, add a large stir bar and start the stirrer at a

rate high enough to create a vortex in the sample. Record the volume spiked on the bench sheet.

10.2.3 Retrieve 1 vial of Easy Seed from the refrigerator. Check the expiration date. Add 2 ml of PBST to the vial and vortex for 20 seconds. Pour this into the vortex of the sample. Repeat this procedure **twice** with 3 ml of DI water. Allow it to mix for 1 minute. Sample is ready for filtration.

10.2.4 **NOTE**: When mixing samples, gradually, turn up the stirrer high enough to create a vortex in the sample but not enough to cause the stir bar to spin off from the center.

10.2.5 **Method Blank:** Randomly select a clean carboy and fill it with 10 L of reagent water. Sit vessel on stir plate, add a stir bar and turn on stirrer high enough to create a vortex. Allow it to mix for 1 minute. Sample is ready for filtration.

10.2.6 **NOTE:** Run Matrix Spike first and the associated field sample second. Matrix Spikes may be spiked, filtered, and refrigerated the day before processing; but not before generating acceptable, documented QC results.

10.2.7 **Field Sample:** Bring filters to room temperature prior to processing. Note: If prior arrangements have been made with a system to submit in bulk form, randomly select a clean carboy and pour in 10 L of the sample. Set the carboy on a stir plate, drop in a stir bar and turn on the stirrer high enough to create a vortex. Allow it to mix for 1 minute. Sample is ready for filtration.

10.2.8 Ensure that the volume of Matrix Spike filtered does not exceed 10% of the filtered volume of the submitted field filtered sample.

10.2.9 **Matrix Spike:** Gently mix cubitainer a few times prior to transfer. Randomly select clean carboy then transfer 10 L (See 10.2.8) of the sample to a randomly selected carboy. Set vessel on a large stir plate, drop in a large stir bar and turn on the stirrer high enough to create a vortex. Record the volume spiked on the bench sheet.

10.2.10 Retrieve 1 vial of Easy Seed from the refrigerator. Check the expiration date. Add 2 ml of PBST to the vial and vortex for 20 seconds. Pour this into the vortex of the sample. Repeat this procedure twice with 3 ml of DI water. Allow it to mix for 1 minute. Sample is ready for filtration.

10.2.11 **PT Samples:** Follow these instructions only if there are no instructions enclosed with the samples. Randomly select a carboy and fill it with 10 L of reagent water. Add a large stir bar to the carboy and place it on a stir plate. Turn on the stirrer allowing it to create a vortex.

10.2.12 Shake the PT sample and mix it well enough to dislodge the pellet. Carefully pour the contents of the tube into the carboy. Add 20 ml of reagent water to the tube; recap it and vortex the sample for 10 seconds. Add this rinsate to the carboy. Repeat with another 20 ml of reagent water. Allow the spiked sample to mix in the carboy for at least one minute.

10.2.13 Remove an aliquot from the carboy and determine the turbidity. Pour the aliquot back into the sample. Fill the tube with reagent water, replace the top,

shake and pour the rinsate into the sample. Repeat the rinsing procedure once more. The sample is ready for filtration.

10.2.14 **IMS Control:** Remove the Dynabeads GC combo from the refrigerator to allow components to come to room temperature.

10.2.15 **Note:** Record on a bench sheet; label it IMS control at the top. Strike through any nonessential information normally associated with field, OPR etc. samples.

10.2.16 Set out one Leighton tube and add 1ml of 10X SL Buffer A and 1 ml of 10X SL Buffer B in that order. Label the tube with the Lab Sample number.

10.2.17 Spike tube with 1 vial of Easyseed.

10.2.18 Retrieve 1 vial of Easy Seed from the refrigerator. Check the expiration date. Add 2 ml of PBST to the vial and vortex for 20 seconds. Using a PBST rinsed pipette, transfer the organisms into the Leighton tube. Repeat this procedure twice with 3 ml of DI water.

10.2.19 Add the volumes of the Easyseed vial + 8 ml (2 ml of PBST and 6 ml of DI water). Using this volume, add enough DI water to bring the testing volume to 10 ml. (It is usually less than a milliliter that needs to be added.)

10.2.20 Proceed as normal starting at 10.5.9 through to completion.

10.3 Filtration

10.3.1 Remove a Filta-Max filter from the pouch. Record the lot and filter numbers on the bench sheet. Place it screw side up into the Filta-Max housing unit and screw on the housing cap.

10.3.2 Retrieve a vented screw cap and attach a short and long tube to the labeled ports on the underside of the cap and screw the cap onto the carboy.

10.3.3 Attach air pressure that has been regulated to a rate of 1 – 4 L/min to the inlet port on the topside of the cap.

10.3.4 Note: Highly turbid samples may filter at a slower rate.

10.3.5 Attach hose from the outlet port on top of the vented cap to the inlet port of the Filta-Max housing unit containing the filter. Position the outlet port of the Filta-Max housing over the glass, graduated carboy (collection carboy).

10.3.6 Note: Set to 15 psi (4^{th} mark on gauge) to achieve an approximate rate of 2 L/ min. Secure the hose with an adjustable tube clamp. Pressure may be adjusted by using the setup minus the filter housing.

10.3.7 Slowly, turn on the pressurized air, forcing the sample through the setup. Once the sample has filtered through, tilt the container so that it is completely emptied then turn off the air supply and record the filtered volume collected in the collection carboy.

10.3.8 Add 1 L of DI water to the sample carboy. Swirl the carboy and filter the rinsate. Do not include this volume as a part of the total volume filtered. This step is for all sample types including the Method Blank.

10.4 Elution and Concentration of Sample

10.4.1 Assemble the Filta-Max concentrator tube unit by placing the filter membrane grill into the center of the concentrator tube base (spout) and placing a membrane filter (rough-side up) on top of it. Screw the concentrator tube base

onto the concentrator tube. Make sure that the spout on the base is in the blocked position. Screw the Quick Connect attachment ring beveled side up onto the top of the concentrator tube.

10.4.2 Measure 600 ml of PBST into a graduated cylinder and pour it into the assembled concentrator tube. Place on bench top.

10.4.3 Screw on a plunger head. Place an o-ring into the groove of the plunger head and lubricate the o-ring with vacuum grease.

10.4.4 Unscrew the top of the filter housing using the mini screwdriver. Aseptically remove the filter module from the housing or bag (field) and screw it into the plunger head.

10.4.5 **NOTE:** Touch the screw and the black encasings only, not the foam portion.

10.4.6 Pour the residual liquid from the filter housing or pipette from the plastic bag into the concentrator tube containing the 600 ml of PBST.

10.4.7 Rinse filter housing with 5 ml of PBST and add rinsate to the concentrator tube. (If field sample, rinse the plastic bag that housed the filter with the 5 ml of PBST.)

10.4.8 Place housing in dirty equipment container.

10.4.9 Assemble the Filta-Max elution tube unit by screwing a second Quick Connect attachment ring onto the bottom of the elution tube with the beveled side down.

10.4.10 Twist/click the assembled elution tube unit into the topside of the large Quick Connect coupler.

10.4.11 Click the coupler into the jaws of the Filta-Max automated wash station. Ensure it is positioned correctly with the pin-locking mechanism.

10.4.12 Release the wash station jaws by turning the release lever counterclockwise. Raise the jaws as high as it will go and lock it into position by turning the lever clockwise.

10.4.13 Press F1 to lower the plunger head.

10.4.14 Insert the Allen wrench through the hole in the elution tube base and remove the filter module bolt. Put bolt in autoclave bag.

10.4.15 Attach the steel tube to the elution tube base.

10.4.16 Attach the assembled concentrator tube (with PBST) to the elution tube base beneath the elution tube.

10.4.17 Press F1 to presoak the filter.

10.4.18 After soaking press F3 for the first wash. Notice the number of times filter is washed. The plunger is moved up and down 20 times. Consult manual if problem occurs.

10.4.19 Detach the assembled concentrator tube and hold it directly below the steel tube in the purge position.

10.4.20 Press F4 for the first purge.

10.4.21 Place clean rubber stopper in the end of the steel tube.

10.4.22 Stand the assembled concentrator tube on a magnetic stirring plate. Attach the magnetic stir bar and turn on to achieve a vortex without splashing.

10.4.23 Look under the cap of the liquid trap bottle prior to connection. Ensure that the tubing connecting to the house vacuum source is that of the port of the shorter tube underneath. Screw the cap onto the bottle and connect the other port to the tap on the concentrator tube base.

10.4.24 Turn on the house vacuum. Do not exceed 30 cm Hg (11.8 in Hg for laboratory gauge) per manufacturer's instructions.

10.4.25 Slowly open the tap so that there is no rush of vacuum pressure exerted on the sample.

10.4.26 Drain the liquid to approximately 20 to 25 ml of sample. Use the stir bar's midway position as a guideline. Slow the stirring speed when close to the 20 to 25 ml. Close the tap.

10.4.27 Immediately, lift the magnetic stir bar out of the sample and rinse it with distilled water into the concentrated sample using the squirt bottle.

10.4.28 Immediately, swirl the concentrator tube and pour the first wash into a 50 ml centrifuge tube.

10.4.29 Label the centrifuge tube with the lab sample number.

10.4.30 **Note:** If the membrane clogs during the process, transfer the trapped sample into a sterile receptacle, remove and place it in a membrane filter bag. Replace the filter **smooth side up.**

10.4.31 Add a second 600 ml of PBST to the concentrator tube for the second wash.

10.4.32 Remove stopper, quick connect the tube back onto wash station and press F3 for the second wash. The plunger is moved up and down 10 times.

10.4.33 Disconnect the concentrator tube and press F4 for the final purge.

10.4.34 Rinse the steel tube with dilution water and repeat the concentration process beginning at step 10.4.20 to 10.4.27 (Drain to approx. 20 ml).

10.4.35 Add 2nd wash to 1st wash. The total volume should not exceed 50ml. Rinse concentrator tube with DI water using squirt bottle and add it to the 50 ml centrifuge tube. Note: use only one 50 ml centrifuge tube per sample.

10.4.36 Using sterile forceps remove the filter membrane from the base and put it into a small plastic bag. Add 5 ml of PBST from the concentrator base and massage membrane for 1 minute. Note: May massage longer if the membrane is heavily debrised. Using the pipette, transfer the filtrate to the 50ml tube. Repeat the membrane wash with another 5 ml of PBST and transfer the filtrate to the 50ml tube.

10.4.37 Note: For multiple membranes, wash each membrane in 5 ml of PBST after changing the membrane. Pipette the filtrate into the 50 ml centrifuge tube.

10.4.38 If at this point there is not a full 50 ml of sample in the centrifuge tube, add the rinse trapped in the bottom of the concentrator tube base to make up the difference.

10.4.39 Using the balance, balance the sample tube and a counterweight centrifuge tube to within 0.5 g of each other before centrifugation. Add weight using DI water.

10.4.40 Centrifuge at 2000 X G for 15 minutes. Note: If you had to replace the membrane filter more than once, centrifuge at 1500XG. Do not use the brake.

10.4.41 Note: Ensure that the sample spins for a full 15mins. For the Beckman GS-6 centrifuge go one click past the 15-minute setting. For the Allegra X-22 and X-14 centrifuges set to 16 min.

10.4.42 Record the pellet volume on the bench sheet using the standard pellets as guides.

10.4.43 Aspirate at the air/water interface from the center of the tube using a Pasteur pipette with the house vacuum system using no more than 11.8 in of Hg. As the target volume (see Sections 10.4.44 – 10.4.47) is approached, reduce the vacuum pressure to ≤ 5 in of Hg.

10.4.44 **If the packed pellet is ≤ 0.5 ml, aspirate down to 5.0 ml to process the one sub-sample**.

10.4.45 **If the packed pellet is > 0.5 ml and ≤ 1.0 ml, aspirate down to 10 ml to process as 2 subsamples of 5.0 ml each**.

10.4.46 **If the packed pellet is >1.0 ml and ≤ 1.5 ml, aspirate down to 15 ml and process as 3 sub-samples of 5 ml each.**

10.4.47 **If the packed pellet is >1.5 ml and ≤ 2.0 ml then aspirate down to 20 ml and process a 4 sub-samples of 5 ml each**. Note: If the packed pellet is >2, discontinue analysis and request a replacement sample.

10.4.48 Vortex the sample vigorously until sample is re-suspended but do not over vortex. Consider the consistency of the pellet and adjust the vortexing procedure accordingly. A grittier pellet would receive less vortexing and more gentle hand swirling.

10.5 Immunomagnetic Separation (IMS)

10.5.1 Prior to performing IMS, remove the Dynabeads GC combo from the refrigerator to allow components to come to room temperature.

10.5.2 Set out one Leighton tube for each subsample to be analyzed. Add to each tube 1 ml of 10X SL Buffer A and 1 ml of 10X SL Buffer B in that order. Label the tube with the Lab Sample number. (For multiple subsamples use the lab sample number followed by A, B, etc.)

10.5.3 Return to the concentrated sample. Pulse the sample once on the vortex mixer and swirl to complete the re-suspension. If the pellet appears to need further agitation, pulse the sample once more on the vortex mixer and swirl again. Record the re-suspended volume on the bench sheet. Note: If the sample is particularly gritty, attempt to re-suspend the pellet by swirling alone so as not to damage any potential organisms.

10.5.4 Rinse a graduated pipette with PBST and use it to transfer the sample to the Leighton tube. Note the volume transferred and subtract this volume from 10. Divide the end value by two. This will be the volume of each of the two DI water rinses to follow.

10.5.5 Add the first rinse of DI water to the 50 ml conical tube. Vortex the tube for 5 seconds and transfer the rinse into the Leighton tube with a pipette. Add the second rinse of DI water to the 50 ml conical tube. Vortex the tube for 5 seconds and transfer with pipette the second rinse into the Leighton tube. Ensure that all of the rinse has been transferred.

10.5.6 At the end of this step each Leighton tube will have 1 ml of 10X SL buffer A, 1 ml of 10X SL buffer B, and 10 ml that consist of re-suspended pellet and the two rinses.

10.5.7 **Calculate Rinse Volume for Multiple Subsamples**: If the concentrated sample will have two subsamples, subtract the measured volume from 20 ml. For three subsamples, subtract from 30 ml and for four subsamples, subtract from 40 ml. Divide the end value by two.

10.5.8 **Note**: When transferring the multiple subsamples, transfer in full 5 ml increments first. Expect the last transferred subsample to be slightly above or slightly below 5 ml.

10.5.9 Vortex Dynabeads anti-*Cryptosporidium* beads for 10 to 15 sec. Visually inspect the beads to ensure that it is thoroughly mixed. Pipette 100µl of the beads into the Leighton tube.

10.5.10 Vortex Dynabeads anti-*Giardia* beads for 10 to 15 sec. Visually inspect the beads to ensure that it is thoroughly mixed. Pipette 100µl of the beads into the same Leighton tube containing the anti-*Cryptosporidium* beads and concentrate. Invert the tube a few times to mix.

10.5.11 Affix tube to rotamix and set the mixer to rotate at 18 rpm for 1 hour. Use a timer.

10.5.12 Use this time to prepare a 1X buffer from 10X SL buffer A. The concentration is 100 µl of buffer A to 900 µl of DI water. Prepare 1.5 ml of 1X buffer per sub-sample. Enough can be made for the day. Label the tube with date, 1X buffer and initials.

10.5.13 When the hour is complete, remove the sample tube(s) from the mixer and place the tube in the magnetic particle concentrator (MPC-1/MPC-6) with the flat side of the tube toward the magnet.

10.5.14 Without removing the tube, position the magnet in a horizontal position with the magnet side of the MPC-1/MPC-6 (and the flat side of the Leighton tube) facing downward.

10.5.15 Hold the tube flush with the magnet and gently rock the tube end-to-end through 90 degrees tilting the cap-end and base-end up and down in turn at a rate of one tilt per second. Continue rocking tube continuously for 2 minutes. Use a timer. Notice the beads collect in the recessed flat area as the magnet pulls them.

10.5.16 **Note:** If the sample is allowed to stand motionless for more than 10 seconds, remove the Leighton tube from the MPC-1/MPC-6, very gently shake the tube to re-suspend the material replace the tube in the MPC-1/MPC-6 and repeat the positioning and process as described.

10.5.17 Return the MPC-1/MPC-6 to the upright position with the sample tube vertical, cap at top. Immediately remove the cap and keeping the flat side of the tube on top, pour off all of the supernatant from the tube held in the MPC-1/MPC-6 into a liquid waste container.

10.5.18 **Important:** Do not shake the tube or remove for the MPC-1/MPC-6 during this step. The goal is to keep the bead attached to the potential organisms. Jostling will cause them to disengage.

10.5.19 Wait for 1 minute allowing more supernatant to collect at the bottom of the tube. Aspirate this additional supernatant with a pipette.

10.5.20 Remove the sample from the MPC-1/MPC-6, remove the cap and pipette 0.5 ml of 1X SL buffer A (prepared earlier) into the tube positioning the stream down the flat side of the tube containing the bead-organism complex.

10.5.21 Mix very gently to re-suspend all of the material in the tube, but do not vortex. (Again, this would cause bead to disengage from potential organisms.)

10.5.22 Pipette all of the liquid into 1.5 micro centrifuge tube labeled with the sample number. Let the tube sit for 1 minute and transfer by pipette any residual into the micro centrifuge tube. Repeat the rinse procedure twice with two 0.5 ml of the 1X SL buffer A.

10.5.23 Place the micro centrifuge tube in the MPC-S with the magnet in place. Notice that it has two positions. Place it in the vertical position first to remove and/or separate as much debris as possible.

10.5.24 Without removing the micro centrifuge tube from the MPC-S, rock the tube gently through a 180-degree rotation. Continue for 1 minute at a rate of one 180-degree rock per second. Notice the bead formation of a distinct brown dot at the back of the tube.

10.5.25 At the end of the minute, keeping the magnetic strip in place, immediately aspirate the supernatant containing the debris from the tube and the residual in the cap. If more than one sample is being processed, conduct three 90-degree rock actions before removing the supernatant from each tube. Do not disturb the bead by touching it with the pipette. Do not shake the tube. Do not remove from the MPC-S while conducting these steps.

10.5.26 **Note for Samples with heavy debris:** Remove the tube from the MPC-S, add 1 ml of PBS and re-suspend very gently. Perform the above procedure again. (10.5.22 to10.5.24)

10.5.27 Remove the magnetic strip from the MPC-S.

10.5.28 Add 50 µl of 0.1N HCl, then vortex at the highest setting for 50 seconds. Use a timer.

10.5.29 **Note:** The vortexing breaks the bead-organism complex and the HCl prevents them from rebinding.

10.5.30 Remove the magnetic strip, allowing the tube to sit in the MPC-S for 10 minutes. Use a timer.

10.5.31 Just before the 10 minutes have expired. Label 2 slides with the sample number, system name, date and analyst initials.

10.5.32 At the end of the 10 minutes, vortex the tube vigorously for 30 seconds. Use a timer.

10.5.33 Ensure that the entire sample is at the base of the tube. Hold the tube at a slant and pulse on the vortex mixer to trap the entire sample at the bottom.

10.5.34 Replace the magnetic strip in the slanted position and allow it to stand for at least 10 to 15 seconds.

10.5.35 Add 5 µl of 1.0N NaOH to the 1st well slide.

10.5.36 Using a pipette of greater than 50 µl volume, transfer the sample onto the 5 µl of NaOH on the 1st well slide. Again do not disturb the bead complex. Ensure that all of the fluid is transferred.

10.5.37 Do not discard the beads, but use them to perform a second dissociation by repeating steps 10.5.27 through 10.5.34.

10.5.38 Add 5 µl of 1.0N NaOH to the 2nd well slide.

10.5.39 Using a pipette of greater than 50 µl volume, transfer the sample onto the 5 µl of NaOH on the 2nd well slide. Again do not disturb the bead complex. Ensure that all of the fluid is transferred

10.5.40 Record the date and time the purified sample was applied to the slide.

10.6 Staining-Easy Stain Procedure

10.6.1 Using the slide warmer set at 37°C, dry the slide. Then immediately perform steps 10.6.4 through 10.6.14.

10.6.2 Prepare positive and negative control slides with each batch of slides. Prepare by adding 20 µl of DI water onto a slide. Gently pulse the Easystain positive control on the vortex mixer, then pipette 30 µl of the control onto the slide, dry using a slide warmer set between 35 – 42°C. For the negative control slide pipette 50 µl of the PBS, not PBST onto a separate slide and dry using slide warmer set between 35-42 °C.

10.6.3 Note: If time does not permit for the whole procedure, then dry the slide (uncovered) overnight at room temperature.

10.6.4 Add 1 to 2 drops of EasyStain to the well. Place slides in humid chamber and store in a dark drawer at room temperature for 30 minutes. Note: the humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed.

10.6.5 Tilt the well slide (long edge down). Gently remove excess EasyStain from below the well with a paper towel strip.

10.6.6 Slowly add 100 - 250 µl ice cold (<2°C) Fixing Buffer to the well and allow flowing over well edges. Leave for 2 minutes. (Following EasyStain Instructions)

10.6.7 **Critical: The Fixing Buffer must be ice cold (<2°C) and left for 2 minutes**.

10.6.8 Tilt well slide (long edge down). Gently remove excess Fixing Buffer from below the well with a paper towel strip.

10.6.9 Add 1 to 2 drops of EasyStain DAPI (or 50 µl of working strength DAPI) solution to the well. Allow standing at room temperature for 2 or more minutes.

10.6.10 Tilt the well slide (long edge down). Gently remove excess DAPI from below the well with a paper towel strip.

10.6.11 Add 50 µl deionized water to the well. Allow to stand for one minute.

10.6.12 Tilt the well slide (long edge down). Gently remove excess water from below the well.

10.6.13 Add 5 µl EasyStain Mounting Medium to the well and apply cover slip.

10.6.14 Alternatively, add 5 µl EasyStain Mounting Medium to the center of a cover slip (placed on the bench). Hold well slide on its edge (long edge down) next to cover slip. Gently lower well slide (sample down) onto cover slip.

10.6.15 Seal the edges with Permount (primary). May use clear fingernail polish as an alternative and record staining completion time on the bench sheet.

10.6.16 **Note: *Do not touch the well during the staining process.***

10.6.17 Store slides in humid chamber in the refrigerator.

10.7 Examination

10.7.1 For detailed instruction on microscope usage, refer to the Microscope SOP. The following steps must be done each time, per analyst per session.

10.7.2 Turn on the microscope power source. **Note:** when using a mercury lamp record hours used.

10.7.3 Before beginning a microscope session, enter name, date, and time started in the Microscope Log Book.

10.7.4 **Note:** Orient each slide with writing to the left. Record coordinates of *Cryptosporidium* and *Giardia* found on field samples.

10.7.5 Characterize 3 *Cryptosporidium* oocysts and 3 *Giardia* cysts using the criteria below and record them in the Positive Slide Characterization Log Book.

10.7.6 Read the Negative Control Slide. Document whether it is acceptable on the sample examination form. It should not contain any oocysts or cysts.

10.7.7 Sample examination - *Cryptosporidium*

10.7.8 **FITC examination** (Must use a minimum of 200X total magnification). Use epifluorescence to scan the entire well for apple-green fluorescence of oocyst and cyst shapes. When brilliant apple-green fluorescing ovoid or spherical objects 4 to 6µm in diameter are observed with brightly highlighted edges, increase magnification to 400X. Switch the microscope to the UV filter block for DAPI (10.7.9), then to DIC (10.7.12) at 1000X.

10.7.9 **DAPI fluorescence examination** (Must use a minimum of 400X total magnification). Using the UV filter block for DAPI, the object will exhibit one of 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.

Note: Look for atypical DAPI fluorescence e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color.

10.7.10 Record oocysts in category a) as DAPI-negative; record oocysts in categories b) and c) as DAPI-positive.

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

10.7.12 **DIC examination** (Must use a minimum of 1000X total magnification-oil immersion lens). Using DIC, 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 SOP Reference 12.5). 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)

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

10.7.14 A positive result is a *Cryptosporidium* oocyst that exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy. All positive results must be characterized and assigned to one of the DAPI and DIC categories in Sections 10.7.9 and 10.7.11. Positive organisms should be photographed using microscopy camera.

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

10.7.16 Report *Cryptosporidium* concentrations as oocysts/L.

10.7.17 Record analyst names

10.7.18 Sample examination – *Giardia*

10.7.19 **FITC examination** (the analyst must use a minimum of 200X total magnification). When brilliant apple-green fluorescing round to oval objects (8 -18 µm long by 5 -15 µm wide) are observed, increase magnification to 400X and switch the microscope to the UV filter block for DAPI then to DIC.

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

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

10.7.21 Record cysts in category (a) as DAPI negative; record cysts in categories (b) and (c) as DAPI positive.

10.7.22 DIC examination (the analyst must use a minimum of 1000X total magnification). Using DIC, 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 atypical structures are not observed, then categorize each object meeting the criteria specified 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

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

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

10.7.25 A positive result is a *Giardia* cyst that exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy. All positive results must be characterized and assigned to one of the DAPI and DIC categories in Sections 10.7.20 and 10.7.22. Positive organisms should photographed using microscopy camera.

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

10.7.27 Report *Giardia* concentrations as cysts/L.

10.7.28 Record analyst names

10.8 Verification of Analyst Performance

10.8.1 On a monthly basis scientists responsible for reading slides must perform a verification of analyst performance on an OPR slide, MS slide, a positive staining control slide or by preparing a separate slide (10.8.2-10.8.4) .

10.8.2 Prepare a slide by spiking approximately 40 ml of PBST with EasySeed using the spiking procedure in 10.2.3. Use a bench sheet to record the preparation of the sample. Fill in the spiking information and then start at centrifugation. Strike through the other fields.

10.8.3 Fill the remainder of the tube to the 50 ml mark and centrifuge at 2000X. Finish analyzing the sample through IMS; however, perform a single dissociation. Stain as usual.

10.8.4 Label the slide as Monthly Verification, the month the date prepared and the preparer's initials.

10.8.5 When reviewing the slide, ensure that there are at least 40 Crypto oocysts, and 40 *Giardia* cysts present and that at least 50% of them are DAPI positive. Prepare another if this is not the case.

10.8.6 Each analyst shall read the slide and determine the number of FITC stained organisms on the slide. Analysts will record their results on both a crypto and *Giardia* report form.

10.8.7 The counts must be within 10% of each other. If this is not the case, analysts must read the slide to determine the source of the error.

10.8.8 This attempt though failed is recorded in the analyst verification book and a new slide is prepared and the test redone. Record results.

10.8.9 These results are also noted in the book.

10.8.10 After the slide has been read and the counts have been determined to be within 10% of each other, analysts will characterize 20 organisms together (10 crypto and 10 *Giardia*). One analyst will be responsible for locating the specimens.

10.8.11 Each person will record what he or she sees and discuss it until a consensus is reached. The end result is recorded on each characterization form.

10.8.12 The results are then recorded on the verification of analyst performance log sheet with all of the sheets stapled together and placed in the verification of analyst performance logbook.

10.8.13 If any analyst weakness is exposed during this session that will comprise field results, that scientist will not read official samples and will be retrained by the lead scientist or supervisor.

10.9 Data Recording and Validation

10.9.1 After paperwork is completed, analyst is to batch the samples for the week

10.9.2 After batching enter results into LIMS by following the data entry SOP 8-002 (SOP reference 12.11).

10.9.3 The batch sheet along with the bench sheets should be given to the supervisor for verification. Supervisor or Senior Scientist will verify all of the information on the sheets.

10.9.4 Supervisor or Senior Scientist will go into LIMS to verify the logged in data, check calculations manually and make any necessary corrections or corrective actions, if applicable.

10.9.5 Supervisor or Senior Scientist will enter a result of complete in the validation field. When verification is over, print reports and give to the manager for final validation.

10.9.6 Manager will generate a QC batch report and check entries made by supervisor.

10.9.7 Reports are checked by analyst against the data on the bench sheets and corrected if necessary. Reports are sent to customers.

10.9.8 Manager then validates and exports the results.

10.10 Method Holding Times Table

<table>
<tr><th>Sample Processing Time</th><th>Maximum Allowable Time Between Breaks</th></tr>
<tr><td colspan="2">Collection</td></tr>
<tr><td colspan="2">Filtration</td></tr>
<tr><td colspan="2">● Up to 96 hours are permitted between sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field) and initiation of elution.</td></tr>
<tr><td>Elution</td><td rowspan="4">These steps must be completed in 1 working day.</td></tr>
<tr><td>Concentration</td></tr>
<tr><td>Purification</td></tr>
<tr><td>Application of purified sample to slide</td></tr>
<tr><td colspan="2">Drying of sample</td></tr>
<tr><td colspan="2">● Up to 72 hours are permitted from application of the purified sample to the slide to staining</td></tr>
<tr><td colspan="2">Staining</td></tr>
<tr><td colspan="2">● Up to 7 days are permitted between sample staining and examination</td></tr>
</table>

Sample Processing Time	Maximum Allowable Time Between Breaks
Examination	

10.10.1 Samples that were not collected the same day they were received, and that are received at >20°C or frozen, or samples that the laboratory has determined exceeded >20°C or froze during shipment, must be rejected.

10.10.2 After receipt, samples must be stored at the laboratory between 1°C and 10°C, and not frozen, until processed.

11.0 Calculations

11.1 Recovery (R) = (Amount Recovered / Amount Spiked) X 100

11.2 Organisms/L = Amount Recovered /Liters Filtered

12.0 References

12.1 EPA Method 1623: *Cryptosporidium and Giardia* in Water by Filtration/IMS/FA, December 2005.

12.2 "Filta-Max ®", Idexx Laboratories, One Idexx Drive, Westbrook, Me. 04092.

12.3 Manual for the Certification of Laboratories Analyzing Drinking Water, Criteria and Procedures Quality Assurance, EPA 815-R-05-004, Fifth Edition, January 2005.

12.4 Supplement 2 to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water.

12.5 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 (1996).

12.6 GA EPD Laboratory SOPs – Initial Demonstration of Capability SOP 6-001, online revision and/or Continuing Demonstration of Capability SOP 6-002, online revision.

12.7 GA EPD Laboratory SOP – EPD Laboratory Procedures for Control Charting and Control Limits, SOP 6-025, online revision.

12.8 GA EPD Laboratory Quality Assurance Plan, online revision.

12.9 GA EPD Laboratory Safety/Chemical Hygiene Plan & Fire Safety Plan, online revision.

12.10 GA EPD SOP 8-016 Epifluoresence Microscope and Digital Imaging System, online revision.

12.11 GA EPD SOP 8-002 Procedure for Data Entry in the Protozoan Section, online revision.

13.0 Waste Management

13.1 See GA EPD Laboratory SOP-EPD Laboratory Waste Management Standard Operating procedures, SOP 6-015, online revision.

14.0 Practical Quantitation Limits (PQLs), Precision and Accuracy Criteria, and Quality Control Approach

14.1 Refer to Appendix A, Table A.1 for precision and accuracy criteria.

Table 14.1 RLs for EPA Method 1623

Parameter/ Method	Analyte	Matrix (aqueous)	
		RL	Unit
EPA 1623	Cryptosporidium	0.1	Org/L
	Giardia	0.1	Org/L

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	*Crypto* Oocysts (IDC)	Initial Demonstration Includes IPR set, MB Test, and MS	One per analyst.	See Below	Identify and Correct Problem Area. Rerun IPRs.	
	IPR Test	4 reagent water samples spike with 100-500 oocysts.	Before field sample analysis, Initial use of method	Limits: Crypto 36-100 Giardia 32-100 Precision: Crypto 34 Giardia37	Identify and Correct Problem Area. Rerun IPRs.	
	MB Test	Method Blank	One per batch (including IPRs)	No cysts or oocysts should be detected.	Locate the source of contamination and clean and rerun method blank.	
	LCS (OPR)	LCS/ LCS -(OPR)	One LCS per batch of 20 samples or less.	Mean Recovery Rates: Refer to SOP 8-001 Appendix A	Identify and Correct Problem Area. Rerun LCS	
	LCS (OPR) Control Charts	LCS (OPR) Control Chart Recoveries	Based on mean recovery and ± 2SD	Recalculate after 10 LCS recoveries	Establish new control limits	Initiate Corrective Action for recoveries outside of acceptable limits

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	MS	MS	One per new system and one per batch of 20 samples or less.	Mean Recovery Rates: Refer to SOP 8-001 Appendix A	Make a notation on the report that there is matrix interference.	
	MS Control Charts	Control Chart MS Recoveries	Based on mean recovery and ± 2SD	Recalculate after 10 MS recoveries	Establish new control limits	Initiate Corrective Action for recoveries outside of acceptable limits
	Spike Values	Spike Values of spiking suspension	Daily as necessary	Record value	Maintain Record	
	Positive Staining Control	Positive Staining Control	Read before microscope field slide examination	3 Crypto oocysts and 3 Giardia cysts on-Positive should have target organisms stained for both FITC and DAPI and intact organelles for DIC- must be acceptable.	Record on microscope log. If controls are with weekly QC, and QC stained properly, then notate on sheet. If control slide fail due to stain malfunction, prepare new slides using another box and/or lot number of stain. If failure is due to organisms, prepare new slides using new organisms.	Discuss differences between analysts

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	Negative Staining Control	Negative Staining Control	Read before microscope field slide examination	Negative should not contain any oocysts or cysts	Record on microscope log. If controls are with weekly QC and QC stained properly, then notate on sheet. If control slide fail due to stain malfunction, prepare new slides using another box and/or lot number of stain. If failure is due to organisms, prepare new slides using new organisms.	Discuss differences between analysts
	Proficiency Evaluation (PT Samples)	Analysis of PE samples	As required by EPA or auditing agency.	All analyte results acceptable per the auditing agency.	Correct the problem.	
	Sample Holding Time and Procedure Requirements	Sample Receipt from Collection or Filtration	As soon as possible, not to exceed 96 hours	Refer to Section 10.10 of SOP.	Sample not analyzed. Reason entered in LIMS.	System requested to resample
		Elution, Concentration, Purification, Application to slide	Must be completed in one day	Refer to Section 10.10 of SOP.	Sample not analyzed. Reason entered in LIMS.	System requested to resample
	Sample Holding Time and Procedure Requirements	Staining	Within 72 hours from application of purified sample to slide for staining	Refer to Section 10.10 of SOP.	Sample not analyzed. Reason entered in LIMS.	System requested to resample
	Sample Holding Time and Procedure Requirements	Examination of Slide	Within 168 hours (7 days) from sample staining to examination	Refer to Section 10.10 of SOP.	Sample not analyzed. Reason entered in LIMS.	System requested to resample

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	Verification of Analyst Performance	Ongoing comparison of slide counts	Performed monthly	Analyst must come within 10% of confirmation analyst.	Discuss and resolve differences.	Discrepancies must be identified and resolved.
	Regent Water	Conductivity	Monthly	>0.5megohms or <2umhos/cm at 25°C	Maintain record	
		Metal concentration	Annually for Pb, Cd, Cr, Cu, Ni, Zn	Must be <0.05mg/L or collectively <0.1mg/L	Maintain record	
		Chlorine Residual	Monthly	<0.1mg/L	Maintain record	
		Heterotrophic Plate Count	Monthly	<500CFU/mL or MPN<500/mL	Maintain record	
	Equipment QC	pH Meter Calibration Slope Determination	Monthly	Slope must be 90-102%	Discrepancies must be identified and resolved. Initiate Corrective Action, Resolve and recalibrate.	
		Balance Calibration	Monthly	Verify and record in log book	Document correction factors if necessary	
		Reference Weight Certification	5 years	Recertify weighs or obtain new certification	Maintain certificate of traceability	
		Balance maintenance calibration and cleaning	Yearly	By qualified independent service technician.	Maintain certificate	

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	Equipment QC	Glass Thermometers, check calibration with NIST traceable	Yearly	Must be <1°C difference. Record: • Serial Number Lab thermometer • NIST thermometer Serial Number • Temp of Lab thermometer • Temp of NIST thermometer • Date of check • Correction or calibration factor Analyst Initials	Dispose	
		Dial and Infrared Thermometers, check calibration with NIST traceable	Quarterly	Must be <1°C difference Record: • Serial Number Lab thermometer • NIST thermometer Serial Number • Temp of Lab thermometer • Temp of NIST thermometer • Date of check • Correction or calibration factor Analyst Initials	Dispose	
		NIST Reference Thermometer, recalibrated and certified	5 years	• NIST certification and validation	Maintain Record	

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

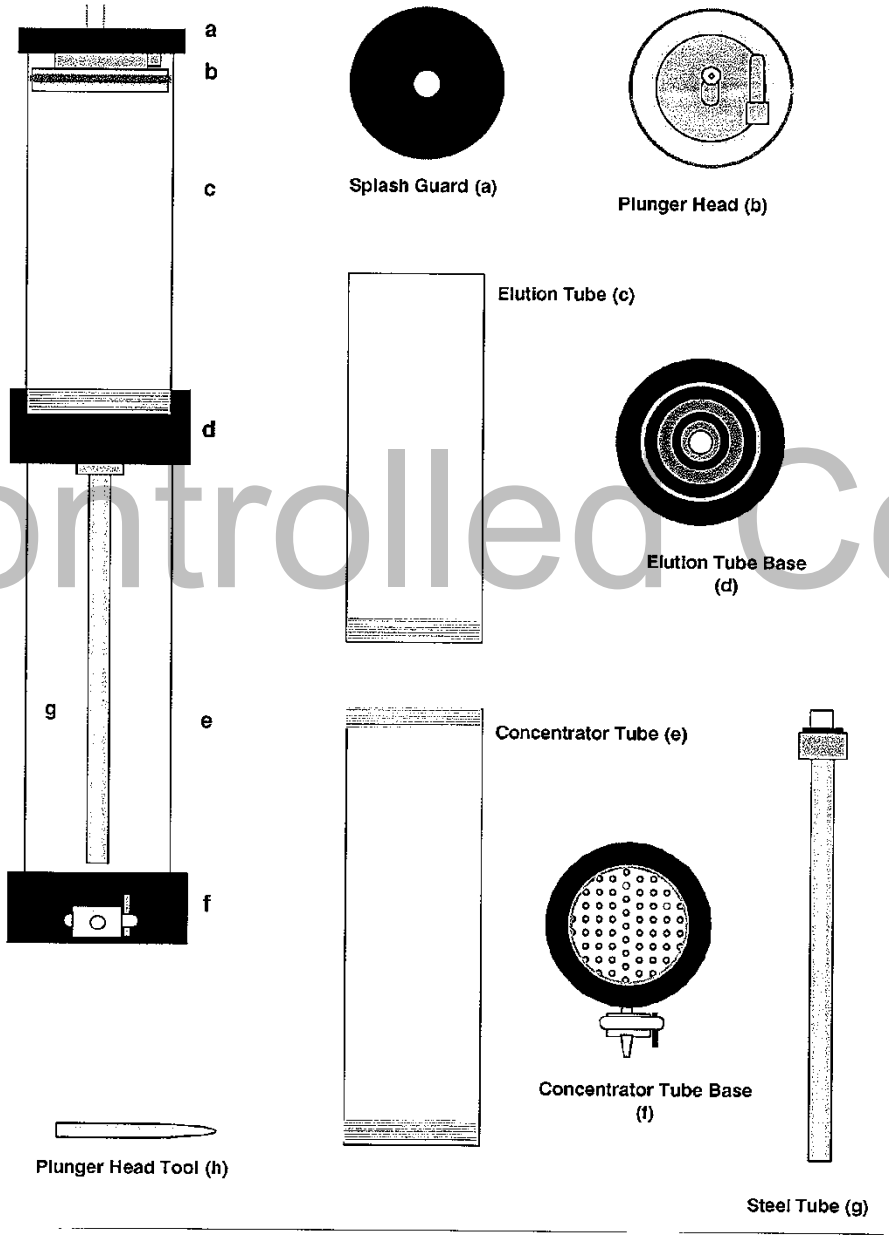
Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	Equipment QC	Incubator and slide warmer temperature correction	Daily	Record temperature on device, include: • Date/Time of reading • Analyst initials	Maintain Record	
		Autoclave use	Daily	Record: • Date • Contents • Sterilization time/temp • Total Time in autoclave Analyst initials	Maintain Record	
		Autoclave spore strips	Daily	Confirm sterilization	Record in log record	
		Autoclave timing mechanisms checked for accuracy	Quarterly	Against a stopwatch or timer. Record results and initial	Adjust as necessary	
		Conductivity Meter Calibration	Daily	• Record Calibration	Recalibrate as necessary	
		Refrigerators	Daily, 1-10°C	• Record date, temperature, analyst initials	Adjust as necessary	
		Micropipettes	Bi-annually	Record date, results	Maintain Record	
		Hand Tally Counter	Quarterly	Confirm Accuracy and operational status	Record Checks, date, analyst initials	
		Glassware and Plasticware	Initially	Confirm Accuracy, within 2.5%	Verify at time of purchase	
		Centrifuge tachometer calibration	Yearly	Record results	Maintain documentation	
		Aspiration System	Yearly	Record aspiration rate and pipette diameter (0.80-1.5mm)	Maintain documentation	

Table 14.2 Quality Control, Equipment Maintenance, and Documentation Requirements for EPA Method 1623

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action or Requirement	Flag Criteria
1623 Analytical QC	Equipment QC	IMS, rotating mixer RPM calibration	Yearly	Record results and initial	Maintain documentation	
		Microscope alignment and adjustment	Yearly	Service contract available for review	Maintain record of alignment and adjustment	
		Microscope alignment and adjustment	Daily as necessary by analyst	Record daily alignment and adjustment	Maintain record of alignment and adjustment	
		Microscope ocular micrometer	First use of microscope and when objective replaced	Calibrate for each objective used	Maintain record of installation	
		Microscope UV bulb use	Record number of hours used	Record daily	Maintain record	

15.0 Appendix (Idexx Components)

Figure 2: Wash Station Components



Appendix A – Quality Assurance Criteria for Method EPA 1623

Table A.1 Quality Assurance Criteria for EPA Method 1623

		Accuracy (%R)			Precision (%RPD)
QC Type	**Analyte**	**LCL**		**UCL**	**Range**
LCS (or OPR)*					
	Cryptosporidium	33	-	100	NA
	Giardia	24	-	100	NA
MS *					
	Cryptosporidium	5	-	100	NA
	Giardia	18	-	100	NA

* LCS/LCSD and MS precision and accuracy limits are developed from control charts of data collected from 1/1/2019 to 1/1/2021. Method 1623 requires control limits based on $\pm 2\ \delta_{n-1}$.

16.0 Updates

16.1 Updated for online revision. Appendix A added.