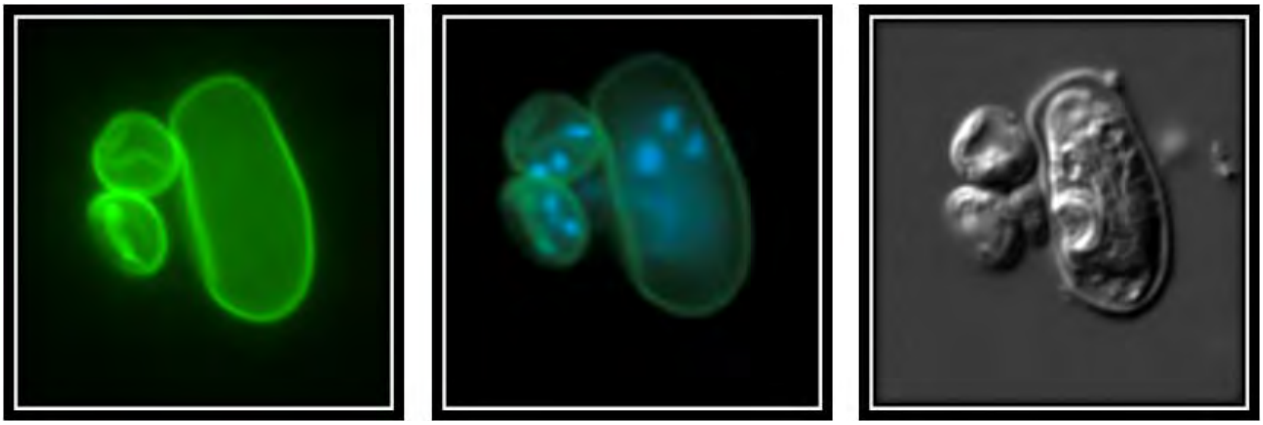
United States
Environmental Protection
Agency

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



Office of Water (MS-140) EPA 815-F-12-006 November 2012 www.epa.gov/safewater

Supplement 2 to
Manual for the Certification of Laboratories Analyzing Drinking Water:
Criteria and Procedures Quality Assurance
EPA 815-R-05-004
January 2005

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***Cryptosporidium* Monitoring**
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***Cryptosporidium* Monitoring**

Supplement to: Chapter I Introduction

The Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) (40 CFR 9, 141, 142) requires public water systems that use surface water or ground water under the direct influence of surface water (GWUDI) to monitor their source water (influent prior to treatment) for *Cryptosporidium* and *E. coli* and turbidity (40 CFR 141.701). Systems serving greater than 10,000 people are required to conduct two years of monthly source water monitoring for *Cryptosporidium*, *E.coli*, and turbidity. Filtered systems serving less than 10,000 people are required to conduct one year of source water monitoring for *E.coli*. If the *E.coli* limits are exceeded, *Cryptosporidium* monitoring is required. The LT2 Rule specifies the methods, approval criteria, and quality assurance practices to be used. The LT2 Rule also requires public water systems to have *Cryptosporidium* samples analyzed by a laboratory approved under EPA's Laboratory Quality Assurance Evaluation Program for analysis of *Cryptosporidium* or a laboratory that has been certified for *Cryptosporidium* analysis by an equivalent State laboratory certification program (40 CFR 141.705).

EPA's Technical Support Center (TSC) in Cincinnati, Ohio, with the assistance of the National Exposure Research Laboratory in Cincinnati, Ohio (NERL-Ci), is responsible for determining the certification status for EPA's Regional laboratories in Parasitology if they are supporting LT2 Rule analyses.

Chapter VII covers the technical criteria to be used during the on-site evaluation of a laboratory for parasitology. Optional audit forms are also included in Chapters VII.

***Cryptosporidium* Monitoring**
Supplement to: Chapter II Responsibilities

Office of Ground Water and Drinking Water (OGWDW) and Office of Research and Development (ORD)

- Conduct triennial on-site audits of each Regional laboratory for parasitology.

***Cryptosporidium* Monitoring**
Supplement to: Chapter III Implementation

17.0 Training

Regions and States should verify that each Certification Officer has passed the *Cryptosporidium* Certification Officer Training in addition to either the Microbiology or Chemistry Certification Officer Training. Observation of an experienced *Cryptosporidium* auditor evaluating a laboratory, and performance of an audit for *Cryptosporidium* under the supervision of an experienced *Cryptosporidium* auditor, are also recommended.

***Cryptosporidium* Monitoring**
New Chapter: Chapter VII Critical Elements for Parasitology (*Cryptosporidium*, *Giardia*)

New Chapter begins on the next page

Chapter VII
Critical Elements for Parasitology (*Cryptosporidium*, *Giardia*)

Note 1: This chapter uses the term 'must' to refer to certification criteria that are required by the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) as part of the Safe Drinking Water Act. The term 'should' is used for procedures that, while not specifically required by the regulations, are considered good laboratory practices. To assure the validity of the data, it is important that laboratories observe both the regulatory criteria and non-regulatory practices. Certification Officers may not recommend certification if the quality control data are judged unsatisfactory or insufficient.

Note 2: Quality control (QC) items, designated by a '**QC**', necessitate written records. Each record should include analyst's initials and date(s).

Note 3: Section references in [ ] refer to U.S. Environmental Protection Agency (EPA) Method 1623, and the corresponding section references in parentheses and italics refer to EPA Method 1623.1.

EPA Method 1623 and 1623.1 Overview

EPA Methods 1623 and1623.1 are for the determination of *Cryptosporidium* and *Giardia* in water. Both methods require filtration, immunomagnetic separation (IMS) of the oocysts and cysts from the sample and enumeration of the target oocysts/cysts based on the results of immunofluorescence assay (IFA or FA) using fluorescein isothiocyanate (FITC), 4',6-diamidino-2-phenylindole (DAPI) staining, and differential interference contrast (DIC) microscopy. EPA Method 1623.1 reflects improvements to Method 1623. Among the key improvements are the incorporation of sodium hexametaphosphate to the elution process and a bead pellet wash step to improve recovery, particularly for samples with traditionally low recovery using Method 1623.

To enhance program-wide data quality and consistency, and guard against the use of sample processing shortcuts that could compromise data quality, Method 1623.1 provides laboratories with the flexibility to select from options for various procedural components that do not require an alternate test procedure study (ATP). However, each option must be performed according to the procedures used during a multi-laboratory validation study. Any additional ATPs should follow a process for conducting side-by-side method comparisons and for conducting quality control acceptance criteria-based method studies.

1. Personnel

The personnel prerequisites for the *Cryptosporidium* laboratory evaluation program are as follows:

1.1 *Principal Analyst/Supervisor (at least one per laboratory)*

The principal analyst/supervisor participates in a monthly analyst verification [Section 10.6 (*Section 9.10*)], supervises and verifies the processing and microscopy in the laboratory and may perform the same duties as an analyst. The principal analyst/supervisor has the responsibility to ensure that all laboratory personnel have

demonstrated the ability to satisfactorily perform the analyses to which they are assigned and that all data reported by the laboratory meet the required quality assurance (QA) and regulatory criteria. The principal analyst/supervisor should have the following experience:

- BS/BA in microbiology or closely related field
- A minimum of 1 year of bench experience with *Cryptosporidium* and FA microscopy
- A minimum of 6 months experience using EPA Method 1623 or 1623.1
- A minimum of 100 samples analyzed using EPA Method 1623 or 1623.1
- "Grandfathering" principal analysts with >10 years experience of protozoan identification duties may be substituted for college education

1.2 ***Analyst***

The analyst participates in a monthly analyst verification [Section 10.6 (*Section 9.10)*], establishes Köhler illumination for the microscope, may perform the same duties as a technician and is able to examine samples using the microscope. An analyst should have the following experience:

- Two years of college in microbiology or equivalent or closely related field
- A minimum of 6 months bench experience with *Cryptosporidium* and FA microscopy
- A minimum of 3 months experience using EPA Method 1623 or 1623.1
- A minimum of 50 samples analyzed using EPA Method 1623 or 1623.1
- "Grandfathering" analysts with >10 years experience of protozoan identification duties may be substituted for college education.

1.3 ***Technician***

The technician filters samples, performs centrifugation, elution, concentration, and purification using IMS, and prepares purified samples on slides for microscopic examination, but does not perform microscopic protozoan detection and identification. A technician should have the following experience:

- A minimum of 3 months of experience in filter extraction and processing of protozoa samples by EPA Method 1623 or 1623.1
- A minimum of 50 samples analyzed using EPA Method 1623 or 1623.1 for the specific analytical procedures they will be using.

1.4 ***Sample Collection Personnel***

If the laboratory also conducts field sampling activities, the personnel who collect samples should have training in the proper collection techniques for all types of samples they collect. The training should include sample holding times, temperature requirements, and chain of custody. Their techniques should be reviewed by experienced sampling personnel prior to independently collecting compliance monitoring samples. All laboratory personnel are encouraged to attend workshops, and training programs that may be available from State and federal regulatory agencies, professional societies, and manufacturers.

1.5 ***Data Produced by Analysts and Technicians in Training***

Data produced by analysts and technicians who have not completed their training or the recommended experience with the method should be considered acceptable only when reviewed and validated by a Principal Analyst/Supervisor. Laboratory supervisors should document the review and the data's acceptability as part of the laboratories' permanent records.

1.6 ***Waiver of Academic Training***

The Certification Authority may waive the need for the recommended academic training on a case by case basis for highly experienced analysts. If a waiver is granted, the Certification Authority should prepare a written and signed justification and the laboratory should have the waiver available for review.

1.7 ***Personnel Records***

Laboratory should have standard operating procedures (SOPs) for how training of all personnel is conducted. Personnel records should be maintained for all laboratory personnel. These records should include documentation of all job related formal education and training which pertains to any aspect of his/her responsibilities including, but not limited to, analytical methodology, laboratory safety, sampling, QA, data analysis, etc.

2. Laboratory Facilities

Laboratory facilities should be clean, temperature and humidity controlled, and should have adequate lighting at bench tops. The laboratory should maintain effective separation between areas where activities are incompatible, minimize traffic flow and ensure that contamination does not adversely affect data quality. Bench tops and floors should be of a material that is easily cleaned and disinfected. Laboratory facilities should have sufficient bench top area for processing samples; storage space for reagents, laboratory supplies, glassware, and portable equipment, floor space for incubators, biological safety cabinet, refrigerators, etc.; and associated area(s) for cleaning glassware and sterilizing materials. Laboratory facilities should have provisions for disinfection and proper disposal of microbiological wastes. Laboratories performing *Cryptosporidium*/*Giardia* analyses should have a room capable of being darkened to near – complete darkness for microscopic examination of slides.

3. Laboratory Equipment and Supplies

Additional information is available in EPA Method 1623 or 1623.1, and in the attached Checklists A, B, and C. Brand names and suppliers are for illustrative, informational purposes only. No endorsement is implied.

Documentation for equipment should be kept by serial number or other unique identifier in a log or QA record book.

3.1 ***pH Meter***

3.1.1 Accuracy and scale graduations should be within pH ± 0.1.

3.1.2 pH buffer aliquots should be used only once.

3.1.3 Electrodes should be maintained according to manufacturer's recommendations.

QC **3.1.4** pH meters should be standardized before each use period with pH 7.0 and either 4.0 or 10.0 standard buffers, whichever range covers the desired pH of the reagent. The dates, pH measurements, and buffers used should be recorded along with the analyst's initials. In addition, a monthly determination of % slope should be performed and recorded. Acceptable % slope is 95 – 105 %.

QC **3.1.5** Commercial buffers should be dated when received, opened, and discarded within the expiration date.

3.2 ***Balance (top loader or pan)***

3.2.1 Balances should have the capability of detection of at least 0.1g for a load of 150 g, and 1 mg for a load of 10 g or less.

QC **3.2.2** Balance calibrations should be verified monthly using ASTM Class 1, 2, or 3 weights. Non-reference weights should be calibrated every six months with reference weights. Verifications and calibrations should be recorded in a log or record book with initials of the individual performing the calibration. Correction values should be used and documented. Damaged or corroded weights should be replaced.

QC **3.2.3** Correction data and certificate of traceability should be available for weights. Reference weights should be re-certified every five years.

QC **3.2.4** Service contracts or internal maintenance protocols and maintenance records should be available. Maintenance calibration and cleaning should be conducted at least annually by a qualified independent service technician.

3.3 ***Temperature Monitoring***

3.3.1 Glass, dial, electronic or infrared thermometers used for refrigerators and sample receipt monitoring should be graduated in at least 1°C increments. The fluid column in glass thermometers should not be separated. Dial thermometers that cannot be calibrated should not be used. The use of mercury thermometers is discouraged because of safety and environmental concerns.

QC **3.3.2** The calibration of glass and electronic thermometers should be checked annually, and dial and infrared thermometers quarterly at the temperature used with a National Institute of Standards and Technology (NIST) certified traceable reference thermometer or one traceable to a NIST reference thermometer. The calibration factor and the date of calibration should be

indicated on the thermometer [Section 8.1.4 (*Section 8.1.4*)]. The laboratory should record the following information:

- Serial number of laboratory thermometer
- Serial number of NIST traceable thermometer or other reference thermometer
- Temperature of laboratory thermometer
- Temperature of NIST traceable thermometer or other reference thermometer
- Date of check
- Correction or calibration factor (for both reference and in-use thermometers)
- Analysts initials

QC **3.3.3** Thermometers that differ by more than 1°C from the reference thermometer should be discarded. Reference thermometers should be recalibrated at least every five years by a qualified service technician. Reference thermometer calibration documentation and correction factors should be maintained by the laboratory.

QC **3.3.4** Continuous recording devices that are used to monitor refrigerator or incubator temperature should be recalibrated at least annually by a qualified service technician.

3.4 *Incubator and Slide Warmer*

3.4.1 Incubators and slide warmers should have an internal temperature monitoring device and maintain temperature specified by the method. Thermometers should be placed on the top and bottom shelves of the use area and immersed in liquid as directed by the manufacturer (except for electronic thermometers).

QC **3.4.2** Calibration corrected temperatures should be labeled on each incubator/slide warmer being used. Documentation should include the date and time of reading, and technician's initials.

3.5 *Autoclave*

3.5.1 The autoclave should have an internal heat source, a temperature gauge with a sensor on the exhaust, a pressure gauge, and an operational safety valve. The autoclave should maintain the sterilization temperature, as specified in the SOP, during the sterilizing cycle.

3.5.2 Pressure cookers should not be used for sterilization because of safety concerns and difficulties maintaining operational control.

QC **3.5.3** The date, contents, sterilization time and temperature, total time in autoclave, and analyst's initials should be recorded each time the autoclave is used.

QC **3.5.4** Copies of the service contract or internal maintenance protocol and maintenance records should be kept. Maintenance should be performed at least annually. A record of the most recent service performed should be on file and available for inspection.

QC **3.5.5** A maximum temperature registering thermometer, electronic temperature readout device, or continuous recording device should be used to ensure that the proper sterilization temperature was reached and recorded. Overcrowding of items in the autoclave to be sterilized should be avoided. Spore strips or ampules should be used monthly as bioindicators to confirm sterilization.

QC **3.5.6** Automatic timing mechanisms should be checked for accuracy quarterly with a stop watch or other accurate timepiece and the results recorded and initialed.

3.5.7 Autoclave door seals should be clean. Autoclave drain screens should be cleaned frequently and debris removed.

3.6 *Conductivity Meter*

3.6.1 Meters should be suitable for checking reagent grade water and readable in either µmhos/cm or µS/cm.

QC **3.6.2** Conductivity meter should be calibrated and recorded at least monthly consistent with manufacturer's recommendations using an appropriate low level certified traceable standard. If the conductivity cannot be calibrated with a commercial standard, the cell constant should be determined monthly using Standard Method 2510, "Conductivity", in *Standard Methods for the Examination of Water and Wastewater, 21st Edition* (Standard Methods).

3.6.3 On-line conductivity units that cannot be calibrated should not be used to monitor conductivity in reagent water.

3.7 *Refrigerator*

3.7.1 Refrigerators should maintain a temperature of 1-10°C. Calibrated thermometers should be graduated in at least 1°C increments with the bulb immersed in liquid. Thermometers should be placed on the top and bottom shelves of the area in use.

QC **3.7.2** Temperatures should be recorded daily for days in use and when laboratory is staffed. Documentation should include temperature, date, and analyst's initials.

3.8 *Pipets, Micropipetters*

3.8.1 Pipets should have legible markings, unbroken tips, no chips or etching. Opened packs of disposable sterile pipets should be resealed between use periods.

QC **3.8.2** Micropipetters must be calibrated annually [Section 9.2 (*Appendix A*)]. The results and dates of calibration checks should be documented.

3.9 *Hand Tally or Digital/Electronic Counter*

3.9.1 The laboratory should have available a hand tally or digital/electronic counter to enumerate oocysts or cysts.

QC **3.9.2** The hand tally or digital/electronic counter should be checked periodically to confirm the accuracy and the operational status. Periodic checks should be documented.

3.10 *Glassware and Plasticware*

3.10.1 Glassware should be borosilicate glass or other corrosion resistant glass and free of cracks, chips, and not etched. Markings should be legible.

3.10.2 Plasticware (polytetrafluorethylene [PTFE], high-density polyethylene [HDPE], low-density polyethylene [LDPE], polystyrene or polypropylene) should be clear, not cracked, not scratched and non-toxic to microorganisms.

3.10.3 Graduated cylinders for measurement should be accurate to within a 2.5% tolerance.

3.11 *Sample Containers*

3.11.1 Sample containers should be capable of collection of 10-L bulk samples (collapsible LDPE cubitainer or equivalent). Fill completely to ensure collection of a 10-L sample. Alternatively, a clean, 10-L carboy with a bottom delivery port may be used after calibration at the 10-L mark. Disposable sample cubitainers should be discarded after one use [Section 6.1(*Section 6.1)*].

3.11.2 If the bulk field sample is transferred to a laboratory carboy, the laboratory carboy should be cleaned and disinfected before use with another sample [Sections 12.2.4.1, Note and 12.3.1.5.1, Note (*Sections 12.2.4.1, Note, and 12.3.1.5, Note*)].

QC **3.11.3** If an empty graduated container is used to measure sample volume, it should be calibrated in 0.5-L increments using a graduated cylinder [Section 6.18 (*Section 6.16*)]. Sample volume is recorded to the nearest estimate of 0.25 L [Sections 12.2.5.2 and 12.3.1.6.2 (*Sections 12.2.4.5 and 12.3.1.5.5*)].

3.12 *Sample Filtration/Concentration*

3.12.1 An approved filter or filtration/concentration device should be used. Listed below are three options that have been demonstrated to be acceptable for use with EPA Methods 1623 and 1623.1 [Sections 6.2.2, 6.2.3, 6.2.4 (*Sections 6.2.8, 6.2.9, 6.2.10*)].

- Envirochek ™ HV, Pall Corporation
- Filta-Max® , IDEXX Corporation
- Portable Continuous Flow Centrifuge (PCFC), Haemonetics Corporation

3.13 *Elution Reagents*

3.13.1 Eluting solution should be prepared consistent with [Section 7.4 (*Section 7.6*)].

3.13.2 Laboratories should store prepared eluting solution for no more than 1 week. Turbid eluting solution should be discarded [Section 7.4, Note (*Section 7.6, Note*)].

3.14 *Laboratory Shaker with Arms*

3.14.1 The laboratory wrist arm shaker should meet the guidelines/specifications described in EPA Method 1623 and 1623.1 [Section 6.2.2.2 (*Section 6.2.8.1 and 6.2.8.2*)].

3.14.2 The clamps (arms) should be extended to their maximum distance from the horizontal rods to maximize shaking. The clamps should be positioned consistent with the elution requirements [Section 12.2.6.1.1 (*Section 12.2.6.1*)].

3.14.3 The shaker speed should be set to maximum (700 - 900 rpm (rotations per minute) or per manufacturer's instructions) [Section 12.2.6.2.3 (*Sections 12.2.7.3 and 12.2.8.3*)].

3.14.4 An accurate laboratory timer or calibrated shaker timer should be used to ensure accurate time measurement [Section 12.2.6.2.3 (*Sections 12.2.7.3 and 12.2.8.3*)].

3.14.5 Shaker should be secured to prevent instrument from moving on laboratory bench.

3.15 *Centrifuge*

3.15.1 The laboratory should have available a nomograph for converting maximal relative centrifugal force (RCF, i.e., g-force) to RPM, a table available from the manufacturer to correlate rpm to g-force, access to manufacturer's website for conversion information, or conversion information programmed into centrifuge.

3.15.2 Swinging bucket centrifuge should be capable of accepting 15- to 250-mL conical centrifuge tubes, achieving 1500 X g, and coasting to a stop without a brake [Sections 6.8 and 13.2.1 (*Sections 6.6 and 13.2.1*)].

3.15.3 The centrifuge should be periodically cleaned and disinfected particularly after any spills/leakage.

3.15.4 To ensure smooth acceleration and deceleration during use, the centrifuge should be loaded symmetrically and the tubes balanced to within manufacture's specifications for the rotor.

QC **3.15.5** The tachometer and timer should be calibrated annually either using an internal maintenance protocol or by a maintenance agreement/contract. The results of the calibration checks should be documented in addition to any other maintenance performed.

3.16 ***Aspiration System***

3.16.1 The laboratory should have available a vacuum source capable of maintaining 25 in. Hg equipped with a shut-off valve and vacuum gauge.

QC **3.16.2** The laboratory should document aspiration rate and internal diameter of pipette (0.80 to 1.5 mm) used for aspiration [(*Section 13.2.2, Note*)].

3.17 ***IMS***

3.17.1 An approved kit/manufacturer of immunomagnetic beads (such as Dynabeads®GC-Combo or equivalent) should be used [Section 7.5 (*Section 7.7.1*)].

3.17.2 The IMS procedure should be performed with all reagents at room temperature (15°C– 25°C) [Section 13.3, Note (*Section 13.3, Note*)].

3.17.3 All reagents/equipment should meet the specifications described in [Section 13.3 (*Section 13.3*)].

QC **3.17.4** The rotating mixer RPM should be calibrated annually and results recorded.

QC **3.17.5** The 0.1 N HCL and 1.0 N NaOH must be purchased at the required normality. Reagents should be dated when received, opened and discarded prior to expiration date. Normality must not be adjusted by the laboratory [Section 7.1.2, Note, 13.3.3.2, Note, 13.3.3.8, Note (*Sections 7.7.3, Note, 13.3.3.2, Note, and 13.3.3.8, Note*)].

3.17.6 Laboratories should use well slides recommended by the stain (direct antibody labeling reagent) manufacturer.

3.18 ***Stains (direct antibody labeling reagents)***

3.18.1 An approved kit/manufacturer of direct antibody labeling reagents, such as those listed below [Sections 7.6.1 – 7.6.4 (*Sections 7.8.1-7.8.4*)], should be used.

- MeriFluor® *Cryptosporidium*/*Giardia*, Meridian Diagnostics
- Aqua-Glo™ G/C Direct FL, Waterborne, Inc.
- Crypt-a-Glo™ and Giardi-a-Glo™, Waterborne, Inc.
- EasyStain™ C&G, BTF Pty Ltd.

3.18.2 Store reagents between 1°C and 10°C. Do not freeze and protect from exposure to light.

3.18.3 If a laboratory wishes to use multiple types of labeling reagents, the laboratory must demonstrate acceptable performance through initial precision and recovery (IPR) test at a minimum for each type and must perform positive and negative staining controls for each batch of slides stained with each product [Section 7.6, Note (*Section 7.8, Note*)]. The performance of each labeling reagent should be monitored in each type of source water.

3.19 ***DAPI Stain***

3.19.1 DAPI stock solution should be prepared as described in EPA Methods 1623 and 1623.1 [Section 7.7.1 (*Section 7.9.1*)]. Only minimal quantities should be prepared, consistent with use. DAPI stock should be stored between 1°C and 10°C in the dark. Do not freeze. DAPI stock should be discarded if positive staining controls are not acceptable.

3.19.2 Antibody kit manufacturer's instructions should be followed for the preparation, application and rinsing of staining solution (working solution). Only minimal quantities of working solution are to be prepared daily, if staining is performed. Store between 1°C and 10°C in the dark on the day of preparation. Do not allow to freeze [Sections 7.7.2 and 14.6 (*Sections 7.9.2 and 14.6*)].

3.20 ***Mounting Medium***

3.20.1 Mounting medium should be prepared, and applied per antibody kit manufacturer's instructions [Section 7.8 (*Section 7.10*)].

3.20.2 Coverslip edges should be sealed with clear nail polish or a self-sealing, hard-set mounting medium should be used [Sections 7.8, 7.9, and 14.9 (*Section 7.10, 7.11, and 14.9*)]

3.20.3 As an alternate to the 1,4-diazabicyclo[2.2.2]octane (DABCO)/glycerol mounting medium or stain manufacturer's mounting media specified in EPA

Methods 1623 and 1623.1, laboratories may wish to evaluate the use of Elvanol® mounting medium [Section 7.8.5 (*Section 7.10.5*)], which hardens and may be useful for archiving slides. EPA recommends QC assessment before changing the mounting medium currently used by the laboratory, including IPR, method blank (MB), matrix spike/matrix spike duplicate (MS/MSD), and unspiked field samples. Commercially prepared mounting media for archiving slides are available.

3.21 *Oocyst/Cyst Suspension*

3.21.1 EPA Methods 1623 and 1623.1 require analysis of spiked QC samples to demonstrate acceptable initial and ongoing laboratory and method performance (IPR samples [Section 9.4 (*Section 9.5*)], MS/MSD samples [Section 9.5 (*Section 9.6*)], and ongoing precision and recovery (OPR) samples [Section 9.7 (*Section 9.8*)]).

3.21.2 The organisms used for QC samples must be enumerated to calculate recoveries and precision and monitor method performance [Section11.1 (*Section 11.1*)].

3.21.3 Samples should be spiked consistent with Methods 1623 and 1623.1 [Section 11.4 (*Section 11.2*)].

QC **3.21.4** The lot number and the spike values of the spiking suspension should be recorded.

3.21.5 Spiking suspensions should be prepared using *Cryptosporidium* oocysts < 3 months old and *Giardia* cysts < 2 weeks old [Section 11.2.2 (*Appendix C.1*)].

3.22 *Microscope*

3.22.1 All analysts must be familiar with all aspects of the operation of their microscope by using the manuals provided with their microscope [Section 10.2 (*Section 10.2*)].

3.22.2 The microscope should be placed in a dark room (room should be near or completely dark) on a surface free of vibration. Sufficient workspace should be available for bench sheets and placement of slides and other ancillary materials [Section 10.1 (*Section 10.1*)].

3.22.3 The microscope should be equipped with appropriate excitation and band pass filters for examining FITC and DAPI slides [Sections 6.9.2 and 6.9.3 (*Sections 6.7.2 and 6.7.3*)].

3.22.4 The microscope should have appropriate objectives and filters for Epifluorescence/DIC and have capability for switching to and from epifluorescence [Section 6.9.1 (*Section 6.7.1*)].

3.22.5 All characterization (DAPI and DIC) and size measurements must be determined using 1000X total magnification and reported to the nearest 0.5 µm [Section 15.2, Note (*Section 15.2, Note)*].

QC **3.22.6** The microscopy segment of EPA Methods 1623 and 1623.1 requires proper alignment and adjustment of sophisticated optics. Without proper alignment and adjustment the microscope will not function at optimum efficiency and reliable identification and enumeration of oocysts/cysts will not be possible. Accordingly, it is important that all aspects of the microscope from the light sources to the oculars be properly adjusted [Section 10.3.1.1 (*Section 10.3*)]. The laboratory should have a service agreement to service the microscope annually in order to ensure the microscope is in alignment. Analysts should be able to make minor adjustments that may be required for use of microscope.

3.22.7 Microscope adjustments (epifluorescent mercury bulb, transmitted bulb, interpupillary distance and oculars, ocular adjustment for microscopes without binocular capability) must be performed consistent with [Sections 10.3.2, 10.3.3 and 10.3.4 (*Section 10.7, Appendix B.1 and B.2*)].

3.22.8 The microscope must be equipped with at least a 20X scanning objective and 100X oil immersion objectives [Section 6.9.1 (*Section 6.7.1*)].

QC **3.22.9** The microscope must have an ocular micrometer or imaging software to measure oocysts/cysts. The laboratory must have a stage micrometer available to calibrate the ocular micrometer or imaging software. The ocular micrometer should be calibrated for each objective used. The calibration procedures must be followed when the microscope is first used and when an objective is replaced [Sections 6.9.1and 10.3.5 (*Section 6.7.1 and Appendix B.3*)]. The calibration information must be available at the corresponding microscope.

3.22.10 Köhler illumination must be established for the 100X oil objective for DIC examination of morphological characteristics of oocysts/cysts. If more than one objective is used for DIC microscopy, Köhler illumination should be reestablished for the new objective lens each time the objective is changed. The Köhler illumination procedure must be followed each time an analyst uses the microscope [Section 10.3.6 (*Section 10.8*)].

3.22.11 The microscope should be cleaned after each period of use following the procedure in [Section 10.4 (*Section10.9*)] or using an equivalent procedure.

QC **3.22.12** The laboratory should maintain a log of the number of hours the UV bulb has been used. Alternatively, a lamp hour meter may be used. Mercury bulbs should not be used longer than they have been rated [Section 10.3.2.11 (*Appendix B.1.11*)].

4. General Laboratory Practices

Laboratories seeking certification should adhere to the laboratory practices described in EPA Methods 1623 and/or 1623.1. Additional information is available in the attached Checklists A, B, and C.

4.1 Laboratory Safety

4.1.1 Achievement of a safe workplace is the responsibility of the organization, the laboratory manger, the supervisory personnel and the employees themselves. All laboratory employees should make every effort to protect themselves and their fellow employees by adhering to the health and safety program that has been developed and documented specifically for their laboratory. Additional information is available in Standard Methods Section 1090, [Sections 5.0 and 20.0 (*Sections 5.0 and 20.0*)].

4.1.2 Health and safety policies and procedures should be posted and/or readily available to all personnel as part of the laboratory's SOPs.

4.1.3 The laboratory should disinfect bench surfaces before and after analysis.

4.1.4 The laboratory should provide and document training of all applicable personnel in the proper use of personal protective equipment (PPE), precautionary measures, and control of chemical and biological hazards. Safety training requires a concerted effort by the laboratory and should be conducted on a routine basis by competent and qualified individuals to be effective.

4.1.5 Laboratory operations that generate aerosols should be conducted in a biological safety cabinet.

4.1.6 Personal protective clothing/equipment should not be worn in non-laboratory areas. Gloves must be changed whenever they are contaminated. Laboratory coats, which have been contaminated with hazardous chemicals/biological materials, should be removed. Laboratory benches should be disinfected before and after analyses and after a spill with a hard surface disinfectant capable of inactivating oocyst/cysts.

4.1.7 Laboratory should record not only accidents, but also "near misses" to permit full evaluation of the effectiveness of the health and safety program.

4.1.8 The laboratory should establish periodic reviews of the plan with documentation of the recommendations and corrective measures implemented.

4.2 Chemicals/Reagents

QC **4.2.1** All chemicals and reagents used should meet the requirements specified in Section 7.0 of EPA Methods 1623 and/or 1623.1. If not specified, "analytical

reagent grade" or American Chemical Society (ACS) grade chemicals or better should be used. All chemicals should be dated when received, opened, and discarded within the expiration date.

QC **4.2.2** All reagents must be prepared using reagent water [Section 7.3 (*Section 7.3*)]. All reagents should be clearly labeled with the identity of the reagent(s), date of preparation, expiration date, and technician's initials. All reagents should be stored consistent with Section 7.0 of EPA Methods 1623 and/or1623.1.

4.3 Reagent Water

4.3.1 Only reagent water meeting the following acceptance criteria must be used for preparing reagents:

Parameter	Limits	Frequency
Conductivity	>0.5 megohms or <2 µmhos/cm (µsiemens/cm) at 25°C	Monthly
Pb, Cd, Cr, Cu, Ni, Zn	Not greater than 0.05 mg/L per contaminant. Collectively not greater than 0.1 mg/L	Annually
Total Chlorine Residual[1]	< 0.1 mg/L	Monthly
Heterotrophic Plate Count[2]	< 500 CFU/mL or MPN < 500/mL[3]	Monthly

[1]DPD (N.N-diethyl-p-phenylenediamine) Method should be used
[2]Pour Plate Method (Standard Methods 9215B) or IDEXX SimPlate® Method
[3]Colony Forming Units (CFU) or Most Probable Number (MPN)

4.3.2 Additional information is available in Standard Methods 1080 and 9020.

QC **4.3.3** The reagent water still or DI unit should be maintained according to the manufacturer's instructions. Maintenance should be documented.

4.4 Glassware Washing

4.4.1 All glassware and plastic ware should be thoroughly cleaned with a detergent designed for laboratory use. Glassware and plastic ware should be cleansed with hot water and detergent, rinsed with hot water to remove all traces of residual detergent, and rinsed with distilled, deionized, or reagent water as the final rinse. All contaminated laboratory glassware should be autoclaved or chemically sterilized before cleaning.

4.4.2 The dishwasher influent and rinse water plumbing system should be made of stainless steel or other nontoxic material.

4.5 Quality Assurance

4.5.1 To ensure that analytical data generated under the rule are technically valid, legally defensible, and of known and acceptable quality, each laboratory must operate a formal QA program and document the scope of the program through a QA plan, as specified in EPA Method 1623 and1623.1 Section 9.1. All laboratory activities including, but not limited to sampling, analytical methods, QC checks, instrument operation, data generation, data validation, corrective action procedures, and recording keeping should be described in the QA plan.

4.5.2 The QA plan should contain a laboratory organization chart or staff listing which identifies staff organization and responsibilities, including QA manager and lab director. All laboratory personnel should be familiar with the contents of the QA plan. It is the responsibility of the laboratory QA manager to ensure the QA plan is current. The laboratory QA manager should be independent from the laboratory management and should have a working knowledge of the statistics involved in QC of laboratory analyses and a basic understanding of the methods the laboratory uses. Ideally, this person should have a staff position reporting directly to upper management, not a line position.

4.5.3 The laboratory QA plan should contain a schedule of all preventative maintenance for equipment.

4.5.4 The laboratory QA plan should be a separately prepared document (stand alone document). However, some information can be incorporated by reference, including laboratory SOPs, analytical methods, QC information, and applicable literature. The QA plan should address the specific analytical/QC requirements of EPA Method 1623 or 1623.1 and any compliance monitoring. The QA plan should address the items listed in Chapter III of the *Manual for the Certification of Laboratories Analyzing Drinking Water (5th Edition)* and Section 2.1 of the *Microbial Laboratory Guidance Manual for the Final Long Term 2 Enhanced Surface Water Treatment Rule* (Lab Guidance Manual). See https://nepis.epa.gov/Exe/ZyPDF.cgi?Dockey=2000UDF3.txt. The QA Plan should be updated at least annually. The QA plan should be reviewed as a part of the on-site audit.

4.5.5 Detailed QC requirements specific to *Cryptosporidium* analyses are discussed in Section 9 of EPA Methods 1623 and 1623.1 and further recommendations are discussed in Section 3 of the Lab Guidance Manual. All laboratories analyzing drinking water compliance samples must adhere to all required QC procedures specified in the approved methods. The Lab Guidance Manual is available for download from https://nepis.epa.gov/Exe/ZyPDF.cgi?Dockey=2000UDF3.txt.

5. Analytical Methodology

5.1 *General*

5.1.1 A laboratory must be certified for all analytical methods that it uses for compliance purposes.

QC **5.1.2** The laboratory is required to maintain records of modifications made to EPA Method 1623 or 1623.1 [Section 9.1.2.2 (*Section 9.3*)]. The documentation must address the items listed in [Section 9.1.2.2 (*Section 9.3*)] and include the results from the following QC tests comparing the modified method to EPA Method 1623 or 1623.1 acceptance criteria listed in Tables 3 and 4 of each Method.

- IPR [Section 9.4 (*Section 9.5*)]
- OPR [Section 9.7 (*Section 9.8*)]
- MS/MSD [Section 9.5 (*Section 9.6*)]
- MB [Section 9.6 (*Section 9.7*)]

Additional guidance is given in EPA Method 1623 Tables 3 and 4 and Tables 2, 3, and 4 for EPA Method 1623.1.

5.2 *IMS Procedure*

5.2.1 Laboratories should follow EPA Method 1623 or 1623.1 or manufacturer's instructions for oocyst/cyst capture and dissociation of beads/oocyst/cyst complex [Section 13.3 (*Section 13.3*)].

5.2.2 The total volume transferred should not be reduced to less than 5 mL above the packed pellet or volume stated by manufacturer of filtration/concentration device [Section 13.2 (*Section 13.2*)].

5.2.3 The maximum amount of pellet that should be processed through an individual IMS reaction is 0.5 mL. If the packed pellet is greater than 0.5 mL, the pellet must be sub-sampled as described in [Section 13.2.4 (*Section 13.2.3*)]. The volume of the packed pellet can be estimated by comparison to a set of pellet standards. Pellet standards may be prepared for long-term usage by measuring appropriate amounts of colored glycerol, sand, or colored floral arranging gel. Pellet standards should range from 0.1 mL to 0.5 mL, in 0.1 mL increments and 0.5 to 3.0 mL in 0.5 mL increments based on the pellet sizes typically observed.

5.2.4 Two acid dissociations are required [Section 13.3.3, Note (*Section 13.3.3, Note*)].

5.2.5 The volumes of IMS reagents listed in the manufacturer's instructions and Methods 1623 and 1623.1 are method requirements [(*Section 13.3.1, Note*)].

5.3 *Staining Procedure*

5.3.1 If a laboratory has more than one option specified for slide drying, the criteria determining when each option is performed should be listed in the staining SOP.

5.3.2 Application of stain to slides should be consistent with manufacturer's instructions [Section 14.2 (*Section 14.2*)].

5.3.3 Incubation of slides should be in accordance with EPA Method 1623/1623.1 or manufacturer's instructions [Section 14.3 (*Section 14.3*)].

5.3.4 Application of wash buffer to slides should be in accordance with EPA Method 1623/1623.1 or manufacturer's instructions [Sections 7.6, 14.5 and 14.7 (*Sections 7.8, 14.5, and 14.7*)].

5.3.5 Laboratories must prepare positive and negative staining controls [Section 14.1(*Section 14.1*)].

5.3.6 Slides that are not read immediately after staining should be stored in a humid chamber in the dark at 1°C to 10°C until read [Section 14.10 (*Section 14.10*)].

5.4 *Sample Examination*

5.4.1 Compliance sample analyses must be performed by a certified laboratory and meet the QC requirements specified in EPA Methods 1623 or 1623.1. These requirements include, but are not limited to, sample temperature requirements, minimum frequencies for OPR, MB, MS samples; acceptable OPR and MB results; holding time requirements; and acceptable staining control results and frequency.

5.4.2 Slide wells should be scanned in a systematic fashion similar to that portrayed in [Section 21.0, Figure 4 (*Section 21.0, Figure 3*)].

5.4.3 All slides should demonstrate clear contrast between the levels of background fluorescence and the organisms when observed using FITC or DAPI.

QC **5.4.4** All slide examination results should be recorded for *Cryptosporidium* oocysts and *Giardia* cysts. Examples of both a bench sheet and slide examination form are attached. All organisms that meet the criteria specified in [Sections 15.2.2 and 15.2.3 (*Sections 15.2.2 and 15.2.3*)] less atypical organisms must be reported. Atypical organisms identified by DIC or DAPI, i.e., possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing organelles, and/or spores, should be noted on the slide examination form.

5.4.5 All sample examinations must be performed with the required magnification as noted below.

QC **5.4.6** Each analyst must characterize a minimum of three *Cryptosporidium* oocysts and three *Giardia* cysts on the positive staining control slide before examining field sample slides. This characterization must be performed by each analyst during each microscopic examination session. FITC examination must be conducted at a minimum of 200X total magnification, DAPI examination must be conducted at a minimum of 400X total magnification, and DIC examination and size measurements must be conducted at a minimum of 1000X total magnification. Size, shape (morphology), and DIC and DAPI characteristics of three *Cryptosporidium* oocysts and three *Giardia* cysts must be recorded by the analyst on a microscope log. The analyst also must indicate on each sample examination form whether the positive staining control was acceptable [Section 15.2.1.1(*Section 15.2.1.1*)].

QC **5.4.7** Negative staining controls should not contain any oocysts or cysts [Section 15.2.1.2 (*Section 15.2.1.2*)]. The analyst must indicate on each sample examination form whether the negative staining control was acceptable.

5.4.8 The analyst must not proceed to field sample examination until the negative staining control demonstrates acceptable results and the positive staining control contains oocysts/cysts within the expected range, and the appropriate fluorescence for both FA and DAPI [Sections 15.2.1 and 15.2.1.3 (*Sections 15.2.1 and 15.2.1.3*)]. Corrective actions should be listed in the QA plan or SOPs if the positive and/or negative staining controls are not acceptable.

5.4.9 Sample Examination – *Cryptosporidium*

QC **5.4.9.1** A positive result is a *Cryptosporidium* oocyst which exhibits all of the following: 1) typical FA fluorescence, 2) typical size and shape, 3) nothing atypical on DAPI, and 4) nothing atypical on DIC microscopy [(*Section 21.0, Figure 4*)]. Each positive result must be characterized and assigned to one of the DAPI and DIC categories described below [Section 15.2.2.4 (*Section 15.2.2.1*)].

5.4.9.2 **FITC** - The analyst must scan the entire well at a minimum of 200X total magnification, for apple-green fluorescing oocyst shapes. If brilliant apple green fluorescing ovoid or spherical objects (4 to 6 µm in diameter) are observed with brightly highlighted edges, the magnification must be increased and the microscope switched to the UV filter block for DAPI [Section 15.2.2.2 (*Section 15.2.2.3*)] and then to DIC [Section 15.2.2.3 (*Section 15.2.2.4*)].

QC **5.4.9.3** **DAPI-** The analyst must use a minimum of 400X total magnification. Using the UV filter block, each object must be categorized with one of the following characteristics;
(a) Light blue internal staining (no distinct nuclei) with green rim

(b) Intense blue staining
(c) Up to four distinct sky blue nuclei

Slides must be examined for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color. If atypical structures are not observed, the analyst must categorize each object meeting the criteria defined as a positive result defined in [Section 15.2.2.4 (*Section 15.2.2.1*)] and record oocysts in category (a) as DAPI-negative; record oocysts in categories (b) and (c) as DAPI-positive.

QC **5.4.9.4** **DIC**- The analyst must use a minimum of 1000X total magnification (oil immersion lens). Using DIC the analyst should look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (spikes, stalks, appendages, pores, one or two large nuclei filling the cell, crystals, spores etc.). If atypical structures are not observed, each of the apple green fluorescing objects must be categorized as follows:
(a) An empty *Cryptosporidium* oocyst
(b) A *Cryptosporidium* oocyst with amorphous structure
(c) A *Cryptosporidium* oocyst with internal structure (one to four sporozoites/oocyst)

Using 1000X total magnification the analyst must 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.

5.4.10 Sample Examination – *Giardia*

QC **5.4.10.1** A positive result is a *Giardia* cyst which exhibits all of the following: 1) typical FA fluorescence, 2) typical size and shape, 3) nothing atypical on DAPI, and 4) nothing atypical on DIC microscopy [(*Section 21.0, Figure 4*)]. Each positive result should be characterized and assigned to one of the DAPI and DIC categories described below [Section 15.2.3.4 (*Sections 15.2.3.1*)].

5.4.10.2 **FITC**- The analyst should use a minimum of 200X total magnification to scan the entire well for apple-green fluorescing cyst shapes. If brilliant apple green fluorescing round to ovoid objects (8-18 µm long by 5-15 µm wide) are observed with brightly highlighted edges, the magnification should be increased and the microscope switched to the UV filter block for DAPI [Section 15.2.3.2 (*Section 15.2.3.3*)]and then to DIC [Section 15.2.3.3 (*Section 15.2.3.4*)].

QC **5.4.10.3 DAPI**- The analyst should use a minimum of 400X total magnification. Using the UV filter block, each object should be categorized with one or more of the following characteristics:
(a) Light blue internal staining (no distinct nuclei) and a green rim
(b) Intense blue internal staining
(c) Up to four sky blue nuclei

Slides should be examined for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color. If atypical structures are not observed, the analyst should categorize each object meeting criteria defined as a positive result in [Section 15.2.3.4 (*Section 15.2.3.*1)] and record cysts in category (a) as DAPI negative; record cysts in categories (b) and (c) as DAPI positive.

QC **5.4.10.4 DIC**- The analyst should use a minimum of 1000X total magnification (oil immersion lens). Using DIC microscopy the analyst should look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, crystals, spores, etc.). If atypical structures are not observed, the analyst should categorize each apple green fluorescing object as one of the following based on DIC examination:
(a) An empty *Giardia* cyst
(b) A *Giardia* cyst with amorphous structure
(c) A *Giardia* cyst with one type of internal structure (nuclei, median body, or axonemes), or
(d) A *Giardia* cyst with more than one type of internal structure

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

5.4.11 It is strongly recommended that positive and interfering organisms detected in field samples be documented by photography.

QC **5.4.12** The date and time of sample examination along with the analyst's name should be recorded on the examination form [Sections 15.2.4 and 15.2.6 (*Section 15.2.4 and 15.2.6*)].

QC **5.4.13** *Cryptosporidium* and *Giardia* concentrations should be reported as oocysts/L and cysts/L respectively [Section 15.2.5 (*Section 15.2.5*)]. If no oocysts or cysts, as defined in [Sections 15.2.2.4 and 15.2.3.4 (*Sections 15.2.2.1 and 15.2.3.1*)] are detected, report zero organisms.

6. Sample Collection, Handling, and Preservation

6.1 *Sample Collection*

6.1.1 Laboratories should ensure proper collection of samples by providing SOP and chain of custody.

6.1.2 Several options are available for public water systems (PWSs) for collection of untreated surface water samples for *Cryptosporidium* analysis which are as follows:

- On site filtration of water samples from unpressurized or pressurized sources using the Pall Envirochek™ or Envirochek™ HV filter.
- On site filtration of water samples from pressurized or unpressurized sources using the IDEXX Filta-Max®foam filter
- Collection of bulk water samples for shipment to the laboratory for filtration and analyses.

Detailed procedures/information for each of these options and packing and shipping information are available in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule* (Source Water Monitoring Guidance Manual). See https://nepis.epa.gov/Exe/ZyPDF.cgi?Dockey=2000CZCJ.txt.

6.1.3 PWSs should collect source water compliance samples from the plant intake prior to chemical treatment, unless certified by the State to collect the source water sample after chemical treatment. Systems that recycle filter backwash water should collect source water samples prior to the point of filter backwash water addition.

6.1.4 The use of multiple sources during monitoring should be consistent with routine operational practice. If there is a tap prior to treatment where sources are combined the sample can be collected at this location. If not, the PWS may collect samples from each source prior to treatment and composite into one sample or collect samples from each source prior to treatment, analyze separately, and calculate a weighted average of the analytical results.

6.1.5 For LT2 Rule compliant samples, there may be instances where a replacement sample is necessary, i.e., the PWS is unable to report a valid *Cryptosporidium* result for a scheduled sampling date. Possible situations may include method holding time exceeded, sample volume requirements not met, QC samples fail acceptance criteria etc. The PWS should submit an explanation for the delayed sampling date to the EPA/State concurrent with the shipment of the replacement sample to the laboratory. The system should collect a replacement sample as close to the required date as feasible but within 21 days of being notified by the laboratory that a result cannot be reported for that date.

6.1.6 A laboratory with multiple PWS clients representing a range of sample volumes is not responsible for performing QC tests with all volumes. However, if the laboratory does analyze both 10 L and 50 L sample volumes for clients then the laboratory should demonstrate acceptable performance in a manner representative of the sample volumes they process. Guidance on initial and ongoing demonstrations of acceptable laboratory performance is provided in Sections 3.3.14.1 through 3.3.14.3 of the Lab Guidance Manual.

6.1.7 Sample collection and/or filtration systems should be well maintained and cleaned appropriately following use. The filtration system should be able to maintain a seal during use with no leaks.

6.2 *Sample Volume Analysis Requirements*

6.2.1 For LT2 Rule compliant samples, the following minimum sample volume requirements must be met:

- 10 L of sample *or*
- 2 mL of packed pellet volume *or*
- As much as two filters can accommodate before clogging (this is applicable only to filters that have been EPA approved for nationwide use).

6.2.2 Systems may analyze larger volumes than 10 L, provided the laboratory has demonstrated acceptable performance on initial and ongoing spiked reagent water and source water samples [Section 9.1.2.1.1 (*Section 9.2*)]. For LT2 Rule compliant monitoring, it is preferred that PWSs analyze similar volumes throughout the monitoring period. However, data sets including different sample volumes are acceptable provided the system analyzes the minimum sample volumes.

6.2.3 MS samples must be collected from the same location as the field sample as split samples or as samples sequentially collected immediately after one another. The MS sample volume analyzed must be within 10% of the volume analyzed for the field sample [Section 9.5.1 (*Section 9.6.2*)].

6.3 *Preservation/Sample Temperature Monitoring*

6.3.1 *Cryptosporidium* oocysts present in samples can degrade, biasing analytical results. *Cryptosporidium* samples must be stored and maintained between 1°C and 10°C to reduce biological activity [Section 8 (*Section 8*)].

6.3.2 Samples for all analyses must remain above freezing at all times. Although this may not pose a significant problem with 10 L water samples, this may be a concern for *Cryptosporidium* filters that are shipped with coolant materials such as wet ice, blue ice, dry ice, or gel packs.

6.3.3 Per EPA Method 1623 and 1623.1, laboratories must reject samples that are received frozen or >20°C unless the sample was collected the same day it was received. In general, same day refers to a typical 8-hour work day [Section 8.1.3 (*Section 8.1.3*)].

6.3.4 Several options to measure sample temperature are available such as temperature control samples/vials, iButton, stick on temperature strips, and infrared thermometers. Temperature measurement devices should be calibrated routinely to ensure accurate measurements [Section 8.1.4 (*Section 8.1.4*)].

6.4 *Holding Time Requirements*

6.4.1 *Cryptosporidium* samples should be analyzed as soon as possible. The laboratory should complete sample filtration, elution, concentration, purification and staining the day the sample is received whenever possible. If it is not possible to complete sample analyses in one day the sample processing can be halted after filtration, application of the purified sample onto the slide or staining. The bullets below summarize the maximum holding times for *Cryptosporidium* samples analyzed with EPA Method 1623 and 1623.1[Section 8.2 and Table 1 (*Section 8.2 and Table 5*)].

- 4 days (96 hours) between collection/filtration and elution
- 1-working day between elution and application of sample to the slide
- 3 days (72 hours) between application of sample to slide and staining
- 7 days (168 hours) between staining and completion of sample examination

6.4.2 Explanation of holding time requirements for samples analyzed by EPA Method 1623 and 1623.1:

- Sample collection and filtration. Sample elution must be initiated within 96 hours of sample collection whether shipped to the laboratory as a bulk sample or filtered in the field.
- Sample elution, concentration, and purification. The laboratory must complete the elution, concentration, purification, and application of the sample to the well slide in one work day. This process ends with the application of the purified sample to the slide for drying. The laboratory should follow the stain manufacturer's instructions for slide drying. The slides must be completely dried before staining and stored to maintain the dried state until stained.
- Staining. The sample must be stained within 72 hours of application of the purified sample to the slide.
- Examination. Although FA, DAPI, and DIC microscopy examination and verification is ideally performed immediately after staining is complete, laboratories have up to 168 hours (7 days) from completion of sample staining to complete the examination and verification of samples. If fading/diffusion of FITC or DAPI staining occurs the laboratory should reduce this holding time. Other options include adjustment of the DAPI staining solution so that fading/diffusion does not occur [Section 14.6

(*Section 14.6*)] or the laboratory may evaluate the use of another mounting medium.

6.5 *Analysis of Complex Samples*

6.5.1 If the sample holding time has not been exceeded and a full volume sample cannot be filtered due to high levels of oocysts, cysts and/or interfering organisms, substances, or materials clogging the filter or precluding acceptable microscopic examination, an aliquot of the sample should be diluted with reagent water and a smaller aliquot filtered. This dilution must be recorded and reported with the results [Section 16.2 (*Section 16.2*)]. If the holding times have been exceeded, the sampling site should be resampled. If this is not possible, the results should be qualified accordingly.

6.5.2 Siliconized or low adhesion centrifuge tubes should be used for samples which are known to adhere to the centrifuge tube walls. Additional options include rinsing centrifuge tubes with phosphate buffered saline Tween® (PBST) elution buffer or Sigmacote® prior to use.

7. *Cryptosporidium* Quality Control

7.1 *EPA Method 1623 and 1623.1 QC*

QC **7.1.1** Laboratories must analyze samples spiked with *Cryptosporidium* oocysts to assess ongoing laboratory and method performance in accordance with EPA Method 1623 and 1623.1 QC requirements. These ongoing spiked sample analyses include an IPR test [Section 9.4 (*Section 9.5*)], and ongoing demonstration of laboratory capability and method performance through the MS test [Section 9.5 (*Section 9.6*)], the MB test [Section 9.6 (*Section 9.7*)], the OPR test [Section 9.7 (*Section 9.8*)], staining controls [Sections 14.1 and 15.2.1 (*Sections 14.1 and 15.2.1*)], and analyst verification tests [Section 10.6 (*Section 9.10*)]. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of [Table 3] in each method. The laboratory should spike samples according to the procedures described in [Section 11.4 (*Section 11.2*)] or according to the instructions provided by the spiking suspension vendor. EPA Method 1623.1 [*Table 2*] lists the required routine QC requirements.

Laboratories are to document/demonstrate a minimum *Cryptosporidium* recovery of 22% prior to analysis of samples at the frequency required in [Section 9.7 (*Section 9.8*)]. See Method 1623 Quality Control Acceptance Criteria update for OPR samples in *Federal Register* Vol. 74, No. 36, February 25, 2009. (The OPR Quality Control Acceptance Criteria for OPR samples using Method 1623.1 is 33% [(*Table 3*)].) This *Federal Register* notice Vol. 74, No. 36, describes the current Lab QA Program for *Cryptosporidium* including details for the basis of downgrading or suspending a laboratory's certification

for analyses of *Cryptosporidium* in support of the Safe Drinking Water Act. This information is available online at http://www.regulations.gov under Docket ID No. EPA-HQ-OW-2002-0011.

7.1.2 QC Batch

All compliance *Cryptosporidium* samples must be associated with an acceptable OPR and MB sample [Section 9.1.7 (*Section 9.8.6*)] through a "QC batch." A QC batch consists of an OPR, and MB, and a maximum of 20 field and MS samples combined that are *eluted, concentrated, and purified* in the same week as the OPR and MB samples using the same reagents. A week is defined as any 168 hour (7 day) period that begins with the processing of the OPR. If more than 20 field and MS samples are analyzed in a week, another OPR and MB must be eluted, concentrated, and purified using the same reagents as the additional field and MS samples [Sections 9.6 and 9.7 (*Sections 9.7 and 9.8*)]. A field sample and its associated MS sample should be analyzed in the same QC batch.

7.1.3 *Cryptosporidium* Spiking Materials

Sources of flow cytometer–counted *Cryptosporidium* spiking suspensions for spiked QC samples include the following vendors, or equivalent:

- Wisconsin State Laboratory of Hygiene - http://www.slh.wisc.edu/
- Bioballs
- Waterborne, Inc. - http://www.waterborneinc.com

The Wisconsin State Laboratory of Hygiene prepares and distributes live *Cryptosporidium* oocysts and *Giardia* cysts that have not been treated to reduce viability. BTF and Waterborne prepare and distribute *Cryptosporidium* oocysts and *Giardia* cysts that have been irradiated to inactivate the organisms. Irradiated organisms may be used for routine QC samples including IPR, OPR and MS samples but should not be used to demonstrate acceptability through a multi-laboratory validation study using multiple matrices.

QC

7.1.4 IPR Test

The IPR test consists of four reagent water samples each spiked with 100 to 500 oocysts. The results of the four analyses are to be used to calculate mean percent recovery and the relative standard deviation (RSD) of the recoveries. The mean *Cryptosporidium* recovery should be from 24% to 100% and the RSD of the four recoveries should be less than or equal to 55% for Method 1623 [Table 3]. (The mean *Cryptosporidium* recovery should be 38 to 100 % with an RSD of 37% for laboratories performing IPR tests using Method 1623.1 [(*Table 3*)]). Characterization of the first three *Cryptosporidium* oocysts and three *Giardia* cysts must be reported on the slide examination form for each IPR sample [Section 9.4.5 (*Section 9.5.5*)].

7.1.5 MB Test

QC **7.1.5.1** The MB test consists of analysis of an unspiked reagent water sample to show freedom from contamination. The method requires that one MB sample must be analyzed each week samples are analyzed or for each QC batch, up to and including 20 samples (MS and field samples combined), whichever is more frequent [Section 9.6 (*Section 9.7*)]. If more than one method variation is used for filtration and/or another technique which uses different apparatus and/or reagents, a separate MB is required for each variation. However, if more than one labeling reagent is used a separate MB is not required [Section 7.6.4, Note (*Section 7.8.4, Note and 14.0, Note*)].

7.1.5.2 MB samples should be analyzed before any field samples in a batch are processed to verify acceptable performance. If oocysts are detected in a MB, the MB is unacceptable and analysis of additional samples is halted until the source of contamination is eliminated and a blank shows no evidence of contamination. If the repeated MB is acceptable, field samples within the holding times can be processed.

7.1.5.3 To demonstrate freedom from contamination and check the efficacy of the cleaning system, the MB should be processed in the same manner as the associated OPR and field samples. The carboy used for the MB is randomly selected from carboy stock or disposable carboys are used for all samples.

7.1.6 OPR Test

QC **7.1.6.1** The OPR consists of analysis of a reagent water sample spiked with 100 to 500 oocysts to demonstrate ongoing acceptable performance. One OPR sample must be analyzed each week samples are analyzed or for each QC batch, up to and including 20 samples (MS and field samples combined), whichever is more frequent [Section 9.7 (*Section 9.8*)]. If more than one method variation is used for filtration and/or another technique which uses different apparatus and/or reagents, a separate OPR is required for each variation. However, if more than one labeling reagent is used a separate OPR is not required [Section 7.6.4, Note (*Section 7.8.4, Note and 14.0, Note*)].

QC **7.1.6.2** OPR samples must be analyzed before any field samples in a batch are processed to verify acceptable performance. OPR *Cryptosporidium* recovery must be from 22% to 100% to be considered acceptable (*Federal Register* Vol. 74, No. 36, February 25, 2009) for EPA Method 1623. OPR *Cryptosporidium* recovery must be from 33 % to 100% for EPA Method 1623.1 [(*Section 9.8.3*)]. Characterization of

the first three *Cryptosporidium* oocysts and three *Giardia* cysts must be reported on the slide examination form [Section 9.7.1.2 (*Section 9.8.1.2*)]. If the OPR *Cryptosporidium* recovery is not acceptable, no samples may be processed. Finding the problem and repeating the OPR to assure the analytical system is "in control," is required before proceeding with sample analysis. If the repeated OPR is acceptable, field samples within holding times can be processed.

QC 7.1.7 OPR Charts

7.1.7.1 Laboratories should maintain a control chart of OPR recoveries, graphically displaying the results of continuing performance when using Method 1623; moreover, this is a requirement for Method 1623.1 [Section 9.7.6 (*Section 9.12.1 and Table 2*)]. The control chart should be developed using the most recent 20 to 30 test results.

7.1.7.2 The control chart should be developed by plotting percent recovery of each OPR sample over time. See Figure 3-2 of The Lab Guidance Manual for an example of an OPR control chart. Based on the mean recoveries, upper control limit should be established as the mean + 2 standard deviations and lower control limit as the mean – 2 standard deviations. After each 5 to 10 new recovery measurements the laboratory should recalculate new control limits using the most recent 20 to 30 data points. If recoveries fall outside the control limits, or declining trends are observed, laboratories should take corrective action to investigate the potential causes of the outlying result. Trouble shooting guidance is available in [Section 9.7.5 (*Section 9.8.7*)].

7.1.8 Staining Controls

QC **7.1.8.1** Positive staining controls are used to verify that the FITC and DAPI stains are fluorescing appropriately. Positive staining controls should be prepared by applying 200 to 400 intact oocysts to a slide and staining the slide with the same reagents and staining procedure used to stain field and MS sample slides. The analyst should examine several fields to verify that the stain is fluorescing at the appropriate intensity and uniformity. Each analyst must characterize a minimum of 3 *Cryptosporidium* oocysts and 3 *Giardia* cysts on the positive staining control slide before examining slides from field samples [Section 15.2.1.1(*Section 15.2.1.1*)]. Control slides and sample slides should be read on the same day. If sample slides from the same staining batch are read over multiple days, the control slide should be rechecked each day before examination of the sample slides. If the laboratory has a large batch of slides that will be examined over several days and is concerned that a single positive control may fade due to multiple examinations, the laboratory should prepare multiple control slides at

the same time with the batch of field slides and alternate between the positive controls when performing the positive control check.

7.1.8.2 Negative staining controls are used to verify that no oocysts or interfering particulates are present. Negative staining controls are prepared by staining and examining a slide with phosphate buffered saline (PBS) solution.

QC **7.1.8.3** The analyst should indicate on each *Cryptosporidium* slide examination form whether the positive staining and negative staining control were acceptable. Each sample must meet the QC criteria for EPA Method 1623 or 1623.1. Positive and negative staining controls must demonstrate acceptable results [Section 15.2.1 (*Section 15.2.1*)].

7.1.9 Verification of Analyst Performance

7.1.9.1 Analyst verifications are ongoing comparisons of slide counts and characterizations used to assess and maintain consistency in slide examination among analysts. The goal is to continually refine/upgrade microscopy skills. When microscopic examinations are being performed, the laboratory must prepare at least monthly one or more slides containing 40 to 200 oocysts. More than 50% of the oocysts must be DAPI positive and undamaged under DIC. Another option is order prepared slides from the Wisconsin State Laboratory of Hygiene, Flow Cytometry Unit (http://www.slh.wisc.edu/) or other approved vendor.

QC **7.1.9.2** For laboratories with multiple analysts, each analyst must determine the DAPI category (DAPI negative, DAPI positive with intense internal blue staining, and DAPI positive with the number of nuclei) and the DIC category (empty, containing amorphous structures, or internal structure characterization) of the same 10 selected oocysts. It is recommended that the DAPI and DIC categorization of the selected oocysts occur with all the analysts at the same time, i.e., each analyst should determine the category independently, then the differences in DAPI and DIC categorizations among analysts are to be discussed, resolved, and the resolutions documented. Alternatively, oocyst coordinates may be recorded for each analyst to locate and categorize the oocysts at different times. Differences among analysts should still be discussed, resolved, and documented.

7.1.9.3 Laboratories should be aware that both FITC and particularly DAPI fluorescence may fade during analyst verification. Accordingly, DAPI comparisons should be performed first. Repeat comparisons with new FITC stained organisms.

7.1.9.4 Each analyst must determine the total number of oocysts by FITC fluorescence at 20X magnification for the entire slide. It is recommended that this count be performed last or on a separate slide than that used for DAPI and DIC characterization so that fading will not influence counts. The total number of oocysts enumerated by each analyst must be within ±10% of each other. If the number is not within this range the analysts must identify the source of any variability between analysts' examination/identification criteria, prepare a new slide, and repeat the performance verification.

7.1.9.5 Laboratories with only one analyst should compare the results of slide examinations to a "protozoa library" of photographs of oocysts and cysts and interfering organisms to verify that examination results are consistent with these established references. These laboratories should perform repetitive counts of a single verification slide for FITC and demonstrate repetitive enumerations within ±10% of each other. These laboratories are encouraged to establish contact with other laboratories to share slides and compare counts.

7.1.10 MS Samples

7.1.10.1 The MS sample entails analysis of an extra bulk water sample spiked with 100 to 500 oocysts. The MS sample is processed at the same time and using the same procedures as the associated field samples to determine the effect of the source water matrix on recovery.

7.1.10.2 For all PWSs, the first MS sample should be collected and analyzed during the first sampling event [Section 9.1.8 (*Section 9.6.1*)]. If it is not possible to analyze an MS sample for the first sampling event, the first MS sample should be analyzed as soon as possible to identify potential method performance issues with the matrix. MS samples must be analyzed once every 20 field samples but may be analyzed more frequently than one every 20 field samples to better characterize method performance in the matrix.

Consistent with this requirement, PWSs falling into the following categories should analyze at least two MS samples during LT2 Rule monitoring:

- Large PWSs that perform monthly monitoring for two years (resulting in 24 samples)
- Small PWSs that are "triggered" into *Cryptosporidium* monitoring and collect semi-monthly samples for one year or monthly samples for two years (resulting in 24 samples)

For large PWSs that perform semi-monthly or more frequent monitoring for two years (resulting in 48 or more samples), a

minimum of three MS samples should be collected and analyzed. If a PWS monitors more frequently or collects more than 60 samples, a minimum of four MS samples should be analyzed.

7.1.10.3 For MS samples associated with field filtered samples, MS samples should be collected as bulk samples and spiked in the laboratory prior to filtration. The volume of the MS sample must be within 10% of the volume of the associated field sample. PWSs that field filter 10 L samples may field filter the monitoring sample but should collect and ship the 10 L MS sample in bulk to the laboratory for spiking, filtering, and analysis.

For PWSs that field filter > 10 L samples, all but 10 L of the MS sample should be filtered in the field. The remaining 10 L of source water for MS analysis should be collected in bulk and shipped to the laboratory. The laboratory should then spike the 10 L bulk sample and pump it through the filter containing the balance of the sample already filtered in the field. The associated monitoring sample should be collected as usual.

QC

7.1.10.4 Similar to OPR control charts, laboratories should assess precision of MS recoveries. This can be accomplished by maintaining a control chart that graphically displays the results of continuing performance. It is recommended in Method 1623 and required for Method 1623.1 that the precision assessment be maintained across all MS samples as well as stratified by source. The control chart should be developed when at least 5 MS samples have been completed [Section 9.5.1.4 (*Section 9.12.2*)].

The control chart should be developed by plotting percent recovery of each MS sample versus time. Based on the mean recoveries on the chart the upper control limit should be the mean + 2 standard deviations and the lower control limit should be the mean – 2 standard deviations. Control charts can be used to compare performance of different method variations and different analysts along with performance in different matrices. If recovery measurements fall outside the control limits, laboratories should take corrective action to investigate the potential causes of the outlying result.

7.2 Certified Laboratories

7.2.1 PWSs must have *Cryptosporidium* samples analyzed by a laboratory that has been certified for *Cryptosporidium* analysis. Details on the elements of the EPA's Laboratory QA Evaluation Program are provided in *Federal Register* Vol. 74, No. 36, February 25, 2009 available online at http://www.regulations.gov under Docket ID No. EPA-HQ-OW-2002-0011),

Chapter III of the *Manual for the Certification of Laboratories Analyzing Drinking Water* (5th Edition), and Section 3.2 of the Lab Guidance Manual. It is suggested that each certified laboratory establish and maintain a relationship with another certified laboratory and develop a protocol to provide backup analyses to clients if needed.

7.2.2 Criteria for Certification Status [February 25, 2009 *Federal Register* Notice available online at http://www.regulations.gov under Docket ID No. EPA-HQ-OW-2002-0011.]

Analysis of a Proficiency Testing (PT) sample is a key component of the certification process. Laboratories seeking certification for *Cryptosporidium* analysis under the LT2 Rule should successfully participate in a PT study. The laboratory should acquire a set of eight initial PT samples from an approved PT provider. Laboratories should suspend these spikes in reagent water to produce simulated source water samples, and analyze the samples using the modifications of EPA Method 1623 or 1623.1 that the laboratory plans to use for *Cryptosporidium* analyses. If a laboratory wishes to be evaluated for more than one modification of the method, the laboratory should acquire a set of eight PT samples for each version. Data submitted by the laboratory from the analysis of the PT samples will be evaluated against the mean recovery and precision (as a RSD) for the PT samples from other laboratories in the same study, and the laboratory will be notified of their results. Failure to successfully participate in two consecutive PT studies should result in the laboratory staff receiving additional training to better perform the method.

Laboratories that meet the above performance criteria should acquire PT samples approximately every four to six months. The Certification Authority should evaluate the precision and recovery data for PT samples to determine if the laboratory continues to meet the performance criteria of the Certification Authority. In addition, the Certification Authority should review raw PT data records to ensure compliance with Method 1623 or 1623.1 requirements.

8. Records and Data Reporting

8.1 Legal and Technical Defensibility

In order to have technically defensible compliance monitoring data, the laboratory should maintain thorough and accurate records. The QA plan and SOPs should describe the policies used by the facility to record and validate data, record retention, and storage. If samples are expected to be part of an enforcement action, chain of custody procedures should be implemented (See Appendix A of the *Manual for the Certification of Laboratories Analyzing Drinking Water* (5th Edition)).

8.2 Recordkeeping

An effective record keeping system provides information on sample collection and preservation, analytical methods, raw data, calculations, reported results, and a record of

persons responsible for sampling and analyses. Laboratories using EPA Method 1623 or 1623.1 should record original data including microscopic examination counts and notes. The data may be recorded on the recommended bench sheets and slide examination forms. Bench sheets and slide examination forms are included in this chapter.

Data should be recorded in ink and a single line drawn through any change with initials, dates and correction entered. Data files may also be microfiche or electronic. Electronic data should be backed up by protected tape, disc, or hard copy. PWSs are required to maintain records of microbiological analyses of compliance samples for five years (40 CFR141.33). A change in ownership, merger, or closure of a laboratory does not negate this requirement. The client water system should be notified before disposing of records so they may request copies if needed. This includes all raw data, calculations, and QC data. If the laboratory changes its computer hardware or software, provisions should be made for transferring old data to the new system so that the data may be retrievable within the record retention period. Data that is expected to become part of litigation may need to be retained longer.

8.3 Data Recording Practices

The following data recording practices should be followed with field, MS, QC, and PT samples for all laboratories performing compliance analyses:

- Record sample identification information, including sample collection and receipt dates and conditions
- Record all raw data (primary measurements) used to calculate final concentrations of oocysts/L for each sample
- Record the date and time of each method step associated with a holding time to verify that all method holding times have been met
- Record the name of the analyst performing each method step to verify that only qualified analysts are performing the method

These data elements are critical to ensuring that final sample concentrations can be verified using primary data and are necessary to demonstrate that all method specified holding times are met. These records should be available for review by Certification Officer.

8.4 Sampling Records

Sampling records provided by the PWS should include the following information:

- PWS name and ID number
- Facility name and number
- Sample collection point name and ID
- Date and start/stop times of collection
- Sampler's name and telephone number (or alternate contact for laboratory)
- Source water temperature and turbidity
- Volume filtered information (if the sample was filtered in the field)
- Whether the filter clogged (if the sample was filtered in the field)
- Analyses requested (e.g., routine field sample analysis or field sample + MS analysis)

Detailed guidance on sample collection data recording as well as forms and sample collection and shipping procedures can be found in the Source Water Monitoring Guidance Manual:
https://nepis.epa.gov/Exe/ZyPDF.cgi?Dockey=2000CZCJ.txt.

8.5 Sample Receipt

Upon sample receipt at the laboratory, the following information should be recorded on a bench sheet, log, or record book. Acceptance criteria for sample receipt should be established and documented in a laboratory's SOPs [Sections 8.1.3 and 8.1.4 (*Sections 8.1.3 and 8.1.4*)]. Laboratories should immediately notify utilities of any deficiencies requiring a resample and document the reasons for rejection.

- PWS name and ID
- Facility name and ID
- Sample collection point name and ID
- Turbidity at the collection point taken immediately after sample collection
- Date and time of sample collection (start and stop times if field filtered)
- Date and time of sample receipt by the laboratory
- Volume filtered (if sample is filtered in the field)
- Name of laboratory person receiving the sample
- Temperature of sample upon receipt
- Any deficiencies (exceeded sample holding time, transport temperature exceeded 20°C or sample leaked during transport, frozen samples, etc.)

8.6 Primary Data Elements for Calculations

Laboratories analyzing compliance samples for *Cryptosporidium* using EPA Method 1623 or 1623.1 should record the following primary data elements required to calculate the final concentrations and percent recoveries for MS, OPR, and PT samples. These data should be recorded on the recommended bench sheet and slide examination form.

- Estimated number of oocysts spiked (MS and OPR samples) based on information provided by the flow–cytometry laboratory producing the spiking suspension
- Sample volume spiked, in L (MS, OPR and PT samples)
- Sample volume filtered, to nearest ¼ L
- Number of filters used (if the filter is clogged)
- Pellet volume after concentration, to the nearest 0.1 mL
- Total volume of the resuspended concentrate transferred to IMS, in mL
- Number of subsamples analyzed
- Total number of oocysts detected in the sample

8.7 **Key QC Data Elements**

The following data should be recorded to determine that all method QC requirements were met and that samples were analyzed by qualified personnel consistent with the requirements of the Laboratory QA Program for the analyses of *Cryptosporidium.*

- Elution date and time (must be within 96 hours of sample collection)
- Slide preparation date and time (must be completed in same working day as elution)

- Sample staining date and time (must be completed within 72 hours of slide preparation)
- Sample examination date and time (must be within 168 hours (7 days) of sample staining)
- Person (PWS or laboratory employee) performing filtration
- Technician or analyst performing elution
- Technician or analyst performing IMS
- Technician or analyst preparing slide
- Technician or analyst staining the slide
- Analyst performing sample examination
- Results of the positive and negative staining controls

The laboratory should also record any additional information that will support the results or aid in identification of problems of sample analyses such as filter type, elution procedure, IMS system used, lots numbers of reagents, detection kits, spiking suspensions, etc. This information should be recorded on bench sheets, and slide examination forms.

9. Calculations for EPA Method 1623 and 1623.1

9.1 The laboratory may choose to report the total oocysts and volume analyzed along with the oocysts/L [Section 15.2.5 (*Section 15.2.5*)] and other data elements described in EPA Method 1623 and 1623.1 to the PWS. The laboratory will need to calculate OPR and MS recoveries to report to their PWS and to maintain QC control charts.

9.2 Calculating Oocyst Concentrations

The following information will be needed to calculate the concentration of *Cryptosporidium* in a sample:

- Number of oocysts in the sample (recorded from slide examination form)
- Volume analyzed

$$\text{Final concentration}\left(\frac{\text{oocysts}}{\text{L}}\right) = \frac{\text{oocysts detected in sample}}{\text{Volume analyzed (L)}}$$

If 100% of the sample volume filtered is examined then the volume analyzed equals the volume filtered. This applies whether one filter or more than one filter was used.

If < 100% of the volume filtered was processed through the remainder of the method then additional calculations are needed [Section 13.2.4.2 (*Section 13.2.3.2*)].

9.3 Determining the Volume of Resuspended Concentrate to use for Packed Pellets > 0.5 mL

Packed pellets with a volume >0.5 mL must be divided into subsamples. The laboratory should use the formula below to determine the total volume of resuspension required in the centrifuge tube before separating the concentrate into two or more subsamples and transferring to IMS [Section 13.2.4 (*Section 13.2.3*)].

$$\text{Total volume of resuspended concentrate (mL) required} = \frac{\text{pellet volume (mL) after centrifugation}}{0.5\ \text{mL}} \times 5\ \text{mL}$$

9.4 MS Recovery Calculations

To determine the percent recovery for a MS sample the following information is needed [Section 9.5.1.2 (*Section 9.6.2.2*)]:

- The number of oocysts counted in the MS sample
- The number of oocysts spiked into the MS sample
- The number of oocysts counted in the unspiked field sample (to correct for background concentration)

$$\text{Percent recovery} = \frac{\text{oocysts counted in MS sample} - \text{oocysts counted in unspiked field sample}}{\text{oocysts spiked in MS sample}} \times 100\%$$

This calculation is based on the assumption that the same sample volume was examined for both the field and MS samples. If the sample volumes examined are different the laboratory should calculate the number of oocysts per L for both the field and MS samples before calculating percent recovery. If both a MS and a MSD are analyzed then the mean recovery and relative percent difference (RPD) should be calculated and compared to the EPA Method 1623 or1623.1 acceptance criteria [Tables 3 and 4 (*Tables 3 and 4*)].

To calculate the mean percent recovery the laboratory should calculate the percent recovery for each sample as described above then perform the following calculation [Section 9.5.2.2 (*Section 9.6.3.2*)]:

$$\text{Mean percent recovery} = \frac{\text{percent recovery of MS sample} + \text{percent recovery of MSD sample}}{2}$$

In order for the laboratory to calculate the RPD, the absolute value (without sign) of the difference between the numbers of oocysts counted in the MS and MSD should be divided by the mean of the oocysts counted in both samples to yield a percentage of the difference. This calculation assumes that the same volume is analyzed for both the MS and MSD. The laboratory should calculate the number counted per L before calculating the RPD if sample volumes analyzed are different [Section 9.5.2.3 (*Section 9.6.3.3*)].

$$\text{RPD} = \frac{|\text{oocysts counted in MS} - \text{oocysts counted in MSD}|}{(\text{oocysts counted in MS} + \text{oocysts counted in MSD})/2} \times 100\%$$

9.5 OPR Sample Calculations

The laboratory should calculate percent recovery of an OPR sample using the following formula [Section 9.7.2 (*Section 9.8.2*)]:

$$\text{Percent recovery} = \frac{\text{oocysts detected}}{\text{oocysts spiked}} \times 100\%$$

OPR recoveries are to be compared to the limits for ongoing recovery in EPA Method 1623 or 1623.1 (See Section 7.1.6.2 of this certification chapter). These recoveries should be tracked/monitored over time to assess precision.

10. Data Archiving

10.1 Hardcopy Data

The following data should be archived:

- Bench sheets and slide examination forms for all compliance monitoring samples, including both field samples and MS samples
- Bench sheets and slide examination forms for all OPR samples and MB samples, and records of compliance monitoring samples associated with each OPR sample and blank sample
- Spike enumeration information received from *Cryptosporidium* spiking suspension vendors
- Bench sheets and slide examination forms for all ongoing PT samples

As part of the Lab QA Program the laboratory should also maintain the same documentation for their IPR and initial PT data for each method variation used for compliance samples.

10.2 Slides

Although not required, laboratories may wish to consider archiving slides and/or photographing slides to maintain for clients. Slides should be stored in the dark between 1°C and 10°C and not frozen in the appropriate environment for the type of mounting medium used.

Acronym List

ACS	American Chemical Society
ATP	Alternate Test Procedure
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DCTS	LT2 RULE/Stage 2 Data Collection and Tracking System
DIC	Differential interference contrast
DPD	N.N-diethyl-p-phenylenediamine
EPA	Environmental Protection Agency
FA	Fluorescence assay or immunofluorescence assay
FITC	Fluorescein isothiocyanate
GLP	Good Laboratory Practice
HDPE	High-density polyethylene
IDC	Initial Demonstration of Capability
IFA	Immunofluorescence assay or fluorescence assay
IMS	Immunomagnetic separation
IPR	Initial Precision and Recovery
Lab Guidance Manual	Microbial Laboratory Guidance Manual for the Final Long Term 2 Enhanced Surface Water Treatment Rule
LDPE	Low-density polyethylene
LT2 Rule	Long Term 2 Enhanced Surface Water Treatment Rule
MB	Method Blank
MPN	Most Probable Number
MS	Matrix Spike
MSD	Matrix Spike Duplicate
NIST	National Institute of Standards and Technology
OPR	Ongoing Precision and Recovery
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween®
PCFC	Portable Continuous Flow Centrifuge
PPE	Personal Protective Equipment
PT	Proficiency Testing
PTFE	Polytetrafluorethylene
PWS	Public Water System
QA	Quality Assurance
QC	Quality Control
RCF	Relative Centrifugal Force
RPD	Relative Percent Difference
RPM	Rotations per Minute
RSD	Relative Standard Deviation
Source Water Monitoring Guidance Manual	Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule
SOP	Standard Operating Procedure

Checklist Item Classification

Below is a description of the categories used to classify each item on Checklists A, B, and C. The categories correspond to information provided in Notes 1-3 at the beginning of Chapter VII.

Recommendation: Indicates suggested improvements for analytical or data recording/QA procedures. Recommendations do not indicate that method or program requirements were not met. Laboratories are not required to implement recommendations but are requested to respond in writing to each recommendation giving details on how they plan to implement the recommendation or why they have decided against implementation.

Critical: Indicates areas that EPA believes are necessary elements of good laboratory practice or may impact method performance. Critical items are not required by the EPA Methods but laboratories seeking approval under the Laboratory QA Program are strongly encouraged to implement all critical items. Laboratories should respond in writing that critical items have been implemented and provide documentation as appropriate. If the item is not implemented, the laboratory should provide an alternative approach for addressing data quality issues or describe why data quality will not be affected if no action is taken.

Requirement: Indicates areas where QC requirements are specified in the EPA Method or LT2 rule and may impact data quality and/or data comparability. Laboratories seeking approval under the Laboratory QA Program must implement all requirements and respond in writing and provide documentation as appropriate.

Method Procedure (1623): Indicates areas where the laboratory's method procedures deviate from the December 2005 version of Method 1622/1623 and may affect method performance. Laboratories seeking approval under the Laboratory QA Program should provide a letter to EPA either: (1) indicating that current procedures will be changed to eliminate method deviations or (2) certifying with data from Tier 1 testing (Section 9.1.2.1.1 of Method 1622/1623, December 2005) that the method modification produced results equivalent or superior to results produced by the method as written. Please note that not all method modifications can be demonstrated to produce equivalent performance through Tier 1 testing; for some modifications the laboratory may need to design a study or provide additional data to demonstrate equivalency and submit the study design to EPA for approval.

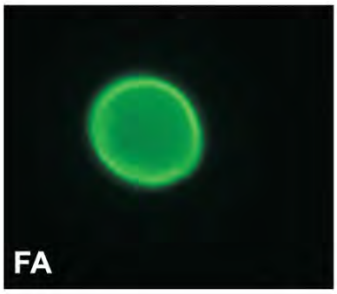
Method Procedure (1623.1): Indicates areas where the laboratory's method procedures deviate from Method 1623.1 and may affect method performance. Table 1 lists the options that can be performed according to the procedures described in the method.

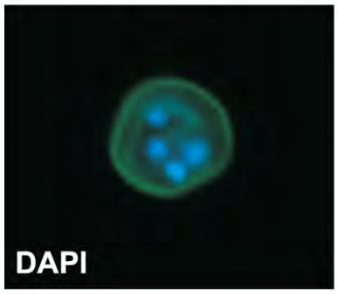
Commendation: Indicates areas where the laboratory has exceeded method or program requirements, shown innovation, or demonstrated excellent laboratory technique. No response is required by the laboratory.

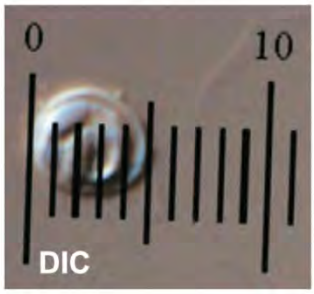
Method 1623/1623.1 Microscopy Visual Guide

Cryptosporidium oocyst criteria:

- Brilliant apple-green fluorescence
- Brightly highlighted edges
- 4 – 6 µm size
- Spherical to ovoid shape
- No atypical characteristics by FA, DAPI fluorescence or DIC microscopy

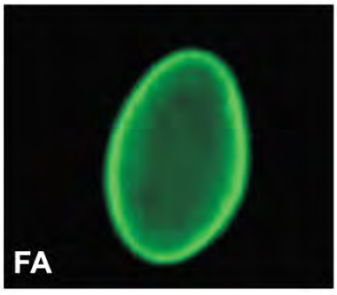


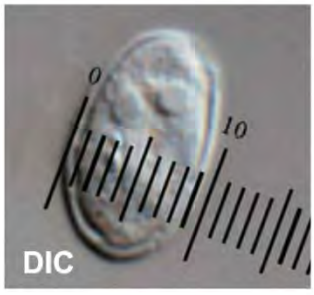




Giardia cyst criteria:

- Brilliant apple-green fluorescence
- Brightly highlighted edges
- 8 – 18 µm long by 5 – 15 µm wide
- Spherical to ovoid shape
- No atypical characteristics by FA, DAPI fluorescence or DIC microscopy





DIC image

Image not in DIC

Example Checklists for Onsite Evaluation of Laboratory Analyzing Drinking Water for *Cryptosporidium* and *Giardia* Using Method 1623/1623.1

Checklist A - Method 1623/1623.1 Audit Package and Data Review

Checklist B - Laboratory SOP Review

Checklist C - Method 1623/1623.1 Technical Review – Sample Processing and Microscopy

Checklist A – Method 1623/1623.1 Audit Package and Data Review

Laboratory Name	Name and Affiliation of Evaluator	Date of Evaluation

Good Laboratory Practice (GLP) is generally defined as a system of management controls for the laboratories to ensure the consistency and reliability of results. Adapted from other federal programs for the purposes of the *Cryptosporidium* Laboratory QA Evaluation Program, GLP includes personnel, equipment, and standard operating procedures appropriate for the program.

Item to be Evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
1 Quality Assurance									
1.1 Is documentation (e.g., resume, sample list) available for all Method 1623/1623.1 staff?	9.1	9.1	1.	Requirement GLP					
1.1.1 Have technicians/analysts analyzed the required number of samples using Method1623 or1623.1?	22.2	22.2	1.1 -1.3	Requirement GLP					
1.2 Are employee training records available and up to date?	9.1	9.1	1.7	Critical GLP					
1.2.1 Have all analysts documented that they have read and understood the QA Plan and SOPs?	-	-	1.7	Critical GLP					
1.3 Is the laboratory performing analyst verification monthly and does the lab have corrective action procedures in place if criteria are not met?	10.6	9.10	7.1.9	Requirement					
1.3.1 If the laboratory has only one analyst, is the analyst demonstrating analyst verification through comparison with photo libraries or repetitive counts?	10.6.4	9.10.4	7.1.9.5	Recommendation					
1.4 Does the quality assurance plan address requirements for *Cryptosporidium* analysis under LT2ESWTR?				Critical					
1.5 Have acceptable initial precision and recovery analyses been performed for each version of the method the laboratory is using?	9.1.2.1.1	9.2	7.1.4	Requirement					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
1.6 Of the field/PT samples reviewed, is each field/PT sample associated with an acceptable method blank?	9.6.1	9.7	7.1.2 7.1.5.1	Requirement					
1.6.1 Were all method blanks (MB) evaluated without contamination?	9.6.2.1	9.7.2	7.1.5	Requirement					# MB reviewed:
1.6.2 Were the same lots of reagents (elution, IMS, and staining) used for the method blank and the associated field/PT samples?	-	-	7.1.5.3	Critical					
1.6.3 Is method blank analyzed prior to the analysis of field/PT samples?	9.6	9.7	7.1.5.2	Requirement					
1.7 Is each field/PT sample associated with an acceptable ongoing precision and recovery (OPR) sample?	9.7	9.8	7.1.2 7.1.6.1	Requirement					
1.7.1 What percentage of OPR samples evaluated met the recovery criteria?	9.7.3 Table 3 Table 4	9.8.3 Table 3 Table 4	7.1.6.2						# OPR reviewed:
1.7.2 Were the same lots of reagents (elution, IMS, and staining) used for the OPR and the associated field/PT samples?	-	-	7.1.6.1	Critical					
1.7.3 Is OPR analyzed prior to the analysis of field/PT samples?	9.7.1	9.8.1	7.1.6.2	Requirement					
1.7.4 Does the laboratory maintain control charts of OPR results?	9.7.6 9.1	9.8.3 9.12.1 Table 2	7.1.7	1623 Recommendation 1623.1 Requirement					
1.7.5 What is the mean and relative standard deviation (RSD), or standard deviation, of the recoveries of the OPR samples included in the control chart?	9.4.3 Table 3 Table 4	9.5.3 Table 3 Table 4	7.1.6.2	QC Criteria					Mean: RSD:
1.8 Were matrix spike (MS) samples analyzed at the minimum frequency of 1 MS per 20 (up to and including) field samples from each source?	9.1.8	9.6.1	7.1.2 7.1.10.2	Requirement					# MS reviewed:
1.8.1 Were MS sample volumes within 10% of their associated field samples' volumes?	9.5.1	9.6.2	7.1.10.3	Requirement					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
1.8.2 Were MS samples analyzed at the same time and using the same method variation as their associated field samples?	Table 2	Table 2	7.1.10.1	Requirement					
1.8.3 What is the mean and relative standard deviation of the MS samples reviewed?	Table 3 Table 4 Table 5	Table 3 Table 4 Table 6	-	QC Criteria					Mean: RSD:
1.8.4 Does the laboratory maintain control charts of MS results?	9.5.1.4 9.1	9.6.2.3 9.12.2 Table 2	7.1.10.4	1623 Recommendation 1623.1 Requirement					
1.9 Were OPR samples spiked with 100 - 500 organisms?	9.7	9.8	7.1.6.1	Requirement					
1.10 Does the laboratory perform IMS controls and maintain IMS control charts? If not, how do they troubleshoot low recoveries?	9.7.5.3	9.8.7.3 9.13	-	Recommendation					
1.11 Does the laboratory have an adequate record system for tracking samples, including unique ID, from collection through log-in, analysis, and data reporting?	-	-	8.0	Critical GLP					
1.12 Is the laboratory using the Method December 2005 version of Method 1623 or Method 1623.1 for LT2 samples?				Requirement					
2 Data Recording Procedures									
2.1 Is shipping information complete, i.e., time/date of sample collection, sampler's name, time/date of sample receipt, receiver's initials, sample condition?	8.1.3	8.1.3	8.5	Requirement					
2.1.1 Were all samples evaluated received at ≤20°C and not frozen?	8.1.3	8.1.3	6.3.3	Requirement					
2.2 Do sample numbers on the chain of custody match the sample numbers on the report forms?	-	-	-	Requirement					
2.3 Are current Method 1623/1623.1 bench sheets used to record sample processing data?	-	-	8.2	Recommendation					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
2.4 Are all primary measurements during each step recorded, including all raw data used in calculations?	9.1.2.2.5	9.3.5	8.0	Requirement					
2.5 Technician/analyst, date, and time of elution is recorded?	12.2.6.2.1	12.2.7.1 12.3.2.1	8.7	Requirement					
2.6 Technician/analyst, date, and time of slide preparation is recorded?	13.3.3.11	13.3.3.11	8.7	Requirement					
2.7 Technician/analyst, date, and time of staining is recorded?	14.10	14.10	8.7	Requirement					
2.8 Are batch and lot numbers of reagents used in the analysis of the sample recorded?	-	-	8.7	Critical					
2.8.1 Lot number for the IMS kit is recorded?	-	-	8.7	Critical					
2.8.2 Lot number of the staining kit is recorded?	-	-	8.7	Critical					
2.8.3 Lot number of the spiking suspensions is recorded?	-	-	3.21.4 8.7	Critical					
2.9 Spike value recorded for all spiked samples?	-	-	3.21.4 8.6	Requirement					
2.10 Are Method 1623/1623.1 *Cryptosporidium* Slide Examination forms used to record sample examination results?	15.2	15.2	8.2	Requirement					
2.11 Name of examining analyst is recorded?	15.2.6	15.2.6	8.7	Requirement					
2.12 Date and time of sample examination is recorded?	15.2.4	15.2.4	8.7	Requirement					
2.13 Are calculations of final concentrations and recoveries complete and correct?	-	-	-	Requirement					
2.14 Is the size of the cysts and oocysts reported to the nearest 0.5 µm?	15.2.2.3 15.2.3.3	15.2.2.4 15.2.3.4	5.4.9.4 5.4.10.4	Requirement					
2.15 Is each reported positive organism detected in a field sample characterized and recorded?	15.2	15.2.2.1 15.2.3.1	5.4.9.1 5.4.10.1	Requirement					
2.16 Do values recorded on the data sheets match the values reported to the client?	-	-	8.1	Requirement					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
2.17 Are mistakes on all forms crossed out with a single line, initialed, and dated?	-	-	8.2	Critical					
2.18 Are data always legible and recorded in pen?	-	-	8.2	Critical					
2.19 Was the final report reviewed by QA manager, lab director or an individual other than the analyst?	-	-	8.1	Critical					
2.20 Do records demonstrate each analyst's characterization of 3 oocysts and 3 cysts from positive control for each microscopy session?	15.2.1.1	15.2.1.1	5.4.6	Requirement					
2.21 Data shows that no more than 0.5 mL of pellet was used per IMS?	13.2.4	13.2.3	5.2.3 8.6	Requirement					
3 Holding Times –Method 1623.1									
3.1 Is sample elution initiated within 96 hours of sample collection or field filtration?	8.2.1 Table 1	8.2.1 Table 5	6.4 8.7	Requirement					
3.2 Are sample elution, concentration, and purification steps completed in one work day?	8.2.2 Table 1	8.2.2 Table 5	6.4 8.7	Requirement					
3.3 Are slides stained within 72 hours of application of the purified sample to the slide?	8.2.3 Table 1	8.2.3 Table 5	6.4 8.7	Requirement					
3.4 Are stained slides read and confirmed within 7 days of staining? [Section 8.2.4 and Table 5]	8.2.4 Table 1	8.2.4 Table 5	6.4 8.7	Requirement					
4 Spike enumeration procedures									
4.1 Source of flow cytometry-enumerated spiking suspensions.	-	11.2	-						
4.2 If 50-L samples are analyzed, what positive control procedure does the laboratory follow for OPR and MS samples: (A) spike entire 50 L, (B) spike and filter 10 L before filtering 40 L, or (C) filter 40 L before spiking and filtering 10 L.	-	-	7.1.10.3						

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

The following items below are optional if the laboratory is NELAC certified. If the laboratory opts to provide NELAC certification, complete the box below by entering the NELAC certification number and date. Provide copy of certification.										
NELAC Certification Number:						**Certification Date:**				
5 Laboratory Equipment and Supplies										
5.1 Reagent-grade water testing										
5.1.1 Is reagent water tested monthly for conductivity and total chlorine residual?	-	-	4.3.1	Critical GLP					
5.1.1.1 Were the results for the above parameters acceptable? Total chlorine residual not greater than 0.1 mg/L, conductivity not greater than 2 µmhos/cm?	-	-	4.3.1	Critical GLP					
5.1.2 Has the reagent water been tested annually for metals – Pb, Cd, Cr, Cu, Ni, Zn?	-	-	4.3.1	Critical GLP					
5.1.2.1 Were the results for the metals testing acceptable; each metal not greater than 0.05 mg/L and collectively not greater than 0.1 mg/L?	-	-	4.3.1	Critical GLP					
5.1.3 Is reagent water tested monthly for heterotrophic plate count?	-	-	4.3.1	Critical GLP					
5.1.3.1 Are the results for the heterotrophic plate count acceptable, < 500 CFU/mL?	-	-	4.3.1	Critical GLP					
5.1.4 Is still or DI unit maintained according to manufacturer's instructions?	-	-	4.3.3	Critical GLP					
5.2 pH meter										
5.2.1 Accuracy ± 0.1 units, scale graduations, 0.1 units?	-	-	3.1.1	Critical GLP					
5.2.2 Is a record maintained for pH measurements and calibrations?	-	-	3.1.4	Critical GLP					
5.2.3 Is pH meter standardized each use period with pH 7, 4 or 10 standard buffers (selection dependent upon desired pH)?	-	-	3.1.4	Critical GLP					
5.2.4 Are all pH buffers dated when received and opened, and discarded before expiration date?	-	-	3.1.5	Critical GLP					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

5.3 Balances (top loader or pan balance)										
5.3.1	Are balance calibrations verified monthly using ASTM Class 1, Class 2 or Class 3 weights or weights traceable to Class 1, Class 2, or Class 3 weights, or equivalent? Non-reference weights should be calibrated every six months with reference weights.	-	-	3.2.2	Critical GLP					
5.3.2	Is correction data and Certificate of Traceability available for weights?	-	-	3.2.3	Critical GLP					
5.3.3	Is preventative maintenance conducted yearly at a minimum?	-	-	3.2.4	Recommendation GLP					
5.4 Temperature recording device										
5.4.1	Are calibration of thermometers checked annually (dial thermometers quarterly) at the temperature used against a reference NIST thermometer or equivalent?	8.1.4	8.1.4	3.3.2	Requirement GLP					
5.4.2	Is the sample storage refrigerator able to maintain temperature of 1 to 10°C?	-	-	3.7.1	Critical GLP					
5.5 Micropipetters										
5.5.1	Have micropipetters been calibrated within the past year?	9.2.1	Appendix A	3.8.2	Requirement GLP					
5.6 Centrifuge										
5.6.1	Is a maintenance contract in place or internal maintenance protocol available?	9.1	9.1	3.15.5	Critical GLP					
5.6.2	Is the centrifuge calibrated yearly?	-	-	3.15.5	Critical GLP					
5.7 Autoclave										
5.7.1	Are date, contents, sterilization time and temperature, and technician initials recorded for each cycle?	-	-	3.5.3	Critical GLP					
5.7.2	Is a maximum registering thermometer or continuous monitoring device used during each autoclave cycle?	-	-	3.5.5	Critical GLP					
5.7.3	Is automatic timing mechanism checked with stopwatch quarterly?	-	-	3.5.6	Critical GLP					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

	5.7.4 Are spore strips or ampules used monthly to confirm sterilization?	-	-	3.5.5	Critical GLP					
6	**Quality Assurance Manual**									
6.1	Does the laboratory have a formal QA laboratory plan prepared and ready for examination?	9.1	9.1	4.5.1	Requirement					
6.2	Is a laboratory organization chart or other information available listing staff organization and responsibilities? Does it identify the QA manager and lab director?			4.5.2	Recommendation					
	6.2.1 Is the QA manager separate from the lab director?	-	-	4.5.2	Recommendation GLP					
6.3	Does the laboratory have a schedule and/or procedure for all preventative maintenance of equipment?	-	-	4.5.3	GLP					

Comments:

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Checklist B - Laboratory SOP Review

Laboratory Name	Name and Affiliation of Evaluator	Date of Evaluation

Good Laboratory Practice (GLP) is generally defined as a system of management controls for the laboratories to ensure the consistency and reliability of results. Adapted from other federal programs for the purposes of the *Cryptosporidium* Laboratory QA Evaluation Program, GLP includes personnel, equipment, and standard operating procedures appropriate for the program.

Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
1 Sample Spiking									
1.1 The suspension vial is vortexed for 30 seconds or per manufacturer's instructions?	11.4.3.1.2	11.2.3.2	-	Method Procedure					
1.2 The carboy used for the method blank is randomly selected from carboy stock to check efficacy of cleaning system or disposable carboys are used for all samples?	-	-	7.1.5.3	Critical					
1.3 The details of the suspension vial rinse, including volumes?	11.4.3.1	11.2.3	-	Method Procedure					
1.4 Acceptable sample spiking procedures, including issues not noted in items 1.1 through 1.3?				Critical GLP					
2 Filtration/Elution									
2.1 Envirochek® HV filtration									
2.1.1 The flow rate is maintained at approximately 2 L/min?	12.2.1.2	12.2.1.2	-	Method Procedure					
2.1.2 The volume filtered is measured using a flow totalizer or calibrated carboy?	12.2.4.2	12.2.4.2	-	Requirement					
2.1.3 The sample is stirred during filtration?	12.2.4.1	12.2.4.1	-	Method Procedure					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

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Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
2.1.4 The details of the carboy rinse after filtration including volume?	12.2.4.5	12.2.4.6	-	Method Procedure					
2.1.5 Appropriate maintenance and cleaning procedures?	-	-	-	Critical					
2.1.6 Acceptable Envirochek® filtration procedures, including issues not noted in items 2.1.1 through 2.1.5?				Critical GLP					
2.2 Envirochek® HV capsule filter elution									
2.2.1 Measurement of the volume of the elution buffer used or that the volume covers the membrane?	12.2.6.2.2	12.2.8.2	-	Method Procedure					
2.2.2 The speed that samples are shaken?	12.2.6.2.3	12.2.8.3	-	Method Procedure					
2.2.3 The dispersant is added to the sample as per Method 1623.1?		12.2.7	-	1623 Recommendation 1623.1 Requirement					
2.2.4 The samples are shaken three times for 5 minutes each time, and each in a different orientation?	12.2.6.2	12.2.8	-	Method Procedure					
2.2.5 Procedures for filter capsule rinse and addition of rinsate to the centrifuge bottle?	12.2.6.2.8	12.2.8.8	-	Method Procedure					
2.2.6 Acceptable Envirochek® capsule filter elution procedures, including issues not noted in items 2.2.1 through 2.2.5?				Critical GLP					
2.3 Filta-Max® filtration									
2.3.1 The flow rate is maintained at ≤4 L per minute for Filta-Max®?	12.3.1.1.3	12.3.1.1.3	-	Method Procedure					
2.3.2 The volume filtered is measured using a flow totalizer or calibrated carboy?	12.3.1.5.2	12.3.1.5.2	-	Requirement					
2.3.3 Appropriate maintenance and cleaning procedures? [Section 12.3.4]	12.3.4	12.3.4	-	Requirement					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
2.3.4 Acceptable Filta-Max® filtration procedures, including issues not noted in items 2.3.1 through 2.3.3?				Critical GLP					
2.4 Filta-Max® filter wash station elution									
2.4.1 The use of PBST to elute the filter?	7.4.2.4	7.6.2.4	-	Method Procedure					
2.4.2 The amount of PBST used for each wash? (approx. 600 mL)	12.3.2.2	12.3.2.2	-	Method Procedure					
2.4.3 The plunger is moved up and down 20 times during the first wash?	12.3.2.2.1 h	12.3.2.2.1 h	-	Method Procedure					
2.4.4 The plunger is moved up and down gently to avoid generating excess foam?	12.3.2.2.1 h	12.3.2.2.1 h	-	Method Procedure					
2.4.5 That during the second wash the plunger is moved up and down 10 times?	12.3.2.2.2 b	12.3.2.2.2 b	-	Method Procedure					
2.4.6 The instructions for cleaning the wash station between samples?	12.3.4.2	12.3.4.2	-	Requirement					
2.4.7 The housing is rinsed after filter is removed and the rinse is included in the sample volume?	12.3.2.2.1 d	12.3.2.2.1 d	-	Method Procedure					
2.4.8 Acceptable Filta-Max® filter wash station elution procedures, including issues not noted in items 2.4.1 through 2.4.7?				Critical GLP					
3 Concentration									
3.1 Filta-Max® filter sample concentration (as an alternative or in addition to Section 3.2)									
3.1.1 The force of the vacuum is maintained below 30 cm Hg?	NOTE pg 43	NOTE pg 34	-	Method Procedure					
3.1.2 That concentration is performed after each of the washes?	12.3.2.2.1 j	12.3.2.2.1 j	-	Method Procedure					
3.1.3 The sample is concentrated so that some liquid remains above the filter (enough to cover the stir bar about half-way)?	12.3.3.2.1 c	12.3.3.2.1 b	-	Method Procedure					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated For each item, does the SOP specify:		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
3.1.4	The stir bar and concentration tube are rinsed after each concentration and the liquid added to the concentrate?	12.3.3.2	12.3.3.2	-	Requirement					
3.1.5	The filter membrane is washed twice with 5 mL of PBST each time?	12.3.3.2.3	12.3.3.2.3	-	Method Procedure					
3.1.6	Acceptable Filta-Max® filter sample concentration procedures, including issues not noted in items 3.1.1 through 3.1.5?				Critical GLP					
3.2	**Envirochek® HV and Filta-Max® filter sample centrifugation**									
3.2.1	The sample is centrifuged at 1500 x G (maximum 2000 x G) using a swinging bucket rotor?	13.2.1 including NOTE	13.2.1 including NOTE	-	Method Procedure					
3.2.2	Instructions to ensure the centrifuge tubes are properly balanced prior to centrifugation?	-	-	3.15.4	Critical					
3.2.3	The sample is centrifuged for 15 minutes with start time beginning when centrifuge reaches the required speed?	13.2.1	13.2.1	-	Method Procedure					
3.2.4	The centrifuge is slowly decelerated at the end without using the brake?	13.2.1	13.2.1	-	Method Procedure					
3.2.5	Acceptable Envirochek® HV and Filta-Max® filter sample centrifugation procedures, including issues not noted in items 3.2.1 through 3.2.4?				Critical GLP					
4	**Purification and Slide Preparation**									
4.1	The centrifuged sample supernatant is aspirated no lower than 5 mL of supernatant above every 0.5 mL of the pellet or portion of 0.5 mL pellet?	13.2.2	13.2.2 13.2.3	5.2.2 5.2.3	Requirement					
4.1.1	The type and internal diameter of pipette used for aspiration of supernatant?	-	NOTE pg 37	-	Recommendation					
4.1.2	The rate of aspiration (i.e., mL/ min or pressure of the vacuum)?	-	13.2.2	-	Recommendation					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
4.2 The tube is vortexed vigorously until pellet is completely resuspended?	13.2.3	13.2.2.1	-	Method Procedure					
4.3 Appropriate procedures for dividing pellets greater than 0.5 mL into subsamples and the analysis of the subsamples?	13.2.4	13.2.3	-	Critical					
4.4 No more than 0.5 mL of pellet is used per IMS?	13.2.4	13.2.3	5.2.3	Method Procedure					
4.5 The resuspended pellet volume is quantitatively transferred to the flat-sided tube (2 rinses) including the determination of the rinse volumes?	13.3.2.1	13.3.2.1	-	Method Procedure					
4.6 SL-Buffer A is used at room temperature or that it is checked for precipitate before use?	NOTE pg 47	NOTE pg 39	3.17.2	Method Procedure					
4.7 The volume of 10x SL-Buffer A is 1 mL?	13.3.1.2	13.3.1.2	5.2.5	Method Procedure					
4.8 The volume of 10x SL-Buffer B is 1 mL?	13.3.1.3	13.3.1.3	5.2.5	Method Procedure					
4.9 Instructions for thorough resuspension of IMS beads prior to addition to the flat-sided tube?	13.3.2.2 13.3.2.4	13.3.2.2 13.3.2.4	-	Method Procedure					
4.10 100 µL of *Cryptosporidium* and *Giardia* beads are used?	13.3.2.3 13.3.2.5	13.3.2.3 13.3.2.5	5.2.5	Method Procedure					
4.11 The flat-sided tube is rotated at 18 rpm for 1 hour at room temperature?	13.3.2.6	13.3.2.6	-	Method Procedure					
4.12 Which magnetic concentrators, MPC®-1 or MPC®-6, are used?				Method Procedure					
4.13 The placement of the flat-sided tube in the magnet and the rock technique and time?	13.3.2.9	13.3.2.8 13.3.2.9	-	Method Procedure					
4.14 The sample is quantitatively transferred from the flat-sided tube to the microcentrifuge tube (2 rinses) including rinse volumes?	13.3.2.13	13.3.2.14	-	Method Procedure					
4.15 The flat-sided tube is allowed to sit one minute after each transfer to accumulate residual sample, then the residual is transferred to microcentrifuge tube?	13.3.2.13	13.3.2.14	-	Method Procedure					
4.16 The magnet is in the vertical position in the MPC®-S?	-	13.3.2.13	-	Method Procedure					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

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Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
4.17 The beads are rinsed with PBS while inside the microcentrifuge tube?	13.3.4	13.3.2.17	-	1623 Recommendation 1623.1 Requirement					
4.18 Standard NaOH (5 µL, 1N) and standard HCl (50 µL, 0.1N) are used?	NOTES pg 49-50	NOTES pg 42	3.17.5	Requirement					
4.19 The sample is vortexed vigorously for 50 seconds immediately after the addition of acid and 30 seconds after the sample has set for 10 minutes at room temperature?	13.3.3	13.3.3	-	Method Procedure					
4.20 The magnet is in the slanted position in the MPC®-S for dissociation steps?	-	13.3.3.6	-	Method Procedure					
4.21 A second dissociation is performed?	13.3.3.10	13.3.3.10	5.2.4	Requirement					
4.22 When the second dissociation is performed, the laboratory: A) uses a second slide, or B) adds the additional volume to the original slide?	13.3.3.10	13.3.3.10 13.4.5	-	Circle one: A B					
4.23 The volume and the timing of the NaOH addition to the wells?	13.3.3.8	13.3.3.8	-	Method Procedure					
4.24 When the slides are dried (e.g., room temperature or slide warmer), the laboratory: A) uses room temperature, or B) uses 35° to 42°C, or C) follows manufacturer's instructions?	13.3.3.12	13.3.3.12	-	Circle one: A B C					
4.25 If the laboratory has more than one option specified for slide drying, are criteria included for when each option will be used?	-	-	5.3.1	Recommendation					
4.26 That positive and negative staining controls are prepared at the same time the slides are prepared?	14.1	14.1.3	-	Requirement					
4.27 Acceptable sample purification and slide preparation procedures, including issues not noted in items 4.1 through 4.26?				Critical GLP					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
5 Sample Staining									
5.1 Which stain to use and to follow manufacturer's instructions for FITC stain application?	14.2	14.2	5.3.2	Method Procedure					
5.2 The slides are incubated in a humid chamber in the dark at room temperature for approximately 30 minutes or per manufacturer's directions?	14.3	14.3	5.3.3	Method Procedure					
5.3 The working DAPI stain is prepared the day it is used?	7.7.2	7.9.2	3.19.2	Method Procedure					
5.4 The stock DAPI is stored at 1 to 10°C in the dark?	7.7.1	7.9.1	3.19.1	Method Procedure					
5.5 The volume of working DAPI applied and the incubation time?	14.6	14.6	-	Method Procedure					
5.6 The technique used to drain the excess stain from the well and to rinse the well?	14.5	14.5	-	Method Procedure					
5.7 What type and amount of mounting media used?	7.8	7.10	-	Method Procedure					
5.8 That all the edges of the cover slip are sealed well with clear fingernail polish, unless Elvanol® is used?	14.9	14.9	-	Method Procedure					
5.9 The finished slides or slides not read immediately are stored in a humid chamber in the dark at 1° to 10°C (humid chamber not required for Elvanol®)?	14.10	14.10	5.3.6	Method Procedure					
5.10 Acceptable sample staining procedures, including issues not noted in items 5.1 through 5.9?				Critical GLP					
6 Microscope and Examination									
6.1 Instructions for ocular and Kohler adjustments?	10.3.4 10.3.6	10.7 10.8	3.22.10	Requirement					
6.2 That all measurements must be recorded to the nearest 0.5 micron?	15.2.2.3 15.2.3.3	15.2.2.4 15.2.3.4	3.22.5	Requirement					
6.3 Microscope cleaning procedures?	10.4	10.9	3.22.11	Requirement					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
6.4 The recording of coordinates of all cysts and oocysts on the worksheet for future reference; and slide orientation on the microscope stage to standardize coordinate recording?	-	-	-	Recommendation					
6.5 The examination and acceptance of positive and negative staining controls before proceeding with examination of field samples?	15.2.1	15.2.1	5.4.6 5.4.7	Requirement					
6.6 That each analyst characterizes 3 oocysts and 3 cysts on the positive staining control at each examination session?	15.2.1.1	15.2.1.1	5.4.6	Requirement					
6.7 Corrective actions if positive and/or negative staining controls are not acceptable?	-	-	5.4.8	Recommendation					
6.8 The criteria for organism identification?	15.2.2	15.2.2 15.2.3	5.4.9 5.4.10	Requirement					
6.9 Every positive organism in a field sample is characterized and recorded?	15.2	15.2.2.1 15.2.3.1	5.4.9.1 5.4.10.1	Requirement					
6.10 Acceptable microscope and examination procedures, including issues not noted in items 6.1 through 6.9?				Requirement GLP					
7 Reagents									
7.1 Procedures for the preparation of all essential chemicals and reagents?	7.0	7.0	4.2	Critical					
7.2 That expiration dates are specified for all reagents prepared by the laboratory?	-	-	4.2.2	Critical					
8 Quality Assurance									
8.1 Training protocol for new employees?	9.1	9.1	1.7	Requirement GLP					
8.2 Procedures for performing analyst verification?	10.6	9.10	7.1.9	Requirement GLP					
8.3 Positive and interfering organisms detected in field samples are documented by photography?	-	-	5.4.11	Recommendation					

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Item to be Evaluated For each item, does the SOP specify:	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
8.4 Acceptable procedures for sample collection for field or utility personnel?	-	-	6.1	Critical GLP					
8.5 Criteria for sample acceptance and corrective action procedures?	8.1.3	8.1.3	6.	Requirement GLP					
8.6 Method required holding times?	8.2	8.2	6.4	Requirement GLP					
8.7 Manual data recording procedures?	-	-	8.0	Critical GLP					
8.8 Procedures for checking the accuracy of data transcriptions, including electronic data entry?	-	-	8.1	Critical GLP					
8.9 Procedures for checking the accuracy of manual calculations?	-	-	8.1	Critical GLP					
8.10 Procedures for electronic data entry and storage?	-	-	8.2	Critical GLP					
8.11 How backup of stored data is performed?	-	-	8.2	Critical GLP					
8.12 Corrective action procedures for OPR failures?	9.7.4	9.8.5	7.1.6.2	Requirement GLP					
8.13 Corrective action procedures for method blank contamination?	9.6.2	9.7.3	7.1.5.2	Requirement GLP					
8.14 Procedures for identifying and assessing declining trends in recovery through review of control charts and/or other recovery data?	-	-	7.1.7.2	Recommendation GLP					
8.15 Corrective action procedures for investigating QC failures or declining trends in recovery?	-	-	7.1.7.2	Recommendation GLP					
8.16 Acceptable glassware washing procedures?	-	-	4.4	Critical GLP					

Comments:

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

Checklist C – Method 1623/1623.1 Technical Review – Sample Processing and Microscopy

Laboratory Name	Name and Affiliation of Evaluator	Date of Evaluation

Good Laboratory Practice (GLP) is generally defined as a system of management controls for the laboratories to ensure the consistency and reliability of results. Adapted from other federal programs for the purposes of the *Cryptosporidium* Laboratory QA Evaluation Program, GLP includes personnel, equipment, and standard operating procedures appropriate for the program.

Item to be evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
1 Laboratory Facilities									
1.1 Does laboratory appear to have established appropriate safety and health practices prior to use of this method?	5.0	5.0	4.1	Critical					
1.2 Do all laboratory personnel wear gloves when handling biohazard and toxic compounds, and change gloves before touching other surfaces and equipment?	5.3 5.4	5.3	4.1.6	Critical GLP					
1.3 Does the laboratory disinfect bench surfaces before and after analyses?	-	-	4.1.3	Critical GLP					
1.4 Does the laboratory have adequate bench space to perform the method?	-	-	2.0	Critical GLP					
1.5 Other than the issues noted in items 1.1 through 1.4 (if any), no other facility issues were observed?									
2 Reagents									
2.1 Is reagent water used to prepare all reagents?	7.3	7.3	4.3.1	Requirement					
2.2 Are all reagents clearly labeled with identity of reagent, date of preparation, technician initials, and expiration date?	-	-	4.2.2	Critical GLP					

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

November 2012

Item to be evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
2.3 Are SOPs available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
3 Sample Spiking				**Technician:**					
3.1 Was spike suspension vial vortexed for 30 seconds or per manufacturer's instructions?	11.4.3.1.2	11.2.3.2	-	Method Procedure					
3.2 Is the carboy used for method blank randomly selected from carboy stock to check efficacy of cleaning system?	-	-	7.1.5.3	Critical GLP					
3.3 Was the suspension vial adequately rinsed?	11.4.3.1	11.2.3	-	Method Procedure					
3.4 Are SOPs for sample spiking available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
3.5 Other than issues noted for items 3.1 through 3.4 (if any) was sample spiking demonstrated successfully?									
4 Filtration/Elution									
4.1 Envirochek® HV filtration				**Technician:**					
4.1.1 Are all components required for sample filtration present and in good condition?	6.1 6.2.1-6.2.2 6.3	6.1 - 6.2.8	6.1.7	Requirement GLP					
4.1.2 Is the filter assembly set up correctly?	Figure 3a	Figure 1	-	Method Procedure GLP					
4.1.3 Is the pump adequate for needs?	6.3.3	6.2.4	-	Requirement GLP					
4.1.4 Is the appropriate flow rate maintained (approximately 2 L/min)?	12.2.1.2	12.2.1.2	-	Method Procedure					

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Item to be evaluated		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
4.1.5	Is the volume filtered measured using a flow totalizer or calibrated carboy?	12.2.4.2	12.2.4.2	-	Requirement					
4.1.6	Is the system well maintained and cleaned appropriately following use?	4.5	4.5	6.1.7	Critical GLP					
4.1.7	Is the system able to maintain seal during use with no leaks?	-	-	6.1.7	Requirement GLP					
4.1.8	Are SOPs for Envirochek® HV filtration available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
4.1.9	Other than issues noted for items 4.1.1 through 4.1.8 (if any) was Envirochek® HV filtration demonstrated successfully?									
4.2 Envirochek® HV capsule filter elution					**Technician:**					
4.2.1	Is the elution buffer prepared as per Method?	7.4.1	7.6.1	-	Method Procedure					
4.2.2	Is the wrist-shaker assembly set up correctly with arms fully extended?	12.2.6.1.1	12.2.6.1	3.14.2	Method Procedure GLP					
4.2.3	Is the dispersant addition performed as per Method 1623.1?	-	12.2.7	-	1623 Recommendation **1623.1 Method Procedure**					
4.2.4	Is volume of elution buffer measured to ensure the use of one 250 mL centrifuge tube?	12.2.6.2.2	12.2.8.2	-	Method Procedure					
4.2.5	Are the samples shaken at an appropriate speed?	12.2.6.2.3	12.2.8.3	3.14.3	Method Procedure					

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Item to be evaluated		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
4.2.6	Are the samples shaken three times for 5 minutes each time, and each in a different orientation?	12.2.6.2	12.2.8	-	Method Procedure					
4.2.7	Are SOPs for Envirochek® HV capsule filter elution available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
4.2.8	Other than issues noted for items 4.2.1 through 4.2.7 (if any) was Envirochek® HV capsule filter elution demonstrated successfully?									
4.3	**Filta-Max® filtration**				**Technician:**					
4.3.1	Which filter is used – Filta-Max® (black end caps) or Filta-Max xpress® (red end caps)?									
4.3.2	Are all components required for sample filtration present and in good condition?	6.1 6.2.1 6.2.3 6.3	6.1 6.2.1-6.2.7 6.2.9	6.1.7	Requirement GLP					
4.3.3	Is the filter assembly set up correctly?	Figure 3b	Figure 2	-	Method Procedure GLP					
4.3.4	Is appropriate flow rate maintained of <4 L per minute for Filta-Max®?	12.3.1.1.3	12.3.1.1.3	-	Method Procedure					
4.3.5	Is the volume filtered measured correctly using a flow meter or calibrated carboy?	12.3.1.5.2	12.3.1.5.2	-	Requirement GLP					
4.3.6	Is system well maintained and cleaned appropriately following use?	12.3.4	12.3.4	6.1.7	Requirement GLP					

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Item to be evaluated		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
4.3.7	Is system able to maintain seal during use with no leaks?	-	-	6.1.7	Requirement GLP					
4.3.8	Does the laboratory indicate on the filter housing the correct direction of flow?	12.3.1.3	12.3.1.3	-	Critical					
4.3.9	Are SOPs for Filta-Max® filtration available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
4.3.10	Other than issues noted for items 4.3.1 through 4.3.9 (if any) was Filta-Max® filtration demonstrated successfully?									
4.4	**Filta-Max® filter wash station elution**				**Technician:**					
4.4.1	Is an automatic or manual wash station used?									
4.4.2	Is the filter wash station set up correctly?	12.3.2.1	12.3.2.1	-	Requirement GLP					
4.4.3	Is residual suspension rinsed from all containers?	12.3.2.2.1d	12.3.2.2.1d	-	Critical					
4.4.4	Is PBST used to elute the filter?	7.4.2.4	7.6.2.4	-	Method Procedure					
4.4.5	Is an appropriate amount of PBST used for each wash? (approx. 600 mL)	12.3.2.2	12.3.2.2	-	Method Procedure					
4.4.6	During the first wash, is the plunger moved up and down 20 times?	12.3.2.2.1h	12.3.2.2.1h	-	Method Procedure					
4.4.7	Is the plunger moved up and down gently to avoid generating excess foam?	12.3.2.2.1h	12.3.2.2.1h	-	Method Procedure					
4.4.8	During the second wash, is the plunger moved up and down 10 times?	12.3.2.2.2b	12.3.2.2.2b	-	Method Procedure					

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Item to be evaluated		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
4.4.9	If the automatic washer is used, is the machine operating properly?	12.3.2.1	12.3.2.1	-	Requirement					
4.4.10	Is the wash station cleaned adequately between samples?	12.3.4.2	12.3.4.2	-	Requirement GLP					
4.4.11	Are SOPs for Filta-Max® filter wash station elution available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
4.4.12	Other than issues noted for items 4.4.1 through 4.4.11 (if any) was Filta-Max® filter wash station elution demonstrated successfully?									
5 Concentration										
5.1 Filta-Max® filter sample concentration					**Technician:**					
5.1.1	Is concentrator set up correctly?	12.3.3.2.1b	12.3.3.2.1a	-	Requirement GLP					
5.1.2	Is the force of the vacuum maintained below 30 cm Hg?	NOTE pg 43	NOTE pg 34	-	Method Procedure					
5.1.3	Is concentration performed after each of the washes?	12.3.2.2.1j	12.3.2.2.1j	-	Method Procedure					
5.1.4	Is the sample concentrated so that some liquid remains above the filter (enough to cover the stir bar about half-way)?	12.3.3.2.1c	12.3.3.2.1b	-	Method Procedure					
5.1.5	Are the stir bar and concentration tube rinsed after each concentration and the liquid added to the concentrate?	12.3.3.2.1c	12.3.3.2.1b	-	Requirement					
5.1.6	Was the filter membrane washed twice with 5 mL of PBST?	12.3.3.2.3	12.3.3.2.3	-	Method Procedure					

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Item to be evaluated		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
5.1.7	Are SOPs for Filta-Max® filter sample concentration available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
5.1.8	Other than issues noted for items 5.1.1 through 5.1.7 (if any) was Filta-Max® filter sample concentration demonstrated successfully?									
5.2 Envirochek® HV and Filta-Max® filter sample centrifugation					**Technician:**					
5.2.1	Is the sample centrifuged at 1500 x G (maximum 2000 x G) using a swinging bucket rotor?	13.2.1 and NOTE pg 46	13.2.1 and NOTE pg 37	-	Method Procedure GLP					
5.2.2	Are the centrifuge tubes properly balanced prior to centrifugation?	-	13.2.1	3.15.4	Critical					
5.2.3	Does lab have easily accessible method for determining relative centrifugal force of centrifuges?	-	-	3.15.1	Critical GLP					
5.2.4	Is the sample centrifuged for 15 minutes, with time beginning when centrifuge reaches desired speed?	13.2.1	13.2.1	-	Method Procedure					
5.2.5	Is the centrifuge slowly decelerated at the end without the brake?	13.2.1	13.2.1	-	Method Procedure					
5.2.6	Is the pellet volume determined?	13.2.1	13.2.1	5.2.3	Requirement					
5.2.7	Is there a set of standards for comparison of pellet size?	-	-	5.2.3	Recommendation GLP					

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Item to be evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
5.2.8 Are SOPs for Envirochek® and Filta-Max® filter sample centrifugation available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
5.2.9 Other than issues noted for items 5.2.1 through 5.2.8 (if any) was Envirochek® HV or Filta-Max® filter sample centrifugation demonstrated successfully?									
6 Purification and Slide Preparation				**Technician:**					
6.1 Is an approved IMS kit/manufacturer used?	7.5	7.7.1	-	Method Procedure GLP					
6.2 Is the supernatant from the centrifuged sample aspirated no lower than 5 mL of supernatant above every 0.5 mL pellet or portion of 0.5 mL pellet?	13.2.2	13.2.2 13.2.3	5.2.2 5.2.3	Requirement					
6.2.1 Are the samples aspirated using the pipette, with the documented internal diameter, as specified in the SOP?	-	NOTE pg 37	-	Critical					
6.2.2 Is the proper rate (mL/min) or pressure (psi) maintained throughout aspiration?	-	13.2.2 13.2.3	-	Method Procedure					
6.3 Is the pellet vortexed a sufficient time for resuspension?	13.2.3 13.2.4.1.3 13.2.4.2	13.2.2.1 13.2.3.1.2 13.2.3.2	-	Method Procedure					
6.4 Is the resuspended pellet volume quantitatively transferred to the flat-sided tube (2 rinses)?	13.3.2.1	13.3.2.1	-	Method Procedure					
6.5 Are the IMS beads thoroughly resuspended prior to addition to the flat-sided tube?	13.3.2.2 13.3.2.4	13.3.2.2 13.3.2.4	-	Method Procedure					

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Item to be evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
6.6 Is the flat-sided tube rotated at 18 rpm for 1 hour at room temperature?	13.3.2.6	13.3.2.6	-	Method Procedure					
6.7 Is the rotating mixer calibrated annually?	-	-	3.17.4	Critical GLP					
6.8 Is flat-sided tube correctly placed in magnet and rocked through 90 degrees about once per second?	13.3.2.7-13.3.2.9	13.3.2.7-13.3.2.9	-	Method Procedure					
6.9 Is all the liquid removed when decanting is performed with the magnet up?	13.3.2.11	13.3.2.11	-	Method Procedure					
6.10 Is the sample quantitatively transferred from the flat-sided tube to the microcentrifuge tube (2 rinses)?	13.3.2.13	13.3.2.14	-	Method Procedure					
6.11 Are the beads rinsed with PBS while inside the microcentrifuge tube?	13.3.4	13.3.2.17	-	1623 Recommendation 1623.1 Requirement					
6.12 Is standard NaOH (5 µL, 1N) and standard HCl (50 µL, 0.1N) used?	NOTE pg 49 & 50	NOTE pg 42	3.17.5	Requirement GLP					
6.13 Is sample vortexed vigorously for 50 seconds immediately after the addition of acid and 30 seconds after the sample has set for 10 minutes at room temperature?	13.3.3.2-13.3.3.4	13.3.3.2-13.3.3.4	-	Method Procedure					
6.14 Is a second dissociation performed?	13.3.3.10 NOTE pg 49	13.3.3.10 NOTE pg 41	5.2.4	Requirement					
6.15 When the second dissociation is performed, does the laboratory: (A) use a second slide (B) add the additional volume to the original slide?	13.3.3.10	13.3.3.10 13.4.5	-	Circle one: A B					
6.16 Are the slides clearly labeled so they can be associated with the correct sample?	13.3.3.7	13.3.3.7	-	Requirement					
6.17 What type of slides is used?				GLP					

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Item to be evaluated		Reference*			Classification	Satisfactory				Comments/ Response Requested
		1623	1623.1	Cert		Yes	No	NA	UNK	
6.18	Is slide dried at: (A) room temperature, (B) 35º to 42ºC, or (C) in the refrigerator?	13.3.3.12	13.3.3.12	-	Circle one: A B C					
6.19	If the slide is warmed, is incubator or slide warmer calibrated and labeled?	-	-	3.4	Critical GLP					
6.20	Are SOPs available in the work area for sample purification and slide preparation, and does laboratory practice reflect written procedures?				Critical GLP					
6.21	Other than issues noted for items 6.1 through 6.20 (if any) was purification and slide preparation demonstrated successfully?									
7	**Sample Staining**				**Technician:**					
7.1	What staining kit/manufacturer is used?	14.2	14.2	3.18.1	GLP					
7.2	Is FITC stain applied according to manufacturer's directions?	14.2	14.2	5.3.2	Method Procedure					
7.3	Are positive and negative staining controls performed?	14.1	14.1	5.3.5	Requirement					
7.4	Are the slides incubated in a humid chamber in the dark at room temperature for approximately 30 minutes or per manufacturer's directions?	14.3	14.3	5.3.3	Method Procedure					
7.5	Are the labeling reagents rinsed away properly after incubation, without disturbing the sample?	14.5	14.5	-	Method Procedure					
7.6	Was the working DAPI stain prepared the day it was used?	7.7.2	7.9.2	3.19.2	Method Procedure					
7.7	Is stock DAPI stored at 1 to 10°C in the dark?	7.7.1	7.9.1	3.19.1	Method Procedure					
7.8	Is the DAPI stain applied properly and allowed to stand for a minimum of 1 minute?	14.6	14.6	-	Method Procedure					

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Item to be evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
7.9 Is the DAPI stain rinsed away properly without disturbing the sample?	14.7	14.7	-	Method Procedure					
7.10 Is the mounting media applied properly?	14.8	14.8	-	Method Procedure					
7.10.1 What type of mounting media is used?	7.8	7.10	-	GLP					
7.10.2 Are all the edges of the cover slip sealed well with clear fingernail polish, unless Elvanol® is used?	7.9 14.9	7.11 14.9	-	Method Procedure					
7.11 Are the finished slides stored in a humid chamber in the dark at 1 to 10°C (humid chamber not required for Elvanol®)?	14.10	14.10	5.3.6	Method Procedure					
7.12 Are SOPs for sample staining available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
7.13 Other than issues noted for items 7.1 through 7.12 (if any) was sample staining demonstrated successfully?									
8 Microscope and Examination									
8.1 Is microscope equipped with appropriate excitation and band pass filters for examining FITC labeled specimens as demonstrated with lab, and auditor provided, positive staining control?	6.9.2	6.7.2	3.22.3	Requirement GLP					
8.2 Is microscope equipped with appropriate excitation and band pass filters for examining DAPI labeled specimens as demonstrated with lab, and auditor provided, positive staining control?	6.9.3	6.7.3	3.22.3	Requirement GLP					
8.3 Does the microscope have appropriate objectives and filters for DIC, which change easily to and from epifluorescence?	6.9.1	6.7.1	3.22.4	Requirement GLP					

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Item to be evaluated	Reference*			Classification	Satisfactory				Comments/ Response Requested
	1623	1623.1	Cert		Yes	No	NA	UNK	
8.4 Are all portions of the microscope, from the light sources to the oculars, properly adjusted?	10.3	10.0 Appendix B	3.22.6	Requirement					
8.5 Is the DIC image appropriate for each laboratory microscope?	-	Figure 4	Visual Guide	Requirement					
8.6 Is microscope cleaned after every session?	10.4	10.9.8	3.22.11	Requirement GLP					
8.7 Does the microscope have a 20X scanning objective?	6.9.1	6.7.1	3.22.8	Requirement GLP					
8.8 Does the microscope have a 100X oil immersion objective?	6.9.1	6.7.1	3.22.8	Requirement GLP					
8.9 Is the microscope equipped with an ocular micrometer?	6.9.1	6.7.1	3.22.9	Requirement GLP					
8.10 Is a stage micrometer available to laboratory?	6.9.1 10.3.5	6.7.1 App. B 3	3.22.9	Requirement					
8.11 Is a calibration table for 100X objective located close to the microscope(s)?	10.3.5.7	App. B 3.7	3.22.9	Requirement					
8.12 Has the mercury bulb been used less than the maximum hours recommended by the manufacturer?	10.3.2.11	App.B 1.11	3.22.12	Requirement					
8.13 Does the laboratory have a preventative maintenance agreement in place to service the microscope annually?	-	-	3.22.6	Critical GLP					
8.14 Are SOPs for sample examination available in the work area, and does laboratory practice reflect written procedures?				Critical GLP					
8.15 Other than issues noted for items 8.1 through 8.13 (if any) was Microscope and Examination demonstrated successfully?									

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

9	Positive Staining Control and OPR Slides									
9.1	Does the laboratory's positive staining control slide contain (oo)cysts at the appropriate fluorescence intensity for FITC?	15.2.1.3	15.2.1.3	5.4.8 5.4.9.2 5.4.10.2	Requirement					
9.2	Does the laboratory's positive staining control slide contain (oo)cysts at the appropriate fluorescence intensity for DAPI?	15.2.1.3	15.2.1.3	5.4.8 5.4.9.3 5.4.10.3	Requirement					
9.3	Does the laboratory's positive staining control slide contain an appropriate level of background fluorescence?	-	-	5.4.3	Recommendation					
9.4	Is concentration of oocysts on the positive staining control slide appropriate?	14.1.1 15.2.1.3	14.1.1 15.2.1.3	7.1.8.1	Requirement					
9.5	Does the laboratory's positive staining control exhibit appropriate contrast and organism features by DIC?	-	Figure 4	Visual Guide	Requirement					
9.6	Does the laboratory's OPR slide contain (oo)cysts at the appropriate fluorescence intensity for FITC?	15.2.2.1 15.2.3.1	15.2.2.2 15.2.3.2	5.4.9.2 5.4.10.2	Requirement					
9.7	Does the laboratory's OPR slide contain (oo)cysts at the appropriate fluorescence intensity for DAPI?	15.2.2.2 15.2.3.2	15.2.2.3 15.2.3.3	5.4.9.3 5.4.10.3	Recommendation					
9.8	Does the laboratory's OPR slide contain an appropriate level of background fluorescence?	-	-	5.4.3	Requirement					
9.9	Does the laboratory's OPR slide exhibit appropriate contrast and organism features by DIC?	9.7.1.1	9.8.1.1 Figure 4	Visual Guide	Requirement					
9.10	Does the technical auditor's count of *Cryptosporidium* oocysts and *Giardia* cysts on the OPR slide sent by the laboratory agree within 10% of laboratory count?	10.6.3.1	9.10.3.1	7.1.9.4	Requirement					

Comments:

** References refer to Section numbers in Method 1623 December 2005 (1623); Method 1623.1 January 2012 (1623.1); Certification Manual Chapter VII Supplement to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water (Cert)*

10 Onsite Sample Processing			
Method Step	**Name**	**Position**	**Demonstrated Technique Successfully** yes/no
Spiking – *(filter type)*			
Filtration - *(filter type)*			
Spiking flat-sided tube, and processing IMS control			
Aspiration and transfer from 250 mL bottle			

11 Onsite Blind Spike Results						
Sample	**Crypto Spike Value**	**Crypto Count**	**Crypto Recovery (%)**	***Giardia* Spike Value**	***Giardia* Count**	***Giardia* Recovery (%)**

12 Evaluation of Onsite Sample Processing and Blind Spike Results – Comments and Recommendations		
Classification	**Comments**	**Response Requested**

13 Was analyst microscope operation acceptable? (yes/no)				
		Requirement *Method 1623: 10.3.4.1* *Method 1623.1: 10.7.1*	**Requirement** *Method 1623: 10.3.4.2-3* *Method 1623.1: 10.7.2-3*	**Requirement** *Method 1623: 10.3.6* *Method 1623.1: 10.8*
Name	**Position**	**Adjust Interpupillary Distance**	**Focus both eyepieces**	**Establish Kohler Illumination**

14 Slide Count and Analyst Verification Results (yes/no)					
	Requirement *Method 1623: 10.6.3.1* *Method 1623.1: 9.10.3.1*	**Requirement** *Method 1623: 10.6.3.1* *Method 1623.1: 9.10.3.1*	**Requirement** *Method 1623: 15.2* *Method 1623.1: 15.2*	**Requirement** *Method 1623: 15.2.2.3 15.2.3.3* *Method 1623.1: 15.2.2.4 15.2.3.4*	**Requirement** *Method 1623: 15.2.2.3 15.2.3.3* *Method 1623.1: 15.2.2.4 15.2.3.4*
Analyst	**Crypto Count Within 10% of Target Count**	***Giardia* Count Within 10% of Target Count**	**Examine and Record Characteristics**	**Measurement (100X)**	**Demonstrated Internal Structures**

15 Evaluation of Analyst Microscopy and Examination Skills – Comments and Recommendations		
Classification	**Comments**	**Response Requested**

Laboratory Name:	Laboratory ID:

Method 1623/1623.1 Bench Sheet

Sample Identification Information

* Lab Sample ID:	Turbidity (NTU):
* PWS ID:	Person Receiving Sample:
* Facility ID:	Temperature (°C) @ sample receipt:
* Sample Collection Point ID:	Date of sample receipt:
* Sample collection date & time:	Time of Sample receipt:
* Sample type (circle one): Initial precision and recovery (IPR) Method blank Field (monitoring) sample Ongoing precision and recovery (OPR) Matrix spike (MS) Proficiency testing (PT)	

Sample Spiking Information (for IPR, OPR, MS, and PT samples only)

* Estimated number spiked:	Crypto:	Giardia:	Spiking time:
* Sample volume spiked (L):			Spiking date:
Spike manufacturer & ID:			Spiking analyst:

Sample Filtration

Filter type (circle one): Envirochek HV Filta-Max PCFC	Method version (circle one): 1623 1623.1
Did filter clog? (circle one): Yes No	Filtration time: Filter lot number:
* Number of filter(s) used?:	Filtration date:
* Volume filtered (L) to nearest 1/4L:	Filtration analyst:

Filter Elution (must be initiated within 96 hours of sample collection/filtration)

Elution procedure (circle one): Wrist shaker Filta-Max wash station	Elution Buffer lot number:
Type of Elution buffer (circle one): NaHMP/LA-12 LA-12 PBST PCFC	
Elution buffer expiration date:	Elution time:
NaHMP lot number:	Elution date:
NaHMP expiration date:	Elution analyst:

Concentration, IMS, and Slide Preparation

(must be completed on same working day that samples are eluted)

Procedure (circle one): Centrifugation Filta-Max concentrator Other (specify)	
* Pellet volume after concentration (mL) to nearest 0.1mL:	Concentration analyst:
* Total volume of resuspended concentrate (mL):	IMS analyst:
* Volume of resuspended concentrate transferred to IMS (mL):	Slide preparation time:
Number of subsamples processed through entire method:	Slide preparation date:
IMS lot number:	Slide preparation analyst:
IMS system (circle one): Dynal GC-Combo Dynal anti-Crypto Other (specify)	
Slides (circle one): Meridian Dynal Waterborne Other (specify)	

Slide Staining (must be completed within 72 hours of application of sample to the slide)

Detection kit (circle one): BTF EasyStain Merifluor Crypt-a-glo Giardi-a-glo Aqua-glo Other (specify)	
Detection kit lot number:	Staining date & time:
Number of slides for this sample:	Staining analyst:

*** Examination Results as Total FA number from all slides for sample Cryptosporidium: Giardia:**

Comments:

* = Data entered into LT2/Stage2 Data Collection and Tracking System

Laboratory name:	Laboratory ID:

Method 1623/1623.1 Slide Examination Form

Sample ID:	Analyst:
Examination/verification completion: (must be completed within 168 hours (7 days) of staining) **Date:** **Time:**	Slide number: Total number of slides for this sample:
Positive staining control acceptable & 3 oocysts and cysts characterized with FITC, Size, Shape, DAPI and DIC ☐ YES ☐ NO	Negative staining control acceptable ☐ YES ☐ NO
FITC, Size, Shape, DIC and DAPI Characteristics Must Be Recorded for all Oocysts Detected in Field Sample ☐ YES ☐ NO	

Cryptosporidium Results

Object located by FA No.	Shape (oval or round)	Size L x W (µm)	DAPI -	DAPI +		D.I.C.		
			Light blue internal staining, no distinct nuclei, green rim	Intense blue internal staining	Number of nuclei stained sky blue	Empty oocysts	Oocysts with amorphous structure	Oocysts with internal structure
								Number of sporozoites
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
Total FA number from this slide:								
Analyst signature: P.A. ☐				Principle Analyst (P.A.)Signature:				
Comments:								

Giardia Results

Object located by FA No.	Shape (oval or round)	Size L x W (µm)	DAPI -	DAPI +		D.I.C.				
			Light blue internal staining, no distinct nuclei, green rim	Intense blue internal staining	Number of nuclei stained sky blue	Empty cysts	Cysts with amorphous structure	Cysts with internal structure		
								Number of nuclei	Median body	Axonemes
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
Total FA number from this slide:										
Comments:										